**Stim1- and Orai1-mediated store-operated calcium entry is critical for angiotensin II-induced vascular smooth muscle cell proliferation**

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**Aim**

Despite the fact that angiotensin (Ang) II is a critical regulator of the proliferation and migration of vascular smooth muscle cells (VSMCs), the effect of Ang II on VSMC proliferation has remained unclear. In this study, we determined whether Stim1- and Orai1-mediated store-operated calcium (Ca^{2+}) entry (SOCE) plays a critical role in Ang II-induced VSMC proliferation and Ang II-accelerated neointimal growth after balloon injury of rat carotid arteries.

**Methods and results**

Knockdown of Stim1 and Orai1, putative calcium sensors/modulators, suppressed Ang II-mediated Ca^{2+} entry and cell proliferation in synthetic VSMCs. Stim1 and Orai1 short interfering RNAs (siRNAs) decreased neointimal growth induced by Ang II in balloon-injured rat carotid arteries. Ang II significantly increased the expression of Stim1 and Orai1 in neoIntima. In addition, our results showed that receptor subtype-1 (AT1) significantly contributed to Ang II-induced Ca^{2+} entry and proliferation of synthetic VSMCs. However, we found that transient receptor potential canonical 1 (Trpc1) had no effect on Ang II-induced SOCE or cell proliferation of synthetic VSMCs.

**Conclusions**

We show for the first time that Stim1- and Orai1-mediated SOCE may be critical for Ang II-induced VSMC proliferation. This provides important information with respect to targeting cardiovascular diseases under the enhanced renin–Ang system.

**Keywords** Angiotensin II • Stim1 • Orai1 • Vascular smooth muscle cells • Proliferation

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**1. Introduction**

Atherosclerosis is one of the most common diseases in developed and developing countries. The proliferation of vascular smooth muscle cells (VSMCs) plays a crucial role in intimal thickening of the arterial wall, a feature commonly associated with atherosclerosis. Despite the fact that percutaneous coronary intervention (PCI) has significantly improved the clinical outcome of atherosclerosis, understanding restenosis after PCI has remained a challenge.¹ Restenosis refers to the reoccurrence of stenosis due to intimal lesions; this process significantly contributes to the proliferation and migration of medial VSMCs.² Therefore, the proliferation of VSMCs is a key event in atherosclerosis and restenosis after vascular injury. To prevent the development of atherosclerosis and restenosis, it is critical to understand the mechanisms leading to the proliferation of VSMCs at the site of atherosclerotic lesions.

Angiotensin (Ang) II is the main final effector molecule of the renin–Ang system. Physiologically, Ang II plays an important role in controlling blood pressure and fluid volume. However, Ang II is also widely recognized as a critical regulator of VSMC proliferation and migration, which is one cause of cardiovascular remodelling, and is associated with the development of hypertension, atherosclerosis, and restenosis.³⁴ The signal transduction and regulatory mechanisms involved in Ang II signalling that lead to hypertrophy, migration, and inflammatory gene expression in VSMCs have been extensively examined.⁵ However, the effect of Ang II on VSMC proliferation, the key event in the pathogenesis of atherosclerosis and restenosis, has remained unclear.

Calcium (Ca^{2+}) channels are of particular interest in cell proliferation because of the profound anti-proliferative effects of removing extracellular Ca^{2+}. In addition, evidence from studies in many cell types has indicated that Ca^{2+} entry mechanisms play an essential role in Ang II-induced VSMC proliferation and migration.
role in this process. Currently, at least three different types of Ca\textsuperscript{2+}-permeable channels have been described in VSMCs: voltage-dependent Ca\textsuperscript{2+} channels, stretch-activated Ca\textsuperscript{2+} channels, and receptor-activated Ca\textsuperscript{2+} channels, which include second messenger-gated channels, ionotropic receptors, and store-operated channels (SOCs). Store-operated Ca\textsuperscript{2+} entry (SOCE), also known as capacitative Ca\textsuperscript{2+} entry, is thought to play an essential role in the regulation of cell proliferation and apoptosis.

Over the years, many genes have been claimed to encode SOCs. Recently, high-throughput RNA silencing screening by several laboratories identified two main components of SOCE: the endoplasmic reticulum (ER)-localized Ca\textsuperscript{2+} sensor protein, Stim1, and the plasma membrane (PM)-localized Ca\textsuperscript{2+} channel, Orai1. Ca\textsuperscript{2+} depletion in the ER is sensed by the luminal EF-hand of Stim proteins, causing their multimerization, and subsequent translocation within close proximity of the PM. The interaction of the cytoplasmic C termini of Stim with PM components, and association with PM-localized Orai1 channels, cause their activation in the PM, and mediates Ca\textsuperscript{2+} influx. Several laboratories have recently showed that Stim1 and Orai1 are expressed in VSMCs.

It is clear that SOCE is initiated by inositol 1,4,5-trisphosphate (IP\textsubscript{3})-mediated depletion of ER Ca\textsuperscript{2+} in response to certain types of stimuli. Ang II stimulates the growth of VSMCs, in which important cellular mechanisms are mediated by the activation of the phospholipase C–IP\textsubscript{3}–1,2-diacylglycerol cascade, mitogen-activated protein kinases, tyrosine kinases, and redox signalling cascades.

Our previous studies showed that abolishing SOCE significantly inhibited VSMC growth, even in the presence of serum. However, it remains unknown if SOC activation is key to Ang II-induced VSMC proliferation. Previously, studies showed that PDI kinase is involved in Ang II signalling. However, Ko et al. reported inhibition of Ang II-induced VSMC proliferation by Ca\textsuperscript{2+} entry blockers. Recently, a study showed that Ang II can induce IP\textsubscript{3}-operated Ca\textsuperscript{2+} influx in cardiomyocytes. Moreover, it has been confirmed that SOCE is involved in mediating Ang II-induced endothelial hyperpermeability. Thus, SOCs could potentially be an important downstream effector for Ang II facilitation of VSMC growth. However, it is still unclear whether Stim1 and Orai1 are involved in Ang II-induced VSMC growth. Therefore, we tested the hypothesis that Stim1- and Orai1-mediated SOCE are critical for Ang II-induced VSMC proliferation.

2. Methods

2.1 Reagents

Ang II, thapsigargin, 2-APB, and PD123319 were purchased from Sigma (MO, USA). The anti-Stim1 antibody was obtained from BD Biosciences (NJ, USA); anti-Orai1, anti-transient receptor potential canonical 1 (Trpc1), and anti-β-actin antibodies were purchased from Sigma. Foetal bovine serum (FBS) was obtained from HyClone (UT, USA). Medium and supplements were purchased from Gibco BRL (NY, USA). Fura-2AM was obtained from Beyotime Chemical Company (Jiangsu, China).

2.2 Construction of adeno viral vectors

Short interfering RNA (siRNA) sequences for rat Stim1 were designed as described, and siRNA sequences for rat Orai1 and Trpc1 were obtained from Dharmaco RNA Technologies (CO, USA). A non-silencing control (NSC) sequence was designed according to the sequence of a negative control. All short hairpin RNAs cloned into the pGS-1 vector were from GenesiL (Wuhan, China). All viruses were titrated using the standard plaque assay, and the titre was 3 × 10\textsuperscript{9} plaque-forming units (p.f.u.)/mL. All adenoviruses expressed green fluorescent protein (GFP) under a separate promoter, allowing verification of infection.

2.3 Cell culture and adeno viral transduction

Rats were killed by intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg), and these protocols fully complied with recommendations in the American Veterinary Medical Association Panel on Euthanasia. VSMCs were isolated from the thoracic aorta of male rats by enzymatic digestion. Briefly, the rat aorta was carefully dissected free from surrounding tissue and rinsed in Hank’s balanced salt solution. The aortas were minced and digested in 10 mL digestion solution at 37°C for 30 min. The cellular digest was filtered, centrifuged, and washed in Dulbecco’s modified Eagle’s medium (DMEM). Isolated VSMCs were maintained in culture (45% DMEM and 45% Ham’s F12 medium with 10% FBS supplemented with l-glutamine) at 37°C, 5% CO\textsubscript{2}, and 100% humidity. Cells were passaged and used within 3–5 passages (synthetic). VSMCs were transduced with Ad-si/Stim1, Ad-si/rOrai1 Ad-si/rTrpc1, or NSC, and protein levels were evaluated in the cell extracts.

2.4 \textsuperscript{3}H-Thymidine incorporation

VSMC DNA synthesis was measured by \textsuperscript{3}H-thymidine incorporation as previously described. Briefly, confluent VSMCs in 100-mm dishes were detached with 0.05% trypsin/EDTA, seeded onto 24-well plates (10 000 cells/well) in 10% FBS/DMEM, and allowed to attach overnight. The cells were then starved for 24 h and incubated for 48 h with adeno viral (Ad) vectors as indicated. During the final 4 h of the assay, 1 μCi of [methyl-\textsuperscript{3}H]-thymidine was added to each well. Incorporated \textsuperscript{3}H-thymidine was precipitated with 10% trichloroacetic acid and counted with a liquid scintillation counter.

2.5 Cell counting

The growth of VSMCs was examined by cell counting. Cells first underwent mitogenic quiescence by serum starvation and viral infection. The cell number under these experimental conditions was used as the baseline. To examine the status of VSMC proliferation, the cells were subsequently stimulated with serum (10% FBS), and the cell number was counted at 12 and 24 h. Each count is an average of three repeats, and each data point is the average of three experiments.

2.6 Measurement of intracellular free [Ca\textsuperscript{2+}]

For calcium experiments, the VSMCs were loaded with the calcium indicator, Fura-2AM (5 μM), in Herpes-buffered saline for 30 min at 20°C. Cells on coverslips were placed in cation-safe medium in the absence or presence of CaCl\textsubscript{2}. For [Ca\textsuperscript{2+}] measurements, fluorescent images of several cells were recorded and analyzed with a digital fluorescence imaging system. Fluorescence emission at 510 nm was monitored using excitation wavelengths of 340 and 380 nm; intracellular [Ca\textsuperscript{2+}] measurements are shown as 340/380-nm fluorescence ratios. All traces are averages from multiple cells, and are representative of several independent experiments.

2.7 Western blotting

The vessels or VSMC proteins were extracted as previously described. Equal amounts of protein were separated by SDS–PAGE (10% polyacrylamide gel) and then transferred onto a polyvinylidene difluoride membrane by electroblotting for 3 h at 150 mA. The membrane was blocked in 5% non-fat milk in Tris-buffered saline along with 0.5% Tween 20. The membrane was incubated with primary antibodies, followed by the appropriate secondary antibodies, and detection of specific proteins was performed by enhanced chemiluminescence following the
manufacturer’s instructions. Densitometric signals were quantified by Quantity One software.

2.8 Balloon angioplasty and the delivery of adenoviruses

Male Wistar rats weighing 250–300 g (Chongqing, China) were anaesthetized by intramuscular injection of 100 mg/kg ketamine and 5 mg/kg xylazine. Periodic observation of respiration, colour of mucous membranes, and pain response was used for monitoring adequacy of the anaesthesia.

The left common carotid artery was exposed through a midline cervical incision, and blood flow to the site was temporarily interrupted by ligation of the left common, internal, and external carotid arteries with vessel clips. The balloon catheter (1.5 F, Cordis, USA) was introduced through the left external carotid artery into the common carotid artery and inflated at 2 atm with a calibrated inflation device. The carotid artery was damaged by passing the inflated balloon catheter back and forth through the lumen three times. Immediately after the injury, 100 μL of a saline solution containing Ad-si/Stim1, Ad-si/Orai1, Ad-si/Trpc1, or NSC, with or without 100 nM of Ang II, were infused and remained in contact with the carotid artery for 1 h. The adenovirus or saline solution was then removed, the carotid artery section was flushed, and blood flow was restored. The incision was closed with sutures. Three or 14 days after the arteriotomy, rats were killed by intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg), and these protocols fully complied with recommendations in the American Veterinary Medical Association Panel on Euthanasia. The carotid arteries were carefully dissected free from surrounding tissue. Animal experiments were approved by the local animal care (No. 00065822, Kunming General Hospital). All studies conformed to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.9 Immunocytochemistry

Carotid arteries were fixed and embedded in paraffin wax. Six cross sections were cut from approximately the middle of the artery, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 min. Endogenous peroxidase activity was quenched and sections were blocked in 10% horse serum in PBS for 2 h. Primary antibody was applied overnight at 4°C and secondary antibody was applied for 1 h at room temperature. Sections were stained using an SP Kit (Beijing, China), which uses a special preparation of biotinylated horseradish peroxidase H reagents to form complexes for immunoperoxidase staining. Parallel control experiments were done using adjacent sections, omitting the primary antibody.

2.10 Morphology

Briefly, 3 and 14 days after angioplasty, animals were anaesthetized as described above, and the carotid arteries were fixed by perfusion through a large cannula placed in the left ventricle, with 100 mL of PBS (pH 7.2), followed by 80 mL of PBS containing 4% paraformaldehyde. The carotid arteries were removed, and six cross sections were cut from approximately the middle of the artery. The sections were stained with haematoxylin and eosin to demarcate the cell types. The sections were photographed under low power, video-digitized, and stored in an image analysis system. The media and neointima were traced carefully, and the neointima and media areas were calculated.

2.11 Statistical analysis

Results are expressed as the mean ± SEM of n rats for the in vivo experiments and the mean ± SEM of multiple experiments for the molecular experiments. SPSS software, v.13.0 was used for statistical analysis. Student’s t tests were used to compare two groups, and ANOVA was used with Tukey’s multiple comparison tests for multiple groups. Values of P < 0.05 were regarded as statistically significant.

3. Results

3.1 Pharmacological characterization of Ang II-mediated Ca2+ entry in synthetic VSMCs

We evaluated pharmacological inhibitors of SOCE to characterize Ang II-mediated Ca2+ entry in synthetic VSMCs. Ang II (0.1 μmol/L) induced Ca2+ release from the sarcoplasmic reticulum (SR) of primary cultured VSMCs in the absence of extracellular Ca2+. When extracellular Ca2+ was added to 2 mM, Ang II activated a Ca2+ entry pathway. In control experiments, replenishment of extracellular Ca2+ in absence of Ang II did not elicit a detectable elevation in Ca2+ entry (data not shown). However, adding Ca2+ in the presence of Ang II might stimulate Ca2+ entry pathways other than SOCE, such as second messenger-operated channels. Next, we investigated the role of second messenger-operated channels in the Ang II-activated Ca2+ entry. Cells were incubated with 2-APB (50 μmol/L) before restoration of extracellular Ca2+, and the results showed that a 20-min pre-incubation with 2-APB essentially abrogated Ang II-activated Ca2+ entry across the PM (Figure 1B and C). This indicated that second messenger-operated channels were not attributable to Ang II-mediated Ca2+ entry. Together, these data suggest that Ang II-mediated Ca2+ entry may be attributed to the release of Ca2+ from the SR. At the same time, Ang II-mediated Ca2+ entry pathway was blocked with 50 μmol/L 2-APB, 10 μmol/L Gd3+, or 50 μM ML-9 inhibitor (Figure 1D and E). These results suggest that Ang II-mediated Ca2+ entry displays classical SOCE features similar to those observed in non-excitable cells, such as HEK-293 and RBL mast cells.

3.2 Knockdown of Stim1 and Orai1 suppresses Ang II-mediated Ca2+ entry in synthetic VSMCs

We used RNA silencing to examine whether Stim1 and Orai1 are critical molecules for Ang II-mediated SOCE in primary cultured VSMCs (synthetic). Rat aortic VSMCs were cultured for use in in vitro experiments. Adenovirus constructs expressing NSC, SiStim1, SiOrai1, and SiTrpc1 were added to rat aortic primary cultured VSMCs (synthetic). The transfection efficiency of adenovirus GFP expression in VSMCs was 90.4 ± 5.6% (Supplementary material online, Figure S1). The expression of Stim1, Orai1, and Trpc1 proteins was studied by western blotting. Transduction of VSMCs with adenovirus (multiplicity of infection 15 p.f.u./cell) effectively decreased Stim1, Orai1, and Trpc1 protein expression 24 h post-transduction (Figure 2A and C). We also used western blot analysis to examine siRNA specificity, and the results showed that SiStim1 did not affect Orai1 levels and vice versa in primary cultured VSMCs (Supplementary material online, Figure S2). Knockdown of Stim1 or Orai1 significantly suppressed Ang II-induced Ca2+ entry in primary cultured VSMCs (synthetic), whereas Trpc1 knockdown had no effect (Figure 2D). Knockdown of Stim1, Orai1, and Trpc1 proteins in VSMCs had no significant effect on Ca2+ release from the SR. These results demonstrated that Stim1 and Orai1, but not Trpc1, are critical molecules for Ang II-mediated SOCE in primary cultured VSMCs (synthetic).
We examined the effects of Stim1, Orai1, and Trpc1 siRNA on Ang II-induced VSMC proliferation by measuring [3H]-thymidine uptake and by cell counting. Compared with cells treated with medium alone, Ang II (100 nmol/L) markedly stimulated VSMC proliferation at 12 and 24 h. Stim1 and Orai1 siRNA resulted in significant inhibition of [3H]-thymidine uptake compared with VSMCs exposed to Ang II stimulation at 12 and 24 h. Nevertheless, the inhibitory effect of cell proliferation had no significant effect on [3H]-thymidine uptake in VSMCs infected with Trpc1 siRNA at 12 and 24 h (Figure 3A and B). Concomitantly, transfection of VSMCs with SiStim1 and SiOrai1 significantly suppressed proliferation of VSMCs in response to Ang II. However, Trpc1 siRNA did not affect Ang II-induced VSMC proliferation (Supplementary material online, Figure S3). These results demonstrate that knockdown of Stim1 and Orai1 can suppress VSMC proliferation in vitro. Furthermore, phosphorylation of cAMP response element-binding (CREB) by Ang II was examined, and knockdown of Stim1 and Orai1 decreased CREB phosphorylation in response to Ang II in primary cultured VSMCs (synthetic) (Figure 3C).

**3.3 Stim1 and Orai1 are critical for Ang II-induced VSMC proliferation**

We examined the effects of Stim1, Orai1, and Trpc1 siRNA on Ang II-induced VSMC proliferation by measuring [3H]-thymidine uptake and by cell counting. Compared with cells treated with medium alone, Ang II (100 nmol/L) markedly stimulated VSMC proliferation at 12 and 24 h. Stim1 and Orai1 siRNA resulted in significant inhibition of [3H]-thymidine uptake compared with VSMCs exposed to Ang II stimulation at 12 and 24 h. Nevertheless, the inhibitory effect of cell proliferation had no significant effect on [3H]-thymidine uptake in VSMCs infected with Trpc1 siRNA at 12 and 24 h (Figure 3A and B). Concomitantly, transfection of VSMCs with SiStim1 and SiOrai1 significantly suppressed proliferation of VSMCs in response to Ang II. However, Trpc1 siRNA did not affect Ang II-induced VSMC proliferation (Supplementary material online, Figure S3). These results demonstrate that knockdown of Stim1 and Orai1 can suppress VSMC proliferation in vitro. Furthermore, phosphorylation of cAMP response element-binding (CREB) by Ang II was examined, and knockdown of Stim1 and Orai1 decreased CREB phosphorylation in response to Ang II in primary cultured VSMCs (synthetic) (Figure 3C).
3.4 Inhibition of AT1 caused a decrease of SOCE in response to Ang II

Ang II signals through seven transmembrane-spanning G protein-coupled receptors that are characterized into subtype-1 (AT1) and subtype-2 (AT2) based on their selective affinity for peptide and non-peptide ligands. Specific non-peptide antagonists, such as losartan and other ‘sartans’, block AT1 receptors, while non-peptide compounds, like PD123317 and PD123319, antagonize AT2 receptors. To examine whether AT1 and AT2 inhibitors influence Ang II-induced Ca\(^{2+}\) entry, phosphorylation of CREB, and VSMC proliferation, we first evaluated the expression levels of AT1 and AT2 in primary cultured VSMCs (synthetic) at passages 3–5 by western blotting, and saw good protein expression of AT1 and AT2 (Supplementary material online, Figure S4). The effects of Ang II on Ca\(^{2+}\) entry, phosphorylation of CREB, and proliferation by VSMCs were mediated by AT1 receptors, since pretreatment with the AT1 receptor antagonist, losartan (1 \(\mu\)M for 30 min) inhibited Ang II effects, while treatment with the AT2 receptor antagonist, PD123319 (1 \(\mu\)M for 30 min) did not (Figure 4 and Supplementary material online, Figure S5). These results suggest that AT1 significantly contributes to Ang II-induced Ca\(^{2+}\) entry and VSMC proliferation.
3.5 Stim1 and Orai1 siRNAs decrease neointimal growth induced by Ang II in balloon-injured rat carotid arteries

The rat carotid balloon-injury model shares numerous identical features with clinical restenosis and arterial injury diseases, and has therefore been used extensively to study the mechanisms underlying restenosis and its prevention.16,30 In this study, we first examined the efficiency of adenovirus transfection in rat carotid arteries by western blot analysis. The levels of GFP expression reached a maximum at Day 3, and remained high until Day 14 after Ad infection. These results suggest that the adenovirus-mediated delivery system was effective in rat carotid arteries (Supplementary material online, Figure S6).

We delivered adenovirus constructs expressing NSC, SiStim1, SiOrai1, and SiTrpc1 to rat carotid arteries. Expression of Stim1, Orai1, and Trpc1 proteins was confirmed by western blotting. Compared with the NSC group, incubation with SiStim1, SiOrai1, and SiTrpc1 significantly attenuated the expression of Stim1, Orai1, and Trpc1, respectively, after 3 days of injury (Figure SA). We delivered adenovirus constructs expressing NSC, SiStim1, SiOrai1, and SiTrpc1 to rat carotid arteries, with or without Ang II (100 nM), for 1 h. Arterial sections after 14 days of injury were used for intimal and media area analysis. The data showed that Ang II significantly increased neointimal growth in balloon-injured carotid arteries. Exposure of injured arteries to SiStim1 and SiOrai1, but not SiTrpc1, suppressed Ang II-induced neointimal growth in balloon-injured vessels and intimal thickening caused by injury (Figure SB). Uninjured carotid arteries are shown in Supplementary material online, Figure S7. These results indicate that knockdown of Stim1 and Orai1 was able to reduce neointimal thickening in balloon-injured carotid arteries in the presence or absence of Ang II.
Figure 4 Effect of AT1 and AT2 inhibitors on Ang II-induced Ca\(^{2+}\) entry (A), phosphorylation of CREB (B), and the uptake of \[^{3}H\]-thymidine (C). Treatment with the AT1 receptor antagonist losartan (1 μM for 30 min) inhibited the Ang II effects, while treatment with the AT2 receptor antagonist PD123319 (1 μM for 30 min) did not. The average SOCE levels in response to 100 nmol/L Ang II after pretreatment for 30 min with losartan or PD123319 are shown (n = 16 cells). Data are representative of at least three independent experiments. \(* P < 0.05\) vs. control and PD123319. Pretreatment with losartan significantly decreased the phosphorylation of CREB by VSMCs in response to Ang II (100 nmol/L) stimulation. The data are presented as the mean ± SEM (n = 3). Pretreatment with losartan significantly decreased the uptake of \[^{3}H\]-thymidine by VSMCs in response to Ang II (100 nmol/L) stimulation at 24 h. The data are presented as the mean ± SEM (n = 6). \(* P < 0.05\) vs. control and Ang II + losartan.
3.6 Expression and localization of Stim1 and Orai1 in the arterial wall at Day 14 after injury

Our group, along with other research groups, recently demonstrated the up-regulation of Stim1 and Orai1 proteins in neointima formation following arterial balloon injury in the rat.\(^{31}\) In this study, Ang II significantly increased neointimal growth in balloon-injured carotid arteries. Compared with the injury alone group, Stim1 and Orai1 protein levels in the left injured carotid increased further with Ang II treatment after balloon angioplasty (Figure 6A). Immunofluorescence analysis performed on injured artery sections without Ang II treatment at Day 14 showed higher levels of Stim1 and Orai1 in the neointima than in the medial. Ang II produced an increase in Stim1 and Orai1 staining in the neointima (Figure 6B). To quantify the expression of Stim1 and Orai1 in the neointima and media 14 days after injury, the expression levels of Stim1 and Orai1 proteins were analysed by western blotting. Compared with the injury alone group, the expression of Stim1 and Orai1 in the neointima did not further increase upon Ang II treatment. However, Ang II significantly increased the expression of Stim1 and Orai1 in the neointima (Figure 6C). Based on these observations, we conclude that the up-regulation of Stim1 and Orai1 expression is followed by neointimal growth.

Figure 5 Stim1 and Orai1 siRNAs affected Ang II-stimulated neointimal growth. (A) Western blot analysis of Stim1, Orai1, Trpc1, and β-actin in rat carotid arteries (whole artery) at 3 days following balloon injury and gene delivery. The data are representative of three different experiments. Densitometric analysis of Stim1, Orai1, and Trpc1 protein expression levels, normalized to the expression levels of the housekeeping β-actin gene, was determined using the Quantity One program. The results are expressed as the mean ± SEM of three experiments. \(*P < 0.05\) vs. NSC. (B) Rats were euthanized after 14 days, and arterial sections were stained with haematoxylin and eosin. Intimal and media areas were compared among rat carotid arteries transfected with adenovirus expressing NSC, SiStim1, SiOrai1, and SiTrpc1, with or without Ang II (100 nM), for 1 h. The results are expressed as the mean ± SEM of six experiments. \(*P < 0.05\) vs. the other groups; \(#P < 0.05\) vs. the corresponding value obtained in the absence of Ang II. All bars represent 200 μm.
Ang II contributes to vascular lesions by promoting VSMC proliferation. This study showed for the first time that Stim1- and Orai1-mediated SOCE play a critical role in Ang II-induced VSMC proliferation, and Ang II accelerated neointimal growth after balloon injury of rat carotid arteries. We also demonstrated that Trpc1 had no effect on Ang II-induced SOCE and cell proliferation in synthetic VSMCs based on several independent lines of evidence. First, knockdown of Stim1 and Orai1 suppressed Ang II-mediated Ca\(^{2+}\) entry and cell proliferation in synthetic VSMCs. Second, Stim1 and Orai1 siRNAs decreased neointimal growth induced by Ang II in balloon-injured rat carotid arteries. Third, Ang II significantly increased the expression of Stim1 and Orai1 in the neointima. In addition, our results showed that AT1 significantly contributed to Ang II-induced Ca\(^{2+}\) entry and proliferation in synthetic VSMCs. These observations suggest that Stim1- and Orai1-mediated SOCE are critical for Ang II-induced VSMC proliferation.

Many lines of evidence have shown that Ang II can induce phosphatidylinositol 4,5-bisphosphate breakdown into two second messengers: IP\(_3\) and diacylglycerol. IP\(_3\) causes Ca\(^{2+}\) release from internal stores, which causes depletion of internal Ca\(^{2+}\) stores. The SOCE pathway is activated by internal Ca\(^{2+}\) store depletion; the major functions of the SOCE pathway are believed to be replenishment of the empty stores, and maintenance of sustained increases in cytoplasmic [Ca\(^{2+}\)] that are necessary for signalling downstream.
to the nucleus.11 In this study, we showed that Ang II-induced SOCE in synthetic VSMCs display classical pharmacological features of SOCE. Depletion of internal Ca\(^{2+}\) stores induced the ER/SR-resident Ca\(^{2+}\) store sensor, Stim1, to translocate to areas near the PM to signal the activation of SOC channels encoded by Orai1 proteins. Stim1 and Orai1 are therefore critical for Ang II-mediated SOCE. However, in this study, we did not observe involvement of Trpc1 in Ang II-induced SOCE despite complete knockdown of its protein expression. Some previous studies have suggested that Trpc1 channels can participate in Ang II-induced SOCE.33,34 These contradictory results may be explained as follows: the variability in subunit complex composition in different cells may confer functional specificity to the SOCE channel. For example, SOCE is mediated by formation of the Trpc1 complex with Trpc3 in human parotid gland ducal cells and hippocampal neuronal cells.35 In this study, we demonstrated the involvement of Stim1 and Orai1 Ang II-induced SOCE in rat aortic synthetic VSMC but the other study implicated Stim1 and Orai1 in human coronary artery smooth muscle cell SOCE. Recently, Potier et al.22 showed that SOCE in rat aortic synthetic VSMCs is mediated by Stim1 and Orai1 independently of other Orai isoforms and Trpc proteins. Zarayskij et al.36 reported normal SOC currents in VSMCs in Trpc1-knockout mice, and questioned the role of Trpc1 as a component of SOCE in smooth muscle. Previous reports have also shown increased Stim1 and Orai1 protein expression and up-regulated SOCE in cultured VSMCs compared with their quiescent freshly isolated counterparts.13 Therefore, the fact must be recognized that cultured VSMCs do not completely resemble VSMCs in situ.

It is clear that increased Ca\(^{2+}\) influx is an important stimulus for VSMC proliferation. In this report, we showed that knockdown of Stim1 or Orai1 inhibited cell proliferation and CREB phosphorylation induced by Ang II in cultured synthetic VSMCs, whereas Trpc1 silencing had no significant effect. These findings suggest that Stim1 and Orai1 are critical for Ang II-induced VSMC proliferation. In addition, we reported that losartan, but not PD123319, inhibited Ang II-induced IP\(_3\)-dependent Ca\(^{2+}\) release, and the decrease in Ca\(^{2+}\) store emptying inhibited Ang II-induced SOCE in VSMCs. These data show that AT1 significantly contributes to Ang II-induced SOCE, and the proliferation of VSMCs.

Our previous studies demonstrated that in vivo silencing of Stim1 in rat balloon-injured vessels inhibits neointima formation. Parmentier et al.37 reported that transient exposure of injured arteries to Ang II leads to acceleration of neointimal growth. Therefore, in this study, we tested whether Stim1 and Orai1 siRNAs decreased neointimal growth induced by Ang II in balloon-injured rat carotid arteries. The results showed that knockdown of Stim1 and Orai1 reduced neointimal thickening in balloon-injured carotid arteries in the presence or absence of Ang II, but knockdown of Trpc1 failed to suppress Ang II-induced neointimal growth in balloon-injured vessels and intimal thickening caused by injury. In addition, Ang II significantly increased neointimal growth in balloon-injured carotid arteries. When compared with the injury alone group, Stim1 and Orai1 protein levels in the left injured carotid increased significantly with Ang II treatment after balloon angioplasty. However, the molecular identity of Ang II-induced Stim1 and Orai1 expression in VSMCs remained unknown. In this report, we showed that in cultured synthetic VSMCs Ang II induced VSMC proliferation via Stim1- and Orai1-mediated SOCE entry. Meanwhile, Ang II increased Stim1 and Orai1 expression in the neointima. This could represent a vicious effect. In fact, we found that the expression of Stim1 and Orai1 in media did not increase further with Ang II treatment, whereas Ang II significantly increased the expression of Stim1 and Orai1 in the neointima. Our data indicate that the up-regulation of Stim1 and Orai1 expression is followed by neointimal growth. Parmentier et al. reported that Ang II did not potentiate neointimal growth by directly stimulating VSMC proliferation, and that Ang II may stimulate the synthesis of VSMC-derived mediators that promote migration of one or more cell types and their subsequent proliferation, leading to acceleration of neointimal growth. Thus, the question of whether Ang II directly induces neointimal growth requires further research.

In summary, we showed that Stim1- and Orai1-mediated SOCE are critical for Ang II-induced VSMC proliferation in vitro, and Stim1 and Orai1 knockdown suppresses Ang II-induced neointimal growth in vivo. Taken together, these studies indicate that Stim1 and Orai1 may be potential therapeutic targets for Ang II-induced cardiovascular disorders.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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