Enhanced store-operated Ca$^{2+}$ entry and TRPC channel expression in pulmonary arteries of monocrotaline-induced pulmonary hypertensive rats

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Liu XR, Zhang MF, Yang N, Liu Q, Wang RX, Cao YN, Yang XR, Sham JS, Lin MJ. Enhanced store-operated Ca$^{2+}$ entry and TRPC channel expression in pulmonary arteries of monocrotaline-induced pulmonary hypertensive rats. Am J Physiol Cell Physiol 302: C77–C87, 2012. First published September 21, 2011; doi:10.1152/ajpcell.00247.2011.—Pulmonary hypertension (PH) is a pathophysiological condition associated with a broad spectrum of diseases of different pathological features and etiological mechanisms (53). Among them, pulmonary arterial hypertension (PAH) is a severe progressive form characterized by vasoconstriction, plexiform formation, and vascular cell proliferation and remodeling, resulting in elevated pulmonary vascular resistance, right ventricular hypertrophy, and eventually right heart failure and death (16). There are different types of PAH, including idiopathic (IPAH) and heritable PAH (53). PH owing to lung diseases and hypoxia is another category of PH, which includes PH associated with chronic obstructive pulmonary disease, sleep disorders, and chronic exposure to high altitude. PH in this group is generally mild to moderate, but it worsens the prognosis of the diseases (9, 41). The etiologic mechanisms of the various forms of PH are different, but they all have the common features of abnormalities in pulmonary vascular function, vascular cell proliferation, and remodeling, suggesting that they may share some important downstream signaling mechanisms in the process of disease development.

Intracellular Ca$^{2+}$ signaling plays pivotal roles in the regulation of numerous physiological and pathophysiological processes, including contraction, proliferation, hypertrophy, and migration, in pulmonary arterial smooth muscle cell (PASMC) (45). Previous studies indicate that there are major alterations in ion channel expression and Ca$^{2+}$ homeostasis, such as membrane depolarization, downregulation of voltage-gated K$^{+}$ channels, increase in Ca$^{2+}$ influx, and elevation of resting intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{i}$), in PASMCs of IPAH patients and animal models of PH (26, 33, 51). Ca$^{2+}$ influx in PASMCs is mainly regulated by voltage-dependent Ca$^{2+}$ channels and voltage-independent nonselective cation channels, such as store-operated Ca$^{2+}$ channels and receptor-operated Ca$^{2+}$ channels (ROCC) (45). Blockers of voltage-gated Ca$^{2+}$ channel, however, are only effective in a small fraction of PAH patients, and the effect can be partial and temporary (2, 3, 10). Nifedipine is also ineffective in reducing pulmonary vascular resistance of chronic hypoxic rats (39). In contrast, the elevated resting [Ca$^{2+}$]$_{i}$ in PASMCs and vascular tone of pulmonary arteries (PAs) of chronic hypoxic rats could be reduced to the control level by the removal of extracellular Ca$^{2+}$ or by using the nonselective cation channel blockers La$^{3+}$, Ni$^{2+}$, and SKF-96365 (26, 51). These results suggest that voltage-independent nonselective cation channels, instead of voltage-gated Ca$^{2+}$ channels, constitute the major Ca$^{2+}$ pathways for the elevated [Ca$^{2+}$]$_{i}$, in PA, at least in the chronic hypoxic rat model.

Members of the canonical transient receptor potential (TRPC) gene family are known to encode different types of nonselective cation channels, which have been implicated as SOCC and ROCC in vascular smooth muscle cells. Multiple TRPC subtypes have been identified in PAs and PASMCs from several different species (13, 26, 37, 57, 60). Using specific small interfering RNA (siRNA), we have previously shown that TRPC1 and TRPC6 are the major constituents for store-operated Ca$^{2+}$ entry (SOCE) and receptor-operated Ca$^{2+}$ entry (ROCE), respectively, in rat intralobar PASMCs. More importantly, the expression of TRPC1 and TRPC6 mRNAs and proteins are upregulated, and SOCE and ROCE are potentiated
in PASMCs of hypoxic PH rats (26). The enhanced SOCE is responsible for the elevated resting [Ca\textsuperscript{2+}]\textsubscript{i} in PASMCs and the increased resting tension of PA of chronic hypoxic rats. Furthermore, upregulation of TRPC6 and TRPC3 expression and enhanced SOCE had also been shown in PASMCs of IPAH patients (64, 69), suggesting that TRPC-dependent SOCE could be a common pathway involved in the development of PH.

To further explore this hypothesis, the present study used a widely used rat model of monocrotaline (MCT)-induced PAH to examine the changes in the expression of TRPC channels and the activity of SOCE, as well as their contribution to the altered pulmonary vascular reactivity to endothelin-1 (ET-1) in PAH.

MATERIALS AND METHODS

**MCT-induced PAH rat model.** Experiments were performed in adult male Sprague-Dawley rats (200–250 g). Rats were given a single intraperitoneal injection of MCT (60 mg/kg) or an equivalent volume of saline (2 ml/kg). MCT (Sigma) was dissolved in 1 N HCl, neutralized to pH 7.4 with 1 N NaOH, and diluted with saline. Twenty-one days after MCT or sham injection, rats were anesthetized with urethane (1 g/kg). Right ventricle systolic pressure (RVSP) and systemic arterial pressure (SAP) were measured by accessing the right ventricle through the jugular vein and by cannulating the right carotid artery, respectively, with polyethylene catheters connected to pressure transducers (YPJ01; Chengyi). Pressure signals were displayed continuously on an RM6240 polygraph (Chengyi). Heart rate was determined from the right ventricle pressure pulse. At the end of hemodynamic measurement, the rat was killed with an overdose of urethane.

The heart was removed, and right ventricular mass index was calculated as the ratio of wet weight of the right ventricle to the left ventricular wall plus septum [RV/(LV + SL)]. All procedures were approved by the Animal Care and Use Committee of Fujian Medical University.

**Histological staining and morphometry.** Lung specimens of control and MCT-treated rats were collected for the determination of pulmonary vascular remodeling. Rats were anesthetized with urethane (1 g/kg body wt ip) 5 min after a heparin injection (500 IU/kg body wt) and then restrained in the supine position. The left lung was isolated, and formaldehyde was injected through the trachea under a pressure of 15 cm\text{H}_2\text{O}, fixed for 24 h, and then embedded with paraffin. Five 6-µm-thick sections were obtained from each lung and stained with hematoxylin and eosin. Images of the lung sections were acquired using an Olympus IMT-2 microscope with \( \times15 \) and \( \times40 \) objectives. The lumen and total area, as well as the wall thickness and arterial radius of the 51- to 100-µm and 101- to 150-µm PAs were quantified using the Image Pro 5.0 software.

**Isolation and culture of PASMCs.** PASMCs were enzymatically isolated and transiently cultured as previously described (46, 52). Briefly, rats were injected with heparin and anesthetized with sodium urethane (1 g/kg). They were exsanguinated, and the lungs were removed and transferred to a petri dish filled with HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Third- and fourth-generation intra-PAs (\( \sim 300 \) to 800 µm) were isolated and cleaned free of connective tissue. The endothelium was removed by gently rubbing the luminal surface with a cotton swab. Arteries were then allowed to recover for 30 min in cold (4°C) HBSS, followed by 20 min in reduced-Ca\textsuperscript{2+} (20 µM) HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 µM Ca\textsuperscript{2+} HBSS containing collagenase (type I, 1.750 U/ml), papain (9.5 U/ml), BSA (2 mg/ml), and dithiothreitol (1 mM), then removed and washed with Ca\textsuperscript{2+}-free HBSS to stop digestion. PASMCs were dispersed gently by trituration with a small-bore pipette in Ca\textsuperscript{2+}-free HBSS at room temperature. The cell suspension was then placed on 25-mm glass cover slips and transiently (16–24 h) cultured in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% FCS, 100 U/ml of streptomycin, and 100 µg/ml of penicillin.

**Measurement of [Ca\textsuperscript{2+}]\textsubscript{i}**. [Ca\textsuperscript{2+}]\textsubscript{i} were monitored using the membrane-permeable Ca\textsuperscript{2+}-sensitive fluorescent dye fluo 3-AM as previously described (26). PASMCs were loaded with 5–10 µM fluo 3-AM (dissolved in DMSO with 20% pluronic acid) for 30–45 min at room temperature (\( \sim 22°C \)) in normal Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed thoroughly with Tyrode solution to remove extracellular fluo 3-AM and rested for 15–30 min to allow for complete deesterification of cytosolic dye. Fluo 3-AM was excited at 488 nm, and emission light at >515 nm was detected using a Nikon TE2000U microscope equipped with epifluorescence attachments and a microfluorometer (PTI). Protocols were executed, and data were collected online with an analog-to-digital interface (FeliX32; PTI). [Ca\textsuperscript{2+}]\textsubscript{i} was calibrated using the equation \([Ca\textsuperscript{2+}]\textsubscript{i} = K_F (F - F_{bg}) (F_{max} - F)\), where \(F_{bg}\) is background fluorescence and \(F_{max}\) is the maximum fluorescence determined in situ in cells superfused with 10 µM 4-bromo-A-23187 and 10 mM Ca\textsuperscript{2+}, and \(K_F\) of fluo 3-AM is 1.1 µM.

**Quantitative real-time RT-PCR.** Deendothelialized PAs frozen in liquid nitrogen were homogenized mechanically. Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA). Genomic DNA contamination was removed by TURBO DNA-free DNase (Ambion, Austin, TX). Total RNA (0.5 µg) was used for first-strand cDNA synthesis using random hexamer primers and Superscript III RNase H - Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Quantitative real-time RT-PCR was used to quantify the changes in the expression of TRPC subtypes. PCR reactions were set up with iQ SYBR Green PCR Supermix (Bio-Rad, Hercules, CA) using 1 µl of cDNA as the template in each 20-µl reaction mixture. Gene-specific real-time PCR primers for TRPC channels are listed in Table 1. The PCR protocol, consisting of an initial step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, was performed using the iQ5 Multicor Color Real-time PCR Detection System (Bio-Rad). Standard curves were generated from serial dilutions of purified PCR products of known copy number. In the experiments for determining the time course of change in TRPC1 and TRPC4 expression, the relative values of TRPC1 and TRPC4 were calculated using the \(\Delta\DeltaCT\) method with \(\beta\)-actin as the internal control. The values measured from samples at various time points were normalized to the averaged value of the control samples.

**Western blotting.** Deendothelialized PAs were quickly frozen in liquid nitrogen. The frozen tissues were crushed and homogenized using a mortar and pestle and then resuspended in ice-cold cell lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% Nonidet P-40, and protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was centrifuged at 4°C with 14,000 g for 15 min, and the supernatant was collected, and the protein concentration was estimated using the bicinchoninic acid assay. The protein sample (40 µg) was resolved in an 8% SDS-PAGE and electrotransferred onto a polyvinylidene membrane. The membrane was blocked with 5% (w/v) nonfat dry milk in PBS containing 0.05% Tween 20 (PBST) for 2 h at room temperature, followed by incubation at 4°C overnight with a monoclonal anti-TRPC1 antibody (ab51255, 1:300 dilution; Abcam) or an anti-TRPC4 antibody (ACC-018, 1:500 dilution; Alomone). The nitrocellulose membrane was then washed with PBST. After being washed, the membrane was incubated with goat-anti-rabbit secondary antibody (no. 7074, 1:3,000 dilution; Cell Signaling Technology) at room temperature for 1 h. Excess secondary antibody was washed again, the bound secondary antibody was detected with enhanced chemiluminescence (Pierce, Rockford, IL), and images were taken using a Gel Logic 200 image system (Kodak, New Haven, CT).
Isometric contraction. Intralobar PAs (300–800 μm OD) were isolated and placed in oxygenated modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.18 KH2PO4, 25 NaHCO3, 10 dextrose, and 2 CaCl2. They were cleaned of connective tissue and cut into 4-mm lengths. Endothelium was disrupted by gently rubbing the lumen with a small wooden stick, and the arterial rings were suspended between two stainless steel stirrups in organ chambers filled with modified Krebs solution for isometric tension recording. The solution was gassed with 95% O2-5% CO2 to maintain a pH of 7.4. Arteries were exposed to 60 mM KCl to establish maximum contraction and to phenylephrine (3 μM) followed by acetylcholine (10 μM) to determine the number of animals, cell samples, or PA rings used. Statistical analysis. Data are expressed as means ± SE, and n indicates the number of animals, cell samples, or PA rings used. Statistical significance was assessed using unpaired or paired Student’s t-tests and ANOVA wherever appropriate. Differences were considered significant at P < 0.05. Curve fitting was performed using the SigmaPlot 8.0 software.

RESULTS

Verification of MCT-induced PAH model. MCT-treated rats exhibited profound PAH and right ventricular hypertrophy when examined on the 21st day after injection. RVSP was increased dramatically (control: 23.6 ± 1.1 mmHg, n = 20; MCT: 52.8 ± 3.2 mmHg, n = 32, P < 0.01), and right heart mass ratio RV/(LV + S) was doubled (control: 29.2 ± 0.7%, n = 20; MCT: 58.3 ± 1.9%, n = 20, P < 0.01) (Fig. 1, A and B). Consistent with previous reports, there was no significant change in mean SAP (control: 105.1 ± 4.0 mmHg, n = 20; MCT: 106.6 ± 3.6 mmHg, n = 21) and the heart rate (control: 368 ± 9 beats/min, n = 22; MCT: 370 ± 7 beats/min, n = 22) in the two groups of rats. Histological examination of lung sections of MCT-treated rats showed that the medial wall of the muscular small PAs (vessel outer diameter of 50–150 μm) was thickened significantly (Fig. 1, C–F). Morphometric analysis of these vessels showed that the ratio of luminal area over total area was diminished markedly (51–100 μm: control 0.585 ± 0.015, n = 39, MCT 0.075 ± 0.008, n = 25, P < 0.01; 101–150 μm: control 0.54 ± 0.016, n = 30, MCT 0.090 ± 0.007, n = 33, P < 0.01) (Fig. 2G), and the ratio of the wall thickness over artery radius was increased notably in MCT-treated PAs (51–100 μm: control 0.292 ± 0.015, n = 39, MCT 0.745 ± 0.030, n = 35; 101–150 μm: control 0.295 ± 0.018, n = 30, MCT 0.712 ± 0.027, n = 39, P < 0.01) (Fig. 2H). These results are consistent with neomuscularization and vascular remodeling in small PAs of MCT-treated rats.

TRPC expression in PA was altered by MCT-induced PAH. The mRNA levels of various TRPC subtypes in endothelium-denuded PAs of control and MCT-treated rats were compared using quantitative real-time RT-PCR (Fig. 2A). TRPC1 and TRPC6 transcripts were predominantly expressed, with a lower level of TRPC3 and almost no expression of TRPC4, TRPC5, and TRPC7 transcripts, in control PAs. The expression of TRPC3 and TRPC6 transcripts was higher in MCT-treated PAs (51–100 μm: control 0.305 ± 0.040, n = 39, MCT 0.714 ± 0.030, n = 35; 101–150 μm: control 0.307 ± 0.027, n = 30, MCT 0.706 ± 0.027, n = 39, P < 0.01) (Fig. 2C). These results are consistent with neomuscularization and vascular remodeling in small PAs of MCT-treated rats.

Table 1. Oligonucleotide sequences of the primers used for RT-PCR

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<th>Target</th>
<th>Primers</th>
<th>Expected Size, bp</th>
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<td>TRPC1</td>
<td>Sense: 5'-CGTGGAGGACAAAGGCTAGAT</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-ACAGACTTTCTGGAA</td>
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<tr>
<td>TRPC3</td>
<td>Sense: 5'-CGTGGCCTTGGTTGTTGAAAC</td>
<td>76</td>
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<tr>
<td></td>
<td>Antisense: 5'-AGTGTAAGGGCTGATGGA</td>
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</tr>
<tr>
<td>TRPC4</td>
<td>Sense: 5'-CCAAATCCAAAGGCTAGAGG</td>
<td>153</td>
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<tr>
<td></td>
<td>Antisense: 5'-CGGAGAACCTCCCAGAATT</td>
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<tr>
<td>TRPC5</td>
<td>Sense: 5'-CGTCTTGCACCACCTTGG</td>
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</tr>
<tr>
<td></td>
<td>Antisense: 5'-CTTGTTGTGCGAACCTTCC</td>
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<tr>
<td>TRPC6</td>
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<td></td>
<td>Antisense: 5'-CTCTGCTCCTCCACTTCCTGG</td>
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<tr>
<td>TRPC1*</td>
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<td></td>
<td>Antisense: 5'-TGATCTGGTCGAAAC</td>
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<td>β-Actin*</td>
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<td>150</td>
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<td></td>
<td>Antisense: 5'-TTTGAATGTCAACGGAGATTTC</td>
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All primers were used for real-time RT-PCR. TRPC, transient receptor potential. *Primers used for the time course experiment.
SOCE were evaluated further by measuring Ca$^{2+}$ transients in PASMCs isolated from control and MCT-treated rats (Fig. 3, C, D, and F). The resting [Ca$^{2+}$]$_i$ was slightly greater in MCT PASMCs (340 ± 23 nM, n = 78) compared with the control PASMCs (247 ± 18 nM, n = 63, P < 0.01). In the presence of nifedipine (3 μM), removal of extracellular Ca$^{2+}$ (Ca$^{2+}$-free Tyrode solution with 0.1 mM EGTA) caused a slight reduction in resting [Ca$^{2+}$]. Application of 10 μM CPA for 10 min caused a slight transient increase in [Ca$^{2+}$], and readmission...
of Ca\textsuperscript{2+} to external solution elicited a mild increase in [Ca\textsuperscript{2+}], in the control PASMCs (233 ± 24 nM, n = 15). In contrast, the magnitude of the Ca\textsuperscript{2+} transient was increased significantly by severalfold (1,832 ± 307 nM, n = 8, P < 0.01) in PASMCs isolated from MCT-treated rats (Fig. 3F). The augmentation of CPA-induced contraction in PAs and Ca\textsuperscript{2+} entry in PASMCs of MCT-treated rats was consistent with the notion that SOCE is enhanced during MCT-induced PAH.

**Time course of increase of CPA-induced PA contraction and TRPC1 upregulation was similar in MCT-treated rats.** The association of the enhanced SOCE and upregulation of TRPC channels was further examined by determining the CPA-induced contraction and the expression of TRPC1 and TRPC4 mRNA in PAs at different time points after MCT injection (Fig. 4). The CPA-induced PA contraction was enhanced significantly in 3 days, reached a plateau in 5 days, and was sustained for 3 wk after MCT injection (Fig. 4, A and B). TRPC1 mRNA level was increased significantly 1 day after MCT treatment, and the increased TRPC expression was maintained over the 3-wk period. In contrast, the expression of TRPC4 mRNA increased gradually, and the maximal level was reached 2 to 3 wk after MCT injection (Fig. 4, C and D). Hence, the time course of the enhanced CPA-induced contraction corresponded more closely to that of the increase in TRPC1 expression, instead of TRPC4 expression, in PA of MCT-treated rats.

**ET-1-induced contraction was enhanced by MCT-induced PAH.** Many vasoactive agonists, including ET-1, bind to G\textsubscript{q} protein-coupled receptors to activate phospholipase C-\(\beta\) to generate inositol trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG), leading to Ca\textsuperscript{2+} influx through SOCC (19, 20, 72). To examine if the enhanced SOCE potentiates agonist-induced vasoconstriction, we compared the ET-1-induced contractile response in PAs of control and MCT-treated rats. ET-1 at concentrations between 0.3 and 30 nM induced concentration-dependent contraction in PAs. The responses elicited by 1 and 3 nM ET-1 were significantly greater in PAs of MCT-treated rats when normalized with the maximal KCl-induced contraction (Fig. 5). The potency of ET-1, based on the EC\textsubscript{50} estimated from the Hill equation, was increased significantly in the MCT-treated group (control: 1.76 ± 0.24 nM, n = 19; MCT: 0.95 ± 0.16 nM, n = 10, P < 0.01).

**SOCE contribution to enhanced ET-1-induced responses in MCT-treated rats.** To examine the contribution of SOCE in the ET-1-induced response, the effective concentration of Gd\textsuperscript{3+} and La\textsuperscript{3+} to inhibit SOCE (26) was first determined in PA rings precontracted by 10 \(\mu\)M CPA followed by cumulative addition of Gd\textsuperscript{3+} or La\textsuperscript{3+} at various concentrations (Fig. 6, A and B). Both Gd\textsuperscript{3+} and La\textsuperscript{3+} caused concentration-dependent relaxation of CPA-precontracted PAs with IC\textsubscript{50} of 2.8 ± 0.1 nM (n = 6) and 5.8 ± 1.1 \(\mu\)M (n = 7), respectively (Fig. 6C), indicating that Gd\textsuperscript{3+} is a thousand times more potent than La\textsuperscript{3+} for the inhibition of SOCE. To evaluate SOCE in the ET-1-induced vasoconstriction, PAs of control and MCT-treated rats were treated with 3 \(\mu\)M nifedipine to block L-type voltage-gated Ca\textsuperscript{2+} channels and precontracted by 10 nM ET-1. Nifedipine had minimal effect on the baseline tension in PAs of control and MCT-treated rats, and the contractions induced by

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**Fig. 3.** Cyclopiazonic acid (CPA)-induced PA contraction and Ca\textsuperscript{2+} transients in control and MCT-treated rats. A and B: typical traces showing CPA-induced PA contraction in control and MCT-treated rats, respectively. External Ca\textsuperscript{2+} was removed (0 Ca\textsuperscript{2+} + 0.1 nM EGTA) with the application of 10 \(\mu\)M CPA for 10 min, and then 2 nM Ca\textsuperscript{2+} was reintroduced to allow store-operated Ca\textsuperscript{2+} entry. C: the average values of CPA-induced PA contraction in control (n = 18) and MCT-treated (n = 21) rats. Data are expressed as a percentage of KCl\textsuperscript{2+} (60 mM)-induced contractile responses. D and E are representative traces of Ca\textsuperscript{2+} transients generated in CPA-precontracted pulmonary arterial smooth muscle cells (PASMCs) from control and MCT-treated rats. F: the average change in Ca\textsuperscript{2+} transients elicited by CPA in PASMCs from control (n = 15) and MCT-treated (n = 8) rats. **Significant difference from control, P < 0.01.**
ET-1 were similar in the two groups (control: 98.3 ± 2.1%, n = 40, MCT: 109.3 ± 7.1%, n = 35). The lanthanides Gd³⁺ (3 nM) and La³⁺ (30 μM), the organic SOCE blocker N-{4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP-2, 1 μM), and the nonselective cation channel blocker SKF-96365 (30 μM) were applied. Vasorelaxation induced by all four blockers was significant greater in PAs of MCT-treated than the control rats (Gd³⁺: control = 50.2 ± 3.3%, n = 9, MCT = 69.9 ± 2.6%, n = 9, P < 0.01; La³⁺: control = 35.7 ± 3.9%, n = 11, MCT = 70.0 ± 5.1%, n = 8, P < 0.01; BTP-2: control = 28.4 ± 2.9%, n = 10, MCT = 62.4 ± 7.0%, n = 10, P < 0.01; SKF-96365: control = 38.2 ± 4.1%, n = 10, MCT = 61.4 ± 3.6%, n = 8, P < 0.01) (Fig. 7). The high potency of Gd³⁺ at a low concentration was consistent with the inhibition of SOCE. The increase in percent relaxation caused by the four SOCE blockers suggested that there was a greater contribution of SOCE to the ET-1-induced contraction in PA of MCT-treated rats.

The participation of SOCE in the ET-1-elicited Ca²⁺ entry was examined further in PASMCs (Fig. 8). In the presence of nifedipine (3 μM), application of 10 nM ET-1 caused a moderate increase in [Ca²⁺]ᵢ in the control group. The Ca²⁺ response was nearly doubled in MCT PASMCs (P < 0.05). Addition of 3 nM Gd³⁺ caused 25.2 ± 3.1% (n = 8) reduction in [Ca²⁺]ᵢ of control PASMCs but a 51.9 ± 4.5% (n = 8, P < 0.01) decrease in PASMCs of MCT-treated rats (Fig. 8, C)

Fig. 4. Time course of change in CPA-induced PA contraction and TRPC1 and TRPC4 mRNA expression in MCT-treated rats. A: typical traces of contraction induced by application of 2 mM Ca²⁺ to PA pretreated with 10 μM CPA in the absence of external Ca²⁺ (0 Ca²⁺ + 0.1 mM EGTA) for 10 min. PAs were isolated from control rats and from rats 3, 5, 7, 14, and 21 days after MCT injection. B: average values of CPA-induced contraction of PA of rats isolated at various time points after MCT injection (n = 8–10 PAs for each time point). Data are expressed as a percentage of K⁺ (60 mM)-induced contractile responses. C and D: relative quantity of normalized TRPC1 and TRPC4 mRNA expression in PA of rats isolated at various time points after MCT injection (n = 8 rats for each time point). *P < 0.05 and **P < 0.01.

Fig. 5. Endothelin-1 (ET-1)-induced concentration-dependent contractile responses in isolated PAs of control and MCT-treated rats. A and B: typical traces generated from PA of control and MCT-treated rats, respectively. The numbers are log molar concentration of ET-1. C: the average concentration-dependent change of contractile tension elicited by ET-1 in control (n = 19) and MCT-treated (n = 10) rats. Data are expressed as the percent contractile responses induced by 60 mM K⁺. **P < 0.05.
and D). Similarly, 30 μM La³⁺ decreased [Ca²⁺], of control PASMCs (39.2 ± 4.8%, n = 14), and the reduction of [Ca²⁺], was apparently larger in PASMCs of MCT-treated rats (59.2 ± 6.2%, n = 11, P < 0.01) (Fig. 8, G and H). These data of Ca²⁺ measurement further confirmed an increased contribution of SOCE in ET-1-induced Ca²⁺ influx in PASMCs of MCT-treated rats.

DISCUSSIONS

There is increasing evidence suggesting that TRPC channels play important roles in PH. We have previously reported that chronic hypoxia-induced PH in rat is associated with the upregulation of TRPC1 and TRPC6 expression and the potentiation of SOCE and ROCE in PASMCs (26). The enhanced SOCE is responsible for the elevated resting [Ca²⁺] in PASMCs and the increased resting tension of PA of chronic hypoxic rats. Subsequent studies found that the increased TRPC expression in hypoxic PASMCs requires full expression of hypoxia-inducible factor-1α (59). Sildenafil treatment, which partially inhibits the development of hypoxic PH, reduces the upregulation of TRPC channels (31). Abnormalities in the regulation of TRPC channels have been reported in cultured PASMCs of patients with IPAH. TRPC3 and TRPC6 were found constitutively overexpressed, and knock down of TRPC6 with siRNA attenuated the accelerated proliferation of these PASMCs (64). Additionally, a functional single-nucleotide polymorphism in the TRPC6 gene promoter region was identified in patients with IPAH. This single-nucleotide polymorphism was found to enhance nuclear factor-κB-mediated promoter activity, stimulate TRPC6 expression, and increase agonist-induced Ca²⁺ entry in PASMCs (65). The dual endothelin receptor A and endothelin receptor B blocker bosentan, which has been used clinically for the treatment of PAH, could reduce the overexpression of TRPC6 and inhibit proliferation of IPAH PASMCs (22). Moreover, bone morphogenetic protein 4, a ligand of the bone morphogenetic protein signaling pathway that is a prevalent factor in hereditary PAH, has also been shown to increase the expression of TRPC1, TRPC4, and TRPC6 in cultured rat PASMCs (30). These findings raise the possibility that TRPC channel upregulation and the associated increase in Ca²⁺ entry could be an important signaling pathway in the development of PH.

In the present study, we further tested this hypothesis by using a widely used experimental model of PAH. MCT causes severe PH through the toxic effects of its metabolite MCT pyrrole on pulmonary endothelium, leading to inflammation, endothelial cell damage and apoptosis, prominent medial thickening, and inflammatory adventitial remodeling, resulting in elevated pulmonary arterial pressure and right heart hypertrophy (8, 54, 61). Consistent with our hypothesis, we found that the expression of TRPC1 and TRPC4 channels, as well as pulmonary vascular response and Ca²⁺ entry in PASMCs elicited by depleting SR Ca²⁺ stores, was enhanced significantly in MCT-induced pulmonary hypertensive rats. Furthermore, MCT treatment significantly potentiated the ET-1-induced contraction of PA and Ca²⁺ entry in PASMCs. These ET-1-induced responses were more susceptible to organic and inorganic SOCE blockers, suggesting that SOCE contributes to the enhanced pulmonary vascular reactivity in MCT-treated rats. Because MCT induces PH through mechanisms distinctive from chronic hypoxia, the enhancement of TRPC1 expression and SOCE in the two different models supports the notion that the TRPC channel-dependent SOCE pathway is a common signaling pathway shared by different types of PH.

Recent studies on SOCE have established that the stromal interacting molecule 1 (STIM1), a transmembrane protein with an NH₂-terminal EF-hand Ca²⁺-binding domain, operates as the Ca²⁺ sensor of endoplasmic reticulum (ER) or SR Ca²⁺ content (28, 48, 70). Depletion of ER/SR Ca²⁺ content (28, 48, 70). Depletion of ER/SR Ca²⁺ content results in the translocation and rearrangement of STIM1 into the form of punctae, which couple with the Ca²⁺ release-activated channel (CRAC) Orai 1 and/or TRPC channels in the plasma membrane to form SOCC complexes, leading to subsequent activation of Ca²⁺ entry. Structure-function studies revealed that STIM1 contains a cytoplasmic ERM domain that binds with TRPC1, TRPC4, and TRPC5 channels (15), an Orai activating region within the ERM domain that gates the Orai channels (42, 66), and a polybasic lysine-rich domain that is required for the clustering of STIM1 at the plasma membrane and the gating of TRPC channels (27, 68). Depending on the cell types, STIM1 interacts with TRPC and Orai proteins either cooperatively to
confer SOCE (21, 24, 25, 56) or independently to activate SOCE or CRAC current, respectively (1, 6, 37, 38, 44). STIM1 mRNA and protein are highly expressed in PAs, and its permissive role in SOCE has been verified by knock down or overexpression of STIM1 in PASMCs (32, 37).

TRPC channels have long been implicated in SOCE since their discovery. Among the various TRPC channels, TRPC1 is considered as a major constituent of SOCE in both systemic cells and PASMCs (5, 26, 37, 62, 63). Inhibition of TRPC1 expression with antisense oligonucleotides blocked SOCE in cultured human PASMCs (55), and overexpression of TRPC1 in rat PA enhanced SOCE-induced vasoconstriction (23). Knock down of TRPC1 in rat PASMCs using siRNA attenuated thapsigargin-activated SOCE without affecting ROCE, and knock down of TRPC6 only inhibited the DAG analog-induced ROCE without altering SOCE. These experiments suggest that TRPC1 is responsible for SOCE and is distinctly independent from the ROCE pathway in PASMCs (26). Additionally, pretreatment of mouse PASMCs with an antibody against TRPC1 inhibited CPA-induced SOCE. TRPC1 was coimmunoprecipitated with STIM1, and the level of coimmunoprecipitation was increased in cells subjected to store depletion (37). Our present study shows that the increase in TRPC1 mRNA and protein level is associated with enhanced SOCE in

Fig. 7. Vasorelaxant effects of Gd³⁺, La³⁺, SKF-96365, and 10 µM BTP-2 on ET-1-preconstricted PAs of control and MCT-treated PAs. A: left and middle show representative tracings of vasorelaxation induced by 3 nM Gd³⁺ in PA rings preconstricted with 10 nM ET-1 in control and MCT-treated rats, respectively. PA rings were pretreated by 3 µM nifedipine for 5 min. Data are expressed as a percentage of K⁺ (60 mM)-induced contractile responses. Right shows the average percent relaxation of ET-1-induced contractions caused by Gd³⁺ in control (n = 9) and MCT-treated (n = 9) groups. NIF, nifedipine. B: representative tracings of vasorelaxation and the average percent relaxation of ET-1-induced contractions caused by 30 µM La³⁺ in PA rings of control (n = 11) and MCT-treated (n = 8) rats. C: representative tracings of vasorelaxation and the average percent relaxation of ET-1-induced contractions caused by 30 µM SKF-96365 in PAs of control (n = 10) and MCT-treated (n = 8) rats. D: representative tracings of vasorelaxation and the average percent relaxation of ET-1-induced contractions caused by 10 µM BTP-2 in PAs of control (n = 10) and MCT-treated (n = 10) rats. BTP-2, N-[4-[[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide. *P < 0.05, differences between control and MCT-treated groups.

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PA of MCT-treated rats, similar to our observations in chronic hypoxia rats (26). Moreover, the time course of the increase in TRPC1 expression was found to correspond with that of the enhanced CPA-induced PAH. This is supported by our results showing augmented responsiveness to ET-1 in endothelium-denuded PAs of MCT rats. The hyperreactivity to ET-1 could be attributable to an increase in ET receptors (18, 29) and their downstream signaling pathways involving SOCE. ET-1 binds to G protein-coupled ET receptors to activate phospholipase C to generate IP₃ and DAG, which can act synergistically to promote Ca²⁺ entry through TRPC channels. IP₃ activates Ca²⁺ release via IP₃ receptors and cross-activates neighboring ryanodine receptors through a Ca²⁺-induced Ca²⁺ release mechanism (71), leading to the reduction of SR Ca²⁺ to activate SOCE. DAG directly activates the ROC (17, 20) and stimulates protein kinase C to enhance L-type Ca²⁺ channel activity (34).

In this study, the contribution of SOCE in ET-1-induced PA contraction was examined using four different inorganic and organic blockers, Gd³⁺, La³⁺, BTP-2, and SKF-96365, in the presence of nifedipine to inhibit L-type Ca²⁺ channels. The lanthanides Gd³⁺ and La³⁺ were used because of their high selectivity for SOCE at low concentration (7, 26, 47). BTP-2 is an organic SOCE inhibitor that has been shown to block TRPC channels (12, 14, 40, 73), and SKF-96365 is a nonselective cation channel blocker that inhibits SOCE in PASMCS (36, 58). The high potency of Gd³⁺ (IC₅₀ = 2.8 nM) for the inhibition of CPA-induced SOCE was verified in our preparation (Fig. 7). Our results of a low concentration of Gd³⁺, as well as the other SOCE blockers, significantly inhibiting the ET-1-activated contraction suggest that the response was mediated at least in part by SOCE. The effective inhibition of ET-1-induced contraction with BTP-2 further suggests that TRPC channels could be involved. This is consistent with reports in other vascular smooth muscles that ET-1 activates TRPC channels (43, 49, 50) and the contraction is mediated by several nonselective cation channel pathways, including SOCE (35, 72). Furthermore, the percent inhibition of ET-1-induced PA contraction by each SOCE blocker was potentiated significantly in the PAs of MCT-treated rats. The increase in the efficacies of the SOCE blockers was apparently related to their ability of reducing [Ca²⁺]ᵢ because both Gd³⁺ and La³⁺ caused greater reduction of the ET-1-activated Ca²⁺ transient

Fig. 8. Inhibitory effects of 3 nM Gd³⁺ and 30 µM La³⁺ on intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) response induced by 10 nM ET-1 in PASMCs of control and MCT-treated rats. A and B: typical tracings showing the blocking effect of 3 nM Gd³⁺ on ET-1-induced Ca²⁺ response in control and MCT PASMCs. PASMCs were pretreated by 3 µM nifedipine for 5 min. C: average change in [Ca²⁺]ᵢ elicited by 10 nM ET-1 and 1 nM Gd³⁺ in control (n = 13) and MCT (n = 12) PASMCs. D: summary data showing percent inhibition of 10 nM ET-1-elicited Ca²⁺ responses by 3 nM Gd³⁺. E and F: typical tracings showing the blocking effect of 30 µM La³⁺ on ET-1-induced Ca²⁺ response in control and MCT PASMCs. G: average change in [Ca²⁺]ᵢ elicited by 10 nM ET-1 and 30 µM La³⁺ in control (n = 14) and MCT (n = 11) PASMCs. H: summary data showing percent inhibition of 10 nM ET-1-elicited Ca²⁺ responses by 30 µM La³⁺. **Statistically significant difference (P < 0.01) between control and MCT cells.
in MCT-treated PASMCs. These results are consistent with a greater contribution of SOCE in the ET-1-induced contraction in PAs of MCT rats.

In summary, the expression of the store-operated TRPC1 and TRPC4 channels and the SOCE activity is enhanced in PAs of MCT-induced PAH rats. The enhanced SOCE plays a significant role in the enhanced vascular reactivity in MCT PAH. Our findings support the hypothesis that TRPC1-dependent SOCE is a common signaling pathway involved in the development of PAH and provide further physiological basis for targeting SOCE and TRPC channels for the treatment of PH.

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REFERENCES


