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Role of TRPC1 and NF-κB in mediating angiotensin II-induced Ca^{2+} entry and endothelial hyperpermeability

Li-xia Yang [a], Rui-wei Guo [a,*], Bei Liu [b], Xian-mei Wang [a], Feng Qi [a], Chuan-ming Guo [a], Yan-kun Shi [a], Hong Wang [a]

[a] Department of Cardiology, Kunming General Hospital of Chengdu Military Area, Yunnan 650032, China
[b] Department of Obstetrics and Gynecology, Kunming General Hospital of Chengdu Military Area, Yunnan 650032, China

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ABSTRACT

Endothelial dysfunction is associated with cardiovascular diseases. The Ca^{2+} influx occurring via activation of plasmalemma Ca^{2+} channels was shown to be critical in signaling the increase in endothelial permeability in response to a variety of permeability-increasing mediators. It has been reported that angiotensin II (AngII) could induce Ca^{2+} signaling in some cells, and transient receptor potential canonical 1 (TRPC1) had an important role in this process. The objective of this study was to examine the mechanism of AngII-induced Ca^{2+} entry and vascular endothelial hyperpermeability. Human umbilical vein endothelial cells (HUVECs) exposed to AngII exhibited dose-dependent increase in $[Ca^{2+}]i$ and endothelial permeability. Quantitative real-time RT-PCR and Western blotting showed that the level of TRPC1 expression had increased significantly at 12 h and at 24 h after treatment of HUEVCs with AngII. The expression of p65 was suppressed using an RNAi strategy. The results showed that the NF-κB signaling pathway and type-1 receptor of AngII was involved in AngII-induced TRPC1 upregulation. Moreover, knockdown of TRPC1 and NF-κB expression attenuates AngII-induced $[Ca^{2+}]i$ and endothelial permeability. NF-κB and TRPC1 have critical roles in AngII-induced Ca^{2+} entry and endothelial permeability.

1. Introduction

Endothelial dysfunction is associated with cardiovascular diseases such as hypertension, atherosclerosis, diabetic vasculopathy and heart failure [23,26]. Recently, the Ca^{2+} influx response occurring via activation of plasmalemma Ca^{2+} channels was shown to be critical in signaling the increase in endothelial permeability in response to a variety of permeability-increasing mediators, including thrombin [27,31]. The resulting elevation of intracellular Ca^{2+} could contribute to barrier disruption because Ca^{2+} entry into endothelial cells is recognized to promote interendothelial gap formation. Members of the transient receptor potential canonical (TRPC) subfamily of channel-forming proteins, including TRPC1, are important for regulating Ca^{2+} entry into endothelial cells [2,21] and mediating the increase in endothelial permeability [15,17,25]. Activation of these channels depends on Ca^{2+} store depletion; hence, it is termed store-operated Ca^{2+} entry (SOCE) [1,28]. It has been shown that TRPC1-mediated Ca^{2+} entry contributes significantly to the thrombin-induced increase in endothelial permeability [15,17]. In addition, endothelial cells expressing TRPC1 demonstrated cytoskeletal changes associated with increased endothelial permeability in response to SOC activation [18].

Angiotensin II (AngII) is a potent vasoconstrictor hormone that is cleaved from angiotensinogen by renin and angiotensin-converting enzyme. AngII is implicated also in pathophysiological processes related to vascular injury and repair, by promoting generation of oxidative stress in the vascular wall that leads to endothelial dysfunction [29]. However, the precise role of AngII in vascular endothelial injury is unknown. Recently, Du et al. reported that angiotensin II could induce Ca^{2+} signaling in glomerular mesangial cells, and TRPC1 had an important role in this process [7]. In addition, there is evidence that the binding sites of NF-κB are located in the 5′-regulatory region of the TRPC1 gene [24,30]. NF-κB is retained in the cytoplasm of cells by an inhibitor subunit, inhibitor of κB (IκB), until cell stimulation results in the sequential phosphorylation, ubiquitination, and degradation of IκB. This exposes a nuclear localization signal on NF-κB, allowing it to translocate into the nucleus and promote gene transcription [13]. Earlier, we showed that AngII stimulates NF-κB nuclear translocation in human umbilical vein endothelial cells via the type-1 receptor of AngII (AT1) and mitogen-activated protein kinase (MAPK) pathways [9]. Therefore, it is possible that NF-κB is required for AngII-induced upregulation of TRPC1.

* Corresponding author. Tel.: +86 871 4774573.
E-mail address: grw771210@163.com (R.-w. Guo).

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In this study, we explored the possibility that AngII induces the expression of TRPC1 via the NF-κB pathway, thereby increasing marked Ca^{2+} influx and endothelial permeability.

2. Methods

2.1. Materials

Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT), and all other cell culture reagents were purchased from Gibco-BRL (Grand Island, NY). AngII, PD123319, Thapsigargin (TG) were all purchased from Sigma (MO, USA). Losartan was a generous gift from Merck. TRPC1 monoclonal antibody came from Abcam Corporation (Abcam, UK), and other antibodies came from Santa Cruz Bio Inc. (Santa Cruz, CA). [γ-^{32}P] ATP was purchased from Amersham/Pharmacia Biosciences (Piscataway, NJ). NF-κB consensus oligonucleotide was obtained from Promega (Madison, WI). The SYBR Green 1 Real-time RT-PCR kit and siRNAs were purchased from TaKaRa Bio Inc. (Dalian, China). Fura-2AM was obtained from Beyotime Chemical Company (Jiangshu, China).

2.2. Cell culture

HUEVC were isolated by 0.1% collagenase digestion from normal umbilical cords, as described previously [16], and cultured in Medium 199 supplemented with 10% FBS, 50 μg/ml endothelial cell growth supplement, 50 μg/ml heparin, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Cells were used in the experiment at passages two through four.

2.3. Real-time RT-PCR analysis

Total RNA was isolated with Trizol reagent according to the manufacturer's protocol, and first-strand cDNA was synthesized using reverse transcriptase. The expression of TRPC1 mRNA was measured by SYBR Green1 fluorescence real-time RT-PCR. Real-time quantitative PCR was performed as previously described [11] using the following primers: TRPC1, 5′-GAGGTGATGGCGCTGAAGG-3′ (sense) and 5′-GCACGCCAGCAAGAAAAGC-3′ (antisense); and GAPDH, 5′-ATTCCATGGCACCGTCAAG-3′ (sense) and 5′-AGGGATGATGTTCTGGAGAGC -3′ (antisense), respectively.

2.4. Analysis of protein by Western blotting

Protein extraction was performed as previously described [9]. The protein concentration was determined by the Lowry's method using bovine serum albumin (BSA) as a standard. Equal amounts of protein were separated on 10% SDS-PAGE gels. The protein was subsequently transferred onto a polyvinylidene difluoride membrane by electroblotting for 3 h at 150 mA. The membranes were blocked in a 5% non-fat milk solution in TBS with 0.5% Tween 20, and incubated with primary antibodies, as indicated. Goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies were used at a ratio of 1:1000 for 1 h, and the blot was developed with a Supersignal chemiluminescence detection kit.

2.5. Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts was performed as previously described [9]. EMSA was used to characterize the binding activity of NF-κB in nuclear extracts. The NF-κB oligonucleotide was 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Protein–DNA binding mixture containing 20 μg of nuclear protein extract, was incubated for 20 min at 4 °C in binding medium containing 5% glycerol, 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 1% NP-40, 10 mM Tris (pH 7.5), and 1 μg of poly (dI–dC). Radiolabeled transcription factor consensus oligonucleotide (20,000 cpm of ^{32}P) was added and the complete mixture was incubated for an additional 20 min at room temperature. DNA-binding complexes were resolved by 8% native polyacrylamide gel electrophoresis with 0.5× TBE (50 mM Tris, 45 mM boric acid, and 0.5 mM EDTA) for 90 min at 200 V. The gel was dried and complexes were established with excess unlabeled oligonucleotide.

2.6. RNA interference (RNAi) production and transfection

The sequences for the RNAi experiments were as follows: for the p65 subunit of NF-κB we used sense strand siRNA: AAGAAGCACAGAUACCACCAAtt and antisense strand siRNA: UUGGUGGUAUCUCUUCUUtt. For human TRPC1, sense strand siRNA: GGAUGUGCGGGAGGUGAAGtt and antisense strand siRNA: CUUCACCUCCCGCACAUCCtt. The siRNA duplex along with a non-specific control duplex was obtained from TAKARA Bio Inc. (Dalian, China). siRNA was transfected directly into the target cells using the GeneSilencer siRNA transfection reagent following the instruction manual provided by the supplier. Briefly, cultured cells were washed with medium 199 without serum or antibiotics on the day of transfection and seeded in six-well plates to be 50–70% confluent (typically 1×10^5 to 2×10^5 cells/35 mm plate incubated for 24 h at 37 °C and 5% CO_2). Transfection reagent and siRNA were diluted separately in serum-free medium, mixed and incubated at room temperature for 10 min to allow the siRNA/lipid complex to form. The siRNA/lipid complex was then added to each well at a final concentration of 60 pmol/well of siRNA. At 24 h and 48 h after transfection, cells were harvested for Western blot analysis to determine the level of the p65 subunit.

2.7. Measurement of intracellular free Ca^{2+}, $[Ca^{2+}]i$

The HUVECs were loaded with the calcium indicator Fura-2AM (5 μM) in Hepes-buffered saline. Changes in $[Ca^{2+}]i$ in individual cells were measured using an Aquacosmos system with band-pass filters for 340 nm and 380 nm. $[Ca^{2+}]i$ was calculated from the Fura-2 fluorescence ratio (F340/F380) using linear regression between adjacent points on a calibration curve generated by measuring F340/F380 in at least seven calibration solutions containing Ca^{2+} at concentrations between 0 nM and 854 nM. The SOC-mediated influx of Ca^{2+} following stimulation with 1 μM TG during the change from Ca^{2+}-free conditions to 2 mM Ca^{2+} was measured as described [19].

2.8. Monolayer permeability to albumin

The transmembrane passage of albumin was performed by placing a high concentration of albumin (40 mg/ml) in the lower Transwell chamber and after a 2-h incubation at 37 °C, the diffusion of the albumin through the endothelial monolayer barrier was measured in the upper chamber [22]. In the control samples, baseline mean diffusion of albumin at 2 h was 94 μg/ml.

2.9. Statistical analysis

The results were presented as means ± S.E. of three independent triplicate measurements. Groups of data were compared with ANOVA followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

3. Results

3.1. AngII increases $[Ca^{2+}]i$ and endothelial permeability

The effect of AngII on endothelial permeability was analyzed after treatment of HUVECs with 10–1000 nmol/l AngII for 24 h. The

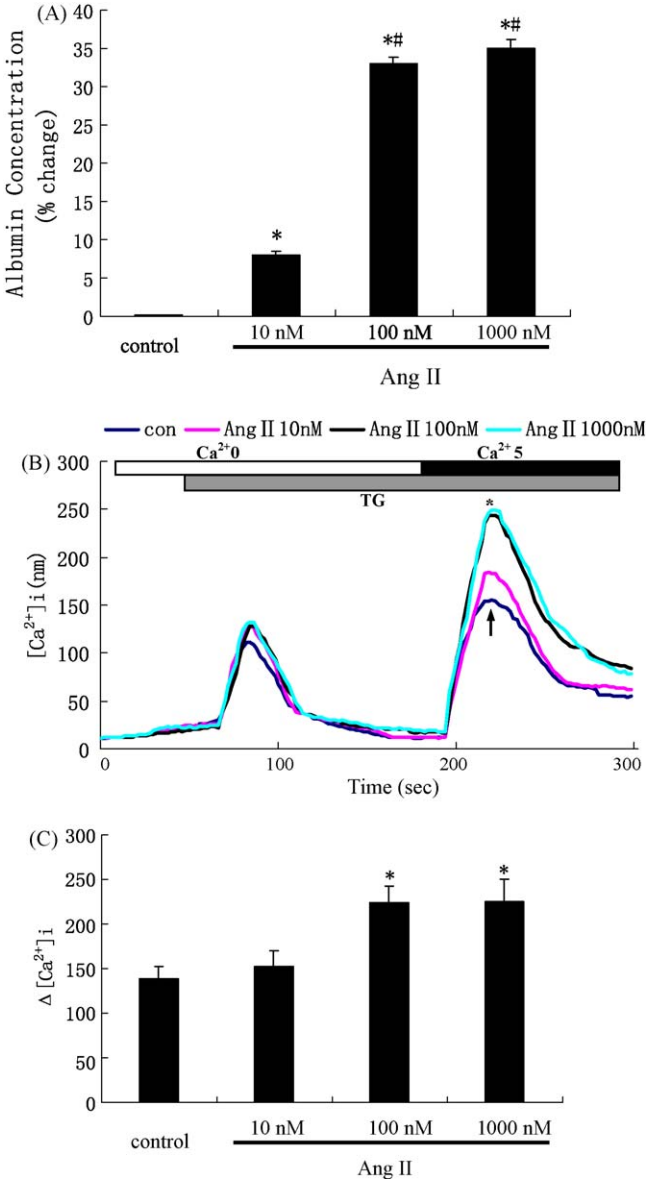

Fig. 1. (A) Diffusion of albumin through an HUVEC monolayer. HUVECs were cultured to confluence on fibronectin-coated 0.45 μm pore size PET inserts and treated with AngII (10–1000 nM) for 24 h. Diffusion of albumin through the tested HUVEC monolayer for 2 h was measured and the percentage change over the control is presented. The data are presented as mean ± SEM ($n = 3$), $^*p < 0.05$ compared to the control group; $\#p < 0.05$ compared to the 10 nM group. (B) We measured the SOC-mediated influx of Ca^{2+} following stimulation with 1 μM TG during the change from Ca^{2+}-free conditions to 5 mM Ca^{2+}. When compared to the control cells, treatment of HUVECs with AngII (100 nmol/l and 1000 nmol/l) for 24 h induced the peak increase in $[Ca^{2+}]i$ caused by SOCE. (C) Statistical analysis of SOC in different groups. The data are presented as the mean ± SEM ($n = 6$), $^*p < 0.05$ versus the control and 10 nM groups.

results showed that AngII increased the endothelial monolayer permeability of HUVECs in a concentration-dependent manner (Fig. 1A). We evaluated the effect of AngII on SOCE, which was activated by the depletion of intracellular Ca^{2+} stores using 1 μM TG in the absence of extracellular Ca^{2+}, followed by the addition of extracellular Ca^{2+} to 5 mM. The TG-mediated SOCE may be attributed to the release of Ca^{2+} from the sarcoplasmic reticulum. When compared to the control cells, treatment of HUVECs with AngII (100 nmol/l and 1000 nmol/l) for 24 h induced the peak increase in $[Ca^{2+}]i$ caused by SOCE (Fig. 1B and C). These results demonstrated that AngII may induce Ca^{2+} entry and endothelial hyperpermeability.

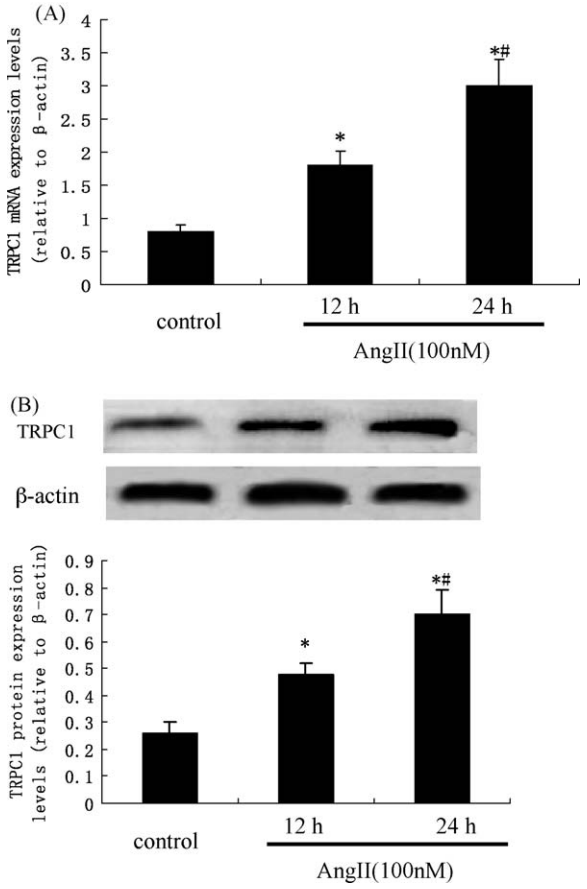

Fig. 2. AngII stimulated the protein and mRNA expression of TRPC1. The protein and mRNA expression of MMP-9 were determined by real-time RT-PCR (A) and Western blotting (B). At 12 h after treatment with AngII (100 nM), the mRNA and protein levels of TRPC1 had increased significantly compared to the control group. The data are presented as the mean ± SE ($n = 3$). $^*p < 0.05$ versus controls. Representative experiments are shown.

3.2. Effects of AngII on the expression of TRPC1 in HUEVCs

There is evidence that TRPC1 is important for regulating Ca^{2+} entry into endothelial cells and mediating the increase in endothelial permeability. Quantitative real-time RT-PCR and Western blotting were used to examine whether AngII affects the expression of TRPC1. TRPC1 mRNA expression was increased in response to AngII (100 nmol/l) stimulation (Fig. 2A). In quantitative real-time RT-PCR, the transcript level increased 3-fold after treatment with AngII for 24 h and reached 6-fold after 48 h of treatment. To determine whether the increased transcription of TRPC1 molecules resulted in a real increase at the protein level, Western blot analysis was performed after treatment with AngII for 12 h and for 24 h. The results showed that the TRPC1 protein level was increased significantly at 12 h and at 24 h after the treatment with AngII (Fig. 2B).

3.3. AngII-induced TRPC1 expression involves NF-κB and AT1

Because AngII induces the expression of genes by activating NF-κB signals in HUEVCs [9], we addressed the possibility that NF-κB is involved in TRPC1. Firstly, the effect of AngII on NF-κB activity was analyzed by EMSA. Treatment of HUVECs with AngII (100 nM) for 0–1 h induced a time-dependent increase in NF-κB DNA binding (Fig. 3A).

We used an RNAi strategy to suppress the expression of p65 in order to examine whether the NF-κB pathway involves AngII-

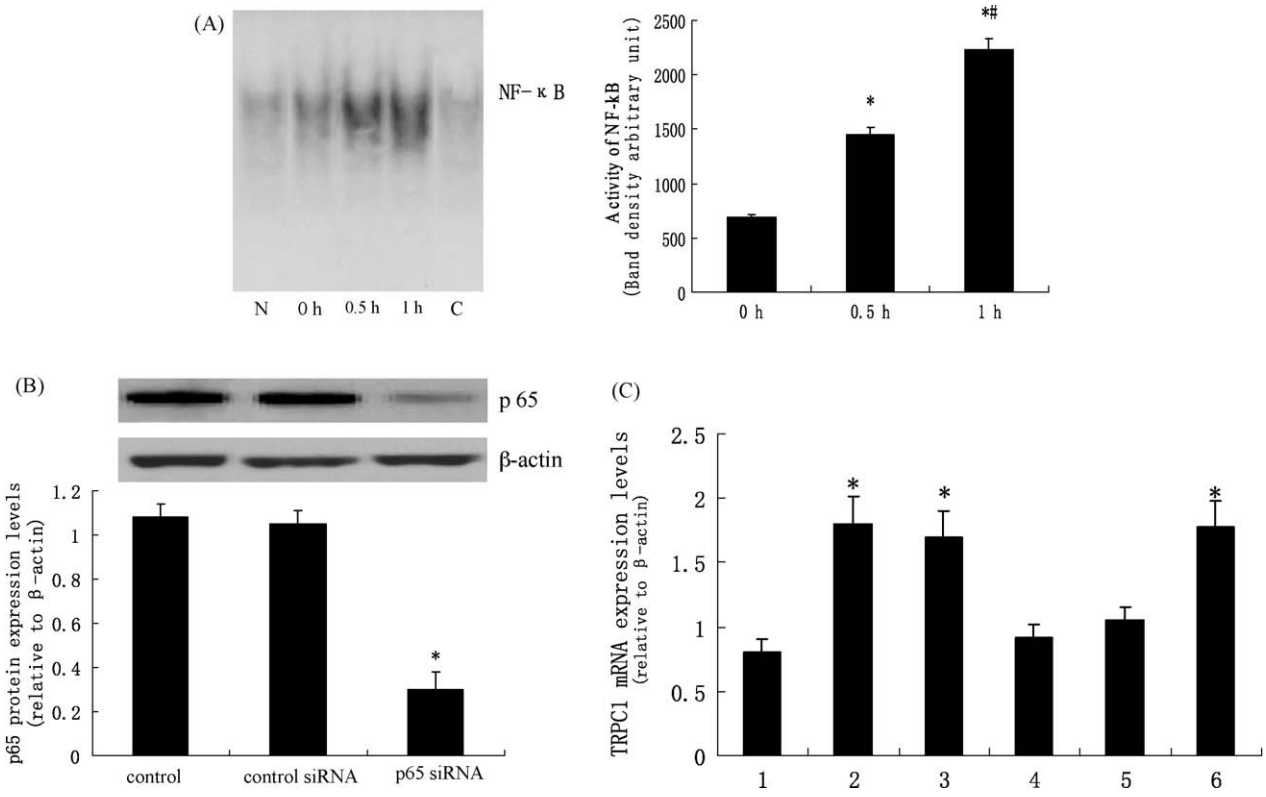

Fig. 3. (A) AngII-induced stimulation of NF-κB DNA binding. Serum-starved HUVECs were incubated with the indicated concentrations of AngII (100 nM). NF-κB DNA binding was measured by EMSA. Equal amounts of protein were loaded in each lane. The data are presented as the mean ± SE ($n = 3$). $^{*}p < 0.05$ versus controls. $^{\#}p < 0.05$ compared to the 0.5 h group. N, negative control without nuclear extract; C, specific competition with 200-fold (molar) non-labeled NF-κB probe. (B) After the expression of p65 was suppressed using an RNAi strategy, the level of expression of p65 was detected by Western blot. The result showed that there was a significant knockdown effect of p65 at 24 h after the RNAi treatment. To rule out the effect of the transfection itself, cells were transfected with a control siRNA. $^{*}p < 0.05$ versus control and control siRNA groups. Cells were treated with losartan (0.1 μM) for 30 min and PD123319 (0.1 μM) for 30 min, and subsequently stimulated the cells with AngII (0.1 μM) for 14 h. In addition, after transfection with control siRNA and p65 siRNA, HUEVCs were induced with AngII (0.1 μM) for 12 h. The real-time RT-PCR (C) and Western blot (D) showed that RNAi-mediated knockdown of p65 abolished AngII-induced expression of TRPC1. Meanwhile, losartan blocked AngII-induced expression of TRPC1, whereas PD123319 did not. Lanes 1, control; 2, AngII; 3, AngII + control siRNA; 4, AngII + p65 siRNA; 5, AngII + losartan; 6, AngII + PD123319. The data are presented as the mean ± SE ($n = 3$). $^{*}p < 0.05$ versus groups 1, 4 and 5. Representative experiments are shown.

induced TRPC1 expression. The Western blot used to detect the expression of p65 revealed that there was a significant knockdown effect of p65 at 24 h after the treatment with RNAi (Fig. 3B). To rule out the effect of the transfection itself, cells were transfected with a control siRNA. There was no apparent effect of the control siRNA transfection on p65 expression. RNAi-mediated knockdown of p65 abolished AngII-induced (100 nM for 24 h) TRPC1 expression (Fig. 3C and D). We pretreated HUEVCs with losartan (100 nM) for 30 min and with PD123319 (100 nM) for 30 min, and subsequently stimulated the cells with AngII (100 nM) for 12 h. The results showed that losartan blocked the TRPC1 expression induced by AngII, whereas PD123319 did not (Fig. 3C and D). These findings suggest that NF-κB and AT_1 involve AngII-induced TRPC1 expression.

3.4. Inhibition of TRPC1 and NF-κB expression attenuates AngII-induced $[Ca^{2+}]i$ and endothelial permeability

We used siRNA against TRPC1 to inhibit endogenously expressed TRPC1 proteins selectively. The inhibitory effect of siRNA on TRPC1 expression was evident at 24 h after siRNA transfection, when the level of the TRPC1 protein level was decreased by 74% (Fig. 4A). Cells treated with AngII but transfected with a random sequence were used as the control group.

We analyzed the effects of TRPC1 and NF-κB on AngII-induced SOCE and endothelial hyperpermeability. The peak increase in $[Ca^{2+}]i$ caused by SOCE and endothelial permeability were decreased significantly in siRNA-treated HUEVCs compared to the control group (Fig. 4B and C). Taken together, these results indicate clearly that TRPC1 and NF-κB molecules have an important role in AngII-induced SOCE and endothelial hyperpermeability. Furthermore, we examined the effect of AT1 and AT2 on AngII-induced SOCE and endothelial hyperpermeability. The cells were treated with losartan (100 nM) for 30 min and with PD123319 (100 nM) for 30 min, and subsequently stimulated with AngII (100 nM) for 24 h. The result showed that losartan blocked AngII-induced SOCE and endothelial hyperpermeability, whereas PD123319 did not (Fig. 4B and C).

4. Discussion

The purpose of this work was to examine the mechanism of AngII-induced Ca^{2+} entry and vascular endothelial hyperpermeability. We found that AngII increased Ca^{2+} entry and endothelial permeability through a mechanism involving the NF-κB pathway and activation of TRPC1-dependent plasmalemmal Ca^{2+} entry. We demonstrated that the stimulatory effects of AngII on TRPC1 require NF-κB activation in HUVECs. This conclusion is based on several independent lines of evidence. First, AngII-upregulated TRPC1 expression was associated with AngII-induced vascular endothelial hyperpermeability. Second, knockdown of endogenous TRPC1 and NF-κB by delivery of siRNA significantly suppressed AngII-induced Ca^{2+} entry and vascular endothelial hyperpermeability. Third, NF-κB knockdown inhibited AngII-induced TRPC1

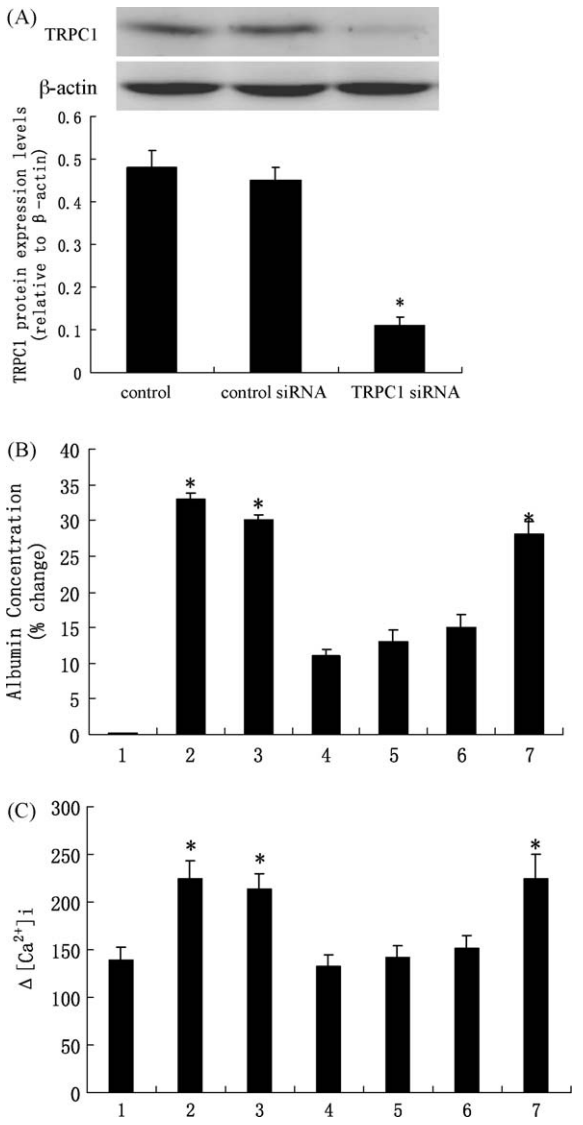

Fig. 4. (A) After the expression of TRPC1 was suppressed using an RNAi strategy, the level of expression of TRPC1 was detected by Western blot. $^*p < 0.05$ versus control and control siRNA groups. (B) Diffusion of albumin through the tested HUVEC monolayer was measured and the percentage change over the control is presented. The data are presented as the mean ± SEM ($n = 3$). Lanes 1, control; 2, AngII; 3, AngII + control siRNA; 4, AngII + TRPC1 siRNA; 5, AngII + p65 siRNA; 6, AngII + losartan; and 7, AngII + PD123319. $^*p < 0.05$ versus groups 1, 4–6. (C) The peak increase in $[Ca^{2+}]i$ caused by SOCE was measured as the SOC-mediated influx of Ca^{2+} following stimulation with 1 μM TG during the change from Ca^{2+}-free conditions to 5 mM Ca^{2+}. Statistical analysis of SOC in different groups. The data are presented as the mean ± SEM ($n = 6$), lanes 1, control; 2, AngII; 3, AngII + control siRNA; 4, AngII + TRPC1 siRNA; 5, AngII + p65 siRNA; 6, AngII + losartan; and 7, AngII + PD123319. $^*p < 0.05$ versus groups 1, 4–6.

expression in HUVECs. In addition, inhibition of AT1 also suppresses AngII-induced Ca^{2+} entry and vascular endothelial hyperpermeability. These observations suggest that TRPC1 and NF-κB have key roles in mediating AngII-induced Ca^{2+} entry and endothelial hyperpermeability.

AngII is recognized as a growth factor that regulates cell growth and fibrosis, as well as a physiological mediator that can restore circulatory integrity [6]. In the last few years, experimental research has demonstrated that AngII is involved in endothelial barrier dysfunction [32]. Work by several groups over the past decade has shown that increasing Ca^{2+} entry is associated with vascular endothelial hyperpermeability [10,12]. In this study, we demonstrated that AngII could induce Ca^{2+} entry and endothelial hyperpermeability in a concentration-dependent manner.

TRP was found first in Drosophila, and has an essential role in the visual response of the fly [5]. Several homologs of the TRP channel family, such as TRPC1 and TRPC6, are expressed in mammalian cells and have intriguing sensing capabilities for a range of factors, including redox status and growth factors [4,8,14]. Recently, TRPC channels have been considered good candidates for Ca^{2+} entry pathways, such as SOCE. On the basis of the importance of TRPC1 in regulating the Ca^{2+} entry-dependent increase in permeability of the human endothelial carrier [15,17], we addressed the role of TRPC1 in the mechanism of AngII-induced permeability modulation. We found that chronic stimulation by AngII increased SOCE together with TRPC1 expression. Moreover, the gene silencing of TRPC1 using siRNA attenuates SOCE. These findings suggest strongly that AngII increases SOCE via the upregulation of TRPC1, which is supported by the results of other studies. Takahashi et al. [30] suggested that TRPC1 was involved in the AngII-induced increase of SOCE in vascular smooth muscle cells. Meanwhile, we demonstrated that gene silencing of TRPC1 inhibited AngII-induced endothelial hyperpermeability.

Recently, Paria et al. showed TNF-α-induced TRPC1 expression via NF-κB activation in endothelial cells and found three NF-κB-binding sequences in the 5′-regulatory region of hTRPC1 [25]. Our earlier work showed that AngII stimulates NF-κB nuclear translocation in HUVEC via the AT1 and p38MAPK pathways. As a result, we asked whether the NF-κB signaling pathway was involved in AngII-induced TRPC1 upregulation. We showed that NF-κB is necessary for AngII-induced TRPC1 expression in HUEVCs. Some studies have explored the mechanisms in depth. Paria et al. showed that the distal region (−1685 to −1221) is important for TRPC1 expression [24]; however, Takahashi et al. showed the proximal NF-κB-binding site (site 3, −709 to −700) may be responsible for AngII-induced TRPC1 upregulation [30]. Further explanation of these discrepancies requires further research. In addition, the knockdown of NF-κB also attenuated AngII-induced Ca^{2+} entry and endothelial hyperpermeability. These findings indicate that AngII and subsequent NF-κB activation increases Ca^{2+} entry and endothelial hyperpermeability through the upregulation of TRPC1.

We have demonstrated that inhibition of AT1 also suppresses AngII-induced Ca^{2+} entry and vascular endothelial hyperpermeability. Our earlier study showed AngII-induced NF-κB activation involved AT1 in HUEVCs. In this study, we show that losartan can abolish AngII-induced TRPC1 expression, Ca^{2+} entry and vascular endothelial hyperpermeability in HUEVC, while the AT_2 antagonist PD123319 cannot. These results demonstrate further that the AT1 antagonist is clinically effective in the management of heart failure and arteriosclerosis [3,20].

In conclusion, we show that the NF-κB and TRPC1 have critical roles in AngII-induced Ca^{2+} entry and endothelial permeability. Furthermore, NF-κB and TRPC1 appear to be potentially useful targets for the treatment of heart failure and arteriosclerosis.

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