TRPC1 stimulates calcium-sensing receptor-induced store-operated Ca\(^{2+}\) entry and nitric oxide production in endothelial cells

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Abstract. Store-operated Ca\(^{2+}\) entry (SOCE) via store-operated Ca\(^{2+}\) channels (SOCC), encoded by transient receptor potential canonical (TRPC) channel proteins, is an important underlying mechanism regulating intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and various intracellular functions in endothelial cells (ECs). TRPC1, the probable candidate for SOCC, is expressed in ECs. Ca\(^{2+}\)-sensing receptor (CaSR) is functionally expressed in vascular endothelium and is important in Ca\(^{2+}\) mobilization and cardiovascular functions. To date, there have been no reports demonstrating an association between CaSR and TRPC1 in ECs. The present study investigated the effects of TRPC1 on CaSR-induced Ca\(^{2+}\) influx and nitric oxide (NO) production in human umbilical vein ECs (HUVECs). TRPC1 and CaSR proteins in HUVECs were measured by immunostaining and western blot analysis. [Ca\(^{2+}\)]\(_i\) levels were measured using the Fura-2-acetoxymethyl ester method. The indicator 3-amino, 4-aminomethyl-2, 7-difluorescein diacetate was used to measure NO production in HUVECs. The expression of TRPC1 protein in HUVECs was silenced by transfecting HUVECs with small interfering RNA (siRNA) against TRPC1. Although changes in extracellular Ca\(^{2+}\) failed to alter [Ca\(^{2+}\)]\(_i\), in HUVECs, the CaSR agonist spermine increased [Ca\(^{2+}\)]\(_i\), and NO production in HUVECs was diminished in Ca\(^{2+}\)-free medium or following treatment with a CaSR negative allosteric modulator (Calhex231), SOCC inhibitor (MRSi845) or TRPC inhibitor (SKF96365). The spermine-induced increases in [Ca\(^{2+}\)]\(_i\), and NO production were reduced in HUVECs transfected with TRPC1 siRNA. These results suggested that TRPC1 is a primary candidate in forming SOCC that stimulates CaSR-induced SOCE and NO production in HUVECs and is a potential therapeutic target for vascular diseases.

Introduction

Alterations in the cytoplasmic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) impact various processes of the vascular endothelium, and have important roles in the regulation of vascular tone, arterial blood pressure and generation of nitric oxide (NO) (1). The alterations in [Ca\(^{2+}\)]\(_i\), are mediated by two primary mechanisms: Ca\(^{2+}\) release from intracellular stores and Ca\(^{2+}\) influx across the plasma membrane via various pathways (2). The dominant mechanism in non-excitable cells is via store-operated Ca\(^{2+}\) entry (SOCE), which is mediated by store-operated calcium channels (SOCCs) (3). SOCE is induced by the activation of phospholipase C by G protein-coupled receptors including Ca\(^{2+}\)-sensing receptor (CaSR), which leads to the production of inositol 1,4,5-trisphosphate (IP3). The subsequent release of Ca\(^{2+}\) from the endoplasmic reticulum (ER) triggers Ca\(^{2+}\) influx by capacitative Ca\(^{2+}\) entry (CCE). Members of the canonical subgroup of transient receptor potential (TRP) proteins constitute tetramers of SOCC (4).

The CaSR is part of an intricate network of calcium channels, pumps and exchangers involved in the control of [Ca\(^{2+}\)]\(_i\), and thereby in the modulation of cardiovascular functions (5). Abnormal Ca\(^{2+}\) handling within blood vessels may contribute to inappropriate contraction, a primary symptom of hypertension. The understanding of how intracellular Ca\(^{2+}\) is regulated

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under physiological and pathophysiological situations forms an important aspect of the search for novel therapeutic targets for the treatment of hypertension. In recent years, major advances in the understanding of Ca\(^{2+}\) homeostasis have been driven in part by the identification of TRP canonical (TRPC) as critical regulators of Ca\(^{2+}\) influx in numerous tissue types (4). It has been reported that TRPC1 is a probable contributor to the formation of SOCC in endothelial cells (ECs) (6), and that TRPC1-mediated Ca\(^{2+}\) entry contributes to the thrombin-induced increase in endothelial permeability (7).

The results from our previous study demonstrated that the CaSR agonist, spermine, stimulated increases in [Ca\(^{2+}\)]\(_e\) and NO production in human aortic ECs (HAECs) (8). However, the molecular mechanisms underlying activation of Ca\(^{2+}\) influx channels by CaSR, their involvement in extracellular Ca\(^{2+}\) influx and their role in CaSR-induced NO production in vascular ECs remain to be elucidated. The present study hypothesized that TRPC1 contributes to CaSR-induced SOCE and NO production in human umbilical vein ECs (HUVECs).

**Materials and methods**

**Materials.** Fetal bovine serum (FBS) was obtained from HyClone; GE Healthcare Life Sciences (Logan, UT, USA), and all other cell culture reagents were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Spermine (a CaSR agonist), Calhex231 (a CaSR negative allosteric modulator), MRS1845 (a SOCC inhibitor) and SKF96365 (a TRPC inhibitor) were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Rabbit anti-TRPC1 monoclonal antibody (catalog no. ACC-010) was obtained from Alomone Laboratories, Ltd. (Jerusalem, Israel). Polyclonal mouse anti-human CaSR antibody was obtained from Abcam (Cambridge, MA, USA; catalog no. ab62653, for western blotting) and from Shanghai Seebio Science & Technology Co., Ltd. (Shanghai, China; catalog no. HL1499 for immunohistochemistry) and other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Small interfering RNA (siRNA) was purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Rabbit anti-mouse IgG (catalog no. BA1101) or TRITC-goat anti-rabbit antibody (catalog no. BA1090) conjugated secondary antibodies (1:30; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min. Cells were washed and examined at green and red wavelengths under a Bio-Rad MRC 1000 confocal microscope (Sanyo Electric Co., Ltd., Moriguchi, Japan). For each experiment, >50 cells were recorded.

**Cell culture.** HUVECs were harvested by 0.25% pancreatin digestion from normal human umbilical cords. The protocol was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The EC culture medium was supplemented with 10% FBS, 50 mg/l EC growth supplement (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), 50 mg/l heparin, 100,000 U/l penicillin and 100 mg/l streptomycin. Cells at passage 3-4 were used in the experiments as described previously (9).

**Drug treatment.** Cells were treated with various concentrations of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_e\)) by sequentially adding 0.5, 2, 4 and 10 mM Ca\(^{2+}\) to cells in a perfusion chamber and monitoring for 5 min at each concentration. For spermine treatment, cells were divided into 6 groups: Spermine, in which cells were monitored for 1 min with Ca\(^{2+}\)-free 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-buffered saline (HBS) of the following composition: 140 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1.2 mM NaHPO\(_4\), 10 mM D-glucose and 20 mM HEPES (pH 7.40), followed by 20 min of continuous perfusion with 2 mM spermine in Ca\(^{2+}\)-free HBS; spermine + Ca\(^{2+}\), in which HBS containing 2 mM Ca\(^{2+}\) was used; Calhex231 + spermine, in which cells were monitored for 1 min with Ca\(^{2+}\)-free HBS, followed by perfusion for 1 min with 1 mM Calhex231 in Ca\(^{2+}\)-free HBS and continuous perfusion for 20 min with 1 mM Calhex231 and 2 mM spermine in Ca\(^{2+}\)-free HBS solution; Calhex231 + spermine + Ca\(^{2+}\), in which HBS containing 2 mM Ca\(^{2+}\) was used; MRS1645 + spermine + Ca\(^{2+}\), in which 5 µM MRS1645 was used instead of Calhex231; and SKF96365 + spermine + Ca\(^{2+}\), in which 5 µM SKF96365 was used instead of Calhex231.

**Immunostaining of CaSR and TRPC1 proteins.** Cultured HUVECs on coverslips were fixed with 95% ice-cold ethanol for 10 min at room temperature and permeabilized with 0.1% Triton X-100 solution in PBS for 10 min at room temperature. The nonspecific binding sites were blocked with 10% goat serum in PBS for 30 min at room temperature. Fixed cells were treated with primary antibodies against CaSR (1:200) or TRPC1 (1:50) for 1 h at room temperature. Following washing, cells were stained with fluorescein isothiocyanate-goat anti-mouse IgG (catalog no. BA1101) or TRITC-goat anti-rabbit (catalog no. BA1090) conjugated secondary antibodies (1:30; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 30 min. Cells were washed and examined at green and red wavelengths under a Bio-Rad MRC 1000 confocal microscope (Sanyo Electric Co., Ltd., Moriguchi, Japan). For each experiment, >50 cells were recorded.

**Western blot analysis.** Cells were rinsed twice with ice-cold PBS and harvested in cell lysis solution (Beijing Biodetech Scientific & Technical Co., Ltd., Beijing, China). Protein concentration was measured using a Bicinchoninic Acid assay kit (Abcam, Shanghai, China) Equal quantities of protein (40 µg) were run on a 10% SDS-PAGE gel and subsequently transferred onto a polyvinylidene difluoride membrane by electroblotting (100 V for 1.5 h). Following incubation with 5% non-fat milk in TBS containing Tween 20 for 2 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against human CaSR (1:500) or TRPC1 (1:200). Goat anti-mouse (1:10,000; catalog no. bs-0296G) or goat anti-rabbit (1:8,000; catalog no. bs-0295G) IgG horse-radish peroxidase-conjugated secondary antibodies (Wuhan Boster Biological Technology, Ltd.) were added to membranes for 1 h. Protein was visualized using an enhanced chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc.). Intensities of the protein bands were quantified using Bio-Rad Quantity One software (version, 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**TRPC1 knockdown by siRNA.** To evaluate the functional role of TRPC1 in CaSR-induced Ca\(^{2+}\) influx and NO production in HUVECs, siRNA was used to reduce TRPC1 expression. For human TRPC1, the sense strand siRNA, 5'-GCG ACA
AGGGUGACUAUUAdTd-3' and antisense strand siRNA, 3'-dTdTTCGCUGUUCCACUGAUAU-5' were used. The selective siRNA duplex and a nonspecific control duplex were obtained from Yangzhou Ruibo Biotech Co., Ltd. Transfection of siRNA into HUVECs was performed using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol. Briefly, cultured cells were washed with Opti-Minimal Essential Medium without serum or antibiotics and seeded in 6-well plates to 30-40% confluence (typically 1x10^5 cells/35-mm plate incubated at 37˚C for 48 h). The transfection reagent and siRNA were diluted separately in serum-free media, mixed and incubated for 10 min at room temperature to form the siRNA/lipid complex. This complex was then added to each well at a final concentration of 70 nM/well of siRNA. At 48 h after transfection, cells were collected to determine TRPC1 protein expression levels by western blot analysis.

Measurement of NO production. NO production was measured with the membrane-permeable indicator dye 3-amino, 4-aminomethyl-2,7-difluorescein (DAF-FM) diacetate (Beyotime Institute of Biotechnology, Shanghai, China), a fluorescent dye sensitive to NO levels (10). A monolayer of HUVECs was seeded onto coverslips and loaded with 0.5 nM DAF-FM in HBS at 37˚C for 30 min in the dark, followed by incubation with DAF-FM-free HBS for an additional 20 min to allow for de-esterification of the indicator. DAF-FM fluorescence was monitored on the aforementioned fluorescence microscopy system at an excitation wavelength of 480±10 nm and an emission wavelength of 510±10 nm. To validate the use of DAF-FM fluorescence as an index of the production of cellular NO induced by agonists, HUVECs were pre-incubated with L-arginine and the specific NO synthase (NOS) inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 1 mM), as described previously (10). For experiments involving a...
Ca\textsuperscript{2+}-free system, Ca\textsuperscript{2+}-free/ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) solution was made by substituting CaCl\textsubscript{2} with 1 mM EGTA in HBS.

**Statistical analysis.** Statistical analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Multiple comparisons were performed using a one-way analysis of variance or the Kruskal-Wallis test, followed by the Student-Newman-Keuls post hoc test or unpaired Student’s t-test. Data are presented as mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Localization of CaSR and TRPC1 expression in HUVECs.** To determine whether the CaSR and TRPC1 are expressed in HUVECs, immunocytochemical analysis with confocal microscopy was performed using polyclonal or monoclonal antibodies against human CaSR and TRPC1. Green fluorescent stained CaSR was localized in the cytosol (Fig. 1A). Red fluorescent stained TRPC1 was localized in the plasma membrane (Fig. 1B). To exclude any non-specific binding, the secondary antibodies alone were used for staining. No immunostaining of the secondary antibodies was observed (Fig. 1C and D). To confirm the expression of CaSR and TRPC1 proteins in HUVECs and the stimulatory effect of spermine on CaSR and TRPC1 expression, western blot analysis was performed using cell lysates isolated from the cytoplasm. The results demonstrated that CaSR and TRPC1 were expressed and that spermine treatment (2 mM, 48 h) did not alter the protein expression levels of CaSR and TRPC1 in HUVECs (Fig. 1F).
CaSR-induced SOCE activation and NO production by TRPC. To demonstrate the presence and function of CaSR in HUVECs, Fura-2AM-loaded HUVECs were stimulated by various known CaSR agonists, including extracellular Ca\(^{2+}\) and spermine (Fig. 2). Treatment with 0.5 to 10 mM [Ca\(^{2+}\)], did not cause any detectable alterations in [Ca\(^{2+}\)]\textsubscript{i} in Fura-2AM-loaded HUVECs (n=7 each; Fig. 2A). Thus, although CaSR is expressed in HUVECs, it does not appear to respond to extracellular Ca\(^{2+}\) as an agonist under these experimental conditions. However, spermine, the other CaSR agonist assessed, induced an increase in [Ca\(^{2+}\)]\textsubscript{i} in HUVECs (P=0.003; Fig. 2B and I) at a concentration (2 mM) that stimulated the CaSR in other cell types (11). Spermine evoked a rapid rise of [Ca\(^{2+}\)]\textsubscript{i}, followed by an elevated plateau phase, which potentially reflected the activation of SOCE. To determine the Ca\(^{2+}\) source in spermine-stimulated HUVECs, cells were treated with 2 mM spermine in Ca\(^{2+}\)-free/EGTA HBS. Spermine stimulated an increase in [Ca\(^{2+}\)]\textsubscript{i} in HUVECs (P=0.002; Fig. 2C) that was reduced compared with that observed in 2 mM [Ca\(^{2+}\)]\textsubscript{o} (P=0.001; Fig. 2I). Spermine resulted in a transient increase in [Ca\(^{2+}\)]\textsubscript{i}, followed by a rapid decay to baseline, which reflected IP3-mediated Ca\(^{2+}\) release from the ER. These results suggested that spermine induced extracellular Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\) release in HUVECs.

To determine whether SOCE responds to spermine stimulation, Fura-2AM-loaded HUVECs were pretreated with 1 mM Calhex231, a highly selective CaSR negative allosteric modulator (12), for 2 min and stimulated with 2 mM spermine in 2 mM [Ca\(^{2+}\)]\textsubscript{o}, medium (Fig. 2D). Calhex231 inhibited the spermine-induced increase in [Ca\(^{2+}\)]\textsubscript{i} in HUVECs (P=0.0001; Fig. 2I). In Ca\(^{2+}\)-free/EGTA media, Calhex231 abrogated spermine-induced changes in [Ca\(^{2+}\)]\textsubscript{i} (P=0.00002; Fig. 2E and I). These findings suggested that SOCE may be triggered by CaSR activation in HUVECs.

To assess the behavior of channels involved in the spermine-induced Ca\(^{2+}\) entry, the effects of SOCC and TRPC nonselective cation channel blockers were examined. Pretreatment with 5 µM MRS1845 (P=0.002) or SKF96365 (P=0.003) significantly inhibited spermine-induced Ca\(^{2+}\) influx (Fig. 2F, G and I). These results suggested that the Ca\(^{2+}\) influx triggered by CaSR activation in HUVECs is associated with TRPC and opening of SOCC.

The production of NO was measured in spermine-treated HUVECs using the fluorescent NO indicator DAF-FM. The spontaneous increment in DAF-FM fluorescence was 281.4±13.7 (n=7) at 20 min (Fig 2J) without Ca\(^{2+}\) mobilizing agents, which indicated the baseline NO production rate. Spermine (2 mM) in Ca\(^{2+}\)-free medium stimulated a significantly slower increase in DAF-FM fluorescence (P=0.004) than in 2 mM Ca\(^{2+}\) medium (P=0.004; Fig. 2J), suggesting that an elevation in [Ca\(^{2+}\)]\textsubscript{o}, by store depletion or CCE, may induce NO production in HUVECs. When 1 mM L-NAME, a selective inhibitor of NOS, was added to 2 mM Ca\(^{2+}\) (P=0.003) or Ca\(^{2+}\)-free media (P<0.0001; Fig. 2J). Pretreatment with 5 µM MRS1845 (P=0.003) or 5 µM SKF96365 (P=0.003) in 2 mM Ca\(^{2+}\) media resulted in inhibition of the spermine-induced increase in DAF-FM fluorescence (Fig. 2J). The results suggested that, via TRPC, CaSR-mediated activation of SOCE stimulates NO production in HUVECs.

TPRC1 serves an important role in CaSR-induced SOCE activation and NO production in HUVECs. It has been previously demonstrated that TRPC1 and/or TRPC4 may function as components of SOCC (13). To determine whether TRPC1 contributes to the response to spermine, selective TRPC1 siRNA was introduced into HUVECs. Western blot analysis demonstrated that TRPC1 siRNA decreased the expression levels of TRPC1 protein by >74% (P=0.0012; Fig. 4A). These results indicated that the SOCE and
NO production triggered by CaSR activation in HUVECs are mediated by TRPC1.

**Discussion**

CaSR was cloned from bovine parathyroid cells in 1993 (14), and it has since been demonstrated that CaSR serves a key physiological role in the cardiovascular system by modulating arterial blood pressure (15). However, the precise mechanisms underlying CaSR-mediated [Ca$^{2+}$]i homeostasis in vascular ECs remain to be elucidated. In HUVECs transfected with selective CaSR siRNA, our previous study revealed that CaSR is important in spermine-evoked Ca$^{2+}$ influx and NO production (16). The present study demonstrated that CaSR activation mediated Ca$^{2+}$ entry through SOCC, and that TRPC1-encoded SOCC was involved in the CaSR-mediated [Ca$^{2+}$]i increase in HUVECs. In addition, the present study demonstrated a role for CaSR in regulating Ca$^{2+}$ entry via SOCC that advances the understanding of the molecular mechanisms underlying CaSR-mediated [Ca$^{2+}$]i increases in human blood vessels. This pattern of Ca$^{2+}$ entry has previously been reported in various cell types, including neonatal ventricular myocytes and the Saos-2 human osteosarcoma cell line following CaSR agonist application (17,18). In these experiments, functionally expressed CaSRs were revealed to respond to extracellular Ca$^{2+}$ as an agonist (17-19) whereas in the present study, a similar protocol did not induce a significant increase in [Ca$^{2+}$]i in HUVECs. This is consistent with our previously published observation in HAECs, in which spermine was the only known CaSR agonist that induced an increase in [Ca$^{2+}$]i in HAECs (8), suggesting that the response of CaSR to agonists may exhibit a cell preference. Generation of NO requires an elevation of [Ca$^{2+}$]i. Although NOS is tightly controlled by multiple underlying mechanisms, including the interaction of endothelium NOS (eNOS) with effector proteins such as heat shock protein 90 and caveolin-1, the elevation of [Ca$^{2+}$]i remains the primary initiator of NO production. However, the results in ECs remain controversial. Certain reports have demonstrated a synergism between NO synthesis and sustained [Ca$^{2+}$]i levels (20), whereas others emphasize a primary role of Ca$^{2+}$ influx (21) or Ca$^{2+}$ release from thapsigargin-sensitive store sites (22). In the present study, [Ca$^{2+}$]i increase and NO production were stimulated by spermine in the presence or absence of extracellular Ca$^{2+}$ in HUVECs. Therefore, it appears that eNOS does not discriminate between the sources of mobilized Ca$^{2+}$ to stimulate NO production. However, the present study revealed that NO production is preferentially linked to Ca$^{2+}$ entry by SOCC activation at comparable stimulation, including 2 mM spermine, indicated by a slight increase in DAF-FM fluorescence in Ca$^{2+}$-free solution, and a marked increase in [Ca$^{2+}$]o in HUVECs. The dependence of CaSR-induced NO production on SOCE was
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