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Sildenafil inhibits chronically hypoxic upregulation of canonical transient receptor potential expression in rat pulmonary arterial smooth muscle

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Lu W, Ran P, Zhang D, Peng G, Li B, Zhong N, Wang J. Sildenafil inhibits chronically hypoxic upregulation of canonical transient receptor potential expression in rat pulmonary arterial smooth muscle. Am J Physiol Cell Physiol 298: C114–C123, 2010. First published November 4, 2009; doi:10.1152/ajpcell.00629.2008.—In pulmonary arterial smooth muscle cells (PASMCs), Ca2+ influx through store-operated Ca2+ channels thought to be composed of canonical transient receptor potential (TRPC) proteins is an important determinant of intracellular free calcium concentration ([Ca2+]i) and pulmonary vascular tone. Sildenafil, a type V phosphodiesterase inhibitor that increases cellular cGMP, is recently identified as a promising agent for treatment of pulmonary hypertension. We previously demonstrated that chronic hypoxia elevated basal [Ca2+]i, in PASMCs due in large part to enhanced store-operated Ca2+ entry (SOCE); moreover, ex vivo exposure to prolonged hypoxia (4% O2 for 60 h) upregulated TRPC1 and TRPC6 expression in PASMCs. We examined the effect of sildenafil on basal [Ca2+]i, SOCE, and the expression of TRPC in PASMCs under prolonged hypoxia exposure. We also examined the effect of sildenafil on TRPC1 and TRPC6 expression in pulmonary arterial smooth muscle (PA) from rats that developed chronically hypoxic pulmonary hypertension (CHPH). Compared with vehicle control, treatment with sildenafil (300 nM) inhibited prolonged hypoxia induced increases of i) basal [Ca2+]i, 2) SOCE, and 3) mRNA and protein expression of TRPC in PASMCs. Moreover, sildenafil (50 mg · kg−1 · day−1) inhibited mRNA and protein expression of TRPC1 and TRPC6 in PA from chronically hypoxic (10% O2 for 21 days) rats, which was associated with decreased right ventricular pressure and right ventricular hypertrophy. Furthermore, we found, in PASMCs exposed to prolonged hypoxia, that knockdown of TRPC1 or TRPC6 by their specific small interfering RNA attenuated the hypoxic increases of SOCE and basal [Ca2+]i, suggesting a cause and effect link between increases of TRPC1 and TRPC6 expression and the hypoxic increases of SOCE and basal [Ca2+]i. These results suggest that sildenafil may alter basal [Ca2+]i, in PASMCs by decreasing SOCE through downregulation of TRPC1 and TRPC6 expression, thereby contributing to decreased vascular tone of pulmonary arteries during the development of CPH.

calcium signaling; store-operated Ca2+ entry; basal intracellular calcium

PULMONARY HYPERTENSION (PH) is a life-threatening disease that may exist either as a primary disorder or as a complication of a variety of cardiopulmonary diseases, progressively leading to heart failure and increased mortality. PH is featured by increased pulmonary artery pressure (PAP) and structural alterations in the walls of the distal pulmonary arteries (PA), known as pulmonary vascular remodeling. Diffusive alveoli hypoxia is thought to be an important trigger that causes these changes. Exposure to chronic hypoxia (CH) leads to chronically hypoxic pulmonary hypertension (CHPH) in animal models, i.e., rat and mouse. Despite the numerous progresses in elucidating the pathophysiological and functional changes of CPH, the underlying cellular and molecular mechanisms remain unclear.

In pulmonary arterial smooth muscle cells (PASMCs), global increases in intracellular Ca2+ concentration ([Ca2+2]i) are associated with increased tone and proliferation (28). We and others have previously found that CH elevated basal [Ca2+]i, in PASMCs due in large part to enhanced store-operated Ca2+ entry (SOCE) through store-operated Ca2+ channels (SOCC) (15, 33). SOCC are thought to be composed of canonical transient receptor potential (TRPC) proteins (19, 20). Of the three most abundantly expressed TRPC isoforms (TRPC1, TRPC4, and TRPC6) in pulmonary arterial smooth muscle (16), mRNA and protein expression of TRPC1 and TRPC6 is selectively upregulated by CH (33).

Sildenafil citrate, a potent and selective type V phosphodiesterase (PDE5) inhibitor, has recently been approved by the Food and Drug Administration as an orally administered drug for treatment of PH. Studies in animal models indicate that sildenafil significantly attenuated the characteristics of CPH, including PAP, pulmonary vascular muscleization, and right ventricular hypertrophy (2, 10, 25, 41). Expanding clinical studies have demonstrated the efficacy of sildenafil in decreasing mean PAP and pulmonary vascular resistance and in improving cardiac index and exercise tolerance in PH patients (3). Sildenafil has been shown to be able to reduce basal [Ca2+]i, acutely in PASMCs and vascular tone in PA smooth muscle from chronically hypoxic rats (21). The molecular mechanisms of those beneficial effects of sildenafil have largely been attributed to its ability to increase the intracellular concentration of cGMP, which exerts relaxation and growth inhibition on vascular smooth muscle cells (18, 31), or to its ability to affect calcium sensitization through inhibition of the RhoA/Rho kinase pathway (10). However, little has been studied about its chronic effect on basal [Ca2+]i, in PASMCs when long-term administration occurs.

This study was designed and performed to test the hypothesis that sildenafil decreases CH-induced changes of PAP through downregulation of TRPC expression, which in turn reduces SOCE and basal [Ca2+]i, in PASMCs.
SILDENAFIL INHIBITS TRPC EXPRESSION IN PASM

METHODS

**Exposure of animals to chronic hypoxia and treatment with sildenafil.** All procedures were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. Adult male Wistar rats (175–300 g) were placed in a hypoxic chamber for 21 days as previously described (27, 33). The chamber was continuously flushed with a mixture of room air and N2 to maintain 10 ± 0.5% O2 and CO2 < 0.5%. Chamber O2 concentration was continuously monitored using a PRO-OX unit (RCI Hudson, Anaheim, CA). Animals were exposed to room air for 10 min every 2 days for replenishing food, drug, and water or for change of cage. Normoxic control animals were kept in room air next to the hypoxic chamber. Four groups were examined: 1) a normoxic control group, 2) a hypoxic control group, 3) a normoxic group treated with sildenafil (50 mg·kg⁻¹·day⁻¹), and 4) a hypoxic group treated with sildenafil (50 mg·kg⁻¹·day⁻¹). Sildenafil (sildenafil citrate tablets, Pfizer, Sandwich, England) was administered in the drinking water and initiated from the day the animals were placed in the chamber. Draining volume was measured daily over a period of 1 wk before the onset of treatment to calculate the uptake of water per day. The concentration of sildenafil in the drinking water was then adjusted accordingly.

**Hemodynamic measurements.** Measurements including right ventricular pressure and right ventricular hypertrophy were done as previously described (26). Briefly, at the end of chronic hypoxic exposure (10% O2 for 21 days), rats were anesthetized with pentobarbital sodium (65 mg/kg ip). An incision was made in the abdomen, resulting in the visualization of the diaphragm. A heparinized saline-filled 23-gauge needle connected to a pressure transducer was inserted through the diaphragm into the right ventricle (RV), the right ventricular systolic pressure (RVSP) was recorded and measured, and the mean right ventricular pressure (RVP) was analyzed by PowerLab systems (ML785 PowerLab/8SP, ADInstruments, Colorado Springs, CO) as described before (26). The right ventricle was dissected from the left ventricle (LV) and septum (S) after removal of the atria. The ventricles were blotted dry and weighed. Total ventricular weight (g), the left ventricle (LV) and septum (S) after removal of the atria. The right ventricle was dissected from intrapulmonary arteries were dissected from lungs of male Wistar rats (300–500 g body wt) anesthetized with pentobarbital sodium (65 mg/kg ip). Adventitia was removed from the isolated PA, and endothelium was denuded by opening the vessel longitudinally and rubbing the luminal surface with a cotton swab. PASMCs were harvested from these PA smooth muscle enzymatically, plated onto 25-mm coverslips in six-well culture dishes, and incubated for 3–4 days in smooth muscle growth media 2 (Clonetics, Walkersville, MD) containing 0.3% serum for 24 h, then exposed to hypoxia (4% O2) for 24 h. In these experiments, KRBS perfusate also contained 2.5 mM CaCl₂, 0.57 mM MgSO₄, 1.18 mM KH₂PO₄, 25 mM NaHCO₃, and 10 mM glucose, was equilibrated with 16% O₂-5% CO₂ at 38°C in heated reservoirs and led via stainless steel tubing and a manifold to an in-line heat exchanger (SF-28, Warner Instrument) before it entered the cell chamber. A dual channel heater controller (TC-344B, Warner Instrument) was connected to the heat exchanger to control its temperature. This system maintained temperature at 37°C and oxygen tension at 12 ± 2.0 mmHg at the coverslip. The cells were perfused for 10 min to remove extracellular dye, and ratiometric measurement of Fura-2 fluorescence was performed at 12- to 30-s intervals using a collimated light beam from a xenon arc lamp filtered by interference filters at 340 and 380 nm and focused onto PASMCs visualized with an electronic shutter (Vincent Associates, Rochester, NY) used to minimize photobleaching. Protocols were executed and data collected on-line with InCyte software (Intracellular Imaging, Cincinnati, OH). [Ca²⁺]i was estimated by linear regression from a calibration curve created by measuring Fura-2 fluorescence in calibration solutions with [Ca²⁺] ranging from 0 to 1,350 nM.

As previously described (32), to assess SOCE, we perfused PASMCs for at least 10 min with Ca²⁺-free KRBS containing 5 µM nifedipine to prevent calcium entry through L-type voltage-dependent Ca²⁺ channels (VDCC) and 10 µM cyclopiazonic acid (CPA) to deplete SR calcium stores. Next, we measured [Ca²⁺]i, at 30 sec intervals before and after restoration of extracellular [Ca²⁺]i to 2.5 mM, and SOCE was evaluated from the increase in [Ca²⁺]i caused by restoration of extracellular [Ca²⁺], in the continued presence of CPA and nifedipine. In these experiments, KRBS perfusate also contained 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) to chelate any residual Ca²⁺. Additionally, we monitored Fura-2 fluorescence excitation at 360 nm at 30-s intervals before and after addition of MnCl₂ (200 µM) to the cell perfusate, and SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn²⁺, which enters the cell as a Ca²⁺ surrogate and reduces Fura-2 fluorescence upon binding to the dye. Fluorescence excitation at 360 nm is the same for Ca²⁺−bound and Ca²⁺−free Fura-2; therefore, changes in fluorescence can be assumed to be caused by Mn²⁺ alone. In the experiment evaluating the effect of membrane potential change on Mn²⁺ quenching, NaCl was replaced with equimolar KCl in the perfusate, with final concentration of KCl at 122.7 mM.

**RNA isolation and measurement by real-time quantitative polymerase chain reaction.** Total RNA was extracted using TRIzol method for deendothelialized distal PA, or using RNaseasy kit (Qiagen, Valencia, CA) for PASMCs, as previously described (16, 32, 33). DNA contamination in RNA preparations was removed by on-column DNase digestion using RNaseasy column and RNase-free DNase (Qiagen). Reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) with reaction mixture containing 1 µg total RNA in a 20-µl volume. cDNA of rat TRPC1, TRPC6, or 18s was amplified by real-time quantitative PCR (qPCR) using QuantiTect SYBR Green PCR Master Mix (Qiagen) in a iCyclerIQ real-time PCR detection system (Bio-Rad), as described previously (16). Primer
sequences in the measurements were 5’-AGGCTTCTGACAAACGAGGA-3’ (sense) and 5’-ACCTGACATCTGTCCGAGC-3’ (antisense) for TRPC1, and 5’-TACCTGTGTTGCTCTTGGAGC-3’ (sense) and 5’-GAGCCTTGGTGGCTTCAATC-3’ (antisense) for TRPC6. Primers for 18s (Rn_Rarl1_1_SG QuantiTect Primer Assay) were from Qiagen. Relative concentration of each transcript was calculated using the Pfaffl method (23). Efficiency for each gene was determined from five-point serial dilutions of an unknown cDNA sample (PA or PASMCs). The expression of TRPC1 and TRPC6 was normalized to 18s in the same sample.

Protein isolation and measurement by Western blotting. Deendothelialized PA or PASMCs samples were homogenized by sonication in Laemmli sample buffer containing 62.5 mM Tris · HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% protease inhibitor cocktail, 1 mM EDTA, and 200 µM 4-(2-aminoethyl) benzene-sulfonate hydrochloride. Total protein concentration in the homogenates was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) using bovine serum albumin as a standard. Homogenates were denatured by addition of dithiothreitol to 150 mM and heating at 95°C for 3 min. Homogenate proteins were resolved by 10% SDS-PAGE calibrated with prestained protein molecular weight markers (Precision Plus, Bio-Rad, Carlsbad, CA). Separated proteins were transferred to polyvinylidene difluoride membranes (pore size 0.45 µM, Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween 20 and blotted with affinity-purified polyclonal antibodies specific for TRPC1 (Sigma, St. Louis, MO), TRPC6 (Alomone Laboratories, Jerusalem, Israel), or monoclonal antibody against α-actin (Sigma). The membranes were then washed for 15 min 3 times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG for 1 h. Bound antibodies were detected using an enhanced chemiluminescence system (ECL, GE Healthcare, Piscataway, NJ).

Materials and drugs. Unless otherwise specified, all reagents were obtained from Sigma Chemical. Sildenafil was a gift from Pfizer. Fura-2 AM (Invitrogen, Carlsbad, CA) was prepared on the day of the experiment as a 2.5 mM stock solution in DMSO. Stock solutions of 30 mM CPA and 30 mM nifedipine were made in DMSO.

Statistical analysis. Data are expressed as means ± SE, and n is the number of experiments, which equals the number of animals providing PA or cells. When Fura-2 fluorescence was measured, the number of cells in each experiment was 20–30, as indicated in RESULTS and figure legends. Statistical analyses were performed using analysis of variance and Students t-test. If significant F-ratios were obtained with the former, pairwise comparison of means was conducted with t-tests. When necessary, a Bonferroni correction was used to adjust level of significance for multiple comparisons. Otherwise, differences were considered significant when P < 0.05.

RESULTS

Sildenafil inhibited the increases of basal [Ca2+]i in PASMCs exposed to prolonged hypoxia. As shown in Fig. 1, ex vivo exposure to prolonged hypoxia (4% O2, 60 h) caused an increase in basal [Ca2+]i from 110 ± 7.4 nM in normoxic control cells to 224 ± 9.0 nM in hypoxic control cells (P < 0.001). Basal [Ca2+]i was attenuated to 157 ± 7.2 nM (P < 0.01 vs. hypoxic control), with attenuation of ~58.8% in PASMCs treated with sildenafil under the hypoxic condition; however, basal [Ca2+]i remained unaltered under the normoxic condition with or without sildenafil treatment (99 ± 10.2 nM, P > 0.05 vs. normoxic control). The morphology of PASMCs was not changed by these treatments (data not shown).

Sildenafil inhibited the increases of SOCE in PASMCs exposed to prolonged hypoxia. We assessed SOCE in two ways. First, we measured the peak increase in [Ca2+]i caused by restoration of extracellular [Ca2+] to 2.5 mM in PASMCs perfused with Ca2+-free KRBS containing CPA, nifedipine, and EGTA. CPA given in the presence of nifedipine caused an initial transient increase in [Ca2+]i, during Ca2+-free perfusion (Fig. 2A). This response was not altered by sildenafil treatment in PASMCs under either normoxic or hypoxic conditions (Fig. 2B). The peak change in [Ca2+]i, caused by restoration of extracellular Ca2+ was greater in hypoxic control PASMCs (386 ± 0.5; n = 9, 0.05) vs. normoxic control cells and §P < 0.01 vs. hypoxic control cells. Cont; control; Sild; sildenafil.

Fig. 1. Changes in basal intracellular free calcium concentration ([Ca2+]i) in pulmonary arterial smooth muscle cells (PASMCs) treated with normoxia control (n = 7 experiments in 205 cells), normoxia plus sildenafil (n = 7 experiments in 196 cells), hypoxia control (4% O2; n = 7 experiments in 210 cells), or hypoxia plus sildenafil (n = 7 experiments in 198 cells) for 60 h. Bar values are means ± SE. *P < 0.001 vs. normoxic control cells and §P < 0.01 vs. hypoxic control cells. Cont; control; Sild; sildenafil.

Because the increase in [Ca2+]i caused by restoration of extracellular Ca2+ can be affected by factors other than SOCE, such as changes in membrane potential, we also measured the rate at which Mn2+ influx more specifically. As seen in Fig. 2C, in the presence of CPA and nifedipine, Fura-2 fluorescence, which is thought to reflect Ca2+ influx more specifically. As seen in Fig. 2C, in the presence of CPA and nifedipine, Fura-2 fluorescence excited at 360 nm (F360) was decreased along time by perfusing PASMCs with Ca2+-free KRBS containing 200 µM Mn2+. Mn2+ quenching, expressed as the percentage decrease in F360 at 10 min, was greater in hypoxic than normoxic control cells (61 ± 5.1% vs. 29 ± 5.7; P < 0.001). Treatment with 300 nM sildenafil decreased the rate of Mn2+ quenching about 65.6% in hypoxic cells (40 ± 3.1% with sildenafil vs. 61 ± 5.1% with vehicle control cells; P < 0.05), but not normoxic cells (28 ± 2.5% with sildenafil vs. 29 ± 5.7% with vehicle control; P = 0.94; Fig. 2, C and D). Changes in membrane potential by complete substitution of NaCl with KCl in the perfusate with a result of final [KCl] at 122.7 mM did not affect the rate of Mn2+ quenching compared with control solution containing 4.7 mM KCl (35 ± 1.5% for high K vs. 38 ± 1.4% for control, P = 0.16; Fig. 2, E and F), suggesting that the effect of
sildenafil on SOCE evaluated in our study is unlikely to be due to changes in membrane potential.

Sildenafil inhibited the increases of TRPC expression in PASMCs exposed to prolonged hypoxia. Compared with normoxic control cells, the mRNA (Fig. 3, A and B) and protein expression (Fig. 3, C and D) of TRPC1 and TRPC6 was increased in PASMCs exposed to prolonged hypoxia. Treatment with sildenafil attenuated these increases in hypoxic cells but did not alter their expression level in normoxic cells (Fig. 3).

Knockdown of TRPC1 or TRPC6 expression reduced the increases of SOCE and basal [Ca\(^{2+}\)] in PASMCs exposed to prolonged hypoxia. To determine the cause and effect relationship between increases of TRPC1 or TRPC6 expression and the hypoxic increases of SOCE and basal [Ca\(^{2+}\)], in PASMCs, we applied loss of function approach by knocking down the endogenous gene expression of TRPC1 or TRPC6 using their specific siRNA, followed by measurement of SOCE and basal [Ca\(^{2+}\)]. Similar to the above observation in nontransfected PASMCs, exposure to prolonged hypoxia increased mRNA and protein expression of TRPC1 and TRPC6 about 50% to 150% in cells treated with siNT (Fig. 4). Compared with siNT-transfected hypoxic cells, treatment of TRPC1 by siT1 under hypoxic conditions reduced the expression of TRPC1 mRNA by 92.4 ± 1.3% (P < 0.01; Fig. 4A) and protein by 81.3 ± 0.2% (P < 0.01; Fig. 4, B and C) without causing significant change in TRPC6 expression; similarly, siT6 treatment reduced TRPC6 mRNA by 93.9 ± 0.6% (P < 0.01; Fig. 4A) and protein by 86.4% ± 1.1% (P < 0.01; Fig. 4, B and C) without altering the level of TRPC1 expression.
In PASMCs exposed to siNT, SOCE assessed by extracellular [Ca\(^{2+}\)] restoration was increased from 152 ± 6.1% in normoxic cells to 391 ± 22.5% in hypoxic cells (P < 0.001; Fig. 5, A and B). Knockdown of TRPC1 by siT1 or of TRPC6 by siT6 under hypoxic conditions did not alter the initial transient increase in [Ca\(^{2+}\)] during Ca\(^{2+}\)-free perfusion; however, SOCE in cells treated with siT1 (211 ± 3.5%), and treated with siT6 (277 ± 13.7%) were both reduced as compared with siNT treated hypoxic cells, although still greater than that in normoxic siNT cells. The reduction by siT1 was greater than that by siT6 (P < 0.05; Fig. 5B). Similarly, SOCE assessed by Mn\(^{2+}\) quenching was increased from 33 ± 1.6% in normoxic cells to 59 ± 2.6% in hypoxic cells (P < 0.01; Fig. 5, C and D). Knockdown of TRPC1 by siT1, or of TRPC6 by siT6, both caused slower rate of Mn\(^{2+}\) quenching compared with siNT-treated hypoxic cells (Fig. 5, C and D); SOCE with siT1 was reduced to 37 ± 0.9% (P < 0.01), and to 43 ± 1.2% with siT6 (P < 0.01). Again, the reductive effect of siT1 on SOCE was greater than siT6 (P < 0.05; Fig. 5D). Somewhat different from SOCE assessed by extracellular [Ca\(^{2+}\)] restoration, Mn\(^{2+}\) quenching in siT1-treated hypoxic cells was not different from that in normoxic siNT cells, whereas in siT6 cells, it was still higher than that in normoxic siNT cells.

Consequently, knockdown of TRPC1 by siT1 and of TRPC6 by siT6 under hypoxic conditions were both associated with reduced levels of basal [Ca\(^{2+}\)], compared with siNT-treated hypoxic PASMCs (162 ± 5.8% in siT1, 182 ± 6.1% in siT6, and 219 ± 9.0% in siNT cells); however, neither of these reductions was sufficient to normalize the basal [Ca\(^{2+}\)], to the level (109 ± 5.3%) in normoxic siNT cells (Fig. 6). The reductive effect of siT1 on hypoxic increase of basal [Ca\(^{2+}\)], was greater than that of siT6.

**Sildenafil inhibited the hemodynamic parameters of CHPH in rats.** Rats exposed to CH (10% O\(_2\) for 21 days) developed CHPH. As shown in Fig. 4, the mean RVP was increased from 10.0 ± 0.45 mmHg (normoxic control) to 27.8 ± 1.08 mmHg (hypoxic control), and RVSP increased from 26.7 ± 1.08 mmHg (normoxic control) to 57.4 ± 2.15 mmHg in hypoxic animals (Fig. 7B). This effect of CH was accompanied by right ventricular hypertrophy, indicated by increases in the ratio of right ventricle to left ventricle plus septum weight [RV/(LV + S)] and the ratio of right ventricle to body weight (RV/body weight). The ratios of RV/(LV + S) increased from 0.261 ± 0.007 (normoxic control) to 0.590 ± 0.035 (hypoxic control; P < 0.01; Fig. 7C); and the ratios of RV/body weight increased from 0.576 ± 0.028 (normoxic controls) to 1.488 ± 0.137 (hypoxic control; P < 0.01; Fig. 7D). Administration of sildenafil significantly reduced CHPH. Accordingly, sildenafil caused a significant decrease of mean RVP to 19.4 ± 1.21 mmHg (P < 0.01 vs. hypoxic control; Fig. 7A), and RVSP to 46.3 ± 1.41 (P < 0.001 vs. hypoxic control; Fig. 7B). The RV/(LV + S) ratio was decreased to 0.434 ± 0.026 (P < 0.01 vs. hypoxic control; Fig. 7C) in chronic hypoxic rats. Treatment with sildenafil did not change mean RVP (10.59 ± 0.39, P = 0.35 vs. vehicle control; Fig. 7A) and RVSP (27.3 ± 0.42 mmHg, P = 0.53 vs. vehicle control; Fig. 7B); however, it increased the RV/(LV + S) ratio to 0.306 ± 0.013 (P < 0.01 compared with vehicle control) in normoxic animals (Fig. 7C).

Weighing of the animals every 2 days revealed that, following an initial weight loss on initiation of sildenafil and hypoxia, all the animals maintained or increased body weight throughout the study (data not shown). In addition, weight ratios (×10\(^{-5}\)) of liver to body (3.76 ± 0.135, 3.79 ± 0.146, 3.75 ± 0.114, and 3.73 ± 0.128, respectively, for normoxia control,
normoxia with sildenafil, hypoxia control, and hypoxia with sildenafil animals; \( n = 6 \) in each group) and ratios (\( \times 10^{-3} \)) of kidney to body (6.41 ± 0.144, 6.08 ± 0.193, 6.36 ± 0.232, and 6.27 ± 0.288, respectively, for normoxia control, normoxia with sildenafil, hypoxia control, and hypoxia with sildenafil animals; \( n = 6 \) in each group) were not changed by hypoxia exposure and/or sildenafil treatment, suggesting good tolerance to these treatments. In contrast, CH caused increases of hematocrit (%) in both control (70 ± 1.18 for hypoxia vs. 43 ± 0.48 for normoxia; \( n = 6 \) each, \( P < 0.05 \)) and sildenafil-treated animals (67 ± 1.54 for hypoxia vs. 44 ± 0.76 for normoxia; \( n = 6 \) each, \( P < 0.05 \)); these hypoxic changes were not different from each other.

**Sildenafil inhibited TRPC1 and TRPC6 expression in distal pulmonary arteries from chronically hypoxic rats.** Similar to our previous observations (33) and to the above results in PASMCs exposed to prolonged hypoxia, mRNA (Fig. 8, A and B) and protein (Fig. 8, C and D) expression levels of TRPC1 and TRPC6 were both increased in endothelium-denuded distal PA from rats exposed to CH (10% \( \text{O}_2 \), 21 days). Treatment with sildenafil markedly suppressed these hypoxic increases in chronically hypoxic animals. The levels of TRPC1 and TRPC6 expression were not different from each other in distal PA from normoxic control animals with or without sildenafil treatment (Fig. 8).

**DISCUSSION**

The present study demonstrated that sildenafil inhibited hypoxia-induced increases in basal \([\text{Ca}^{2+}]_i\) in PASMCs likely through its inhibitory action on the expression of TRPC proteins, contributing to its beneficial effects in the treatment of CPH.

Exposure to prolonged hypoxia ex vivo (4% \( \text{O}_2 \), 60 h) led to increase of basal \([\text{Ca}^{2+}]_i\) in PASMCs from normoxic rats (Fig. 1), consistent with our previous observations (33). Treatment with sildenafil (300 nM) under hypoxic conditions significantly inhibited this increase, without affecting basal \([\text{Ca}^{2+}]_i\) under normoxic conditions. We subsequently demonstrated that this inhibitory effect on the hypoxic increase of basal \([\text{Ca}^{2+}]_i\) in PASMCs by sildenafil likely represents an additional mechanism to what has been suggested by others. For example, Pauvert et al. (21) observed an acute effect of sildenafil on decreasing basal \([\text{Ca}^{2+}]_i\) in PASMCs obtained from hypoxic rats and suggested that the acute effect was due to a decrease in calcium release from internal store and activation of calcium reuptake mechanisms. Michelakis et al. (17) demonstrated that sildenafil could activate large-conductance calcium-sensitive potassium channels in PASMCs; thus it may exert restoration of membrane potential and decrease the hypoxic increase of \([\text{Ca}^{2+}]_i\).

In vascular smooth muscle, global increases in \([\text{Ca}^{2+}]_i\) can be caused by \( \text{Ca}^{2+} \) release from internal storage sites, such as sarcoplasmic reticulum (SR), and/or \( \text{Ca}^{2+} \) influx from extracellular fluid through VDCC, receptor-operated \( \text{Ca}^{2+} \) channels (ROCC), or SOCC (11, 28, 33). In cultured PASMCs exposed to prolonged hypoxia ex vivo, both SOCC and VDCC were found to contribute to the hypoxic increase in basal \([\text{Ca}^{2+}]_i\) (37); however, in PASMCs isolated from chronically hypoxic rats, previous studies suggest that the maintenance of elevated \([\text{Ca}^{2+}]_i\) requires \( \text{Ca}^{2+} \) influx through pathways other than...
Fig. 5. Effects of TRPC1 and TRPC6 knockdown on hypoxic increases of SOCE in PASMCs as assessed by [Ca^{2+}]i restoration. (A and B) and Mn^{2+} quenching (C and D) are shown. A: representative traces of [Ca^{2+}]i responses to restoration of extracellular [Ca^{2+}]i to 2.5 mM after perfusion with Ca^{2+}-free KRBS (0 Ca^{2+}) containing 10 μM CPA, 5 μM Nifed, and 1 mM EGTA in PASMCs treated with siNT, siT1, or siT6 for 60 h under normoxic or hypoxic (4% O_{2}) conditions. B: average maximum increase in [Ca^{2+}]i after restoration of extracellular [Ca^{2+}]i in normoxic siNT, hypoxic siNT, siT1, or siT6 PASMCs. *P < 0.001 vs. normoxic siNT, **P < 0.01 vs. hypoxic siNT, and §P < 0.05 vs. hypoxic siT1. C: time course of Fura-2 fluorescence excited at 360 nm before and after addition of 200 μM Mn^{2+} in Ca^{2+}-free KRBS (0 Ca^{2+}) perfusates containing 10 μM CPA and 5 μM Nifed in PASMCs treated with siNT, siT1, or siT6 for 60 h under normoxic or hypoxic (4% O_{2}) conditions. D: average quenching of Fura-2 fluorescence by Mn^{2+} shown in C. Data are expressed as the percentage decrease in fluorescence at time 10 min from time 0. *P < 0.01 vs. normoxic siNT, **P < 0.01 vs. hypoxic siNT, and §P < 0.05 vs. hypoxic siT1 and normoxic siNT. Bar values are means ± SE; n = 4 in each group.

L-type VDCC (27). Moreover, we demonstrated that the enhanced SOCE contributed to this elevation of [Ca^{2+}]i (33, 34). We therefore focused on determination of the effect of sildenafil on SOCE in this study.

Consistently, we found that SOCE, assessed by measuring Ca^{2+}-bound Fura-2 fluorescence as direct indicator of [Ca^{2+}]i, increase following store depletion and extracellular [Ca^{2+}]i restoration, was greater in PASMCs exposed to prolonged hypoxia ex vivo compared with that measured in normoxic PASMCs (Fig. 2). Because this approach for SOCE assessment could be affected by other factors, such as membrane potential, cytoplasmic calcium buffering, and Ca^{2+} efflux through plasmalemmal Ca^{2+}-ATPases and Na^{+}/Ca^{2+} exchange, we also applied Mn^{2+} quenching method, which was thought to be a more specific evaluation for Ca^{2+} entry (20). In the absence of Mn^{2+}, no significant decrease in F_{360} was observed in PASMCs cultured under either normoxic or hypoxic conditions. Following depletion of intracellular Ca^{2+} stores with CPA, which maximally activates SOCC, addition of Mn^{2+} caused significantly greater quenching of F_{360} in PASMCs exposed to hypoxia (Fig. 2, C and D). The increased rise in [Ca^{2+}]i, following restoration of extracellular [Ca^{2+}]i and faster rate of Mn^{2+} quenching of F_{360} in PASMCs exposed to hypoxia indicate enhanced Ca^{2+} influx through SOCC. Treatment with sildenafil inhibited SOCE assessed via either [Ca^{2+}]i restoration or Mn^{2+} quenching following store depletion in cells exposed to prolonged hypoxia.

According to previous reports, Ca^{2+} entry could be altered by membrane potential independent of VDCC, or store depletion in skeletal muscle cells and endothelial cells (7, 8, 38). In this case, the SOCE assessment via Mn^{2+} quenching could also be influenced by changes of membrane potential. It is unclear whether this happens in PASMCs. We therefore tested it through substitution of Na^{+} with K^{+} in the perfusate, which provided a chemical clamp of membrane potential (E_{K}) at about −5 mV according to Nernst equation: E_{K} = 58 mV log ([K^{+}\text{extracellular}]/[K^{+}\text{intracellular}]) (35). The K^{+} substitution did not cause significant change in the rate of Mn^{2+} quenching, suggesting that the inhibitory effect of sildenafil on hypoxic increases of SOCE is unlikely to be due to influences of membrane potential change.

The decrease in SOCE by sildenafil treatment could be through decreases in 1) Ca^{2+} release from SR, 2) the activation...
of SOCC, and/or 3) the number of SOCC. Several lines of evidence in cGMP-mediated effects, including decrease of inositol trisphosphate formation (IP3) by inhibiting phospholipase C, phosphorylation of IP3 receptor (12), and activation of sarco(endo)plasmic reticulum Ca2+/H11001-ATPase (7), suggest an inhibitory role of sildenafil in agonist (i.e., angiotensin II and phenylephrine)-stimulated calcium release from SR (21); however, in our data (Fig. 2), the transient increase in [Ca2+/H11001i] caused by CPA during perfusion with Ca2+/H11001-free KRBS was not different between sildenafil and vehicle treatment during either normoxia or hypoxia exposure, suggesting similar SR Ca2+ release and therefore similar store depletion. One possible explanation for this discrepancy is that the effect of sildenafil on calcium release might be covered by CPA treatment, which caused complete depletion of SR Ca2+ stores. On the other hand, it is also likely that the decrease in SOCE in cells treated with sildenafil resulted from reduced activation of SOCC through effects of sildenafil on the channels themselves.

Fig. 7. A and B: mean right ventricular pressure (RVP; A) and right ventricular systolic pressure (RVSP; B) in normoxic control rats and animals treated with normoxia plus sildenafil (50 mg · kg−1 · day−1), chronic hypoxia (CH; 10% O2 for 21 days), or CH plus sildenafil. C and D: ratio of right ventricle to left ventricle plus septum (RV/LV + S) (C) and ratio of right ventricle to body weight (RV/body weight) (D) in normoxic control rats and animals treated with normoxia plus sildenafil (50 mg · kg−1 · day−1), CH (10% O2 for 21 days), or CH plus sildenafil. Bar values are means ± SE; n = 7 in each group. *P < 0.01 and **P < 0.05 vs. respective normoxic control and §P < 0.01 vs. respective CH control.

Fig. 8. A and B: expression of TRPC1 (A) and TRPC6 (B) mRNA relative to 18s in distal pulmonary arteries (PA) from rats exposed to normoxia or CH (10% O2 for 21 days) and treated with or without sildenafil (50 mg · kg−1 · day−1) as determined by real-time quantitative PCR. C and D: expression of TRPC1 and TRPC6 protein relative to α-actin in distal PA from rats exposed to normoxia or CH (10% O2 for 21 days) and treated with or without sildenafil (50 mg · kg−1 · day−1) as determined by Western blotting. C: representative blots for TRPC1, TRPC6, and α-actin. D: mean protein expression for TRPC1 and TRPC6 relative to α-actin. Bar values are means ± SE; n = 4 in each group. *P < 0.05 vs. respective normoxia control and §P < 0.05 vs. respective CH control.
Increased expression of TRPC caused by CH is associated with rise in \([\text{Ca}^{2+}]_i\), which in turn may cause increased tone, proliferation, and migration of PASMCs (28), thus contributing to the development of CHPH. Sildenafil has been shown to inhibit the development of CHPH in animal models (2, 10, 25, 38). To confirm the therapeutic effect of sildenafil, we exposed rats to CH (10% O2 for 21 days). Compared with the measurements in normoxic control animals, CH induced a rise in RVP and a significant increase of right ventricular hypertrophy. These increases were accompanied by increases of mRNA and protein expression of TRPC1 and TRPC6 in distal PA, consistent with our previous results (33). Initiation of sildenafil (50 mg · kg\(^{-1}\) · day\(^{-1}\)) at the same time of CH exposure significantly inhibited the increases of RVP and the progression of right ventricular hypertrophy. These inhibitory effects of sildenafil were associated with decreases in the mRNA and protein expression of TRPC1 and TRPC6 in distal PA. The dose of 50 mg · kg\(^{-1}\) · day\(^{-1}\) of sildenafil was selected from the effective range of 10–75 mg · kg\(^{-1}\) · day\(^{-1}\) shown by others in attenuating rat CHPH (2, 10, 25), and a previous safety study indicating “no-toxic-effect” dose level of sildenafil under 60 mg · kg\(^{-1}\) · day\(^{-1}\) in rats (1). Indeed, we did not observe significant changes either in body weight or in the weights of major organs (liver and kidney) with sildenafil treatment under both normoxic and CH conditions, suggesting that the inhibitory effects of sildenafil on TRPC expression were unlikely due to toxicity. Interestingly, while treatment with sildenafil for 21 days decreased right ventricular hypertrophy in CH rats, it actually caused increases of mass ratio of RV/(LV + S) and RV/body weight in normoxic rats. The mechanism accounting for this change is currently unknown.

In conclusion, we found that sildenafil inhibited CH-induced increases of basal \([\text{Ca}^{2+}]_i\], and SOCE in PASMCs. These functional effects of sildenafil were correlated with decreased expression of TRPC1 and TRPC6 transcripts and proteins. Moreover, sildenafil inhibited chronically hypoxic upregulation of TRPC1 and TRPC6 in distal pulmonary arterial smooth muscle from rats that developed CHPH. Our results from this study provide additional mechanistic information of sildenafil in regulating calcium signaling in PASMCs, and in treatment of CHPH, suggesting TRPC1 and TRPC6 as potential molecular targets in the clinical management of PH.

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DISCLOSURES

The authors have no conflicts of interest.

REFERENCES


