Sildenafil Inhibits Human Pulmonary Artery Smooth Muscle Cell Proliferation by Decreasing Capacitative Ca\(^{2+}\) Entry

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Abstract. Ca\(^{2+}\) is a pivotal signal in human pulmonary artery smooth muscle cells (PASMCs) proliferation. Capacitative Ca\(^{2+}\) entry (CCE) via the store-operated channel (SOC), which encoded by the transient receptor potential (TRP) gene, is an important mechanism for regulating intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in PASMCs. Sildenafil, a potent type 5 nucleotide-dependent phosphodiesterase (PDE) inhibitor, has been proposed as a therapeutic tool to treat or prevent pulmonary arterial hypertension (PAH); however, the mechanism of its antiproliferative effect on PASMCs remains unclear. This study was designed to investigate the possible antiproliferative mechanism of sildenafil on human PASMCs, namely, its effect on the Ca\(^{2+}\)-signal pathway. Cultured normal PASMCs were treated with endothelin-1 (ET-1) or ET-1 plus sildenafil separately. Cell number and viability were determined with a hemocytometer or MTT assay. [Ca\(^{2+}\)]\(_i\) was measured by loading PASMCs with fura 2-AM. Expression of the TRPC1 gene and protein was detected by RT-PCR and Western blot, respectively. The results show that sildenafil dose-dependently inhibited the proliferation of PASMCs, the enhancement of basal [Ca\(^{2+}\)]\(_i\) level, increase of CCE, and upregulation of TRPC expression induced by ET-1. These results suggest that sildenafil potently inhibits ET-1–induced PASMCs proliferation and down-regulation of CCE and TRPC expression may be responsible for its antiproliferative effect.

Keywords: store-operated channel, capacitative Ca\(^{2+}\) entry, transient receptor potential channel, pulmonary artery smooth muscle cell

Introduction

The development of pulmonary arterial hypertension (PAH) involves a complex constellation of multiple genes and molecules, which interact with each other and subsequently activate intracellular signaling pathways that eventually result in pulmonary remodeling. The final manifestation of vascular remodeling is characterized largely by medial hypertrophy due to enhanced pulmonary arterial smooth muscle cell (PASMC) proliferation and attenuated apoptosis that will lead to lumen obliteration in the end (1, 2).

Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) as the vital intracellular ubiquitous second messenger induces the hyper-proliferation response, in addition to being the fundamental regulator of contractile machinery in vascular smooth muscle cells, via its effects on transcription factors, mitogens, and cell cycle components. Elevated [Ca\(^{2+}\)]\(_i\) in PASMCs had been observed in the PAH animal model and patients, which is the key triggering point for cell proliferation. There are mainly two kinds of calcium channels in human PASMC membrane: i) voltage-dependent calcium channels (VDCC) that open in response to membrane depolarization and ii) voltage-independent calcium channels (VICC) that include receptor-operated channels (ROC) and store-operated channels (SOC) and can be activated at normal membrane potential with membrane-receptor binding to growth

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factors and mitogenic agonists (ROC) or by Ca\(^{2+}\)-store depletion (SOC). Ca\(^{2+}\) influx via VDCC represents the major pathway in most excitable cells and muscle cells. However, Ca\(^{2+}\) influx via VICC (especially SOC and ROC) can also be a determinant in the regulation of vascular tone and arterial wall structure (3). In particular, Ca\(^{2+}\)-store depletion–mediated Ca\(^{2+}\) entry via SOC in PASMCs has been demonstrated to be involved in the pulmonary vascular remolding during PAH.

Endothelin-1 (ET-1) has been implicated in the pathogenesis of pulmonary hypertension. Its levels correlate closely with the severity of pulmonary vascular remodeling and PASMCs proliferation by increasing the [Ca\(^{2+}\)]. ET-1 binds to G protein–coupled receptors (GPCR) in PASMC membrane, activates phospholipase C (PLC), and produces the secondary messengers (GPCR) in PASMC membrane, activates phospholipase C (PLC), and produces the secondary messengers (IP3) and diacylglycerol (DAG). IP3-induced Ca\(^{2+}\) release from ER produces a transient increase in [Ca\(^{2+}\)]. The subsequent depletion of intracellular Ca\(^{2+}\) stores triggers a sustained Ca\(^{2+}\) flux called capacitative calcium entry (CCE). The store-operated Ca\(^{2+}\) entry (SOCE) via SOC, which is caused by ER depletion, is the dominated component of CCE (4). Transient receptor potential (TRP) channels, a large superfamily of channels permeable to Ca\(^{2+}\), have been progressively identified and characterized to be the SOC candidates. The involvement of TRPC1 in SOCE and its contribution to the development of pulmonary vascular remodeling has been demonstrated in human PASMCs in PAH patients.

Sildenafil, a potent and selective phosphodiesterase type 5 (PDE5) inhibitor, has been proposed as a novel strategy for the treatment of pulmonary hypertension (5). Sildenafil exerts its pharmacological effect by increasing the intracellular concentration of cGMP (6). The mechanism involved in the therapeutic benefits has been attributed to its ability to induce acute pulmonary vascular vasodilatation and its antiproliferative effect. In the chronic hypoxia PAH rat model, sildenafil dose-dependently inhibited the [Ca\(^{2+}\)] response induced by GPCR agonists such as angiotensin II in PASMCs. The relaxant effect of sildenafil appears mainly related to action on calcium signaling via the IP3-dependent calcium release pathway and calcium reuptake mechanisms (7, 8). However, the mechanism of its antiproliferative effect in PASMCs has not been studied.

Therefore, the current study was designed to test the hypothesis that PASMC proliferation mediated by ET-1, which plays a substantial role in the development of pulmonary hypertension and subsequent pulmonary remodeling, is involved in the upregulation of TRPC1 channel expression and CCE function. The antiproliferative effect of sildenafil is due, at least, partially to its down regulatory effect on TRPC1 channels and subsequently decreasing of the CCE function.

Materials and Methods

Cell preparation and culture

Human PASMCs from normal subjects were purchased from Cascade Biologics, Inc. (Portland, OR, USA) and used at passage 4 – 9. PASMCs were cultured in smooth muscle growth medium (SMGM), which consists of smooth muscle basal medium and smooth muscle growth supplement (SMGS) (Cascade Biologics, Inc.). SMGS, in final concentration, contains 4.9% fetal bovine serum (FBS), 2 ng/ml basic fibroblast growth factor, 0.5 ng/ml epidermal growth factor, 5 ng/ml heparin, 5 mg/ml insulin, and 0.2 mg/ml bovine serum albumin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO\(_2\) in air (21% O\(_2\), 74% N\(_2\)) and passaged by trypsinization with trypsin (0.25%; Sigma-Aldrich, St. Louis, MO, USA) – EDTA (0.02%; Amresco, Solon, OH, USA) after 80% – 90% confluence. The growth of the cells was arrested by replacing SMGM with FBS-free SMBM for 24 h, and then the cells were incubated with low-serum SMBM (2% FBS), ET-1, or ET-1 + sildenafil separately for 72 h.

Determination of cell proliferation

Cell proliferation was quantified by directly counting the cell number and MTT assay. Briefly, PASMCs were seeded in 24-well microplates at a density of 1 × 10\(^4\) cells/well. Cell number was determined with a hemocytometer by using 0.45% trypan blue (Sigma-Aldrich, USA). For MTT measurement, cells were plated into 96-well microplates at the concentration of 5 × 10\(^3\) cells/well and treated with the different drugs as described above. After incubation, 20 μl of the MTT reagent was added to each well and the multiwell plates were incubated in a humidified atmosphere for 4 h. Then the supernatant was removed from each well and 150 μl/well of dimethyl sulfoxide added (DMSO; Sigma-Aldrich, L’Isle d’Abeau, France) to solubilize the formed formazan salt crystals. The solubilized formazan product was spectrophotometrically quantified at 570 nm using an ELISA reader (Biorad, Hercules, CA, USA). Data were expressed as % of control.

Measurement of [Ca\(^{2+}\)].

PASMCs were seeded on the coverslips for the calcium imaging experiment. [Ca\(^{2+}\)], in a single cell was measured using the Ca\(^{2+}\)-sensitive fluorescent indicator fura 2-AM (Invitrogen, Carlsbad, CA, USA). Cells were loaded with 3 μM fura 2-AM for 30 min in the dark at
room temperature and then transferred to glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA), fixed on a microscope stage and superfused with physiological salt solution (PSS) for 30 min to remove the extracellular fura 2-AM and to activate intracellular fura 2-AM. [Ca$^{2+}$], of individual cells was measured using an xenon lamp (Lambda DG4; Sutter Instrument Company, Novato, CA, USA) equipped with an epifluorescence microscope (TE2000-U; Nikon, Tokyo) and band-pass filters for wavelengths of 340 and 380 nm. Based on the equation: \[ [\text{Ca}^{2+}] = K_d \times (S_{2+} / S_{2+}) \times (R - R_{\text{min}}) / (R_{\text{max}} - R) \]
the [Ca$^{2+}$]$_i$ of individual cells was measured by the ratio (R) of 340/380 nm fluorescence images (9). Resting [Ca$^{2+}$]$_i$, cyclopiazonic acid (CPA; Sigma-Aldrich, Rehovot, Israel)-induced ER Ca$^{2+}$ release and SOC-mediated Ca$^{2+}$ entry, upon changing perfusion from Ca$^{2+}$-free PSS to 1.8 mM Ca$^{2+}$ PSS, were measured in three groups. In most experiments, multiple (5 – 10) cells were imaged in a single field, and a selected peripheral cytosolic area from each cell was analyzed.

**RT-PCR**

Total RNA was isolated from PASMCs by using TRIzol reagent (Sigma-Aldrich, USA) according to the manufacturer’s instructions. RNA was reverse-transcribed to synthesize first-strand cDNA. The specific primers were designed from the coding regions of human TRPC1 (forward primer: 5’-CAAGATTTTGG AAAATTTCTTG-3’, reverse primer: 5’-TTTGTCTTC ATGATTTGCTAT-3’). As a control for the integrity of RNA, the primers of glyceraldehydes phosphate dehydrogenase (GAPDH) were used (forward primer: 5’-ACCACAGTCCATGGCATAC-3’ and reverse primer: 5’-TCCACCACTCTTGCTGTGTA-3’). PCR was performed by iCycler Thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: the PCR reaction mixture was denatured at 94°C (0.5 min), annealed at 55°C (0.5 min), and extended at 72°C (0.5 min) for 30 cycles. This was followed by a final extension at 72°C (5 min) to ensure complete product extension. Amplified products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. PCR product bands were visualized by UV light, and the intensity values were measured by densitometric analysis with the Quantity One programme (Bio-Rad) and normalized to the intensity values of GAPDH for quantitative comparisons. The PCR product was sequenced, and the amplified production of TRPC1 and GAPDH were 371 and 452 bp, respectively.

**Western blotting**

At the end of the incubation periods, cells were rinsed twice with ice-cold PBS and harvested in cell lysis solution (BioDev-Tech. Company, Beijing, China), then protein was extracted. The protein concentrations were determined by the Bradford assay. The extracts were diluted in 5 × loading buffer [Tris · HCl 0.25 M (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, and 0.5 M dithiothreitol] and heated at 95°C for 5 min. TRPC1 protein was detected using a standard Western blot protocol (“Molecular Cloning”, the second edition). Briefly, 40 μg proteins were separated by 8% SDS-PAGE at 80 V for 0.5 h and 120 V for 1.5 h and then transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA) at 4°C, 100 V for 1.5 h by a Western blot apparatus (Bio-Rad). The transferred membrane was washed for 10 min in TTBS [20 mM Tris · HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20] and then blocked with 10% skimmed milk for 1 h at room temperature. The blocked membrane was incubated with a primary antibody against TRPC1 (dilution 1:1000; Alomone Labs, Jerusalem, Israel) and actin (dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, respectively. After incubation with the horseradish peroxidase–conjugated secondary antibody (dilutions of 1:2000; Beijing Zhong Shan-Golden Bridge Biological Technology Company, Beijing, China) for 1 h at room temperature, the immunoblotting signals were visualized by using a Western Luminescent Dection kit (Vigorous Biotechnology, Beijing, China). The results were quantified by densitometry and the density of immunoblotting was analyzed by scanning X-ray film with Quantitative One software. The value of the relative density of the TRPC1 band was normalized to the density of actin to represent the amount of TRPC1 protein. The ratio of the Blank group was regarded as 100%, and the ET-1 and ET-1 + Sil groups results were expressed as a percentage of the value from Blank group.

**Drugs and reagents**

The PSS contained: 141 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, 10 mM HEPES, and 10 mM glucose (pH 7.4). For Ca$^{2+}$-free PSS, CaCl$_2$ was replaced by equimolar MgCl$_2$, and 1 mM EGTA was added to chelate the residual Ca$^{2+}$ (10). CPA, fura 2-AM, SKF96365 (SKF; Sigma-Aldrich, USA) and nifedipine (Nif; Sigma-Aldrich, Shanghai, China) were dissolved with DMSO to make stock solutions. Sildenafil from Pfizer Global Research and Development (Sandwich, Kent, UK) was dissolved in distilled water (pH 5.3 with HCl).

**Statistical analysis**

Data are each presented as the mean ± S.E.M. All experiments were performed at least with six independent
PASMC cultures. Comparisons between groups of data evaluated via the unpaired Student’s t-test. Differences were considered to be statistically significant when $P<0.05$.

**Results**

SOCE plays an essential role in ET-1–induced PASMCs proliferation

The mitogenic effect of ET-1 on human PASMCs was first examined. Figure 1A shows the time course of PASMCs proliferation mediated by ET-1 (1 μM). The cultured PASMCs were in the exponential growth phase from the 2nd to 5th day. ET-1 (0.01 – 1 μM) increased cell proliferation in a dose-dependent manner after 72-h treatment. At 1 μM, ET-1 significantly increased the cell number by 47% (Fig. 1B).

Chelating the extracellular Ca$^{2+}$ by EDTA (2 mM) or blocking the SOCE by SKF (10 μM), a blocker of SOC and GdCl$_3$ (1 μM), significantly inhibited ET-1–induced PASMCs proliferation compared with the basal group. Whereas, nifedipine (1 μM), a blocker of VDCC, has no obviously inhibitory effect on ET-1–induced cell proliferation. These data suggested that extracellular Ca$^{2+}$ is the vital source of intracellular Ca$^{2+}$ supply to regulate cell growth, and sustained extracellular Ca$^{2+}$ entry via SOC is the main pathway of maintaining the high [Ca$^{2+}$]$_i$ in PASMCs, whereas VDCC-mediated Ca$^{2+}$ entry has little effect on PASMCs growth (Fig. 1C).

Sildenafil inhibits ET-1–mediated human PASMC proliferation

As shown in Fig. 2, sildenafil markedly inhibited ET-1 (1 μM)-mediated increase of PASMC number and viability. Since at the concentration of 100 nM sildenafil reduced the cell number to approximately the unstimulated level, in all subsequent experiments, this concentration was used as the appropriate inhibitory dose as previously described (7, 8). The observed antiproliferative effect of sildenafil was not due to a toxic effect because PASMC viability, assessed by trypan blue exclusion, was not affected by sildenafil.

Sildenafil inhibits ET-1–mediated increase of [Ca$^{2+}$]$_i$ in PASMCs

Having proved that sildenafil could inhibit ET-1–mediated PASMCs proliferation, we next attempted to identify the effect of sildenafil on the change of [Ca$^{2+}$]$_i$ evoked by ET-1 incubation. Perfusion with Ca$^{2+}$-free PSS containing 10 μM CPA, a blocker of SR Ca$^{2+}$-Mg$^{2+}$ ATPase, triggered a transient [Ca$^{2+}$]$_i$ rise in human PASMCs (Fig. 3A) due to leakage of Ca$^{2+}$ from the SR to the cytosol. The CPA-induced [Ca$^{2+}$]$_i$ transient declined back to the original baseline level after 5 – 10 min as the SR Ca$^{2+}$ was depleted. Subsequent restoration of extracellular [Ca$^{2+}$] to 1.8 mM induced a rise in

![Fig. 1. ET-1–mediated human PASMCs proliferation. A: Time course of ET-1 (1 μM)-induced PASMCs proliferation. B: Cell numbers were determined before (Basal) and after 72-h incubation in SMBM (2% FBS) with or without (Blank) ET-1 (0.01 – 1 μM), n = 7, *$P<0.05$ vs Blank, ***$P<0.001$ vs Blank. C: Effect of EDTA, nifedipine, and SKF96365 on ET-1–induced human PASMC proliferation. Cell numbers were determined before (Basal) and after 72-h incubation with SMBM (2% FBS, Blank) without ET-1 (Blank) or with ET-1 (1 μM), ET-1 + EDTA (2 mM), ET-1 + nifedipine (1 μM), and ET-1 + SKF96365 (10 μM), ET-1 + GdCl$_3$ (1 μM). n = 9, ##$P<0.01$ vs Blank, ###$P<0.001$ vs ET-1.](image-url)
[Ca$^{2+}$]$_i$ again, which was obviously due to CCE (Fig. 3A). As shown in Fig. 3B, compared with the blank group, the PASMCs incubated with ET-1 for 72 h have significantly higher resting [Ca$^{2+}$]$_i$ level (0.641 ± 0.013 vs 0.899 ± 0.064, $P<0.001$), higher CPA-induced [Ca$^{2+}$]$_i$ transient peak due to Ca$^{2+}$ release from the SR (1.161 ± 0.034 vs 1.371 ± 0.0367, $P<0.001$), and higher [Ca$^{2+}$]$_i$ peak increase due to CCE (1.235 ± 0.061 vs 2.020 ± 0.069, $P<0.001$). After the PASMCs were treated with 100 nM sildenafil, ET-1-mediated increase
of resting \([\text{Ca}^{2+}]_i\), CPA-induced \([\text{Ca}^{2+}]_i\), release peak, and CCE were partly decreased (resting \([\text{Ca}^{2+}]_i\) from 0.899 ± 0.064 to 0.645 ± 0.014, \(P<0.001\); CPA-induced peak from 1.371 ± 0.037 to 1.173 ± 0.038, \(P<0.001\); CCE from 2.020 ± 0.069 to 1.320 ± 0.057, \(P<0.001\)).

Since resting \([\text{Ca}^{2+}]_i\), SOC and CCE play an essential role in ET-1–induced cell proliferation, these results suggest that sildenafil may exert its antiproliferative effect by inhibiting the SOC-mediated \([\text{Ca}^{2+}]_i\) influx, which influences the resting \([\text{Ca}^{2+}]_i\) and CCE.

**Sildenafil inhibits ET-1–mediated upregulation of TRPC1 expression level in PASMCs**

It has been reported that TRPC1 protein was the subunit of SOC in human PASMCs and its activity and expression level can affect SOCE. Inhibition of TRPC channel expression inhibited PASMC proliferation (11–13). The next set of experiments was designed to test whether the antiproliferative effect of sildenafil is related to the attenuation of TRPC channel expression. As shown in Fig. 4A, the upregulation of mRNA expression level of TRPC1 induced by ET-1 was partly decreased by sildenafil (Fig. 4: A and B), and the ET-1–induced increase of TRPC1 protein expression in PASMCs was also partially inhibited by sildenafil (Fig. 5). These data indicate that sildenafil inhibited TRPC1 expression at both the transcription and translation level. Diminished \([\text{Ca}^{2+}]_i\) influx via SOC due to TRPC1 expression decrease may be the mechanism involved in sildenafil-mediated antiproliferative effect.

**Discussion**

PAH is a progressive disease of pulmonary arteries characterized by a sustained increase in pulmonary pressure and vascular remodeling. The typical pathological changes include muscularization and thickening of pulmonary arteries, intimal proliferation, obliterative lesions, and thrombosis in situ (14). Pulmonary vascular remodeling begins primarily with uncontrolled and inappropriate proliferation of human PASMCs (3). Increased production of mitogenic agonists, such as ET-1 in the blood plasma and lung tissues, has been linked to the development of pulmonary vascular remodeling (2). The medical treatment of PAH has been revolutionized by the availability of new therapeutic agents. Sildenafil, a potent type 5 nucleotide-dependent PDE inhibitor, has been used recently as a therapeutic tool to treat or prevent PAH.

Intracellular \([\text{Ca}^{2+}]_i\) is intimately involved in cell proliferation, as an essential factor in steps of the cell cycle and promoting transcription factor binding activity with mitogenic genes. Extracellular \([\text{Ca}^{2+}]_i\) influx is the important mechanism in maintenance of \([\text{Ca}^{2+}]_i\), and...
blockade of Ca\textsuperscript{2+} influx via SOC can decrease ET-1–induced PASMC proliferation, but VDCC play little role in PASMC growth (Fig. 1). ET-1–induced PASMC proliferation is accompanied by significant increase of resting [Ca\textsuperscript{2+}], calcium release from ER and CCE release via SOC in cytoplasm (Fig. 3). Human PASMC overproliferation is concerned with strengthening expression and opening of SOC (15, 16) and can be attenuated by knockdown or inhibition of the TRP isoform (13).

Since TRPC1 channel subunits participate in forming functional SOC, upregulated TRPC1 gene expression in human PASMCs would be predicted to increase the number of functional SOC, enhance vasoconstrictor and mitogen-mediated increases in [Ca\textsuperscript{2+}], stimulate vasoconstriction, and promote cell growth. It also has been demonstrated that TRPC1 is involved in the forming of SOC that contributes to the development of cardiac hypertrophy (17). The upregulation of TRPC1 and increase of CCE were also observed in cardiomyocytes with chronic treatment of a GPCR agonist such as ET-1, angiotensin II, and phenylephrine. Our results suggest that ET-1–induced PASMC proliferation is related to promoting TRPC1-encoded SOC expression on the genetic level, activating the SOC calcium signal pathway, and leading to the enhancement of [Ca\textsuperscript{2+}] ultimately.

PDE and cGMP are important signaling pathways that play pivotal roles in many physiological processes such as vascular tone control, progression of the cell cycle, and so on. Sildenafil, as an orally active, potent, and selective inhibitor of cGMP-PDE5, can elevate intracellular cGMP level and activate PKG, and the subsequent substrate phosphorylation will produce the vascular relaxation effect. Recent reports demonstrated the therapeutic effect of sildenafil in PAH patients. It can improve the 6-minute walk distance and reduce the pulmonary vascular resistance (18 – 20). Increased PDE5 expression and enzyme activity have been reported in several experimental models of pulmonary hypertension (21, 22) and in remodeled pulmonary arteries from patients with pulmonary arterial hypertension (6), which may contribute to the impairment of vasodilator responses in the hypoxic lung. Studies in experimental animals have also demonstrated that oral treatment with sildenafil significantly reduces neo-muscularization in both hypoxia and monocrotaline models of pulmonary hypertension (23, 24) and limits the increase in pulmonary artery medial thickness induced by a left-to-right arterial shunt (5). In cultured human PASMCs, it was found that that sildenafil exerts an antiproliferative effect induced by PDGF that is mediated by an interaction between the cGMP-PKG and the cAMP-PKA activated pathways, leading to inhibition of PDGF-mediated activation of the ERK. In chronic hypoxic rat, the relaxant effect of sildenafil on pulmonary vasculature is related to action on the IP3-dependent calcium release pathway and calcium reuptake mechanisms (7). Our results in this experiment demonstrate that sildenafil inhibits human PASMCs proliferation induced by ET-1 in a dose-dependent manner (Fig. 3), while it decreases ET-1–induced increase of resting [Ca\textsuperscript{2+}], Ca\textsuperscript{2+} release from ER, and SOC-mediated CCE (Fig. 4) in human PASMCs. Most importantly, sildenafil inhibited ET-1–induced upregulation of TRPC1 mRNA and protein level combined with decrease of SOC-mediated CCE in human PASMCs (Fig. 4). It is plausible to speculate that TRPC1 may serve as a downstream effector in the antiproliferative effect of sildenafil.

The regulation of TRPC channels presents multi-mechanisms, including Ca\textsuperscript{2+} stores depletion, protein–protein interactions [e.g., stromal interacting molecule 1 (STIM1) and orali], and protein phosphorylation (e.g., PKG) (3, 25 – 27). The activity of TRPC3 can be inhibited by PKG-1α via phosphorylation at Thr-11 and Ser-263 of TRPC3. These two PKG phosphorylation sites in human TRPC3 are conserved in the TRPC3/6/7 subfamily, and human TRPC1, 4, and 5 contain other potential PKG phosphorylation sites, which indicate that these TRPC isoforms may be the substrates for PKGs (27, 28). In the present experiment, the specific signaling pathway responsible for the effect of downregulating SOC expression for sildenafil is not elucidated. Further experiments will be carried out to detect whether the inhibitory effect of sildenafil on TRPC1 expression is mediated by a cGMP–PKG-dependent or independent pathway. Whereas, from the results of previous reports and this experiment, it is reasonable to speculate that the therapeutic action of sildenafil may involve multiple targets and downstream signaling pathways that lead to the progression of pulmonary vascular remodeling in PAH.

In conclusion, in this study, the results show that sildenafil exerts an inhibitory effect in mitogen-stimulated human PASMCs proliferation. Its antiproliferative effect is partly related to the action on calcium signaling via downregulation of TRPC1 gene expression, which regulates intracellular Ca\textsuperscript{2+} homeostasis that govern human PASMCs growth. The present work also suggests that inhibition of TRPC1 expression in PASMCs may be a potential target for the treatment of pulmonary arterial hypertension, and it simultaneously provides a new theoretical basis for the mechanism of sildenafil in the management of pulmonary hypertension in the clinic.
References


