Chronic Hypoxia-Induced Upregulation of Store-Operated and Receptor-Operated Ca\textsuperscript{2+} Channels in Pulmonary Arterial Smooth Muscle Cells

A Novel Mechanism of Hypoxic Pulmonary Hypertension

Mo-Jun Lin, George P.H. Leung, Wei-Min Zhang, Xiao-Ru Yang, Kay-Pong Yip, Chung-Ming Tse, James S.K. Sham

Abstract—Chronic hypoxic pulmonary hypertension is associated with profound vascular remodeling and alterations in Ca\textsuperscript{2+} homeostasis in pulmonary arterial smooth muscle cells (PASMCs). Recent studies show that transient receptor potential (TRPC) genes, which encode store-operated and receptor-operated cation channels, play important roles in Ca\textsuperscript{2+} regulation and cell proliferation. However, the influence of chronic hypoxia on TRPC channels has not been determined. Here we compared TRPC expression, and store- and receptor-operated Ca\textsuperscript{2+} entries in PASMCs of normoxic and chronic hypoxic rats. Reverse-transcription polymerase chain reaction (RT-PCR), Western blot, and immunostaining showed consistently that TRPC1, TRPC3, and TRPC6 were expressed in intralobar pulmonary arteries (PAs) and PASMCs. Application of 1-oleoyl-2-acetyl-sn-glycerol (OAG) to directly activate receptor-operated channels, or thapsigargin to deplete Ca\textsuperscript{2+} stores, caused dramatic increase in cation entry measured by Mn\textsuperscript{2+} quenching of fura-2 and by Ca\textsuperscript{2+} transients. OAG-induced responses were ≈700-fold more resistant to La\textsuperscript{3+} inhibition than thapsigargin-induced responses. siRNA knockdown of TRPC1 and TRPC6 specifically attenuated thapsigargin- and OAG-induced cation entries, respectively, indicating that TRPC1 mediates store-operated entry and TRPC6 mediates receptor-operated Ca\textsuperscript{2+} entry. In hypoxic PAs, there were 2- to 3-fold increases in TRPC1 and TRPC6 expression. They were accompanied by significant increases in basal, OAG-induced, and thapsigargin-induced cation entries in hypoxic PASMCs. Moreover, inhibition of store-operated Ca\textsuperscript{2+} entry with La\textsuperscript{3+} and SK&F-96365 reversed the elevated basal [Ca\textsuperscript{2+}], in PASMCs and vascular tone in PAs of chronic hypoxic animals, but nifedipine had minimal effects. Our results for the first time to our knowledge show that both store- and receptor-operated channels of PASMCs are upregulated by chronic hypoxia and contribute to the enhanced vascular tone in hypoxic pulmonary hypertension. (Circ Res. 2004; 95:000-000.)

Key Words: pulmonary hypertension ■ transient receptor potential channels ■ store-operated channel ■ receptor-operated channel

Prolonged exposure to alveolar hypoxia causes pulmonary hypertension with profound vascular remodeling and increase in vasomotor tone. The increase in vascular tone is in part attributable to alterations in vasoconstricting and vasorelaxing influences imposed by the endothelially derived and circulating factors. Recent evidence indicates that chronic hypoxia also causes intrinsic changes in ionic balance and Ca\textsuperscript{2+} homeostasis in pulmonary arterial smooth muscle cells (PASMCs), including membrane depolarization, elevation in resting [Ca\textsuperscript{2+}], and changes in electrophysiological and Ca\textsuperscript{2+} responses to vasoconstrictors and vasodilators. The mechanism for alteration in Ca\textsuperscript{2+} homeostasis in hypoxic PASMCs is controversial. Previous studies found significant suppression of voltage-gated K\textsuperscript{+} (K\textsubscript{v}) currents and K\textsubscript{v} channel expression in PASMCs isolated from chronic hypoxic animals or cultured under hypoxic conditions. Because K\textsubscript{v} channel is a major conductance controlling resting membrane potential, it has been postulated that chronic hypoxia inhibits K\textsubscript{v} channels, causing membrane depolarization, activation of L-type Ca\textsuperscript{2+} channels, and increase in [Ca\textsuperscript{2+}]. The involvement of L-type Ca\textsuperscript{2+} channel activation has gained support from some studies showing that
Ca<sup>2+</sup> channel antagonists, nifedipine and verapamil, attenuated hypoxia-induced pulmonary hypertension.<sup>6,8</sup> However, other studies showed that the effects of Ca<sup>2+</sup> channel blockers were often partial, temporary, and sometimes complicated by changes in cardiac output.<sup>10,11</sup> In chronic hypoxic rats, nifedipine was ineffective in reducing pulmonary hypertension, despite another vasodilator, NIP-121, causing significant reduction in pulmonary arterial pressure and vascular resistance.<sup>12</sup> More dramatically, the elevated resting [Ca<sup>2+</sup>]<sub>i</sub> in PASMCs of chronic hypoxic rats was unaffected by nifedipine but was reduced instantaneously to the level of control PASMCs by removal of extracellular Ca<sup>2+</sup>.<sup>5</sup> These results suggest that other Ca<sup>2+</sup> influx pathway(s) in additional to L-type Ca<sup>2+</sup> channel are involved in chronic hypoxic pulmonary hypertension.

Nonselective cation channels, encoded by the canonical transient receptor potential (TRPC) gene family, constitute the alternative pathways of Ca<sup>2+</sup> entry in vascular myocytes. Recent evidence suggests that TRPC1 channel is related to store-operated Ca<sup>2+</sup> entry, which can be activated by depletion of Ca<sup>2+</sup> stores using cyclopiazonic acid (CPA) or thapsigargin;<sup>13,14</sup> TRPC3 and TRPC6 channels are involved in receptor-operated entry, which can be activated directly by diacylglycerol (DAG) via a protein kinase C (PKC)-independent mechanism.<sup>15–17</sup> Multiple TRPC subtypes have been identified in canine pulmonary arteries (PAs), fresh or cultured rat main PASMCs, and cultured human PASMCs.<sup>14,18–21</sup> They have been implicated to mediate store-operated Ca<sup>2+</sup> influx.<sup>19,20</sup> However, receptor-operated Ca<sup>2+</sup> entry has not been demonstrated in PASMCs, and the effects of chronic hypoxia on TRPC-dependent Ca<sup>2+</sup> entries have not determined. Because hypoxic pulmonary hypertension is associated with PASMC proliferation<sup>14,22</sup> and enhanced reactivity to vasoconstrictors,<sup>12,23,24</sup> both processes are related to store- and receptor-operated Ca<sup>2+</sup> channels; it is possible that the activity/expression of these channels are altered by chronic hypoxia. In the present study, we sought to identify the TRPC channels expressed in rat intralobar PASMCs, characterize the store and receptor-operated cation entries, determine changes in TRPC expression, store-operated and receptor-operated cation entries, and their involvement in the elevated basal [Ca<sup>2+</sup>]<sub>i</sub>, and vasomotor tone induced by chronic hypoxia.

### Materials and Methods

#### Chronic Hypoxic Exposure

Male Wistar rats (150 to 250 grams) were placed in a hypoxic chamber and exposed to either normoxia or normobaric hypoxia 10% O<sub>2</sub> for 3 to 4 weeks to induce hypoxic pulmonary hypertension as described previously.<sup>5</sup>

#### Isolation and Culture of PASMCs

PASMCs were enzymatically isolated from de-endothelialized intralobar PAs.<sup>6</sup> PASMCs from chronic hypoxic and normoxic animals were cultured transiently (16 to 24 hours) inside a modular incubator chamber (Billups-Rothenberg, Inc) under 4% O<sub>2</sub>/5% CO<sub>2</sub> and 21% O<sub>2</sub>/5% CO<sub>2</sub>, respectively, before used.

#### Measurement of Intracellular [Ca<sup>2+</sup>] and Mn<sup>2+</sup>

[Ca<sup>2+</sup>]<sub>i</sub>, was monitored using fluo-3 AM as previously described.<sup>25</sup> Ca<sup>2+</sup> entry through TRPCs was quantified by quenching of fura-2 with Mn<sup>2+</sup>. PASMCs were loaded with fura-2 AM and bathed in a Ca<sup>2+</sup> free (with 0.1 mmol/L EGTA) nifedipine (10 μmol/L) containing Tyrode solution. Fura-2 was excited at 360 nm, and emission light was recorded at >510 nm. After a stable baseline fluorescence was attained, 500 μmol/L Mn<sup>2+</sup> was applied through a multibarrel pipette positioned <50 μm from PASMCs. Maximum rate of quenching of fura-2 fluorescence was determined.

#### Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from intralobar PAs and PASMCs, and reverse-transcription polymerase chain reaction (RT-PCR) was performed using standard methods. Specific sense and antisense primers for TR-PCR were designed according to the strategies described in the online data supplement available at http://circres.ahajournals.org. PCR products of TRPC were quantified using β-actin as an internal standard.

### Western Blotting and Immunostaining of TRPCs

Proteins were extracted from PAs and PASMCs, and TRPC subtypes were detected using a standard Western blot protocol. Polyclonal rabbit anti-TRP1, anti-TRP3, anti-TRP4, or anti-TRP6 antibodies (Alomone Labs, Jerusalem, Israel) were used as the primary antibodies. Rat brain membrane proteins were used as positive controls. Immunostaining of TRPC in PASMCs was performed as described previously.<sup>26</sup> Coverslip without exposing to primary antibodies were used as negative control.

#### siRNA Knockdown of TRPC

PASMCs were isolated and cultured for ~24 hours and then transiently transfected with siRNA specific for TRPC1, TRPC6, or a control nonselective cation channel blocker. siRNA Knockdown of TRPC was determined using Tyrode solution. Fura-2 was excited at 360 nm, and emission light was recorded at >510 nm. After a stable baseline fluorescence was attained, 500 μmol/L Mn<sup>2+</sup> was applied through a multibarrel pipette positioned <50 μm from PASMCs. Maximum rate of quenching of fura-2 fluorescence was determined.

#### Results

### Identification of TRPC Subtypes

TRPC subtypes were detected using a standard Western blot protocol. Polyclonal rabbit anti-TRP1, anti-TRP3, anti-TRP4, or anti-TRP6 antibodies (Alomone Labs, Jerusalem, Israel) were used as the primary antibodies. Rat brain membrane proteins were used as positive controls. Immunostaining of TRPC in PASMCs was performed as described previously.<sup>26</sup> Coverslip without exposing to primary antibodies were used as negative control.

#### Identification of TRPC Subtypes

Figure 1A shows RT-PCR amplified products of TRPCs in normoxic PASMCs after 30 cycles. TRPC1 and TRPC6 were the major, and TRPC3 was the minor, TRPC mRNA expression. TRPC1 and TRPC6 proteins were undetectable. TRPC2 and TRPC5 mRNA were simultaneously detected along with positive controls. All RT-PCR amplified products from PASMCs had sizes corresponding to the predicted sequences and matched with positive controls generated from brain or heart. Same results were obtained when mRNA were extracted from freshly isolated intralobar PAs (Figure 4A and 4B).

Expressions of TRPC proteins in normoxic PASMCs were examined using Western blot analysis (Figure 1B). TRPC1, TRPC3, and TRPC6 proteins of ~140, 150, and 115 kDa, respectively, were clearly detected along with positive controls from rat brain, whereas TRPC4 proteins were undetectable using specific antibodies from 2 different companies. Immunostaining confirmed that the TRPC subtypes identified by RT-PCR and Western blot analysis were indeed expressed in PASMCs and were not attributable to contaminations of endothelial cells. Strong immunofluorescent signals were detected in PASMCs using specific anti-TRPC1, anti-TRPC3, and anti-TRPC6 antibodies, but not with the anti-TRPC4 antibody (Figure 1C).
Thapsigargin-Induced and Diacylglycerol-Induced Ca\(^{2+}\)/Cation Entry

The functional activities of store-operated and receptor-operated channels were quantified by quenching fura-2 fluorescence with Mn\(^{2+}\). In the absence of extracellular Ca\(^{2+}\) (0.1 mmol/L EGTA) and in the presence of 10 \(\mu\)mol/L nifedipine, application of 500 \(\mu\)mol/L Mn\(^{2+}\) caused a slow quenching of fura-2 fluorescence (0.04±0.004%/second).

Figure 2. Characterization of thapsigargin- and OAG-induced cation entry by Mn\(^{2+}\) quenching of fura-2 in PASMCs. A, Representative tracings recorded in control, thapsigargin, OAG, and OAG+staurosporine (stau)-treated PASMCs. B and C, Effects of La\(^{3+}\) on thapsigargin- and OAG-induced cation entries. D, Concentration-dependent inhibition of thapsigargin- and OAG-induced Mn\(^{2+}\) quenching by La\(^{3+}\). Lines are the least-square fit of the data using the Hill equation; 7 to 12 experiments from at least 3 different animals were performed for each concentration of La\(^{3+}\).
Depleting SR Ca\(^{2+}\) stores after 15 minutes of exposure of PASMCs to 10 \(\mu\)mol/L thapsigargin caused a dramatic 25-fold increase in the maximal rate of Mn\(^{2+}\)-induced quenching (1.01±0.19%/second; \(n=8\), \(P<0.001\)) (Figure 2A). Activation of receptor-operated cation entry by 10 minutes pretreatment with 100 \(\mu\)mol/L 1-oleoyl-2-acetyl-sn-glycerol (OAG) also accelerated significantly the rate of quenching from 0.05±0.01 to 0.43±0.09%/second (\(n=9\), \(P<0.001\)). OAG-induced cation entry was not impeded by PKC inhibition using staurosporine. Hence, it was not mediated via PKC-dependent phosphorylation but by direct activation of TRPCs, a hallmark of receptor-operated cation entry.\(^{15}\) Thapsigargin-induced store-operated cation entry was highly sensitive to La\(^{3+}\), which inhibited Mn\(^{2+}\) quenching at submicromolar concentrations with an IC\(_{50}\) of 0.27±0.08 \(\mu\)mol/L (Figure 2B and 2D). By contrast, the OAG-induced cation entry was rather insensitive to La\(^{3+}\). Significant inhibition by La\(^{3+}\) was observed only at concentrations of \(\geq100\ \mu\)mol/L, with an IC\(_{50}\) of 183±54 \(\mu\)mol/L.

Store-operated and receptor-operated Ca\(^{2+}\) entries were further evaluated by recording the Ca\(^{2+}\) transients (Figure 3). In the presence of nifedipine (10 \(\mu\)mol/L), removal of extracellular Ca\(^{2+}\) for 10 minutes caused a slight decrease in resting [Ca\(^{2+}\)]. Re-introduction of Ca\(^{2+}\) resulted in a minimal increase of [Ca\(^{2+}\)] (57.9±25.7 mmol/L) to the basal level. When SR Ca\(^{2+}\) was depleted by thapsigargin, a large over-shoot of Ca\(^{2+}\) transient (772.51±120 mmol/L, \(n=18\)) was elicited immediately on switching back to Ca\(^{2+}\) containing solution. This store-operated Ca\(^{2+}\) entry was abolished by 30 \(\mu\)mol/L La\(^{3+}\) (87.37±68 mmol/L, \(n=13\), \(P<0.005\)). Pretreatment of PASMCs with OAG also activated a Ca\(^{2+}\) entry transient (293±51 mmol/L, \(n=9\)), which was unaffected by 100 \(\mu\)mol/L La\(^{3+}\) (343±54 mmol/L, \(n=9\)). These results suggest that PASMCs possess two functionally distinctive thapsigargin-activated and OAG-activated Ca\(^{2+}\) pathways, which have different sensitivity to the inorganic blocker La\(^{3+}\).

**Chronic Hypoxia–Altered TRPC Expression and Cation Entry**

TRPC expression in intralobar PAs of normoxic and chronic hypoxic rats was quantified by RT-PCR, using \(\beta\)-actin as the internal standard. TRPC1 and TRPC6 mRNA levels were approximately tripled in hypoxic, compared with normoxic PAs, but TRPC3 levels were the same (Figure 4A and 4B). Western blot analysis showed that TRPC1 and TRPC6 protein levels were increased by \(\approx150\%\), whereas TRPC3 protein expression was unaltered, in chronic hypoxic PAs. The increase in the expression of TRPC mRNA and protein in hypoxic PA was accompanied by enhanced cation entry. The rate of thapsigargin-induced Mn\(^{2+}\) quenching was increased from 1.01±0.19%/0.04±0.004%/second (\(n=8\)) in normoxic to 2.23±0.37%/second (\(n=8\), \(P<0.01\)) in hypoxic PASMCs, whereas OAG-induced cation entry was accelerated from 0.31±0.05%/second (\(n=9\)) in normoxic to 0.98±0.31%/second (\(n=9\)) in hypoxic cells. Moreover, the basal rate of cation entry was slightly, but significantly, higher in hypoxic PASMCs (normoxia: 0.041±0.004%/second, \(n=23\); hypoxia: 0.053±0.003%/second, \(n=24\), \(P<0.05\)). Ca\(^{2+}\) entry transients also showed that both thapsigargin- and OAG-induced Ca\(^{2+}\) entries were significantly enhanced in hypoxic PASMCs (Figure 6). After pretreatment with thapsigargin in nifedipine containing Ca\(^{2+}\) free solution, reintroduction of Ca\(^{2+}\) to hypoxic PASMCs elicited a large Ca\(^{2+}\) transient (1.71±0.39 mmol/L, \(n=6\)) of twice the magnitude of Ca\(^{2+}\) entry transients elicited in normoxic PASMCs (772.51±120 mmol/L, \(n=18\)). OAG-induced Ca\(^{2+}\) entry transients were also increased by \(>200\%\) (normoxia: 0.29±0.05 \(\mu\)mol/L, \(n=9\); hypoxia: 0.99±0.26 \(\mu\)mol/L, \(n=8\)) in hypoxic PASMCs. These results clearly indicate that the expression of putative store-operated and receptor-operated Ca\(^{2+}\) channels and their functional activities are proportionately augmented in PASMCs after chronic exposure to hypoxia.

**siRNA Knockdown of TRPC1 and TRPC6**

To verify whether TRPC1 and TRPC6 expressions indeed dictated the store- and receptor-operated cation entries in PASMCs, the channels were knockdown specifically using siRNA. Transfection of normoxic PASMCs with siRNA against TRPC1 resulted in 70% reduction in mRNA and protein of TRPC1, whereas TRPC3 levels were the same (Figure 4A and 4B). Western blot analysis showed that TRPC1 and TRPC6 protein levels were approximately tripled in hypoxic, compared with normoxic PAs, but TRPC3 levels were the same (Figure 4A and 4B).
n=14, P<0.01). OAG-induced Mn\textsuperscript{2+}-quenching was similar in
the two groups of myocytes. Transfection of PASMCs with
TRPC6 siRNA caused a 50% reduction of mRNA and protein of
TRPC6, without affecting the expression of TRPC1. The reduc-
tion of TRPC6 expression was associated with a comparable
reduction in the rate of OAG-induced Mn\textsuperscript{2+} quenching (control:
0.50±0.08%/second; TRPC6 siRNA: 0.19±0.05%/second, n=9, P<0.05), whereas the store-operated cation entry was unaf-
fected. These results indicate that TRPC1 and TRPC6 are the
major determinants of the store- and receptor-operated Ca\textsuperscript{2+}
entries, respectively, in rat PASMCs.

**Roles of Store-Operated and Receptor-Operated Ca\textsuperscript{2+} Entry on Baseline [Ca\textsuperscript{2+}] and Vasomotor Tone**

As reported previously,\textsuperscript{2} basal [Ca\textsuperscript{2+}], was significantly ele-
vated in chronic hypoxic PASMCs (normoxia: 176±9
nmol/L, n=23; hypoxia: 267±30 nmol/L, n=9, P<0.05)
hypoxic cells. **Significance**

**Figure 6.** Comparison of thapsigargin- and OAG-induced Ca\(^{2+}\) transients in normoxic and chronic hypoxic PASMCs. A, Representative traces of thapsigargin-induced Ca\(^{2+}\) transients generated in normoxic (upper panel) and hypoxic (lower panel) PASMCs. B, The averaged change in peak Ca\(^{2+}\) transients elicited by thapsigargin in 18 normoxic and 6 hypoxic cells. C, Representative traces of OAG-induced Ca\(^{2+}\) transients generated in normoxic and hypoxic PASMCs. D, The averaged change in peak Ca\(^{2+}\) transients elicited by OAG in 9 normoxic and 8 hypoxic cells. **Significant difference between normoxic and hypoxic PASMCs.**

(Figure 8). It was quickly reversed by the removal of extracellular Ca\(^{2+}\). Application of 10 \(\mu\)mol/L La\(^{3+}\) to block store-operated Ca\(^{2+}\) channels caused a similar reduction in [Ca\(^{2+}\)], to 159±17 nmol/L (n=12, \(P<0.05\)), whereas complete inhibition of L-type Ca\(^{2+}\) channels with 1 \(\mu\)mol/L nifedipine only caused a 30% to 40% reduction, as induced by Ca\(^{2+}\) removal or La\(^{3+}\). In the presence of nifedipine, 10 \(\mu\)mol/L La\(^{3+}\) caused a further decrease in basal [Ca\(^{2+}\)]; increase [La\(^{3+}\)] to 300 \(\mu\)mol/L to inhibit receptor-operated Ca\(^{2+}\) entry failed to cause additional decrease in [Ca\(^{2+}\)], (n=4). In contrast, removal of Ca\(^{2+}\), application of La\(^{3+}\), or nifedipine caused only minor reduction in basal [Ca\(^{2+}\)] in normoxic PASMCs. These results suggest that store-operated Ca\(^{2+}\) entry is the major Ca\(^{2+}\) entry pathway responsible for the elevated [Ca\(^{2+}\)], in chronic hypoxic PASMCs.

Parallel experiments were performed to evaluate basal vascular tone in PA rings. SK&F-96365 was used to inhibit nonselectively the store- and receptor-operated cation entry because La\(^{3+}\) precipitates in Krebs bicarbonate solution. Removal of extracellular Ca\(^{2+}\) or 30 \(\mu\)mol/L SK&F-96365 caused a 10% to 12% reduction in resting tension in hypoxic PAs but had minimal effects on normoxic PAs. Nifedipine, which has no effect on resting tension in normoxic PAs, relaxed the chronic hypoxic PAs by 3.8±1.4%; and subsequent application of SK&F-96365 further relaxed the hypoxic PA to 9.1±1.5% (n=7, \(P<0.05\)), a reduction comparable to Ca\(^{2+}\) removal.

**Discussion**

The present study demonstrated the coexistence of functionally distinctive receptor-operated and store-operated cation entry pathways in PASMCs. The evidence includes: (1) the putative store-operated Ca\(^{2+}\) channel TRPC1 and receptor-operated Ca\(^{2+}\) channel TRPC6 are coexpressed in rat PASMCs and PAs; (2) the cation/Ca\(^{2+}\) entry can be elicited by depleting SR Ca\(^{2+}\) stores using thapsigargin and by direct activation of receptor-operated channel using OAG; (3) thapsigargin- and OAG-induced cation/Ca\(^{2+}\) entries are distinguishable pharmacologically by a large difference in the sensitivity to La\(^{3+}\); and (4) siRNA knockdown of TRPC1 and TRPC6 inhibited specifically the thapsigargin and OAG-induced cation entry, respectively. As far as we are aware, these are the first direct evidence of receptor-operated cation entry in PASMCs.

The expression of TRPC subtypes has been reported in several types of vascular smooth muscle cells including PASMCs, but their specific expression vary among species and cell preparations. RT-PCR detected TRPC4, TRPC6, and TRPC7 mRNAs in canine main PAs, with TRPC4 being the major expressed isoform.\(^{21}\) In cultured rat main PASMCs, TRPC1, TRPC2, TRPC4, TRPC5, and TRPC6 mRNAs were found highly expressed, whereas TRPC3 and TRPC7 were undetectable.\(^{19}\) In contrast, a similar study using freshly isolated PASMCs from rat main PAs found prominent expression of TRPC1 and TRPC6, less expression of TRPC3, and exceedingly low levels of expression of TRPC4 and TRPC5 mRNA.\(^{20}\) The expression profile of TRPC in our freshly isolated intralobar PAs and transist cultured PASMCs (<1 day) is similar to those of freshly isolated rat main PASMCs, suggesting that TRPC1, TRPC3, and TRPC6 are the major TRPCs expressed in rat pulmonary vasculature. The expression of other TRPC subtypes (eg, TRPC2, TRPC4, TRPC5) in cultured rat main PASMCs could be related to prolonged cell culture and/or contamination by endothelial cells, because TRPC expression changes during PASMC proliferation.\(^{14,18,22}\) and TRPC4 is prominently expressed in pulmonary endothelium.

Detection of the putative receptor-operated channel TRPC6,\(^{15-17}\) in addition to TRPC1, provides the hint that receptor-operated Ca\(^{2+}\) entry pathway is present in PASMCs. Previous studies using vasoactive agonists to activate receptor-operated Ca\(^{2+}\) entry in native tissues were complicated by the fact that they activate both store- and receptor-operated channels, and common blockers of nonselective cation channel inhibit both types of Ca\(^{2+}\) entries. To circumvent these complications, OAG was used as the stimulant to activate directly the receptor-operated TRPC channels,\(^{15}\) without causing SR Ca\(^{2+}\) depletion or IP3s and RyRs activation in PASMCs.\(^{25}\) Indeed, OAG elicited a dramatic cation entry, which was unaffected by PCK inhibition with stauorosporine and was highly resistant to La\(^{3+}\) (Kd \(\approx\)200 \(\mu\)mol/L), in contrast to the La\(^{3+}\)-sensitive thapsigargin-induced cation entry (Kd \(\approx\)0.3 \(\mu\)mol/L) in the same
PASMCs. The several hundred-fold difference in La$^{3+}$ sensitivity is consistent with reports that lanthanides, La$^{3+}$ and Gd$^{3+}$, have a higher affinity to store-operated than to receptor-operated cation channels and can be used as a "yardstick" for distinguishing the two Ca$^{2+}$ entry pathways in PASMCs.

The thapsigargin-induced cation entry in rat intralobar PASMCs is mediated by TRPC1. Using TRPC1 siRNA in this study and using TRPC1 specific antisense oligonucleotides or specific antibody in other studies have demonstrated unequivocally that inhibition of TRPC1 expression/function selectively blocks store-operated Ca$^{2+}$ entry.$^{13,14,27}$ Pharmacological evidence also shows that a low concentration of lanthanides ($<1$ μmol/L), which effectively blocks store-operated cation entry in rat PASMCs, PAs, and systemic myocytes,$^{26,28}$ also blocks TRPC1 channels in heterologous expression systems.$^{29}$ More importantly, the present study demonstrated that TRPC6 is a major constituent for receptor-operated Ca$^{2+}$ entry in PASMCs, because TRPC6 knockdown in siRNA transfected myocytes caused a proportional reduction, and TRPC6 upregulation in chronic hypoxic PASMCs resulted in a comparable increase in OAG-induced cation/ Ca$^{2+}$ entry. This is consistent with findings that TRPC6 mediates agonist induced receptor-operated Ca$^{2+}$ entry in systemic myocytes.$^{16,17}$ Moreover, the observations that TRPC1 siRNA had no effect on OAG-induced cation entry and TRPC6 siRNA did not alter the thapsigargin-induced response provided the molecular evidence that the store- and receptor-operated pathways are mutually independent in rat PASMCs. This is coherent with functional studies that DAG/OAG activates directly TRPC3/6/7 channels but not TRPC1,$^{15}$ and with biochemical studies that TRPCs are divided into two subfamilies of TRPC1/2/5 and TRPC3/6/7, in which members within a subfamily, but not between different subfamilies, can form heterotetrameric channels.$^{30,31}$

It is noteworthy, however, that a study shows that TRPC6 expression is closely related to CPA-induced Ca$^{2+}$ entry in proliferating PASMCs.$^{22}$ It is unclear if TRPC6 exhibits a different phenotype in proliferating PASMCs, eg, by hetero-merization with proteins such as TRPC3, which has been implicated as a receptor- and store-operated Ca$^{2+}$ channel$^{15,32}$ or if CPA activates TRPC6 through mechanism(s) other than SR Ca$^{2+}$ depletion. These interesting possibilities require future investigations.

The major finding of the present study is that chronic hypoxia upregulates TRPC expression and enhances both
store-and receptor-operated Ca\textsuperscript{2+} entries in PASMCs. TRPC upregulation by hypoxia is subtype-specific, such that TRPC1 and TRPC6 expressions are doubled or tripled, whereas other TRPCs are unaltered. Hypoxia may directly promote TRPC expression through the activation of O\textsubscript{2}-regulated transcription factors, including hypoxia inducible factor, which upregulates dozens of target genes in hypoxic cells, including pulmonary cells.\textsuperscript{33} Previous studies showed that partial deficiency of hypoxia inducible factor-1\textalpha in mice annihilated the hypoxia-induced structural and physiological changes in pulmonary vasculature and PASMCs.\textsuperscript{34} However, evidence for hypoxia inducible factor-1\textalpha directly regulates TRPC transcription is unavailable. Alternatively, hypoxia may regulate TRPC expression indirectly through mitogenic/growth factors. It has been reported that hypoxia stimulates the release of PDGF, fibroblast growth factor, vascular endothelial growth factor, and endothelin-1 (ET-1). In vitro studies show that TRPC1 mRNA is upregulated, resting [Ca\textsuperscript{2+}]\textsubscript{i} is elevated, and store-operated Ca\textsuperscript{2+} entry is enhanced during serum-induced proliferation in cultured PASMCs.\textsuperscript{18} and TRPC6 expression is increased by PDGF-induced PASMC proliferation.\textsuperscript{22} Moreover, PASMC proliferation can be blocked by antisense oligonucleotides against TRPCs,\textsuperscript{14,22} suggesting that TRPC upregulation is a required step for the growth processes. Even though these observations may not apply equivalently to in vivo situations, nevertheless the release of mitogens and the massive medial thickening in chronic hypoxic pulmonary hypertension raise the likely possibility that TRPC upregulation is related at least in part to PASMC proliferation.

The enhanced TRPC expression and activity play a critical role in the increase in vascular tone of hypoxic PAs. It is evident in the present study that the basal Ca\textsuperscript{2+} influx via TRPC channels, as indicated by Mn\textsuperscript{2+} quenching, was significantly augmented in chronic hypoxic PASMCs. More dramatically, La\textsuperscript{3+} and the nonselective cation channel blocker,
SK&F-96365, reduced the elevated [Ca\(^{2+}\)] and vascular tone in hypoxic PAs to a level similar to Ca\(^{2+}\) removal. The prominent effects of La\(^{3+}\) and SK&F-96365 are not attributable to the inhibition of voltage-gated Ca\(^{2+}\) channels, because nifedipine at a concentration that completely abolishes voltage-gated Ca\(^{2+}\) entry\(^5\) only had a small effect, and subsequent application of La\(^{3+}\) and SK&F-96365 in the presence of nifedipine caused further reduction in basal [Ca\(^{2+}\)], and vascular tone. In addition, 10 \(\mu\)M/L of La\(^{3+}\) is sufficient to cause the maximum reduction in basal [Ca\(^{2+}\)], suggesting that the store-operated Ca\(^{2+}\) entry is likely the major pathway responsible for the enhanced basal Ca\(^{2+}\) entry in chronic hypoxic PASMCs. TRPC upregulation may contribute to vasomotor tone through several mechanisms. Ca\(^{2+}\) influx via TRPC increases [Ca\(^{2+}\)] to initiate actin–myosin interactions; Ca\(^{2+}\) influx via store-operated channel replenishes SR Ca\(^{2+}\) stores to allow further Ca\(^{2+}\) release;\(^35\) and Na\(^{+}\) influx via TRPCs because of their nonselective nature may cause subsarcolemmal increase in [Na\(^{+}\)] to promote Ca\(^{2+}\) influx via reverse Na\(^{+}\)–Ca\(^{2+}\) exchange.\(^36\) Moreover, activation of TRPC can also lead to membrane depolarization and further activation of L-type Ca\(^{2+}\) channels,\(^37\) a process that has previously been attributed solely to the downregulation of \(K_v\) channel in chronic hypoxic PASMCs.\(^6\),\(^7\)

Increase in store- and receptor-operated Ca\(^{2+}\) entries in chronic hypoxic PASMCs may contribute to alterations in pulmonary vasoactivity.\(^1\) Previous studies showed that hypoxic PAs exhibit dramatic increase in contractile response to vasoconstrictors, such as endothelin-1, angiotensin II, and serotonin,\(^1\),\(^2\),\(^3\),\(^4\),\(^5\) presumable because of an increase or a shift to vasoconstrictors, such as endothelin-1, angiotensin II, and serotonin.\(^6\),\(^7\),\(^8\) Hypoxic pulmonary myocytes are associated with reduced delayed rectifier K\(^{+}\) current in rat pulmonary artery muscle cells.\(^9\)

References


Chronic Hypoxia-Induced Upregulation of Store-Operated and Receptor-Operated Ca$^{2+}$ Channels in Pulmonary Arterial Smooth Muscle Cells. A Novel Mechanism of Hypoxic Pulmonary Hypertension

Mo-Jun Lin, George P.H. Leung, Wei-Min Zhang, Xiao-Ru Yang, Kay-Pong Yip, Chung-Ming Tse and James S.K. Sham

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MATERIALS AND METHODS

Chronic hypoxic exposure. Chronic hypoxic pulmonary hypertension was produced by the established method (2). Male Wistar rats (150-250 g) were placed in a hypoxic chamber and exposed to either normoxia or normobaric hypoxia for 3-4 weeks. The chamber was continuously flushed with either room air or a mixture of room air and N₂ to maintain oxygen level to 10%. Chamber O₂ and CO₂ were monitored continuously (OM-11 oxygen analyzer). Rats were exposed to room air for 10 min twice a week to clean the cages, and to replenish food and water supplies. The procedures were performed in accordance with guidelines of the Johns Hopkins University Animal Care and Use Committee.

Isolation and culture of PASMCs. PASMCs were enzymatically isolated and transiently cultured as previously described (3). Briefly, male Wistar rats (150 – 200 g) were injected with heparin and anesthetized with sodium pentobarbital (130 mg/kg i.p.). 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 MgCl₂, 1.5 CaCl₂, 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH). Third and fourth generation intrapulmonary arteries (~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-Ca²⁺ (20 µM) HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 µM Ca²⁺ HBSS containing collagenase (Type I, 1750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (1 mM), then removed and washed with Ca²⁺-free HBSS to stop digestion. PASMCs were gently dispersed by trituration with a small-bore pipette in Ca²⁺-free HBSS at room temperature. The cell suspension
was then placed on 25 mm glass coverslips in Ham’s F-12 medium (with L-glutamine) supplemented with 0.5% fetal calf serum, 100 U/ml of streptomycin, and 0.1 mg/ml of penicillin. PASMCs from chronic hypoxic and normoxic animals were transiently (~24 hours) cultured inside a modular incubator chamber (billups-rothenberg, Inc.) under 6% O$_2$/5% CO$_2$ and 21% O$_2$/5%CO$_2$, respectively.

**Measurement of intracellular [Ca$^{2+}$].** Intracellular [Ca$^{2+}$] of PASMCs was monitored using the membrane permeable Ca$^{2+}$-sensitive fluorescent dye, fluo-3 acetoxymethyl ester (fluo-3 AM). PASMCs were loaded with 5 - 10 µM fluo-3 AM (dissolved in DMSO with 20% pluronic acid) for 30 - 45 min at room temperature (~22°C) in normal Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed thoroughly with Tyrode’s solution to remove extracellular fluo-3 AM, and rested for 15-30 min in a cell chamber to allow for complete de-esterification of cytosolic dye. Fluo-3 was excited at 488 nm, and emission light at >515 nm was detected using a Nikon Diaphot microscope equipped with epifluorescence attachments and a micro-fluorometer (Biomedical Instrument Group, University of Pennsylvania). Protocols were executed and data collected on-line with a Digidata analog-to-digital interface (Axon Instruments, Inc., Foster City, CA) and the pClamp software package (Axon Instruments Inc., Foster City, CA). Intracellular Ca$^{2+}$ concentration was calibrated by the equation $[\text{Ca}^{2+}]_i = K_D.(F-F_{bg})/F_{\text{max}}-F$, where $F_{bg}$ is background fluorescence and $F_{\text{max}}$ is the maximum fluorescence determined in situ in cell superfused with 10 µM 4-Bromo A-23187; or by a pseudo-ratio method (1), using the following equation: $[\text{Ca}^{2+}]_i = (K_D·R)/[((K_D/[[\text{Ca}^{2+}]_{\text{rest}})+1)–R]$, where R is $F/F_0$, $K_D$ of Fluo-3 is 1.1 µM.
Values of resting Ca\(^{2+}\) ([Ca\(^{2+}\)]_{rest}) of normoxic and chronic hypoxic PASMCs were determined in separate experiments.

*Mn quenching of Fura-2.* Rate of Ca\(^{2+}\) entry through TRPCs was quantified by quenching of fura-2 with Mn\(^{2+}\). PASMCs from normoxic and chronic hypoxic rats were loaded with fura-2 AM as described above. Fura-2 was excited at the Ca\(^{2+}\)-insensitive isobestic point of 360 nm, and emission light was recorded at 510 nm. PASMCs were then bathed in a Ca\(^{2+}\) free (with 0.1 mM EGTA) Tyrode solution containing 10 \(\mu\)M nifedipine. After a stable baseline fluorescent measurement was attained, 500 \(\mu\)M Mn\(^{2+}\) was applied through a concentration-clamp system with the multi-barrel pipette positioned <50 \(\mu\)m from PASMCs. The rates of quenching of fura-2 fluorescence in PASMCs with/without drug treatments were determined and compared.

*Reverse Transcription-Polymerase Chain Reaction (RT-PCR).* Total RNA was isolated from rat intralobar PASMCs using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer’s protocol. Genomic DNA contamination was removed by DNA-free™ DNase Treatment and Removal Reagents (Ambion, Austin, TX). Briefly, 2 units of DNase I were added to the isolated RNA and incubated for 30 min at 37°C. Then, DNase Inactivation Reagent was added and incubated for 2 min at room temperature. The samples were centrifuged at 10,000x g for 1 minute to pellet the DNase Inactivation Reagent. The supernatants were collected and the amounts of RNA were determined by measuring the optical density at 260nm. Total RNA was also isolated from heart and brain as positive controls. Two micrograms of total RNA were used for first-strand cDNA synthesis using random hexamer primers and Superscript II RNase H⁻ Reverse Transcriptase (SuperScript Preamplification System; Gibco BRL). The resulting first-
strand cDNAs were directly used as templates for PCR amplification. Specific sense and antisense primers for RT-PCR were designed according to the following strategies: (i) rat cDNA sequence of TRPC were used, (ii) regions that have high homology among different members of TRPC channels were avoided, and (iii) sequences were selected from regions that are common to the multiple isoforms of the same TRPC subtype (e.g. rTRPC6 has 3 isoforms)(see Table 1). The specificities of the primers were checked with the BLAST program. Reactions were carried out using PCR SuperMix (Gibco BRL) with the following parameters: denaturation at 94°C for 45s, annealing at 50-60 °C for 45s, and extension at 72°C for 1.5 min. A total of 30 cycles were performed. This was followed by a final extension at 72°C for 10 min. The PCR products were analyzed by 1 % agarose gel electrophoresis and visualized by staining with ethidium bromide. To quantify the PCR products of TRPs, β-actin was used as an internal standard and coamplified in the PCR reactions. The two primers used for amplifying β-actin, as listed in table 1, yielded a PCR product of 240 base-pairs. Optical density (OD) of each band on the gel was measured by Eagle Eye II still video system (Strategene, Lajolla, CA). The OD values of TRPC signals were normalized to the OD values in the β-actin. The normalized values are expressed as arbitrary units for quantitative comparison.

Western blotting. Cultured rat PASMCs were rinsed and then scraped into ice-cold PBS. The cells were spun at 16,000 g for 30s and the pellets were resuspended in 5mM sodium phosphate buffer containing 1% protease inhibitor cocktail (Sigma, St. Louis, MO). The samples were vortexed and boiled in Laemmli buffer for 30s, respectively. This step was repeated 3-4 times until the samples were completely dissolved. Whole cell lysate proteins were separated by 10 % (w/v) SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes. The
membranes were subsequently blocked with 5% (w/v) nonfat dry milk in PBS for 1 hour at room temperature followed by incubation with polyclonal rabbit anti-TRP1, anti-TRP3, anti-TRP4 or anti-TRP6 antibody (Alomone labs, Jerusalem, Israel) (1:200 [v/v] dilution in blocking solution) overnight at 4°C. Nitrocellulose membranes were then washed extensively with 0.02% (v/v) Triton X-100 in PBS. After washing, the membranes were incubated with horseradish-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) (1:5000 [v/v] dilution in blocking solution) for 1 hour at room temperature. Excess secondary antibody was again washed, and the bound secondary antibody was detected by enhance chemiluminescence (Western Lightning™ Chemiluminescence Reagent Plus; PerkinElmer Life Science Products, Boston, MA) according to the manufacturer’s protocols. Rat brain membrane proteins were used as positive controls. The tissues were sonicated at 4°C in 5mM sodium phosphate buffer containing 1% protease inhibitor cocktail and centrifuged at 2,500x g for 15 min. The supernatants were vortexed and boiled with Laemmllli buffer, and then analyzed by Western blotting as described above.

**Immunostaining of TRPCs.** PASMCs were fixed in 2% paraformaldehyde for 10 min. The cells were washed with 0.75% glycine in PBS for 30 min, followed by permeabilization with 0.05% Triton-X 100 for 15 min. The cells were then incubated with a combination of unconjugated donkey anti-rabbit IgG, anti-mouse IgG or anti-mouse IgM antibodies (1:20) to block non-specific binding for 2 hour, and then incubated with isoform specific anti-TRPC1, TRPC3, TRPC4, and TRPC6 primary antibodies (Alomone Labs) overnight. Antibody-specific binding were visualized with either donkey CY2-conjugated (1:100, Jackson Immunoresearch Laboratory), which were affinity-purified for multiple labeling. BSA (0.2%) and Triton X-100
(0.05%) were included in PBS for antibody dilution. All incubations were carried out in a moistened chamber at 4 °C. Immunostaining was examined with a MRC-1000 confocal scanning unit (Bio-Rad) equipped with a krypton-argon laser. Coverslip without exposing to primary antibodies were used as negative control.

Transfection of PASMCs with siRNA. PASMCs were isolated and maintained in Ham’s F-12 containing 0.5% fetal bovine serum (FBS) and antibiotics for about 24h before transfection. PASMCs were transiently transfected with the use of RNAiFect Transfection Reagent (Qiagen), according to the manufacturer’s instruction. Briefly, 2µg siRNA was diluted in Ham’s F-12 to give a final volume of 100µl for each well of cells. 6 µl of RNAiFECT Transfection Reagent (Qiagen Inc., CA) was added to the diluted siRNA and was mixed by pipetting. The mixture was then incubated for 15 min at room temperature to allow formation of transfection complexes. At the same time, cells were washed twice with fresh medium and 600 µl of Ham’s F-12 was added to each well at last. The transfection complexes were then dropped onto the cells after it has formed, and the plate was swirled gently to ensure uniform distribution of the transfection complexes. The transfection mixture was removed after incubation with cells for 10-12h at 37ºC. Then cells were further incubated under normal growth conditions for 24 h before use in experiments. Chemically synthesized siRNA oligonucleotides (Dharmacon Research Inc, CO) were used for transfection. The sense sequences of siRNA against rat TRPC1, TRPC6, and a non-silencing control sequence were listed in table 2.

Isometric contraction. Intralobar pulmonary arteries (300-800 µm O.D.) were isolated and placed in oxygenated modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO₄, 1.18 KH₂PO₄, 25 NaHCO₃, 10 dextrose and 2.5 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 16% O$_2$-5% CO$_2$ to maintain a pH of 7.4 and the temperature at 37°C. Isometric contraction was measured using a strain gauge connected to a Grass polygraph. The resting tension was adjusted to 2 g. The arteries were exposed to 80 mM KCl to establish maximum contraction, and to phenylephrine (3x10$^{-7}$ M) followed by acetylcholine (10$^{-6}$ M) to verify complete disruption of endothelium.
Table 1. Oligonucleotide sequences of the primers used for RT-PCR

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<th>primers</th>
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<td>TRPC1</td>
<td>AF061266</td>
<td>5’-ATGGGACAGATGTTACAAGATTTTGG-3’ (sense) 5’-AGCAAACTCCATTCTTTATCCTCATG-3’ (antisense)</td>
<td>1561-1587 1936-1962</td>
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<td>TRPC2</td>
<td>AF136401</td>
<td>5’-GGAGAAAGGGTGCAAAAAGTAGAGC-3’ (sense) 5’-CTCAGTCTCAGTACTCATCCTGCCGACG-3’ (antisense)</td>
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<td>TRPC4</td>
<td>AF288407</td>
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<td>1643-1666 2039-2065</td>
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<td>TRPC5</td>
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<td>1153-1171 1958-1983</td>
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<td>TRPC6</td>
<td>NM053559</td>
<td>5’-CAGCGCGCGCGAGAACGTTCTCTCC-3’ (sense) 5’-CTGCTGGAGGCTCAGACTGGCGG-3’ (antisense)</td>
<td>193-215 583-607</td>
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<td>β-actin</td>
<td>BC063166</td>
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<td>124-144 344-363</td>
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Table 2. Sense sequences of siRNA against TRPC1 and TRPC6, and control non-silencing sequence.

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