Biochemical and Biophysical Research Communications 421 (2012) 750–756

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The upregulation of TRPC6 contributes to Ca^{2+} signaling and actin assembly in human mesangial cells after chronic hypoxia

Chang Liao, He Yang, Rui Zhang, Hui Sun, Bo Zhao, Chunshou Gao, Furong Zhu, Jundong Jiao *

Department of Nephrology, The Second Affiliated Hospital, Harbin Medical University, PR China

ARTICLE INFO

Article history:
Received 5 April 2012
Available online 25 April 2012

Keywords:
Hypoxia
Mesangial cells
TRPC6
Actin cytoskeleton

ABSTRACT

There is increasing evidence that mesangial cells are important targets of chronic hypoxia injury. Impaired Ca^{2+} signaling has been found in mesangial cells (MCs) subjected to chronic hypoxia. However, the mechanisms underlying this phenomenon have not yet been defined. In the present study, we found that chronic hypoxia enhanced the expression of TRPC6 and TRPC6-dependent Ca^{2+} entry, and TRPC6 knockdown inhibited the chronic hypoxia-induced increase in $[Ca^{2+}]i$, suggesting that TRPC6-mediated Ca^{2+} entry is responsible for the elevated $[Ca^{2+}]i$ induced by chronic hypoxia in MCs. In addition, TRPC6 knockdown attenuated chronic hypoxia-induced actin assembly and actin reorganization. We concluded that the upregulation of TRPC6 is involved in the Ca^{2+} signaling and actin assembly in human MCs after chronic hypoxia. These findings provide new insight into the mechanisms underlying the cellular response of MCs to hypoxia.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Chronic hypoxia appears to play an important role in the initiation and progression of chronic renal disease [1]. The pathogenic role of chronic hypoxia in tubulointerstitial injury has been investigated intensively, but little is known about its implications for glomerular damage. Some studies have indicated that hypoxia or ischemic stress also affects glomerular cells in various kidney diseases [2–4]. One recent study found that the glomerular expression levels of the majority of hypoxia-inducible factor (HIF)-regulated genes are significantly altered in human nephrosclerosis [5]. Mesangial cells (MCs) are highly specialized smooth muscle-like cells located in the renal glomerulus. Despite the growing evidence that chronic hypoxia alters MC functions including proliferation [6], extracellular matrix synthesis [7], prostaglandin synthesis [8], inflammatory mediator production [9] and dedifferentiation [3], the signaling mechanisms through which chronic hypoxia alters cellular behavior remains poorly defined.

Ca^{2+} signaling, one of the most important and universal intracellular signaling pathways, is involved in the mediation and regulation of the hypoxic response. In many instances, one of the constant responses to hypoxia is an increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) via the activation of various plasma membrane Ca^{2+} conductances, such as voltage-gated calcium channels, ligand-operated Ca^{2+} channels, and non-specific cation channels [10]. Consistent with this hypothesis, an increase in basal $[Ca^{2+}]i$ induced by chronic hypoxia has been documented in MCs [6]. The source for this increase was extracellular Ca^{2+}, as demonstrated by experiments showing that incubation in calcium-free medium blocked the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$ [6]. However, the Ca^{2+} entry pathway has not yet been identified. Recently, the canonical transient receptor potential 6 (TRPC6) channel, a member of the TRPC family of nonselective cation channels, has been implicated in the hypoxia-induced increase in $[Ca^{2+}]i$ in pulmonary arterial smooth muscle cells [11], glioblastoma multiforme cells [12], and endothelial cells [13]. Several TRPC subunits are related to store-operated Ca^{2+} entry (SOCE), which can be activated by the depletion of the cellular Ca^{2+} store using thapsigargin (TG), and others are involved in receptor-operated Ca^{2+} entry (ROCE), which can be activated directly by diacylglycerol (DAG) via a protein kinase C (PKC)-independent mechanism. It is widely accepted that TRPC6 is responsible for endogenous ROCE in most if not all cell types [14]. However, the functional role of TRPC6-mediated Ca^{2+} entry is not well understood. In endothelial cells [15], kidney podocytes [16] and fibroblasts [16], TRPC6-mediated Ca^{2+} entry promotes actin assembly, stress fiber formation and actin reorganization, thereby regulating cell migration and endothelial permeability. Cellular actin is generally present in interchangeable monomeric and polymeric forms; the globular-(G-) actin subunits assemble into long filamentous F-actin polymers. In many cell types, such as smooth muscle cells [17] and endothelial cells [18], actin assembly and reorganization are well-characterized cytoskeletal changes in response to hypoxia. Recently, TRPC6-mediated Ca^{2+} entry has been impli-

* Corresponding author. Address: Xuefu Road 246, Harbin 150081, Heilongjiang Province, PR China. Fax: +86 451 86662640.
E-mail address: jiaojundong@yahoo.com.cn (J. Jiao).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.04.075

cated in hypoxia-induced cell migration and actin cytoskeleton remodeling in tumor cells [12].

It has been demonstrated that human MCs selectively express isoforms of TRPC1, 3, 4, and 6 proteins [19]. However, the role of TRPC6 in MCs in response to chronic hypoxia has not been established. In the present study, we sought to investigate changes in TRPC6 expression and activity induced by chronic hypoxia and their involvement in subsequent elevated basal $[Ca^{2+}]i$ and actin reorganization.

2. Materials and methods

2.1. Cell culture and chronic hypoxia treatments

An established stable human mesangial cell line was cultured as described previously [20]. Briefly, these cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Cells between passages 3 and 15 were used. To assess the effects of hypoxia, MCs were subcultured in either hypoxic (2% O_2 or 10% O_2) or normoxic (21% O_2) environments for 6–48 h. The cells were cultured in serum-deprived (1% FBS) for 12 h before use.

2.2. Transient transfection in vitro

Transient transfection with TRPC6 siRNA (sc-42672, Santa Cruz, USA) was carried out using the X-treme GENE siRNA Transfection Reagent (Roche, Penzberg, Germany) and PLUS Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were used for experiments 24–48 h after transfection.

2.3. Real-time RT-PCR

RT-PCR was performed using standard methods. The primers sequences used to amplify TRPC1, TRPC3, TRPC4 and TRPC6 were (5′–3′): TRPC1 sense, CGCCGAACGAGGTGAT and antisense GCACG CCAGCAAGAAA; TRPC3 sense, CGGCAACATCCCAGTG and antisense, CGTAGAAGTCGTCGTCCTG; TRPC4 sense, CTCTGGTTGTTCT ACTCAACATG and antisense, CCTGTTGACGAGCAACTTCTTCT; TR PC6, sense, GCCAATGAGCATCTGGAAAT and antisense, TGGAGT CACATCATGGGAGA. GAPDH was used as the loading control. Melting curve analysis was used to verify specificity.

2.4. Western blot

Western blot was performed using a standard protocol. Polyclonal rabbit anti-TRPC1, -TRPC3, -TRPC4, or -TRPC6 antibodies (Alomone Labs, Israel) were used as the primary antibodies. Fluorescence-conjugated goat anti-rabbit IgG (Invitrogen, USA) secondary antibodies were used. The band densities were quantified using the Odyssey infrared imaging system (LI-COR, Lincoln, NE).

2.5. Fluorescence measurement of $[Ca^{2+}]i$

MCs were grown on coverslips and loaded in physiological saline solution containing Fluo-3/AM (3 μM, Molecular Probes Eugene, OR, USA) and Pluronic F-127 (0.03%, Sigma, St. Louis, MO, USA) at 37 °C for 45 min. Fluorescence changes in Fluo-3/AM-loaded cells were detected by laser scanning confocal microscopy (Olympus, Tokyo, Japan). The intracellular calcium level ($[Ca^{2+}]i$) was expressed as a pseudo-ratio value of the actual fluorescence intensity divided by the average baseline fluorescence intensity. The Ca^{2+}-free bath solution contained no $CaCl_2$ and 0.5 mM EGTA. For measurements of basal $[Ca^{2+}]i$ levels, $[Ca^{2+}]i$ was calculated using the formula described by Grynkiewicz et al. [21]. Calibrations were performed at the end of each experiment. High $[Ca^{2+}]i$ conditions were achieved by adding 4 μM ionomycin, and low $[Ca^{2+}]i$ conditions were achieved by adding 10 mM EGTA. Data from 20 to 40 cells were summarized in a single run, and at least three independent experiments were conducted.

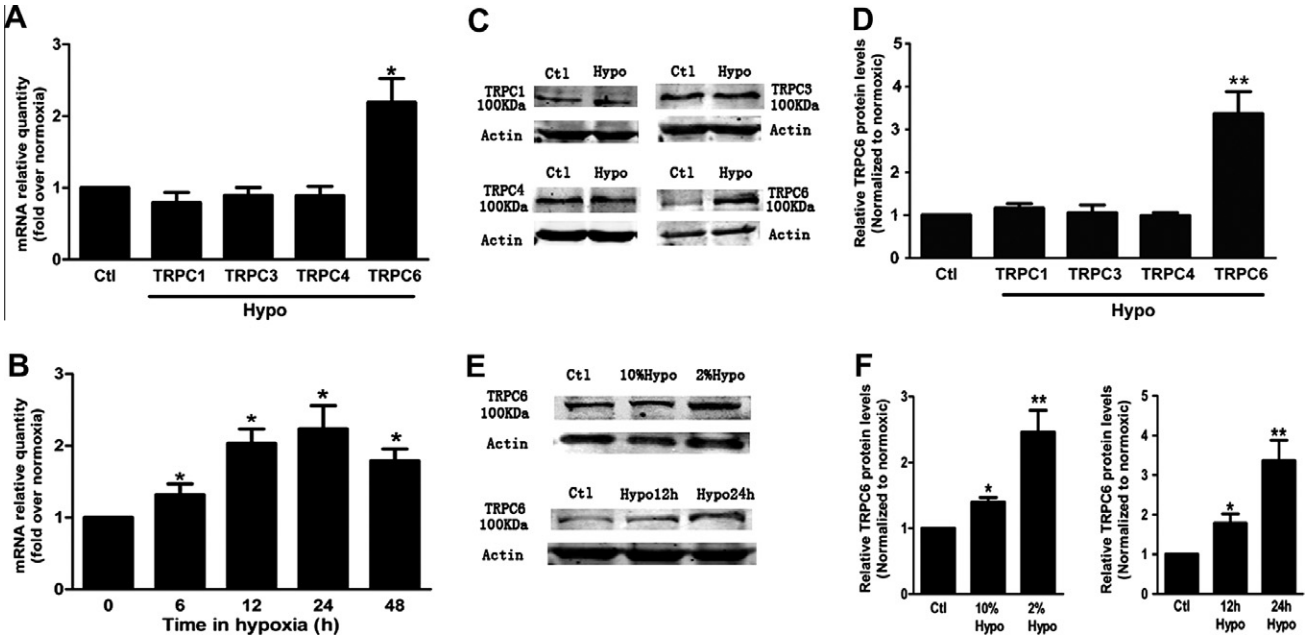

Fig. 1. The influence of chronic hypoxia on TRPC6 mRNA and protein expression. (A) Chronic hypoxia caused a dramatic increase in TRPC6 mRNA level but did not affect TRPC1, TRPC3 or TRPC4 expression ($^{*}p < 0.05$ vs. Ctl, $n = 3$). (B) The time-dependent reduction in TRPC6 mRNA in response to chronic hypoxia ($^{*}p < 0.05$ vs. Ctl, $n = 3$). Representative Western blot (C) and corresponding quantitative analysis (D) showing that chronic hypoxia increased TRPC6 protein expression but did not affect TRPC1, TRPC3 or TRPC4 ($^{**}p < 0.01$ vs. Ctl, $n = 4$). Representative Western blot (E) and corresponding quantitative analysis (F) showing that chronic hypoxia reduced TRPC6 protein expression in a time- and dose-dependent manner ($^{*}p < 0.05$ vs. Ctl, $n = 3$). Asterisks indicate the statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$) with respect to normoxic conditions.

2.6. Direct fluorescence staining of F-actin

MCs prepared for F-actin staining were transferred onto coverslips 24 h before use. The cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with blocking solution (1% BSA in PBS). Cells were stained with phalloidin-FITC (5 μg/ml in PBS; Sigma–Aldrich, St. Louis, MO) and visualized with laser scanning confocal microscopy (Olympus, Tokyo, Japan) using an oil immersion objective lens for high resolution.

2.7. F-/G-actin ratio assay

Protein extracts from MCs were subjected to an F-/G-actin in vivo assay (Cytoskeleton, CO, USA) carried out according to the manufacturer's protocol. Briefly, cells were lysed in a cell lysis and F-actin stabilization buffer. To separate F- and G-actin, samples were centrifuged at 37 °C for 1 h at 100,000*g*. Then, the G-actin-containing supernatants were separated from the F-actin-containing pellets. The pellets were resuspended to the same volume as the supernatants using ice-cold ddH_2O containing 1% cytochalasin D. Aliquots of the supernatant and pellet fractions were separated on 12% SDS–PAGE gels and then Western blotted with monoclonal anti-actin antibody. The F-/G-actin ratio was determined by scanning densitometry.

2.8. Statistical analysis

Data are expressed as the mean ± SEM of the indicated number (n) of experiments. Statistical analysis was performed using unpaired t-test (SPSS 16.0), and graphs were constructed in Adobe Photoshop or plotted in GraphPad Prism 5 (GraphPad Software, Inc.). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic hypoxia upregulated TRPC6 mRNA and protein expression

As shown in Fig. 1A, exposure of MCs to 2% O_2 for 24 h caused a significant increase in the mRNA levels of TRPC6 but not TRPC1, TRPC3, or TRPC4. The upregulation of TRPC6 mRNA transcripts by

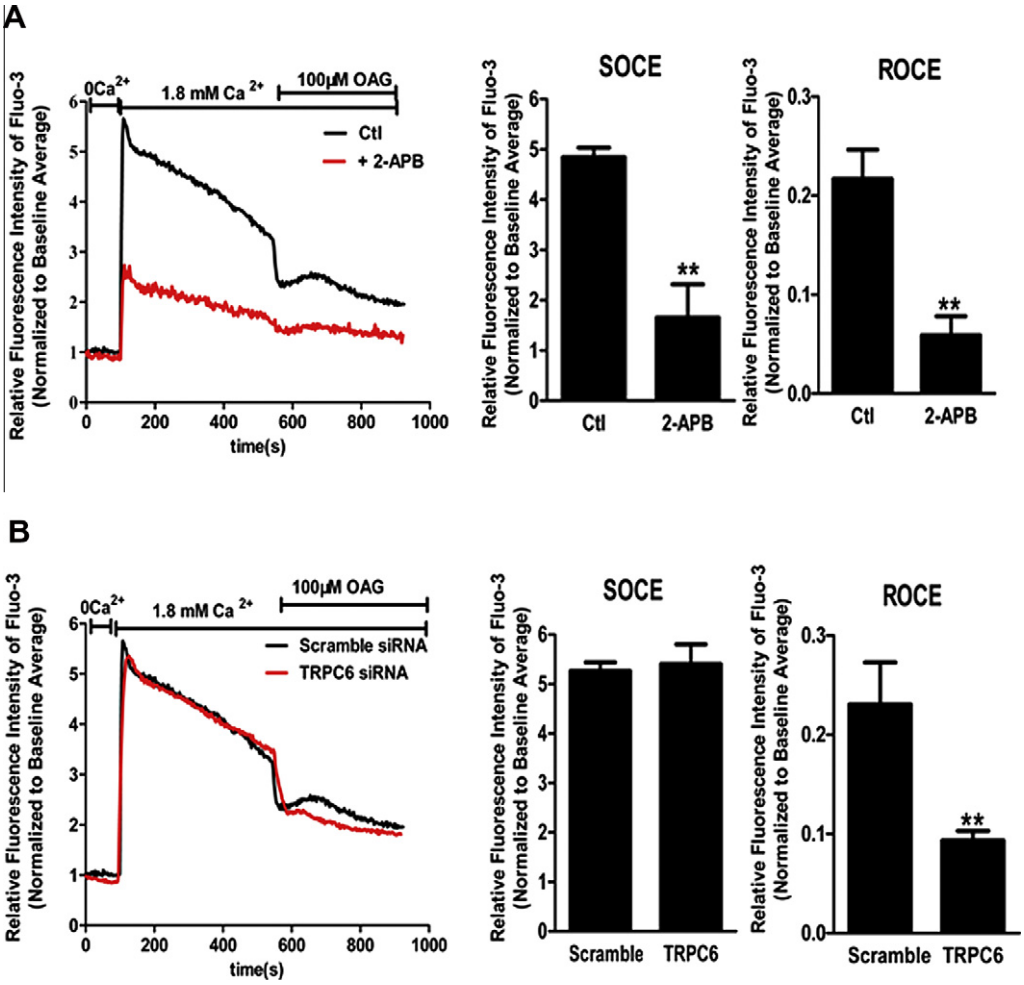

Fig. 2. The effects of TRPC6 knockdown and chronic hypoxia on TG-induced SOCE and OAG-induced ROCE. [Ca^{2+}]i dynamics were monitored using Fura-3 fluorescence methods. All bath solutions used in these experiments contained 10 μM nifedipine. (A) Representative traces (left) and summary data (right) showing that pretreatment with 100 μM 2-APB inhibited TG-induced SOCE and OAG-induced ROCE, respectively (**$p < 0.01$ vs. control, $n = 4$). (B) Representative traces (left) and summary data (right) showing that transfection with TRPC6 siRNA significantly inhibited OAG-induced ROCE compared with transfection with scrambled siRNA (**$p < 0.01$, $n = 3$), whereas TG-induced SOCE was unaffected ($p > 0.05$, $n = 3$). (C) Real-time PCR experiments showing that the TRP6 siRNA significantly reduced the mRNA expression of TRPC6 (*$p < 0.05$ vs. control, $n = 3$) but not TRPC1, TRPC3, or TRPC4 ($p > 0.05$, $n = 3$). Western blot experiments showing that transfection with TRPC6 siRNA significantly reduced TRPC6 protein expression compared to transfection with scramble siRNA (**$p < 0.01$, $n = 3$). (D) Representative traces (left) and summary data (right) showing that chronic hypoxia enhanced OAG-induced ROCE in a time-dependent manner (*$p < 0.01$ vs. normoxia, $n = 5$) but did not affect TG-induced SOCE ($p > 0.05$, $n = 5$). Asterisks indicate the statistical significance (*$p < 0.05$, **$p < 0.01$).

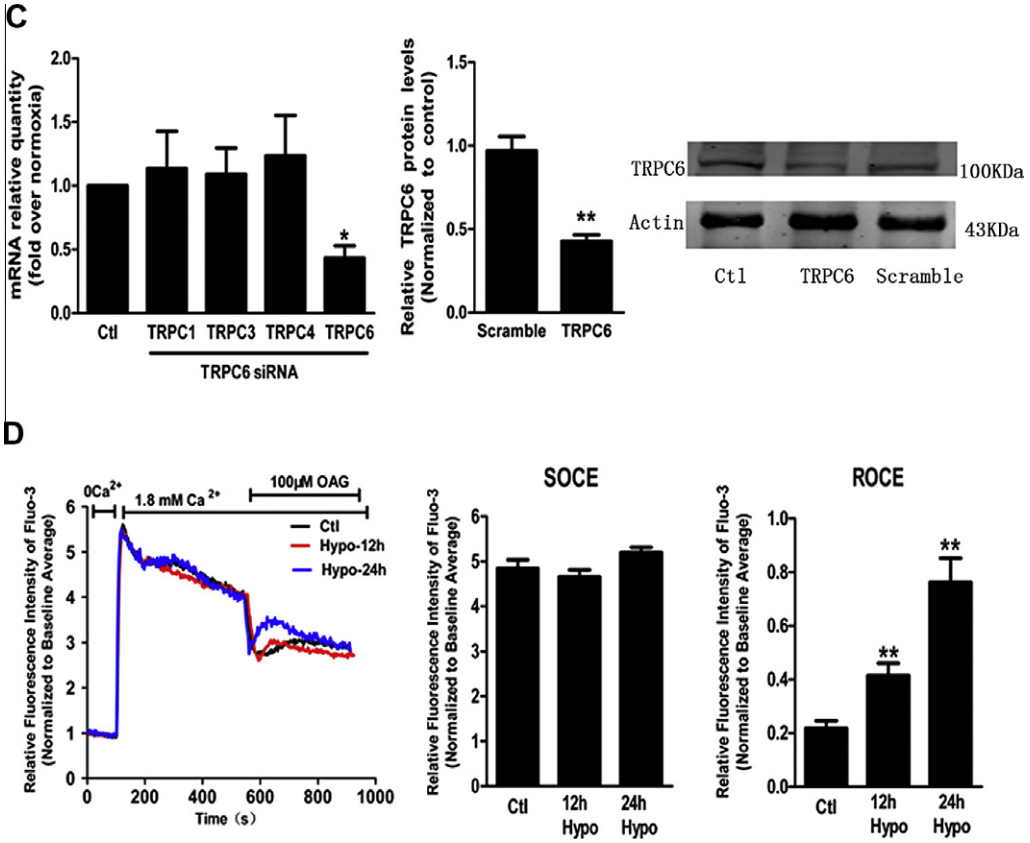

Fig. 2 (*continued*)

chronic hypoxia was markedly time dependent, with onset at 6 h, a peak at 24 h and reversion to some extent at 48 h (Fig. 1B). Consistent with the mRNA expression results, Western blot experiments showed that the exposure of MCs to 2% O_2 for 24 h induced a increase in TRPC6 protein expression, but had no effects on TRPC1, TPRC3 or TRPC4 protein expression (Fig. 1C and D). Furthermore, the upregulation of TRPC6 protein levels was dose and time dependent, as shown in Fig. 1E and F.

3.2. Chronic hypoxia enhanced TRPC6-dependent Ca^{2+} entry

It is well known that TRPC6 can be activated by DAG, resulting in Ca^{2+} entry via ROCE. Therefore, we tested the effects of chronic hypoxia on the Ca^{2+} entry induced by 1-oleoyl-2-acetyl-sn-glycerol (OAG), a membrane permeable DAG analogue. Internal Ca^{2+} stores were depleted by incubating MCs with 1 μM TG for 5 min in Ca^{2+}-free solution. As shown in Fig. 2A, the addition of 1.8 mM Ca^{2+} into the bathing solution induced a remarkable increase in $[Ca^{2+}]i$. The TG-induced SOCE was quantified as the increase in $[Ca^{2+}]i$ upon the readdition of Ca^{2+} to the bath solution after store depletion with TG. After TG-induced SOCE, the application of 100 μM OAG induced an additional substantial $[Ca^{2+}]i$ elevation under conditions in which Ca^{2+} stores are already depleted (Fig. 2A). Thus, OAG-induced Ca^{2+} influx was carried by plasmalemmal channels other than, or at least in addition to, SOCE. MCs exhibited no spontaneous $[Ca^{2+}]i$ changes during these experiments, and the readdition of extracellular Ca^{2+} in the absence of agonists had no effect on $[Ca^{2+}]i$ (data not shown). Additionally, voltage-operated calcium channels can be excluded as mediators of the Ca^{2+} influx because all bathing solutions in these experiments contained 10 μM nifedipine, which blocks both L-type and T-type calcium channels [22]. OAG-induced ROCE was significantly inhibited by 100 μM of 2-aminoethoxydiphenyl borate (2-APB), a nonselective TRPC channel blocker (Fig. 2A). In lieu of selective pharmacological blockers of TRPC6, siRNA knockdown was used to determine whether OAG-induced Ca^{2+} influx requires TRPC6. As shown in Fig. 2B, transfection with TRPC6 siRNA significantly reduced OAG-induced Ca^{2+} influx by 60% compared with transfection with scrambled siRNA ($p < 0.01$, $n = 3$), whereas TG-induced SOCE was unaffected ($p > 0.05$, $n = 3$). Transfection with scrambled siRNA did not alter TG-induced SOCE or OAG-induced ROCE compared with non-transfection (data not shown). The specificity and efficiency of TRPC6-siRNA were confirmed by real-time RT-PCR and Western blot, as shown in Fig. 2C. Taken together, these results strongly suggest that OAG-induced Ca^{2+} influx is TRPC6-dependent.

As shown in Fig. 2D, the exposure of MCs to 2% O_2 for 12 h and 24 h significantly increased OAG-induced ROCE, by 90% and 250%, respectively, but had no effect on TG-induced SOCE ($p < 0.01$, $n = 5$). These results suggest that chronic hypoxia enhances TRPC6-dependent Ca^{2+} influx.

3.3. TRPC6 is involved in the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$

As reported previously [6], basal $[Ca^{2+}]i$ was elevated, from 61.5 ± 11.9 in normoxic MCs to 160.7 ± 10.2 in MCs exposed to chronic hypoxia for 24 h ($p < 0.01$; $n = 3$; Fig. 3A). Next, we examined the role of TRPC6 in the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$. As shown in Fig. 3B, chronic hypoxia induced a significant increase in basal $[Ca^{2+}]i$ in MCs transfected with scramble siRNA ($p < 0.01$; $n = 3$), similar to the increase observed in non-transfected MCs. In contrast, the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$ was significantly inhibited in MCs transfected with TRPC6 siRNA ($p > 0.05$). However, knockdown of TRPC6 did not alter the basal $[Ca^{2+}]i$ in normoxic MCs. Incubation with 10 μM

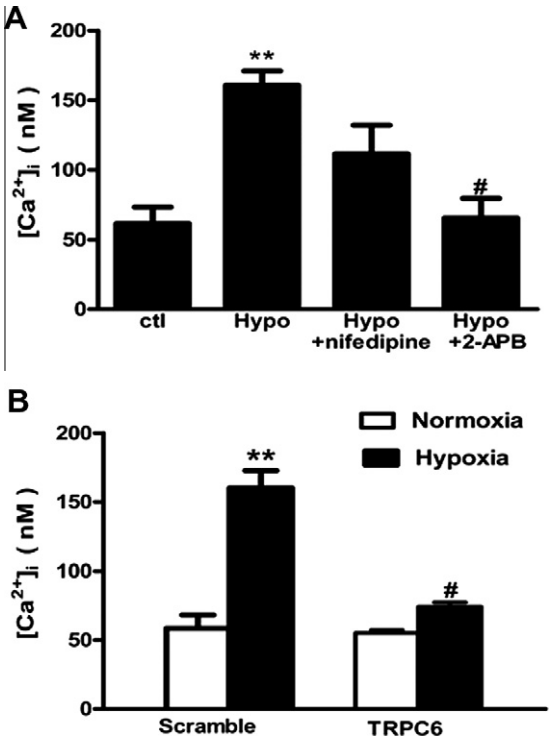

Fig. 3. TRPC6 was involved in the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$. (A) Chronic hypoxia significantly increased basal $[Ca^{2+}]i$ compared with normoxia ($^{**}p < 0.01$; $n = 3$). Pretreatment with 100 μM 2-APB ($^{\#}p < 0.05$ vs. hypoxia, $n = 4$), but not 10 μM nifedipine ($p > 0.05$ vs. hypoxia, $n = 4$), inhibited the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$. (B) In MCs transfected with TRPC6 siRNA, the chronic hypoxia-induced elevation of basal $[Ca^{2+}]i$ was significantly inhibited ($p > 0.05$ vs. normoxia, $n = 4$). $^{**}p < 0.01$ compared with normoxia; $^{\#}p < 0.05$ compared with hypoxia or scramble.

nifedipine did not prevent the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$, whereas pretreatment with 100 μM 2-APB, a nonselective blocker of TRPC channels, attenuated the effect of chronic hypoxia on basal $[Ca^{2+}]i$ (Fig. 3A).

3.4. TRPC6 mediated chronic hypoxia-induced actin assembly and reorganization

Actin assembly was assessed by measuring the F-/G-actin ratio. As shown in Fig. 4A, the F-/G-actin ratio increased significantly from 0.8 ± 0.05 in normoxic MCs to 1.6 ± 0.2 in MCs exposed to 2% O_2 for 24 h ($p < 0.01$; $n = 3$). Actin structures were analyzed by confocal microscopy using phalloidin staining of F-actin. In normoxic MCs, F-actin staining revealed a striking organization of stress fibers (Fig. 4B). Exposure to 2% O_2 for 24 h produced a dramatic increase in the number of stress fibers, the formation of a network of thick stress fibers, and a concomitant decrease in membrane ruffling in MCs (Fig. 4B). The chronic hypoxia-induced actin assembly and reorganization were significantly inhibited by TRPC6 knockdown with siRNA (Fig. 4C and D). As shown in Fig. 4C, chronic hypoxia induced a significant increase in the F-/G-actin ratio in MCs transfected with scrambled siRNA and in non-transfected MCs. The chronic hypoxia-induced increase in F-/G-actin ratio was significantly inhibited in MCs transfected with TRPC6 siRNA ($p > 0.05$). However, TRPC6 knockdown did not affect the F-/G-actin ratio in normoxic MCs. Furthermore, TRPC6 knockdown attenuated chronic hypoxia-induced actin reorganization, as shown in Fig. 4D. Incubation with 10 μM nifedipine did not prevent the chronic hypoxia-induced increase in the F-/G-actin ratio or actin reorganization, whereas incubation with 2-APB attenuated the effect of chronic hypoxia (Fig. 4A and B). These results demonstrated the role of TRPC6 in actin assembly and reorganization in response to chronic hypoxia.

4. Discussion

A growing body of evidence indicates that the renal glomerulus is an important target of chronic hypoxic injury [2,4,5]. One previous study showed that chronic hypoxia induced an increase in $[Ca^{2+}]i$ in MCs and that this increase was related to extracellular Ca^{2+} influx [6]. However, the Ca^{2+} entry pathway induced by chronic hypoxia remained unknown. In the present study, we found that chronic hypoxia enhanced the expression of TRPC6 and TRPC6-dependent Ca^{2+} entry and that knockdown of TRPC6 inhibited the chronic hypoxia-induced increase in $[Ca^{2+}]i$. These results suggest that TRPC6-mediated Ca^{2+} entry is responsible for the elevated $[Ca^{2+}]i$ induced by chronic hypoxia in MCs. Interestingly, the previous study also showed that the chronic hypoxia-induced increase in $[Ca^{2+}]i$ was PKC-independent; incubation with a PKC inhibitor H-7 (50 μM) had no effect on the chronic hypoxia-induced increase in $[Ca^{2+}]i$ [6]. It is well known that TRPC6 activation occurs via PKC independent mechanisms in a variety of cell types [14]. Our study provides further evidence for their findings. In addition, we demonstrate that TRPC6 may play a functional role in actin assembly and reorganization following chronic hypoxia.

It is well known that Ca^{2+} signaling plays an important role in the regulation of the cellular response to hypoxia. However, the mechanism by which Ca^{2+} signaling is altered by hypoxia is controversial. The hypothesis that voltage-gated Ca^{2+} channels are involved in the hypoxia-induced increase in $[Ca^{2+}]i$ is supported by several studies showing that chronic hypoxia induced significant changes in voltage-gated Ca^{2+} channel activity, consistent with an increased expression of these channels [23,24]. However, other studies showed that the blockage of voltage-gated Ca^{2+} channels with either verapamil or nifedipine did not prevent the hypoxia-induced increase in $[Ca^{2+}]i$ [25], and the effects of other Ca^{2+} channel blockers were often partial and temporary [26], suggesting that Ca^{2+} influx pathways other than voltage-gated Ca^{2+} channels are involved in the chronic hypoxia-induced elevation of $[Ca^{2+}]i$. There is increasing evidence that TRPC6 mediates Ca^{2+} influx following chronic hypoxia. For instance, in pulmonary arterial smooth muscle cells [11,27], chronic hypoxia induces a significant increase in TRPC6 expression and activity. Similar results were obtained in other cell types, such as endothelial cells [13] and tumor cells [12]. Our findings are consistent with those of these previous studies. Chronic hypoxia enhances TRPC6 expression in MCs. This TRPC6 upregulation is accompanied by a significant increase in basal and OAG-induced Ca^{2+} entry in hypoxic MCs. However, the chronic hypoxia-induced increase in basal $[Ca^{2+}]i$ cannot be related to the changes in voltage-gated Ca^{2+} channel activity because nifedipine had no effects even at a concentration that completely abolishes voltage-gated Ca^{2+} entry [22], but both the application of 2-APB, a TRPC channel inhibitor, and siRNA knockdown of TRPC6 were able to inhibit the increase in basal $[Ca^{2+}]i$ induced by chronic hypoxia. A previous study showed that the exposure of MCs to hypoxia for 1 h caused an increase in basal $[Ca^{2+}]i$ and showed that this increase can be inhibited by verapamil treatment [6]. Comparing with their results, our data suggest that the acute and chronic hypoxia-induced increase of basal $[Ca^{2+}]i$ might be through different mechanisms. It is noteworthy, however, that TRPC6 does not seem to be involved in the regulation of basal $[Ca^{2+}]i$ under normoxic conditions, i.e., the knockdown of TRPC6 had no effect on basal $[Ca^{2+}]i$ in normoxic MCs. This observation is consistent with the finding that TRPC6 knockdown had no effect on the basal TRPC6 activity of MCs [28].

The functional role of TRPC6-mediated Ca^{2+} entry in the response to hypoxia is unclear. In endothelial cells, TRPC6-mediated

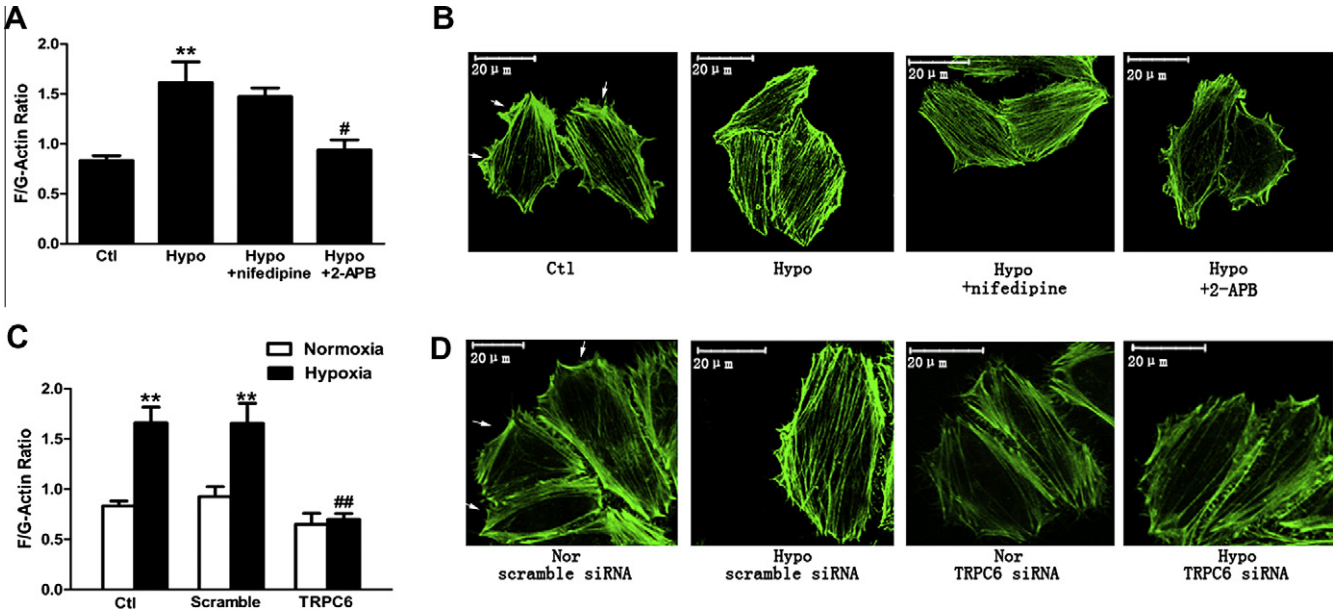

Fig. 4. The role of TRPC6 in chronic hypoxia-induced actin assembly and reorganization. (A) Chronic hypoxia significantly increased the F-/G-actin ratio ($^{**}p < 0.01$, vs. normoxia, $n = 6$). Pretreatment with 100 μM 2-APB attenuated the effect of chronic hypoxia ($^{\#}p < 0.05$ vs. hypoxia, $n = 5$), but 10 μM nifedipine had no effect ($p > 0.05$, vs. hypoxia, $n = 4$). (B) Under normoxic conditions (Ctl group), stress fibers and membrane ruffling (arrows) were observed. Chronic hypoxia led to a dramatic increase in F-actin and a concomitant decrease in membrane ruffling. Pretreatment with 100 μM 2-APB attenuated these morphological changes, whereas pretreatment with 10 μM nifedipine had no effect. (C) The knockdown of TRPC6 prevented the chronic hypoxia-induced increase in the F-/G-actin ratio. (D) Knockdown of TRPC6 attenuated chronic hypoxia-induced actin reorganization. $^{**}p < 0.01$ compared with Ctl; $^{\#}p < 0.05$ compared with hypoxia; $^{\#\#}p < 0.01$ compared with scramble.

Ca^{2+} entry via ROCE is required for thrombin-induced RhoA activation [15]. In the presence of extracellular Ca^{2+}, OAG induced RhoA activation, whereas TG, which directly activates SOCE, failed to activate RhoA [15]. Rho family GTPases including RhoA, Rac1 and Cdc42 control several pathways that modulate actin cytoskeleton dynamics. RhoA promotes the formation of stress fibers and reduces the formation of membrane ruffles, generating a contractile phenotype, whereas Rac1 and Cdc42 promote cell motility by inducing membrane ruffling [29]. The involvement of TRPC6-mediated Ca^{2+} entry in the RhoA-dependent regulation of actin cytoskeleton has been demonstrated in endothelial cells [15] and kidney podocytes [16]. Furthermore, a recent study found that lung endothelial cells from TRPC6-deficient mice exhibited attenuated ischemia-induced Ca^{2+} influx, cellular shape changes and impaired barrier function [13]. The suppression of TRPC6 also greatly inhibited glioma cell migration in response to hypoxia [12]. The data in the present study are consistent with these results in that the knockdown of TRPC6 attenuated the chronic hypoxia-induced formation of stress fibers and reorganization of actin cytoskeleton. Although the link between TRPC6-mediated Ca^{2+} influx and actin cytoskeleton reorganization induced by chronic hypoxia has been established, the underlying mechanism remains poorly understood and should be investigated further.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (31071016), the Research Fund for the Doctoral Program of Higher Education of China (20092307110008), the Nature Science Foundation of Heilongjiang Province (LC2009C03) and the Nature Science Foundation of Harbin City (2008RFLXS013).

References

[1] I. Mimura, M. Nangaku, The suffocating kidney: tubulointerstitial hypoxia in end-stage renal disease, Nat. Rev. Nephrol. 6 (2010) 667–678.

[2] K. Brukamp, B. Jim, M.J. Moeller, V.H. Haase, Hypoxia and podocyte-specific Vhlh deletion confer risk of glomerular disease, Am. J. Physiol. Renal Physiol. 293 (2007) F1397–F1407.

[3] S. Kishi, H. Abe, H. Akiyama, T. Tominaga, T. Murakami, A. Mima, K. Nagai, F. Kishi, M. Matsuura, T. Matsubara, N. Iehara, O. Ueda, N. Fukushima, K. Jishage, T. Doi, SOX9 protein induces a chondrogenic phenotype of mesangial cells and contributes to advanced diabetic nephropathy, J. Biol. Chem. 286 (2011) 32162–32169.

[4] H. Nishi, R. Inagi, H. Kato, M. Tanemoto, I. Kojima, D. Son, T. Fujita, M. Nangaku, Hemoglobin is expressed by mesangial cells and reduces oxidant stress, J. Am. Soc. Nephrol. 19 (2008) 1500–1508.

[5] M.A. Neusser, M.T. Lindenmeyer, A.G. Moll, S. Segerer, I. Edenhofer, K. Sen, D.P. Stiehl, M. Kretzler, H.J. Grone, D. Schlondorff, C.D. Cohen, Human nephrosclerosis triggers a hypoxia-related glomerulopathy, Am. J. Pathol. 176 (2010) 594–607.

[6] A. Sahai, C. Mei, T.A. Pattison, R.L. Tannen, Chronic hypoxia induces proliferation of cultured mesangial cells: role of calcium and protein kinase C, Am. J. Physiol. 273 (1997) F954–F960.

[7] S.B. Kim, S.A. Kang, J.S. Park, J.S. Lee, C.D. Hong, Effects of hypoxia on the extracellular matrix production of cultured rat mesangial cells, Nephron 72 (1996) 275–280.

[8] W. Jelkmann, A. Kurtz, U. Forstermann, J. Pfeilschifter, C. Bauer, Hypoxia enhances prostaglandin synthesis in renal mesangial cell cultures, Prostaglandins 30 (1985) 109–118.

[9] C. Petry, A. Huwiler, W. Eberhardt, M. Kaszkin, J. Pfeilschifter, Hypoxia increases group IIA phospholipase A(2) expression under inflammatory conditions in rat renal mesangial cells, J. Am. Soc. Nephrol. 16 (2005) 2897–2905.

[10] E.C. Toescu, Hypoxia sensing and pathways of cytosolic Ca^{2+} increases, Cell Calcium 36 (2004) 187–199.

[11] M.J. Lin, G.P. Leung, W.M. Zhang, X.R. Yang, K.P. Yip, C.M. Tse, J.S. Sham, Chronic hypoxia-induced upregulation of store-operated and receptor-operated Ca^{2+} channels in pulmonary arterial smooth muscle cells: a novel mechanism of hypoxic pulmonary hypertension, Circ. Res. 95 (2004) 496–505.

[12] S. Chigurupati, R. Venkataraman, D. Barrera, A. Naganathan, M. Madan, L. Paul, J.V. Pattisapu, G.A. Kyriazis, K. Sugaya, S. Bushnev, J.D. Lathia, J.N. Rich, S.L. Chan, Receptor channel TRPC6 is a key mediator of Notch-driven glioblastoma growth and invasiveness, Cancer Res. 70 (2010) 418–427.

[13] N. Weissmann, A. Sydykov, H. Kalwa, U. Storch, B. Fuchs, M.M. Schnitzler, R.P. Brandes, F. Grimminger, M. Meissner, M. Freichel, S. Offermanns, F. Veit, O. Pak, K.H. Krause, R.T. Schermuly, A.C. Brewer, H.H. Schmidt, W. Seeger, A.M. Shah, T. Gudermann, H.A. Ghofrani, A. Dietrich, Activation of TRPC6 channels is essential for lung ischaemia–reperfusion induced oedema in mice, Nat. Commun. 3 (2012) 649.

[14] L. Birnbaumer, The TRPC class of ion channels: a critical review of their roles in slow, sustained increases in intracellular Ca(2+) concentrations, Annu. Rev. Pharmacol. Toxicol. 49 (2009) 395–426.

[15] I. Singh, N. Knezevic, G.U. Ahmmed, V. Kini, A.B. Malik, D. Mehta, Galphaq-TRPC6-mediated Ca^{2+} entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin, J. Biol. Chem. 282 (2007) 7833–7843.

[16] D. Tian, S.M. Jacobo, D. Billing, A. Rozkalne, S.D. Gage, T. Anagnostou, H. Pavenstaedt, H.H. Hsu, J. Schlondorff, A. Ramos, A. Greka, Antagonistic regulation of actin dynamics and cell motility by TRPC5 and TRPC6 channels, Sci. Signal. 3 (2010) ra77.

[17] J. Fediuk, A. Gutsol, N. Nolette, S. Dakshinamurti, Thromboxane-induced actin polymerization in hypoxic pulmonary artery is independent of Rho, Am. J. Physiol. Lung Cell Mol. Physiol. 302 (2012) L13–L26.

[18] U.S. Kayyali, C.M. Pennella, C. Trujillo, O. Villa, M. Gaestel, P.M. Hassoun, Cytoskeletal changes in hypoxic pulmonary endothelial cells are dependent on MAPK-activated protein kinase MK2, J. Biol. Chem. 277 (2002) 42596–42602.

[19] S. Sours, J. Du, S. Chu, M. Ding, X.J. Zhou, R. Ma, Expression of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells, Am. J. Physiol. Renal Physiol. 290 (2006) F1507–F1515.

[20] J.D. Jiao, C.Q. Xu, P. Yue, D.L. Dong, Z. Li, Z.M. Du, B.F. Yang, Volume-sensitive outwardly rectifying chloride channels are involved in oxidative stress-induced apoptosis of mesangial cells, Biochem. Biophys. Res. Commun. 340 (2006) 277–285.

[21] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.

[22] N. Akaike, P.G. Kostyuk, Y.V. Osipchuk, Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypothalamic neurones, J. Physiol. 412 (1989) 181–195.

[23] D.K. Hirenallur-S, S.T. Haworth, J.T. Leming, J. Chang, G. Hernandez, J.B. Gordon, N.J. Rusch, Upregulation of vascular calcium channels in neonatal piglets with hypoxia-induced pulmonary hypertension, Am. J. Physiol. Lung Cell Mol. Physiol. 295 (2008) L915–L924.

[24] T.R. Del, K.L. Levitsky, J. Lopez-Barneo, M.D. Chiara, Induction of T-type calcium channel gene expression by chronic hypoxia, J. Biol. Chem. 278 (2003) 22316–22324.

[25] T.P. Robertson, D. Hague, P.I. Aaronson, J.P. Ward, Voltage-independent calcium entry in hypoxic pulmonary vasoconstriction of intrapulmonary arteries of the rat, J. Physiol. 525 (Pt 3) (2000) 669–680.

[26] P. Agostoni, E. Doria, C. Galli, G. Tamborini, M.D. Guazzi, Nifedipine reduces pulmonary pressure and vascular tone during short- but not long-term treatment of pulmonary hypertension in patients with chronic obstructive pulmonary disease, Am. Rev. Respir. Dis. 139 (1989) 120–125.

[27] C. Tang, W.K. To, F. Meng, Y. Wang, Y. Gu, A role for receptor-operated Ca^{2+} entry in human pulmonary artery smooth muscle cells in response to hypoxia, Physiol. Res. 59 (2010) 909–918.

[28] S. Graham, M. Ding, S. Sours-Brothers, T. Yorio, J.X. Ma, R. Ma, Downregulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired Ca^{2+} signaling in glomerular mesangial cells in diabetes, Am. J. Physiol. Renal Physiol. 293 (2007) F1381–F1390.

[29] S. Etienne-Manneville, A. Hall, Rho GTPases in cell biology, Nature 420 (2002) 629–635.