

Original Paper

Alpha1-Adrenergic Receptor Activation Stimulates Calcium Entry and Proliferation via TRPC6 Channels in Cultured Human Mesangial Cells

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Key Words

TRPC6 • Mesangial cells • Adrenoceptor • ERK signaling

Abstract

Background and Aims: There is accumulating evidence that sympathetic nervous hyperactivity contributes to the pathogenesis of glomerular sclerosis independent of blood pressure effects. A previous study showed that α_1 -adrenoceptor (α_1 -AR) antagonists inhibit mesangial cell (MC) proliferation. However, the underlying mechanism remains unclear. **Methods and Results:** We found that α_1 -AR is expressed in a human mesangial cell line. The α_1 -AR agonist phenylephrine (PE) induced Ca^{2+} influx as well as release from intracellular Ca^{2+} stores. Blockade of TRPC6 with siRNA, anti-TRPC6 antibodies and a TRPC blocker attenuated the PE-induced $[\text{Ca}^{2+}]_i$ increase. Additionally, the PE-induced $[\text{Ca}^{2+}]_i$ increase was phospholipase C dependent. Furthermore, PE induced a $[\text{Ca}^{2+}]_i$ increase even when the intracellular Ca^{2+} stores were already depleted. This effect was mimicked by an analog of diacylglycerol. These results suggested that, upon α_1 -AR stimulation, TRPC6 mediates Ca^{2+} influx via a receptor-operated Ca^{2+} entry mechanism. Finally, TRPC6 contributes to the PE-induced MC proliferation. The mechanisms are associated with the extracellular signal-regulated kinase (ERK) signaling pathway because blockade of TRPC6 and chelation of extracellular Ca^{2+} abrogated PE-induced ERK1/2 phosphorylation. **Conclusion:** TRPC6 channels are involved in α_1 -AR activation-induced Ca^{2+} entry, which mediates proliferation via ERK signaling in human MCs.

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Introduction

There is convincing evidence that the hyperactivity of the sympathetic nervous system is a hallmark of chronic kidney disease (CKD) [1]. The increased sympathetic activity contributes to the progression of CKD and to the pathogenesis of hypertension. Several studies have shown that the inhibition of sympathetic excitation, either by renal denervation or pharmacologic agents, attenuates renal damage independent of blood pressure effects in animal models of CKD [2-5]. The role of renal sympathetic nerves has important clinical implications, and percutaneous catheter-based renal denervation has recently become available to disrupt renal sympathetic nerves. However, the precise role of sympathetic nervous system activation in the pathogenesis of glomerulosclerosis is poorly understood. Mesangial cell (MC) proliferation and extracellular matrix expansion play a pivotal role in the pathogenesis of glomerulosclerosis. A variety of signaling molecules are involved in proliferation and extracellular matrix expansion of MCs [6-9]. However, there have been relatively few studies to investigate the role of sympathetic hyperactivity in MCs.

A previous study showed that doxazosin, prazosin and dicentrine, all of which are α_1 -adrenoceptor (α_1 -AR) antagonists, cause a dose-dependent inhibition of the mesangial cell growth induced by serum and platelet-derived growth factor (PDGF) [10]. Furthermore, the α_1 -AR antagonist doxazosin reduces mesangial cell matrix production [11]. However, the underlying mechanisms remain poorly defined. The gene expression of all three α_1 -AR subtypes (α_{1A} , α_{1B} , and α_{1D}) was identified in the kidney cortex [11, 12]. Using in situ hybridization, α_1 -AR mRNA staining was detected in glomerular MCs [12]. Prototypically, stimulation of α_1 -AR leads to the activation of G protein-coupled phospholipase C β (PLC β), which catalyzes the formation of inositol 1,4,5-triphosphate (IP $_3$) and diacylglycerol (DAG), thereby causing a release of internal Ca $^{2+}$ stores and an accompanying sustained Ca $^{2+}$ influx [13, 14]. In many cases, transient receptor potential canonical (TRPC) channels are thought to contribute to the sustained Ca $^{2+}$ influx through store-operated Ca $^{2+}$ entry (SOCE) or receptor-operated Ca $^{2+}$ entry (ROCE) [15-20].

Our previous studies demonstrated that TRPC6 is an important pathway of Ca $^{2+}$ influx and is involved in mesangial cell proliferation and Ca $^{2+}$ entry induced by hypoxia and calcium-sensing receptors [21, 22]. We therefore sought to investigate whether α_1 -AR activation stimulates Ca $^{2+}$ influx and proliferation via TRPC6 in human MCs and, if so, to identify the underlying mechanisms.

Materials and Methods

Cell culture and plasmid transfection

An established, stable, human mesangial cell line was cultured as previously described [21]. Briefly, the cells were cultured in RPMI1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone, USA) in 5% CO $_2$ at 37°C. Human MCs between passages 3 and 15 were used. Human MCs were transiently transfected with TRPC6 siRNA or scrambled siRNA (Santa Cruz, USA) using the Xtreme GENE siRNA transfection reagent (Roche, Germany) according to the manufacturer's instructions. Cells were used for experiments 48-72 h after transfection.

Reverse transcription PCR

Reverse transcription was performed using standard methods. The specific primer sequences used to amplify α_{1A} -AR, α_{1B} -AR and α_{1D} -AR were as follows (5'-3'): α_{1A} -AR Sense: TGA TTT CAA GCC CTC TGA AAC AG; α_{1A} -AR Antisense: ATG TCC TTG TGT TGC CCT TCC AC; α_{1B} -AR Sense: CTT CAC CTT CAA GCT CCT GAC CGA G; α_{1B} -AR Antisense: TGC CAG AAA AGC AGCTGC CCC TCT C; α_{1D} -AR Sense: TGT CGC ACA AGA TCG CCG CC; α_{1D} -AR Antisense: CCA GCA CAC TCC GCG GCC TA.

Western Blotting

Western blots were performed using a standard protocol. The membrane was probed with the following primary antibodies: polyclonal rabbit anti-TRPC6 antibodies (Abcam, USA), rabbit anti- α_1 -AR

antibodies (Abcam, USA), phospho-ERK1/2 or total-ERK1/2 antibody (Cell Signaling) or anti-actin antibody (Santa Cruz, USA). The membranes were washed extensively and incubated with fluorescence-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (Invitrogen, USA). Western blot bands were quantified using the Odyssey infrared imaging system (LI-COR Bioscience, USA).

Fluorescence measurement of $[Ca^{2+}]_i$

MCs were grown on coverslips and loaded with Pluronic F-127 (0.03%; Sigma, USA) and Fluo-3/AM (0.03%; Sigma) at 37°C for 45 min. Fluorescence changes in the Fluo-3/AM-loaded cells were detected by laser scanning confocal microscopy (FV300; Olympus, Japan). The Ca^{2+} -free bath solution contained no $CaCl_2$ and 0.5 mM EGTA. MCs were then stimulated with agonists or inhibitors as described in the results. $[Ca^{2+}]_i$ was expressed as a pseudo-ratio value of the actual fluorescence intensity divided by the average baseline fluorescence intensity. The calcium concentration was calculated using the formula described by Grynkiewicz et al [23]. Calibrations were performed immediately following each experiment. High $[Ca^{2+}]_i$ conditions were achieved by adding 4 μ M 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.

Cell proliferation assay

Cell proliferation was measured using a Cell Proliferation ELISA BrdU kit (Roche, Germany) according to the manufacturer's protocol. Cells were seeded in a 96-well plate (5000 cells/well) and cultured for 24 h. After starvation for another 12 h in serum-free medium, cells were incubated in the same medium supplemented with different treatments for 24 h. Six hours before the end of incubation, BrdU was added to the medium, and the cells were incubated for 6 h. The absorbance at 450 nm (reference wavelength: 630 nm) was measured with a scanning multi-well spectrophotometer (Amersham Pharmacia Biotech). The absorbance values correlated directly with the amount of DNA synthesis and therefore with the number of proliferating cells in culture. Stimulation was expressed as the fold proliferation over the basal growth of the control set as unity.

Statistical analysis

Data are presented as the mean \pm SEM with the number (n) of experiments indicated. Statistical analyses were performed using an unpaired t-test (SPSS16.0), and graphs were prepared with Adobe Photoshop or were plotted in GraphPad Prism 5 (GraphPad Software, Inc.). A P-value less than 0.05 was considered to be statistically significant.

Results

α_1 -AR is expressed in human mesangial cells.

We first sought to examine whether α_1 -AR is expressed in the human mesangial cell line used in this study. RT-PCR experiments revealed that α_{1A} -AR mRNA (275 bp) and α_{1D} -AR mRNA (202 bp) were not detected in cultured human MCs, although they were detected in mouse kidney tissues (data not shown). α_{1B} -AR mRNA (151 bp) was detected in cultured human MCs and mouse kidney tissues (data not shown), a result that was consistent with previous studies [11, 24]. Experiments without reverse transcriptase confirmed that the PCR products originated from mRNA and not from genomic DNA. Western blot analysis showed a 60 kDa band corresponding to α_1 -AR in human MCs and the positive control of mouse kidney tissues (Fig. 1). Taken together, these data indicate that α_1 -AR is present in cultured human MCs.

Fig. 1. Expression of α_1 -AR protein in a human mesangial cell line. Western blotting showed that α_1 -AR protein expression, as a 60 kDa band, was found in both the cultured human MCs and the positive control of mouse kidney tissues (MK).

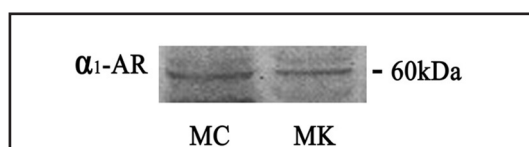
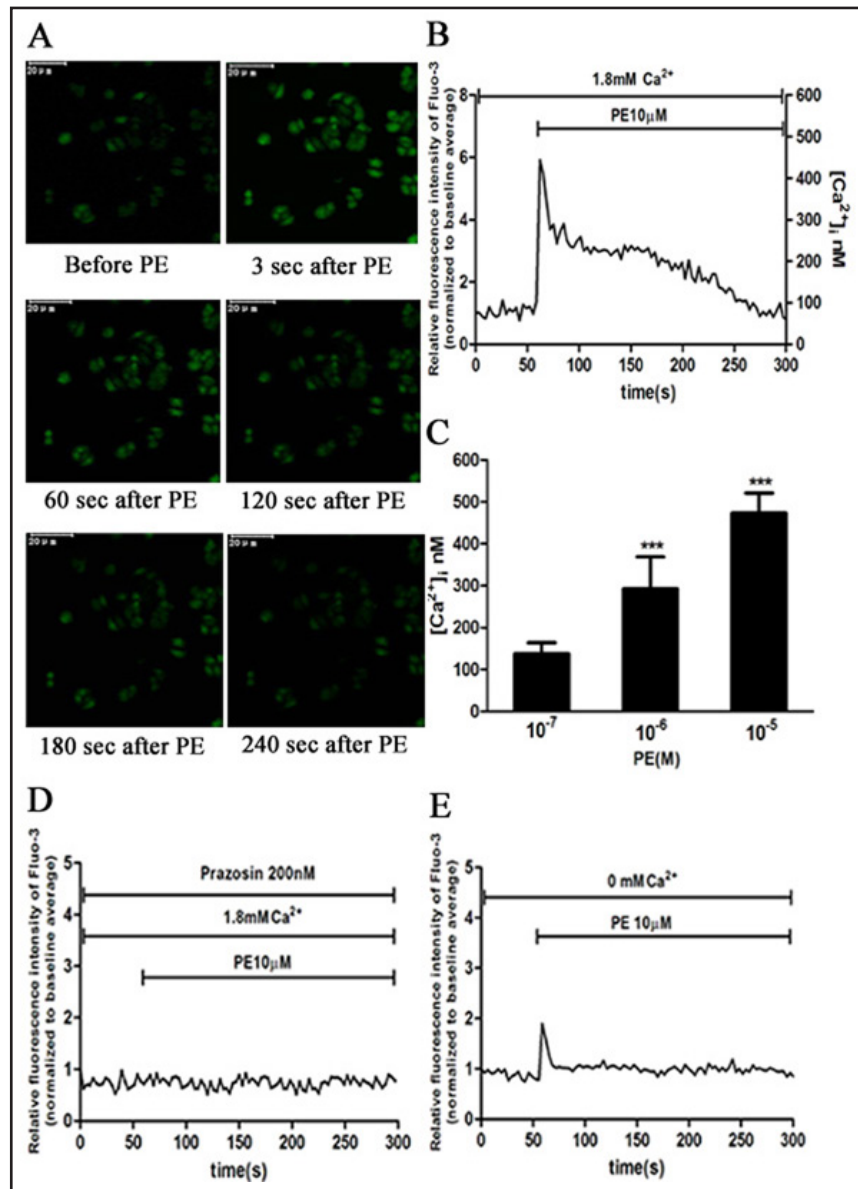


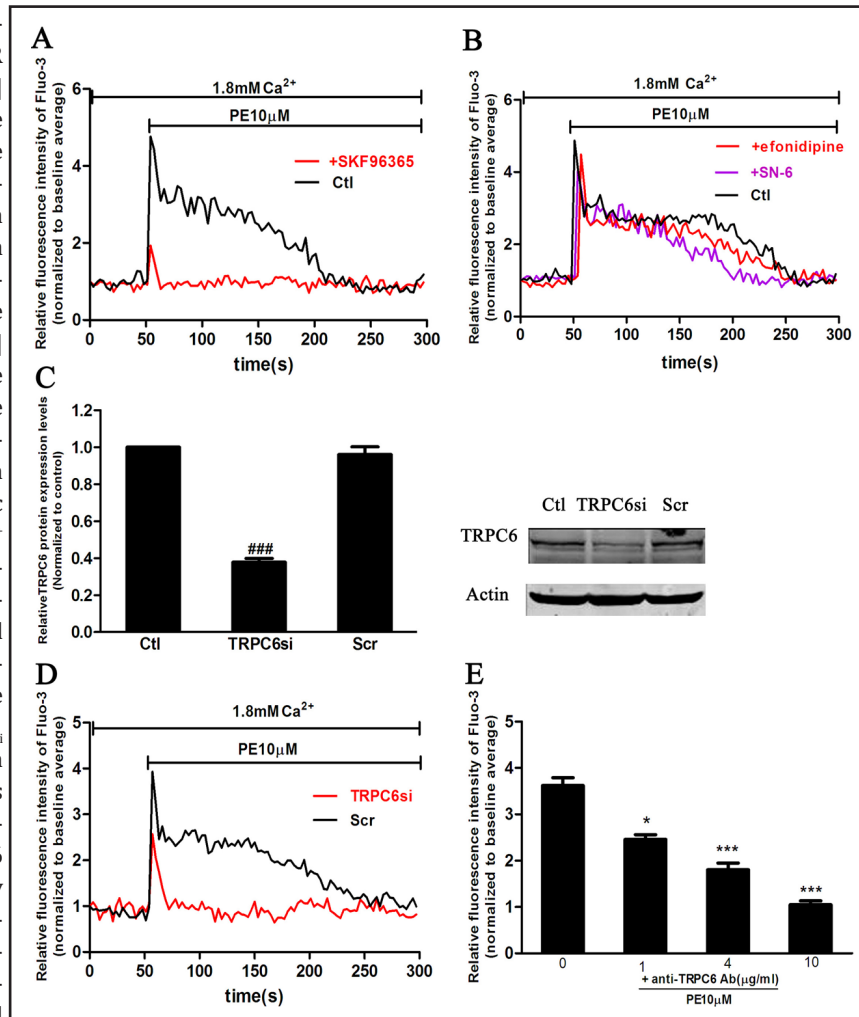
Fig. 2. Activation of α_1 -AR induces an increase in $[Ca^{2+}]_i$ in human MCs. The representative image (A) and trace (B) showing that PE induced a $[Ca^{2+}]_i$ increase in the presence of 1.8 mM extracellular Ca^{2+} . (C) PE induced a $[Ca^{2+}]_i$ increase in a concentration-dependent manner. The average $[Ca^{2+}]_i$ responses to PE are shown as the means \pm SEM (n=3). (D) The representative trace showing that the PE-mediated $[Ca^{2+}]_i$ increase was prevented by pretreatment with prazosin. (E) The representative traces of the effect of PE on $[Ca^{2+}]_i$ in Ca^{2+} -free solution. The results were from at least three independent experiments, and each experiment measured 20 to 40 cells.



α_1 -AR agonists stimulate an $[Ca^{2+}]_i$ increase in human MCs.

The effect of α_1 -AR agonists on the $[Ca^{2+}]_i$ in human MCs was assessed using Fluo-3/AM assays. As shown in Fig. 2A and 2B, the presence of 1.8 mM extracellular Ca^{2+} and 10 μ M phenylephrine (PE), a known α_1 -AR agonist, evoked a rapid increase in $[Ca^{2+}]_i$ and a subsequent sustained increase in $[Ca^{2+}]_i$. MCs exhibited no spontaneous $[Ca^{2+}]_i$ changes during these experiments (data not shown). The PE-induced $[Ca^{2+}]_i$ increase occurred in a concentration-dependent manner, as shown in Fig. 2C. Pretreatment of MCs with 200 nM prazosin, a selective α_1 -AR antagonist, significantly inhibited the PE-induced $[Ca^{2+}]_i$ increase (Fig. 2D), indicating that the PE-induced $[Ca^{2+}]_i$ elevation is specific for α_1 -AR. To examine whether Ca^{2+} influx is involved in the PE-induced $[Ca^{2+}]_i$ increase, MCs were stimulated by PE in the absence of extracellular Ca^{2+} . As shown in Fig. 2E, PE induced a transient increase in $[Ca^{2+}]_i$ in the Ca^{2+} -free solutions, which was due to the Ca^{2+} release from intracellular Ca^{2+} stores through α_1 -AR activation by PE. However, it is not likely that there is residual Ca^{2+} in the medium because under our experimental conditions, the medium contained no Ca^{2+} and 0.5 mM EGTA was added to chelate the possible trace amounts of Ca^{2+} . These data suggest that α_1 -AR activation induces Ca^{2+} release from intracellular Ca^{2+} stores. However, the PE-

Fig. 3. TRPC6 is involved in the α_1 -AR agonist-induced $[Ca^{2+}]_i$ increase. (A) The representative trace showing that pretreatment for 30 min with SKF96365, a TRPC channel blocker, inhibited the PE-induced $[Ca^{2+}]_i$ increase. (B) The representative trace showing that pretreatment for 30 min with SN-6, a specific inhibitor of Na^+/Ca^{2+} exchangers, or efonidipine, a voltage-gated calcium channel blocker, had no apparent effect on the PE-induced $[Ca^{2+}]_i$ increase. (C) Western blot experiments showing that transfection with TRPC6 siRNA significantly reduced TRPC6 protein expression compared with transfection with scrambled



siRNA (Scr). Data are shown as the means \pm SEMs (### p <0.001 vs Scr group; $n=3$). (D) The representative trace showing that transfection with TRPC6 siRNA significantly reduced the PE-induced $[Ca^{2+}]_i$ increase. (E) Summary of data showing that pretreatment with anti-TRPC6 antibody (0, 1, 4, or 10 μ g/ml) produced a concentration-dependent inhibition of the PE-induced $[Ca^{2+}]_i$ increase. Data are shown as the means \pm SEM (* p <0.05, *** p <0.001 vs 0 μ g/ml anti-TRPC6 antibody group; $n=3$). The results were from at least three independent experiments, and each experiment measured 20 to 40 cells.

induced $[Ca^{2+}]_i$ increase in the absence of extracellular Ca^{2+} was significantly smaller than that observed in the presence of 1.8 mM extracellular Ca^{2+} , suggesting that extracellular Ca^{2+} influx is also involved in the $[Ca^{2+}]_i$ increase stimulated by α_1 -AR activation in human MCs.

TRPC6 is required for the α_1 -AR agonist-induced $[Ca^{2+}]_i$ increase.

Because TRPC channels, voltage-gated calcium channels and Na^+/Ca^{2+} exchangers are the main pathways for Ca^{2+} influx in MCs, we examined the role of these pathways in the PE-induced $[Ca^{2+}]_i$ increase. As shown in Fig. 3A and 3B, pretreatment with 30 μ M SKF96365, a nonselective TRPC channel blocker, significantly inhibited the PE-induced $[Ca^{2+}]_i$ increase. However, pretreatment with 10 μ M efonidipine, a voltage-gated calcium channel blocker, or 10 μ M SN-6, a specific inhibitor of Na^+/Ca^{2+} exchangers, had no apparent effect (Fig. 3B). These data suggest that TRPC-dependent Ca^{2+} entry is involved in the PE-induced $[Ca^{2+}]_i$ increase. To investigate whether TRPC6 contributes to the PE-induced $[Ca^{2+}]_i$ increase, we used siRNA technology to down-regulate TRPC6 expression. As shown in Fig. 3C, the specificity and efficiency of TRPC6-siRNA were confirmed by western blot analyses. Consistent

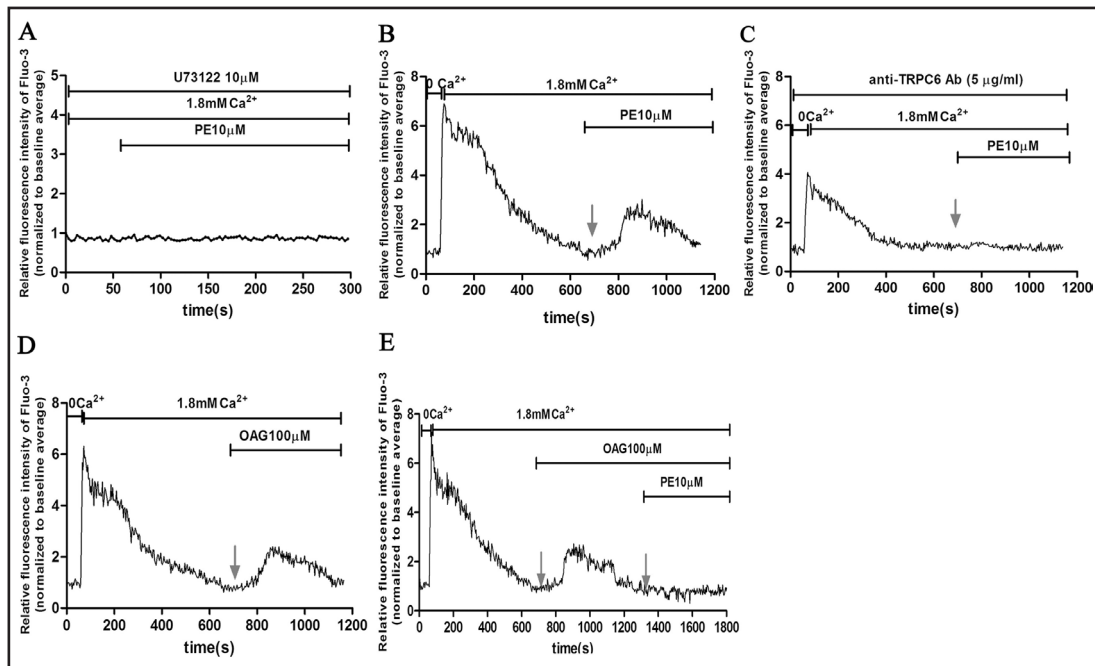


Fig. 4. PLC and ROCE are involved in the α_1 -AR agonist-induced $[Ca^{2+}]_i$ increase. (A) The representative trace showing that pretreatment for 30 min with U73122, a PLC inhibitor, almost completely abolished the PE-induced $[Ca^{2+}]_i$ increase. (B) Cells were stimulated with TG for 10 min in Ca^{2+} -free solution, and then, 1.8 mM Ca^{2+} was re-added to the solution. The representative trace showed that PE evoked an additional increase in $[Ca^{2+}]_i$. (C) The representative trace showing that the PE-induced $[Ca^{2+}]_i$ increase was inhibited by pretreatment for 30 min with anti-TRPC6 antibodies (5 μ g/ml). (D) The representative trace showing that, using the same protocol, OAG induced a similar $[Ca^{2+}]_i$ increase. (E) The representative trace showing that PE did not produce a subsequent $[Ca^{2+}]_i$ increase after the application of OAG. The results were from at least three independent experiments, and each experiment measured 20 to 40 cells.

with our previous reports [21, 22], knockdown of TRPC6 did not affect the expression of other isoforms of TRPC6 expressed in human MCs (data not shown). Compared with cells transfected with scrambled siRNA, transfection with TRPC6 siRNA significantly inhibited the PE-induced $[Ca^{2+}]_i$ increase ($p < 0.05$, $n = 3$; Fig. 3D). Additionally, blockade of TRPC6 with the anti-TRPC6 antibody inhibited the PE-induced $[Ca^{2+}]_i$ increase in a dose-dependent manner (Fig. 3E). These results strongly demonstrated that TRPC6 is essential for the $[Ca^{2+}]_i$ increase mediated by α_1 -AR activation.

PLC and ROCE are involved in the α_1 -AR agonist-induced $[Ca^{2+}]_i$ increase.

TRPC6 predominantly induces ROCE via PLC signaling pathways upon GPCR activation [25]. Thus, we examined whether the PE-induced $[Ca^{2+}]_i$ increase is PLC dependent and whether TRPC6 is involved in the PE-induced Ca^{2+} influx via ROCE. As shown in Fig. 4A, pretreatment of cells with U73122 (10 μ M), a PLC inhibitor, almost completely abolished the PE-induced $[Ca^{2+}]_i$ increase, whereas U73343 (10 μ M), its inactive analog, had no inhibitory effect (data not shown). To demonstrate that ROCE is involved in the PE-induced Ca^{2+} increase, we used a Ca^{2+} depletion/ Ca^{2+} re-addition protocol. The intracellular Ca^{2+} stores were depleted with 1 μ M thapsigargin (TG), an endoplasmic reticulum Ca^{2+} -ATPase inhibitor, in the absence of extracellular Ca^{2+} . Once $[Ca^{2+}]_i$ had returned to the basal level, extracellular Ca^{2+} was restored to 1.8 mM. As shown in Fig. 4B, the re-addition of extracellular Ca^{2+} initiated an expected $[Ca^{2+}]_i$ rise, which was due to SOCE, and the subsequent application of PE (10 μ M) evoked another $[Ca^{2+}]_i$ increase, which was regarded as ROCE because, under this condition, the intracellular Ca^{2+} stores were already depleted. The PE-induced Ca^{2+} influx was abolished by pretreatment with TRPC6 antibodies, suggesting that PE-induced Ca^{2+}

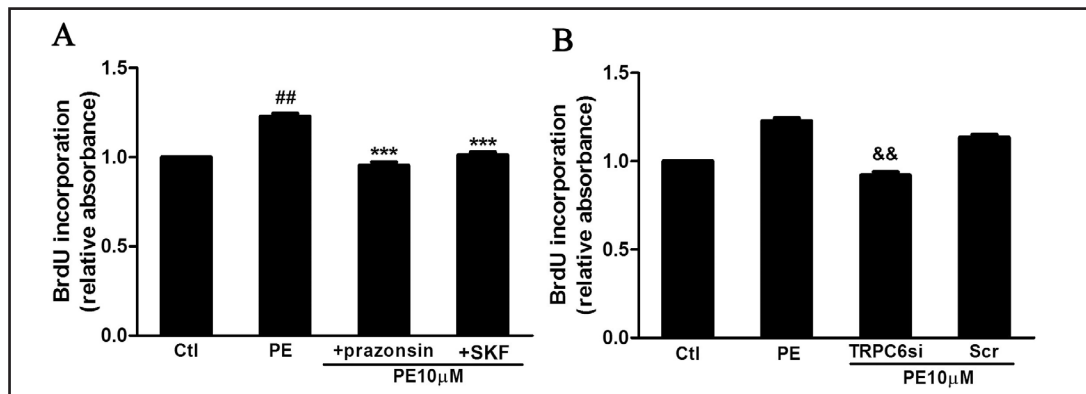


Fig. 5. α_1 -AR activation mediates cell proliferation via TRPC6. Cells were starved for 12 h and then were incubated in the same medium in the presence or absence of various inhibitors or agonists for 24 h. (A) PE (10 μ M) induced the proliferation of human MCs compared with the control group. However, PE-induced cell proliferation was significantly inhibited by pretreatment with prazosin (200 nM) and SKF96365 (30 μ M). (B) Compared with the transfection of scrambled siRNA (Scr), transfection of TRPC6 siRNA significantly attenuated the PE-induced proliferation. Data are shown as the means \pm SEM of three experiments. (## p <0.01 vs control group; *** p <0.001 vs PE group; && p <0.01 vs Scr group, $n=3$).

influx is specific for TRPC6 (Fig. 4C). Using the same protocol, the effect of PE on Ca^{2+} influx was mimicked by the application of OAG (100 μ M), a membrane-permeable DAG analog (Fig. 4D). Moreover, PE was added following the application of OAG, and no further $[Ca^{2+}]_i$ increase was observed (Fig. 4E). These data demonstrate that ROCE via TRPC6 is involved in the Ca^{2+} influx mediated by α_1 -AR activation in a PLC-dependent manner.

α_1 -AR agonists promote cell proliferation via TRPC6.

Previous studies have shown that α_1 -AR activation promotes cell proliferation in many cell types, including MCs [10], and we investigated the contribution of TRPC6 to the α_1 -AR agonist-mediated proliferation of MCs. Consistent with previous reports, incubation of cells for 24 h with PE (10 μ M) enhanced their proliferation by 22.8% compared with the control group (Fig. 5A, p <0.01; $n=3$). The cell viability by MTT assessment was not affected under our experimental conditions (data not shown). PE-mediated cell proliferation appeared to act through α_1 -AR activation because pretreatment with an α_1 -AR selective antagonist, prazosin (200 nM), significantly attenuated PE-mediated cell proliferation (Fig. 5A, p <0.001; $n=3$). PE-mediated cell proliferation was significantly inhibited by pretreatment with a TRPC channel blocker, SKF96365 (30 μ M; Fig. 5A, p <0.001; $n=3$). Furthermore, transfection of TRPC6 siRNA significantly attenuated the promotion of proliferation by PE compared with scrambled siRNA (Fig. 5B). In the absence of PE, transfection of TRPC6 siRNA or scrambled siRNA did not affect the cell proliferation activity (data not shown). Taken together, these data indicate that TRPC6 contributes to the cell proliferation mediated by α_1 -AR activation.

TRPC6 is linked to α_1 -AR agonist-mediated cell proliferation via ERK1/2 signaling.

There is evidence that extracellular signal-regulated kinases 1 and 2 (ERK 1/2) contribute to α_1 -AR-stimulated mitogenic responses [13, 26, 27]. Recently, ERK signaling has been shown to be regulated downstream of TRPC6 [28-30]. Therefore, we examined whether TRPC6 mediates mesangial cell proliferation via ERK 1/2 in response to α_1 -AR stimulation. As shown in Fig. 6A, treatment with PE (10 μ M) resulted in time-dependent ERK1/2 phosphorylation, whereas total ERK1/2 was not affected by PE stimulation. The PE-induced ERK1/2 phosphorylation was specific for α_1 -AR because this effect was almost completely abolished by the α_1 -AR antagonist prazosin (Fig. 6B). The selective ERK1/2 inhibitor PD98059 (100 μ M) abolished the ERK1/2 phosphorylation stimulated by PE (Fig. 6C). In parallel, the PE-mediated cell proliferation was attenuated by PD98059 (Fig. 6D), indicating that phosphorylation of ERK1/2 is involved in PE-mediated cell proliferation. As

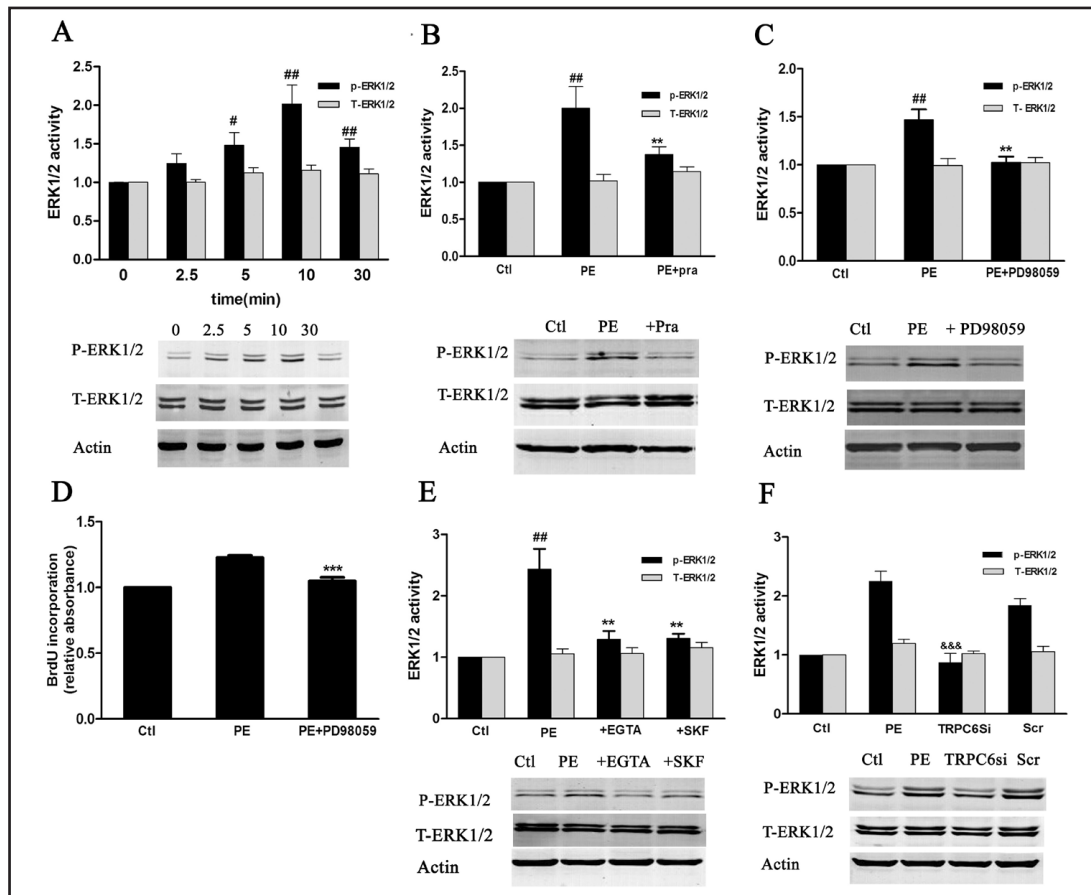


Fig. 6. TRPC6 is linked to α_1 -AR agonist-mediated cell proliferation via ERK1/2 signaling. Cells were starved for 12 h and were then treated with 10 μ M PE for 10 min with or without pretreatment with various inhibitors for 30 min. The total and phosphorylated ERK1/2 levels were measured by western blot analysis. (A) PE increased ERK1/2 phosphorylation in a time-dependent manner. (B) The PE-induced ERK1/2 phosphorylation was inhibited by 200 nM prazosin (pra). (C) PD98059 (100 μ M), a selective ERK1/2 inhibitor, inhibited PE-induced ERK1/2 phosphorylation. (D) PD98059 (100 μ M) inhibited PE-induced cell proliferation. (E) SKF96365 (30 μ M) or EGTA (100 μ M) inhibited PE-induced ERK1/2 phosphorylation. (F) Compared with transfection with scrambled siRNA (Scr), transfection with TRPC6 siRNA attenuated PE-induced ERK1/2 phosphorylation. The results were from at least three independent experiments. Asterisks indicate the statistical significance (** $p < 0.01$ vs PE group; # $p < 0.05$; ## $p < 0.01$ vs control group; &&& $p < 0.001$ vs Scr group).

shown in Fig. 6E, the PE-induced phosphorylation of ERK1/2 was significantly inhibited by pretreatment with SKF96365 (30 μ M), a TRPC blocker. Chelation of extracellular Ca^{2+} with EGTA (100 μ M) also abrogated the ERK1/2 phosphorylation stimulated by PE (Fig. 6E). Furthermore, transfection of TRPC6 siRNA significantly attenuated the PE-induced phosphorylation of ERK1/2 compared with scrambled siRNA (Fig. 6F). These results demonstrate that TRPC6 is involved in cell proliferation upon α_1 -AR activation via ERK1/2 signaling.

Discussion

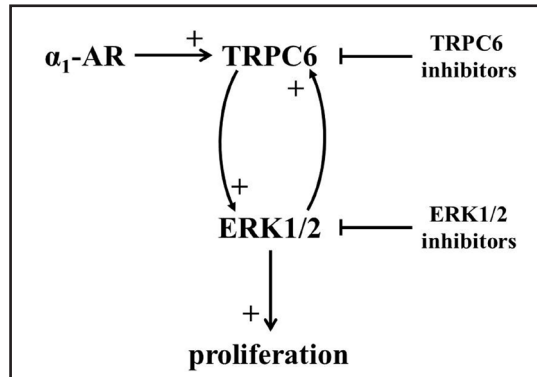
Recent studies have shown that renal denervation has a blood pressure-independent protective effect against renal injury [2, 5]. Renal sympathetic nerves are involved in unilateral ureter obstruction, a nonhemodynamic model of renal injury [31], suggesting

that local nerve-derived signaling molecules contribute to renal injury. However, the underlying mechanisms are poorly understood. Several studies have revealed that the mechanisms may be associated with inflammation [5], oxidative stress [2] and the intrarenal renin-angiotensin system [2, 31]. To date, few studies have investigated the potential effects of renal sympathetic nerves on glomerular MCs. In the present study, we demonstrate that α_1 -AR activation induces TRPC6-dependent Ca^{2+} influx, which is involved in the proliferation of human MCs via ERK signaling. This implies that renal sympathetic activation could directly regulate mesangial cell proliferation through neurotransmitters, such as norepinephrine. Other neurotransmitters may also contribute to mesangial cell proliferation. ATP, a co-transmitter of norepinephrine, mediates the proliferation of human MCs [32].

In many cell types, α_1 -AR utilizes intracellular Ca^{2+} as a second messenger to modulate cellular function [13, 14]. As with most $G_{q/11}$ -coupled receptors, the Ca^{2+} influx by the stimulation of α_1 -AR is often mediated by TRPC channels via ROCE or SOCE rather than by voltage-gated Ca^{2+} channels, which may be dependent upon the particular cell type and α_1 -AR subtype [13, 14]. Similar to cardiac myocytes [19], vascular smooth muscle cells [33], prostate smooth muscle cells [18], and PC12 cells [20], our results demonstrate that activation of α_1 -AR mediates Ca^{2+} influx via TRPC6 in human MCs because the α_1 -AR agonist-induced $[\text{Ca}^{2+}]_i$ increase was attenuated by the administration of TRPC6 siRNA, anti-TRPC6 antibodies and TRPC blocker but not by the voltage-gated Ca^{2+} blocker or the inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchangers. As a receptor-operated channel, TRPC6 can be activated by the DAG produced by activated PLC without involvement of Ca^{2+} stores [25]. We demonstrate that upon stimulation of α_1 -AR, TRPC6-mediated Ca^{2+} influx occurs at least via ROCE because the α_1 -AR agonist induces a $[\text{Ca}^{2+}]_i$ increase when the intracellular Ca^{2+} stores are already depleted by TG. Moreover, this effect was mimicked by OAG, an analog of DAG, which can directly activate TRPC6 independently of Ca^{2+} store depletion [25]. The concept is supported by recent studies that snapin, a regulator of receptor signaling, augments α_1 -AR-induced Ca^{2+} influx through TRPC6 [19, 20]. However, the possibility that other TRPC channels expressed in MCs are also involved in α_1 -AR agonist-induced Ca^{2+} influx could not be excluded, given that TRPC6 can form heteromeric channel complexes with other TRPC channels [25].

α_1 -AR activates mitogenic responses in various cell types via the mitogen-activated protein kinase (MAPK) family, including ERK, c-Jun N-terminal kinases (JNKs) and p38 kinases [13]. However, the underlying mechanism is not completely clear. Among three canonical MAPK signaling pathways, the ERK pathway plays a central role in regulating cell proliferation in various cell types, including MCs. Indeed, our study showed that α_1 -AR stimulation promotes mesangial cell proliferation, a finding that was consistent with that of a previous study [10]. In the present study, we demonstrate that α_1 -AR activation-induced proliferation of MCs is associated with ERK1/2 phosphorylation. We found that TRPC6 contributes to ERK signaling because the application of TRPC6 siRNA, the TRPC blocker, or chelation of extracellular Ca^{2+} attenuated α_1 -AR activation-induced ERK1/2 phosphorylation. This idea is supported by recently accumulating evidence that TRPC6 induces ERK activation in podocytes [30], neurons [28] and islet cells [29]. In podocytes, enhancement of TRPC6 activity resulted in increased ERK1/2 phosphorylation through a cell-autonomous and a non-cell-autonomous mechanism in a Ca^{2+} -dependent manner [30]. In addition, in MCs and HEK293 cells, ERK1/2 may directly activate TRPC6 by phosphorylation at Ser-281 [34]. Thus, the interaction of TRPC6 with ERK1/2 provides positive feedback to Ca^{2+} influx through TRPC6 (Fig. 7). Several other signaling pathways were shown to be regulated downstream

Fig. 7. Scheme illustrating a possible mechanism by which α_1 -AR stimulation activates ERK1/2 via TRPC6.



of TRPC6. For example, TRPC6 activates the calcineurin-NFAT (nuclear factor of activated T-cells) and RhoA pathways, both of which are involved in TRPC6-mediated mitogenic responses [25]. Therefore, the possibility that TRPC6 mediates mesangial cell proliferation in response to α_1 -AR activation via other signaling pathways could not be excluded and should be further explored.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81441023).

Disclosure Statement

The authors declare that no conflict of interest exists.

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