Hypoxia increases AP-1 binding activity by enhancing capacitative Ca\textsuperscript{2+} entry in human pulmonary artery endothelial cells

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Fantozzi, Ivana, Shen Zhang, Oleksandr Platoshyn, Carmelle V. Remillard, Randy T. Cowling, and Jason X.-J. Yuan. Hypoxia increases AP-1 binding activity by enhancing capacitative Ca\textsuperscript{2+} entry in human pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 285: L1233–L1245, 2003. First published August 8, 2003; 10.1152/ajplung.00445.2002.—Activating protein (AP)-1 transcription factors modulate expression of genes involved in cell proliferation and migration. Chronic hypoxia increases pulmonary artery smooth muscle cell proliferation by up-regulating AP-1-responsive genes encoding for endothelium-derived vasoactive and mitogenic factors implicated in pulmonary hypertension development. The expression of AP-1 transcription factors is sensitive to changes in cytosolic free [Ca\textsuperscript{2+}]\textsubscript{cyt} (\([\text{Ca}^{2+}]\text{_{cyt}}\)). Capacitative Ca\textsuperscript{2+} entry (CCE) via store-operated Ca\textsuperscript{2+} channels (SOC) is an important mechanism for raising [Ca\textsuperscript{2+}]\textsubscript{cyt} in pulmonary artery endothelial cells (PAEC). Using combined molecular biological, fluorescence microscopy, and biophysical approaches, we examined the effect of chronic hypoxia (3% O\textsubscript{2}, 72 h) on AP-1 DNA binding activity, CCE, and transient receptor potential (TRP) gene expression in human (h) PAEC. EMSA showed that AP-1 binding to hPAEC nuclear protein extracts was significantly enhanced by hypoxia, the increase being dependent on store-operated Ca\textsuperscript{2+} influx and sensitive to La\textsuperscript{3+}, an SOC inhibitor. Hypoxia also increased basal [Ca\textsuperscript{2+}]\textsubscript{cyt}, the amount of CCE produced by store depletion with cyclopiazonic acid, and the amplitude of SOC-mediated currents (\(I\text{_{SOC}}\)). The increases of CCE amplitude and \(I\text{_{SOC}}\) current density by hypoxia were paralleled by enhanced TRPC4 mRNA and protein expression. Hypoxia-enhanced CCE and TRPC4 expression were also attenuated by La\textsuperscript{3+}. These data suggest that hypoxia increases AP-1 binding activity by enhancing Ca\textsuperscript{2+} influx via La\textsuperscript{3+}-sensitive TRP-encoded SOC channels in hPAEC. The Ca\textsuperscript{2+}-mediated increase in AP-1 binding may play an important role in upregulating AP-1-responsive gene expression, in stimulating pulmonary vascular cell proliferation and, ultimately, in pulmonary vascular remodeling in patients with hypoxia-mediated pulmonary hypertension.

activating protein-1; store-operated channels; transient receptor potential genes

PULMONARY VASCULAR REMODELING in patients and animals with hypoxia-mediated pulmonary hypertension is characterized by pulmonary arterial medial hypertrophy, which is due primarily to increased cell proliferation and/or reduced apoptosis in smooth muscle cells (13, 62). One of the mechanisms by which chronic hypoxia induces pulmonary artery smooth muscle cell (PASMC) proliferation is by promoting transcriptional activation of genes encoding vasoactive agonists, such as endothelin-1 (ET-1), and mitogens, such as vascular endothelial (VEGF) and platelet-derived (PDGF) growth factors (7, 59). Indeed, it has been demonstrated that chronic hypoxia increases the synthesis and production of ET-1 (8, 33), VEGF (9, 35, 40, 68), and PDGF (31) in pulmonary arterial endothelial cells (PAEC). However, the cellular mechanisms involved in hypoxia-induced upregulation of mitogens in human PAEC are still incompletely understood.

AP-1, or activating protein-1, refers to a family of homo (e.g., Jun/Jun)- or hetero (e.g., Jun/Fos)-dimeric transcription factors composed of Jun (v-Jun, c-Jun, JunB, and JunD), Fos (v-Fos, c-Fos, FosB, Fra1, and Fra2), or activating transcription factor (ATF2, ATF3/ LRF1, B-ATF) subunits that bind to a common DNA site, the AP-1 binding site (5’-TGACTCA-3’ (TRE) or 5’-TGACGTCA-3’ (CRE)) (45). AP-1 transcription factors control gene expression directly by regulating the genes that contain the AP-1 binding sites in their promoters or indirectly by forming heterodimers with other types of transcription factors (e.g., STAT) that regulate AP-1-responsive genes (76). The physiological function of AP-1 transcription factors greatly depends on the different target genes and is usually involved in the regulation of cell proliferation, migration, and apoptosis (30). Overexpression of AP-1 oncogenes accelerates cell proliferation and promotes tumor cell growth (27, 42, 74), suggesting that regulation of AP-1 binding activity may be key in modulating cell growth.

The genes expressing AP-1 transcription factors are Ca\textsuperscript{2+}-sensitive (3, 23, 24), i.e., rise in cytosolic ([Ca\textsuperscript{2+}]\textsubscript{cyt}) and/or nuclear free Ca\textsuperscript{2+} concentrations up-regulate AP-1 transcription factors (18, 19, 23, 24). For example, the expression of c-fos and c-jun proto-oncogenes is upregulated by a sustained increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} due to Ca\textsuperscript{2+} influx (3, 23, 24, 28, 60). Ca\textsuperscript{2+} in different areas of the cell and the different amplitude...
and frequency of [Ca\(^{2+}\)]\(_{\text{cyt}}\) increases result in different transcriptional responses (11, 12, 38). Enhanced [Ca\(^{2+}\)]\(_{\text{cyt}}\) due to influx via sarcotomal Ca\(^{2+}\) channels or release from intracellular stores [e.g., sarcoplasmic reticulum (SR)] activates different genes encoding for transcription factors and signal transduction proteins (3).

Of particular interest, Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels (SOC) appears to play an important role in refilling the depleted SR stores and in maintaining a sustained [Ca\(^{2+}\)]\(_{\text{cyt}}\) increase during agonist stimulation via a mechanism known as capacitance Ca\(^{2+}\) entry (CCE) (6, 50, 56). Native SOC channels are believed to be formed by subunits encoded by a novel family of transient receptor potential (TRP) channel genes. Members of this TRP channel family have been identified in both pulmonary artery smooth muscle and endothelial cells (16, 20, 29, 43, 49, 69, 73). It has been recently reported that endothelial canonical or short/transient TRP (TRPC) 4 expression contributes to lung microvascular permeability in mice (16, 69). SOC-mediated Ca\(^{2+}\) entry, regulated by TRP expression, is therefore of great physiological relevance not only in vascular remodeling, but also in the regulation of vascular tone (6, 20, 43, 67, 73, 75).

Hypoxia-enhanced expression of many AP-1-responsive gene products in PAEC, including VEGF, PDGF, and ET-1, has been implicated in hypoxia-induced pulmonary hypertension (4, 14). Because AP-1-induced expression of mitogens and growth factors is regulated by increased [Ca\(^{2+}\)]\(_{\text{cyt}}\), we hypothesized that chronic hypoxia increases AP-1 DNA binding activity by enhancing Ca\(^{2+}\) influx through SOC channels. The subsequent increase in expression of AP-1-responsive genes may contribute to the development of pulmonary hypertension.

**MATERIALS AND METHODS**

**Cell culture and hypoxic treatment.** Human PAEC from normal subjects were purchased from Clonetics. The cells were maintained in culture at 37°C in an endothelial growth medium (EGM, Clonetics) composed of endothelial basal medium (EBM) supplemented with 2% fetal bovine serum, 0.5 ng/ml human epidermal growth factor (EGF), 2 ng/ml human fibroblast growth factor, and 5 μg/ml insulin. Cells were cultured and plated onto coverslips (for electrophysiological and fluorescence microscopy experiments) or petri dishes (for molecular biology experiments) using trypsin/EDTA buffer (Clonetics) to 70%–90% confluence. Cells cultured between passages 4 and 6 were used for these experiments. We arrested the growth of the cells by replacing EGM with serum-free EBM 48 h before experimentation and divided them into two groups. One group of cells (normoxic) was incubated at 5% CO\(_2\) in air (21% O\(_2\), 74% N\(_2\)). A second group of cells (hypoxic) was incubated at 3% O\(_2\), 5% CO\(_2\), and 92% N\(_2\) for 24 h.

**Nuclear protein extraction preparation.** Subconfluent (75%) human PAEC cells were washed with phosphate-buffered saline (PBS) and harvested in 0.5 ml cold 1× EMSA cytoplasmic extraction buffer [10 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 0.4% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), and serine/cysteine/metalloprotease (e.g., pronase, thermolysin, chymotrypsin, and papain) inhibitors] and centrifuged at 2,500 rpm for 3 min at 4°C. The cell pellet was resuspended in 0.25 ml of cold 1× EMSA cytoplasmic extraction buffer and left on ice for 5 min. After centrifugation the pellet was resuspended in 50 μl of cold 1× EMSA nuclear extraction buffer [containing 50 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl\(_2\), 25% glycerol, 0.5 mM sodium orthovanadate, and protease inhibitors] and was incubated on ice for 10 min. The cell suspension was centrifuged at 14,000 rpm for 10 min at 4°C; the supernatant was aspirated, aliquoted, and stored at −80°C for use in EMSA. Protein concentration was determined by BCA Protein Assay Reagents (Pierce Biotechnology) using bovine serum albumin (BSA) as a standard.

**EMSA probe.** The double-stranded AP-1 oligonucleotides used in these experiments had the following sequences: 5′- CGCTTGATGACTCGCCGGA-3′ (standard AP-1 probe) and 5′-CGCTTGATGACTTGGCCGGAA-3′ (mutated AP-1 probe). The standard AP-1 probe contained a consensus AP-1 binding site (underlined above) for c-Jun homodimeric and Jun/Fos heterodimeric complexes (Santa Cruz Biotechnology).

We prepared double-stranded oligonucleotide probes by annealing equimolar amounts of complementary single-stranded oligonucleotides in a 10× annealing buffer: 1 M Tris-HCl (pH 7.5), 5 M NaCl, 0.5 M EDTA, in double-distilled water; 100 mM/ml final concentration for each oligonucleotide) in a 65°C water bath for 10 min and allowing it to cool slowly at room temperature. For mobility shift assays, the oligonucleotides (100 ng each) were end-labeled with 25 μCi of [γ-32P]ATP (3,000 Ci/mM; Amer sham) and T4 polynucleotide kinase (GIBCO), purified with the Nucleotide Removal Kit (Qiagen), and eluted with 100 μl of elution buffer.

**EMSA assay.** Binding reactions were performed in a 20-μl reaction volume of 1× EMSA binding buffer [10 mM Tris-HCl (pH 7.9), 0.5 mM EDTA, 50 mM NaCl, 1 mM DTT, and 0.5 mM sodium orthovanadate] containing 0.3 μg poly(dI-dC), 5 μg BSA, 0.01 μg sheared salmon sperm DNA, 0.5 μl of the 32P-labeled probe (0.3 ng) and 0.2 μg nuclear extraction protein. The binding reaction was allowed to proceed at room temperature for 20 min. Then, 4 μl of DNA gel loading buffer were added to each sample, and protein-DNA complexes were resolved by electrophoresis on nonnaturating 5% polyacrylamide gels and were visualized by autoradiography. Trans Cruz supershift antibody (1 μg/μl) was added to the reaction mixture and incubated for an additional 30 min at room temperature for gel supershift assays. The antibodies used in these experiments included rabbit polyclonal IgG raised against c-Jun, JunB, c-Fos or JunD, and a polyclonal antibody raised against all Fos proteins, including c-Fos, Fra-1, and Fra-2. General (G) antibodies against Fos and Jun were also used. These can cross-react with all members of their respective subfamilies [i.e., Fos(G)] will recognize Fos, FosB, Fra1, and Fra2, and Jun(G) will recognize JunD, JunB, and c-Jun in our experiments. All gel supershift antibodies were purchased from Santa Cruz Biotechnology.

**Measurement of [Ca\(^{2+}\)]\(_{\text{cyt}}\).** [Ca\(^{2+}\)]\(_{\text{cyt}}\) was measured with the Ca\(^{2+}\)-sensitive fluorescent indicator fura 2 as described previously (20). Briefly, cells on coverslips were loaded with membrane-permeable fura 2-acetoxyethyl ester (fura 2-AM, 3 μM for 30 min) in the dark at room temperature (22–24°C) under an atmosphere of 5% CO\(_2\)-95% air. The coverslips were transferred to a recording cell chamber on the microscope stage and superfused (2–3 ml/min) with physiological salt solution (PSS) for 30 min to remove extracellular dye and to allow intracellular cleavage of fura 2-AM to active fura 2. The PSS contained (in mM) 141 NaCl, 4.7 KCl, 1.8 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPEs, and 10 glucose (pH 7.4 with 10.220.32.247 on July 10, 2017 http://ajplung.physiology.org/ Downloaded from
5 M NaOH). In Ca²⁺-free PSS, CaCl₂ was replaced by equimolar MgCl₂, and 0.1 mM EGTA was added to chelate residual Ca²⁺. Fura 2 fluorescence (340 and 380 nm excitation) was detected at 32°C using a Nikon UV-FLuor objective lens and a charge-coupled device camera. The fluorescence signals emitted at 510 nm were monitored using a fluorescence microscopy system (Intracellular Imaging) and recorded on an IBM-compatible computer for later analysis. The 340/380 nm ratios (R) of the fluorescence images used to calculate [Ca²⁺]cyt using the following equation: [Ca²⁺]cyt = [Kd x (S2/S0) x (R – Rmin)/Rmax – R], where S2 and S0 are the emission fluorescence values at 380 nm excitation in the presence of EGTA and Triton X-100, respectively; Kd (225 mM) is the dissociation constant of the Ca²⁺-fura 2 complex; and Rmax and Rmin were calculated according to Grynkiewicz et al. (21).

Measurement of macroscopic store-operated Ca²⁺ currents. Whole cell store-operated Ca²⁺ currents (ISOC) were recorded with an Axopatch-1D amplifier and a DigiData 1200 interface (Axon Instruments) by patch-clamp techniques (20, 22, 67, 75). Patch pipettes (2–4 MΩ) were fabricated on a Sutter P-97 pipette puller using borosilicate glass capillary tubing and fire polished on a MF-83 microforge (Narishige Instruments). Whole cell ISOC were recorded during 300-ms voltage steps ranging between −100 and +100 mV (20-mV increments) from a holding potential of 0 mV (to inactivate voltage-gated Ca²⁺ and Na⁺ currents). Traces recorded before the activation of SOCs were used as a template to subtract leak currents. SOCs were activated by passive depletion of the SR with cyclopiazonic acid (CPA), an inhibitor of the SR Ca²⁺-Mg²⁺ ATPase pump. All experiments were performed at room temperature (22–24°C).

The bath solution for recording ISOC contained (in mM) 120 sodium methanesulfonate, 20 calcium aspartate, 0.5 3,4-diaminopyridine, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). As the SR conditions eliminated the currents through K⁺ or Cl⁻ channels. For a Ca²⁺-free bath solution, CaCl₂ was replaced by equimolar MgCl₂ and 1 mM EGTA was added to chelate residual Ca²⁺.

RNA extraction and RT-PCR. Total RNA was isolated from explanted human PAEC using the RNeasy Mini Kit (Qiagen). Human brain total RNA was purchased from GIBCO-BRL. Explanted human PAEC using the RNeasy Mini Kit (Qiagen). The bound antibody was detected with an enzyme-linked immunosorbent assay (ELISA) overnight at 4°C. Finally, the membranes were washed and exposed to anti-rabbit horseradish peroxidase-conjugated IgG for 90 min at room temperature. The bound antibody was detected with an enhanced chemiluminescence detection system (Amersham). The monoclonal anti-α-actin antibody (Upstate Laboratories) was used as a control.

Synthesis and transfection of small interfering RNA. Three 21-nucleotide small interfering RNA (siRNA) sequences specifically targeting human TRPC4 were synthesized and purchased from Sequitur. A scrambled TRPC4 siRNA was also synthesized for use as a control. Human PAEC were plated at 2 × 10³ cells per well in a 24-well plate and grown in EGM-filled plates to 90% confluence before transfection. PAEC were transfected with 20 nM of each siRNA using the Gene Porter 2 transfection reagent kit (Gene Therapy Systems). After transfection, cells were incubated at 37°C in serum-free (EBM) culture media. Fifteen hours posttransfection, fresh growth medium was added, and the cells were left to recover at 37°C under normoxic conditions. For RT-PCR experiments, cells were allowed to recover for 8 h before mRNA extraction. For Western blot analysis from hypoxic cells, the 8-h recovery period in normoxia was followed by a 72-h incubation in a hypoxic (3% O₂) before protein extraction was performed.

Reagents. CPA (Sigma) was prepared as a 30-mM stock in DMSO and diluted 1:3,000 in the bath solution or culture medium to a final concentration of 10 μM. Nifedipine (Sigma) and La³⁺ were directly dissolved in the bath solutions on the day of use. The pH was adjusted to 7.4 after addition of any drugs. In [Ca²⁺]cyt and ISOC measurement experiments, the same amount of DMSO (0.03% final) used for dissolving CPA was added to control solutions. DMSO alone (vehicle) had negligible effects on [Ca²⁺]cyt and ISOC in human PAEC.

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed using unpaired Stu-
nuclear protein extracts (lane 3) or mutated (lane 4) AP-1 probes confirmed the specificity of the AP-1 probe in our experiments; AP-1 binding by the radiolabeled DNA probe was abolished by the addition of the AP-1 competitor probe but not its mutated isofron.

RESULTS

AP-1 binding in human PAEC nuclear protein extracts. First, we tested for AP-1 binding activity in normoxic human PAEC using an EMSA. Figure 1A shows that the 32P-labeled double-stranded DNA probe containing the AP-1 binding consensus sequence (described in METHODS AND MATERIALS) bound nuclear protein extracts (lane 2). Incubation of the protein extracts and 32P-labeled AP-1 probe with 100-fold more concentrated unlabeled native (lane 3) or mutated (lane 4) AP-1 probes confirmed the specificity of the AP-1 probe in our experiments; AP-1 binding by the radiolabeled DNA probe was abolished by the addition of the AP-1 competitor probe but not its mutated isofrom.

Because two bands were present in the band shift assay shown in Fig. 1A, we performed band supershift assays to identify the band corresponding to specificity of the AP-1 probe in our experiments; AP-1 binding by the radiolabeled DNA probe was abolished by the addition of the AP-1 competitor probe but not its mutated isofrom.

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antibodies against c-Jun, c-Fos, and JunD were incubated along with AP-1 binding activity (Fig. 2, lane 2 and lane 3). In extracts of human PAEC (lane 4), nuclear protein extracts were incubated with the same [32P]AP-1 probe and a mutated nonradiolabeled AP-1 competitor. Antibodies against c-Jun and JunD antibodies resulted in a shift in AP-1 binding (Fig. 2, lane 5 and 8), suggesting that Fos and Jun transcription factors are involved. We further characterized the AP-1 members involved by incubating the hypoxic PAEC nuclear protein extracts with specific antibodies against selected members of the AP-1 Jun and Fos families, namely JunD, JunB, c-Fos, c-Jun, Fra1, and Fra2. The dense band corresponding to AP-1 binding was supershifted in the presence of specific antibodies against only JunD, c-Jun, and Fra-1 (lanes 3, 7, and 10), suggesting that these AP-1 transcription factors may be upregulated by hypoxia. Incubation with specific JunB, c-Fos, and Fra2 (lanes 4 and 6) antibodies did not result in a supershift.

Chronic hypoxia enhances [Ca\textsuperscript{2+}]\textsubscript{cyt} via increased CCE. Having shown that hypoxia-mediated AP-1 binding was dependent on Ca\textsuperscript{2+} influx, we next sought to identify the source of the sarcolemmal Ca\textsuperscript{2+} influx triggered by hypoxia. When extracellular Ca\textsuperscript{2+} was removed, blockade of SR Ca\textsuperscript{2+}-Mg\textsuperscript{2+} ATPase, a Ca\textsuperscript{2+}

### Figure 1

**Electrophoretic mobility shift assay (EMSA) of activating protein-1 (AP-1) binding activity in normoxic human pulmonary artery smooth muscle cells (PAEC).** A: [32P]-labeled probe containing the activating protein (AP)-1 consensus sequence (see METHODS AND MATERIALS for sequence) (lane 1) was incubated with nuclear protein extracts of human PAEC (lane 2). In lane 3, [32P]AP-1 probe and nuclear protein extracts were incubated with a competitor nonradio-labeled 100 times more concentrated than the 32P-labeled probe. In lane 4, nuclear protein extracts were incubated with the same [32P]AP-1 probe and a mutated nonradiolabeled AP-1 competitor. B: antibodies against c-Jun, c-Fos, and JunD were incubated along with [32P]-labeled AP-1 probes under similar conditions to confirm AP-1 binding specificity.

### Figure 2

**Hypoxia increases AP-1 binding activity in human PAEC.** A: [32P]AP-1 probe was incubated with nuclear protein extracts from cells cultured under normoxic (Nor, 21% O\textsubscript{2}) and hypoxic (Hyp, 3% O\textsubscript{2}) conditions for 72 h in the absence or presence (EGTA) of extracellular Ca\textsuperscript{2+} (2 mM). B: summarized data (n = 5 experiments) showing AP-1 DNA binding activity (arbitrary units) in human PAEC cultured under Nor (open bar) and Hyp conditions with (gray bar) or without (solid bar) EGTA treatment. ***P < 0.001 vs. Nor, ++P < 0.01 vs. Hyp. C: Fos and Jun composition of AP-1 heterodimers. Specific (S) antibodies against JunD, JunB, c-Fos, c-Jun, Fra-2, and Fra-1, and general (G) Fos and Jun antibodies were incubated along with [32P]-labeled AP-1 probes with nuclear protein extracts from Hyp human PAEC.
sequestration pump in the SR, with 10 μM CPA, evoked a transient \([\text{Ca}^{2+}]_{\text{cyt}}\) increase (Fig. 3A). Restoration of extracellular \([\text{Ca}^{2+}]\) to 1.8 mM in the continued presence of CPA evoked a second \([\text{Ca}^{2+}]_{\text{cyt}}\) increase of similar magnitude. The latter increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) represents CCE incurred as a result of store depletion by CPA.

In comparison to normoxic cells, exposure of human PAEC to 3% O\(_2\) (hypoxia) caused a significant increase in resting \([\text{Ca}^{2+}]_{\text{cyt}}\) levels (from 141 ± 6 to 223 ± 15 nM, \(P < 0.001\); Fig. 3B). The \(\text{Ca}^{2+}\) transients evoked by CPA in the absence (from 334 ± 17 to 668 ± 85 nM, \(P < 0.001\)) or presence (from 249 ± 17 to 749 ± 165 nM, \(P < 0.001\); Fig. 3, C and D) of extracellular \(\text{Ca}^{2+}\) (1.8 mM) were also significantly increased by hypoxia. The enhanced \([\text{Ca}^{2+}]_{\text{cyt}}\) may thus be attributed partially to increased CCE.

**SOC activity underlies the CPA-induced CCE.** SOC can be activated by intracellular store depletion by CPA and thapsigargin (56). To verify whether this was the case in our experiments, we treated isolated human PAEC with 10 μM CPA under normoxic and hypoxic conditions (Fig. 4). Using whole cell patch-clamp techniques, we stimulated cells for 300 ms at voltages ranging between −100 and +100 mV (20-mV increments) from a holding potential of 0 mV (which inactivated all voltage-gated cation channels). Although they were small in control conditions, \(I_{\text{soc}}\) were substantially increased in the presence of CPA regardless of the \(O_2\) tension. However, the amplitude and current density of \(I_{\text{soc}}\) were much greater in hypoxic cells (Fig. 4B, \(P < 0.01\)). Combined with the enhanced CCE observed under similar conditions, it is plausible that the enhanced \(\text{Ca}^{2+}\) influx (CCE) induced by chronic hypoxia occurs via SOC.

Blockade of SOC with 1 μM La\(^{3+}\), a potent blocker of SOC (20) and more specifically of TRPC4 (16), significantly decreased the CPA-induced CCE (Fig. 5A) to 166 ± 11 from 360 ± 36 nM in normoxic control and markedly inhibited the hypoxia-mediated increase in AP-1 binding activity (Fig. 5B, \(P < 0.05\)). Augmentation of \(\text{Ca}^{2+}\) influx by 10 μM ionomycin, a \(\text{Ca}^{2+}\) ionophore, significantly increased AP-1 binding activity under normoxic conditions (Fig. 5C, \(P < 0.01\)). These results suggest that the increased \([\text{Ca}^{2+}]_{\text{cyt}}\) due to \(\text{Ca}^{2+}\) influx promotes AP-1 binding and that the \(\text{Ca}^{2+}\) entry via SOC plays an important role in the hypoxia-induced increase in AP-1 binding activity in human PAEC.

**Molecular identity of TRP channels in human PAEC: effect of chronic hypoxia.** TRP genes are believed to encode for subunits that form heterotrameric SOC channels in a variety of cell types (6, 10, 50, 56, 66). Having measured \(I_{\text{soc}}\), we determined the molecular identity of TRP channels in human PAEC and verified whether chronic hypoxia alters the expression of TRPC (Fig. 6A), long/melastatin TRP (TRPM) (Fig. 6B), and
increases in AP-1 binding activity in cells treated with (La3+).

Summarized data (5 experiments) showing the effect of La3+ on AP-1 DNA binding activity (arbitrary unit). M, 1-kb Plus DNA ladder marker (GIBCO-BRL). Primers used for human PAEC cultured under Nor and Hyp conditions. Expression of TRPC4 gene (Fig. 6A) with little effect on other TRP channels. The increase in TRPC4 mRNA expression was dependent on the duration of the hypoxic exposure (Fig. 7A), increasing with time and reaching a maximum after 48–72 h. Western blot analyses of total protein extracts from normoxic and hypoxic PAEC confirmed that TRPC4 protein expression was enhanced during chronic hypoxia (Fig. 7B). The lower-density second band apparent in Fig. 7B may correspond to a TRPC4 splicing variant, TRPC4α or TRPC4β (58, 70). Anti-TRPC4 antibody specificity was assessed by co-incubating with the TRPC4 antibody with a 17-amino acid epitope corresponding to residues 943–958 of mouse TRPC4 (Fig. 7C). In the absence of this competing peptide, a clear doublet was visible corresponding to the ~112-kDa TRPC4 (as in Fig. 7B, a doublet is visible, suggesting the presence of TRPC4 splice variants). In the presence of the competing peptide, the TRPC4 band was not observed, confirming the anti-TRPC4 antibody specificity.

TRPC4 gene silencing inhibits hypoxia-induced increase in AP-1 binding activity. Gene silencing in mammalian and human cells can be achieved by introducing siRNA specifically targeting a gene of interest. To further test the role of TRPC4 in the hypoxia-mediated increase in AP-1 binding activity, we examined and compared TRPC4 protein level and AP-1 binding activity in control PAEC and cells transfected with TRPC4-specific siRNA. Efficiency of siRNA transfection was determined with fluorescence-labeled siRNA; cells were loaded as described in METHODS AND MATERIALS. Fluorescence was visi-

Fig. 6. Molecular identification of transient receptor potential (TRP) channels and effect of chronic hypoxia on TRP mRNA expression in human PAEC. RT-PCR amplified products for human TRPC1–7 (A), TRPM1–8 (B), and TRPV1–6 (C) channels and GAPDH (D) are shown for human PAEC cultured under Nor and Hyp conditions. Expression of TRPC channel mRNA in human brain tissue (Br) is used as a positive control. M, 1-kb Plus DNA ladder marker (GIBCO-BRL). Primers used for the RT-PCR experiments are listed in Table 1.
ble only in siRNA-transfected cells (Fig. 8A). Two of the three synthesized siRNA oligonucleotides (siRNA1 and -3) effectively decreased TRPC4 mRNA expression in normoxic PAEC (Fig. 8); siRNA2 and a scrambled siRNA oligonucleotide (sense strand: 5'-H11032-GUGUCGUAGU-CAUCCGATT-3' and antisense strand: 5'-H11032-UCGGAU-GAACUUACGACACTT-3') had no effect on TRPC4 mRNA levels. The third siRNA, siRNA3 (sense strand: 5'-H11032-ACUCUUGUGUACAAAGGATT-3' and antisense strand: 5'-H11032-UCCUUUCUGAACAGAGUTT-3') was further used to evaluate its effect on TRPC4 protein levels and on AP-1 binding activity in hypoxic PAEC. siRNA3 significantly decreased both the hypoxia-enhanced TRPC4 protein expression (Fig. 9A, \( P < 0.01 \) vs. the scrambled siRNA control) and AP-1 binding activity (Fig. 9B, \( P < 0.001 \) vs. normoxia and siRNA) in PAEC. The use of siRNA oligonucleotides further supports our conclusion that TRPC4 mRNA and protein expression are significantly increased by hypoxia and the resultant increases in CCE and \([Ca^{2+}]_{\text{cyt}}\) may serve as an important mechanism for hypoxia-mediated increase in AP-1 binding activity.

**DISCUSSION**

Hypoxia-induced pulmonary vascular remodeling involves intimal and medial hypertrophy of pulmonary...
understanding how hypoxia affects Ca²⁺-mediated pulmonary vascular wall thickening and on Ca²⁺-sensitive AP-1 transcription factors in hypoxia-mediated pulmonary vascular remodeling and pulmonary hypertension (Fig. 10).

arteries. Many of the growth factors that contribute to this remodeling process are derived in the endothelium. Hypoxia not only influences the secretion and synthesis of vasoactive substances, mitogens, and growth factors in PAEC but also affects their gene regulation. The latter is modulated by second messengers, protein kinases, and transcription factors (7, 14, 27, 59, 64, 65). In patients and animals with hypoxic pulmonary hypertension, many of the vasoactive and mitogenic substances are upregulated in lung tissues and blood. Our study focused on elucidating the role of Ca²⁺-sensitive AP-1 transcription factors in hypoxia-mediated pulmonary vascular wall thickening and on understanding how hypoxia affects [Ca²⁺]cyt in human PAEC. The results from the present study demonstrate that chronic hypoxia in human PAEC 1) upregulates mRNA and protein expression of TRPC4, 2) increases the amplitude of CCE and the current density of I_SOc (elicited by passive store depletion with CPA), and 3) enhances AP-1 DNA binding activity. In addition, we have shown that the upregulation of TRPC4 expression by chronic hypoxia is significantly blocked by La³⁺, an SOC/TRPC4 inhibitor (16), suggesting that increased Ca²⁺ influx via TRPC4 channels during hypoxia may be responsible for the enhanced AP-1 binding activity. PAEC express many genes that contain the AP-1 binding sites in their promoters. The hypoxia-mediated increase in [Ca²⁺]cyt and subsequent augmentation of AP-1 binding activity would be an important mechanism involved in hypoxia-mediated pulmonary vascular remodeling and pulmonary hypertension (Fig. 10).

Modulation of transcription factors by hypoxia and [Ca²⁺]cyt. Because of their physical location in the arterial intima, endothelial cells are in a unique position to respond to circulating factors and products of environmental stresses carried in the circulation. Sensing any physiological perturbations, endothelial cells serve as signal transducers to modulate vascular reactivity by producing and releasing vasoactive, mitogenic, and remodeling factors. The pulmonary circulation, in particular, is sensitive to changes in O₂ tension, and pulmonary endothelial cells play an essential role in modulating the remodeling and vasoconstrictive

Fig. 9. The TRPC4-specific siRNA treatment decreases TRPC4 protein expression and AP-1 binding activity. Cells treated with siRNA3 or a scrambled oligonucleotide (Cont) were cultured under Nor or Hyp conditions for 72 h before total (A) or nuclear (B) protein, as indicated, was extracted. A: Western blot analysis showing siRNA3-induced decrease in TRPC4 total protein expression (arbitrary units) in a sample gel (left) and in summarized data (right) (**P < 0.01 vs. Control). α-Actin was used as a control. B: the AP-1 probe was incubated with nuclear proteins extracts from cells cultured under Nor and Hyp conditions. Hyp cells had been treated with scrambled siRNA (Cont) or siRNA3. The sample gel and bar graph of summarized data show the increased AP-1 DNA binding activity (arbitrary units) in Hyp cells treated with scrambled siRNA (solid bar) and the significant decrease in AP-1 binding activity caused by siRNA3 treatment (gray bar). ***P < 0.001 vs. Nor and siRNA.

Fig. 10. Schematic diagram summarizing the potential role of TRP-encoded Ca²⁺ channels and AP-1 transcription factors in hypoxia-induced pulmonary vascular remodeling and pulmonary hypertension. Hypoxia upregulates the gene expression of TRPC channels (TRPC4 genes specifically in human PAEC) and enhances the activity of SOC and receptor-operated Ca²⁺ channels (ROC) by increasing the number of functional subunits that are involved in forming SOC and ROC. Upon agonist stimulation, the inositol 1,4,5-trisphosphate (IP₃)-mediated store depletion activates SOC and induces CCE. The agonist-induced production of IP₃ and diacylglycerol may also activate ROC. The increased SOC/ROC activity raises [Ca²⁺]cyt and enhances AP-1 expression and binding, thereby upregulating the expression of AP-1-responsive genes, such as endothelin-1, PDGF, and VEGF. The latter stimulate pulmonary vascular smooth muscle and endothelial cell proliferation, causing increased pulmonary vascular resistance (PVR) and arterial pressure (PAP), both of which contribute to the development of pulmonary hypertension.

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processes in this vascular bed. In particular, a variety of vasoactive genes are regulated by hypoxia in mammalian PAEC, including ET-1, PDGF-B, VEGF, endothelial nitric oxide synthase (eNOS), insulin-like growth factor (IGF), thromboxane A2, EGF, prostacyclin synthase, heparin sulfates, thrombospordin-1, and serotonin (14, 27, 74). During hypoxia-induced pulmonary vascular remodeling, the expression of proliferative factors (e.g., VEGF, PDGF-B, EGF, IGF, thrombospordin-1, ET-1) is upregulated (14, 15), while the expression of antiproliferative factors (e.g., eNOS) may be downregulated (14). The hypoxic regulation of gene expression is likely a result of transcription factor activity or mRNA instability. There is evidence that proteins with heme-containing O2 sensors may play a role in O2 sensing, thereby leading to the transcriptional regulation of O2-regulated gene expression (7, 59). However, it is generally accepted that O2 tension changes, it stands to reason that the two phenomena might account for the hypoxia-induced increase in AP-1 binding activity in human PAEC. Therefore, the evidence strongly suggests that increased Ca2+ influx via SOC channels, at least in part, mediates enhanced AP-1 binding during chronic hypoxia.

**Physiological implications of TRP gene upregulation by hypoxia.** TRP genes encode for a wide variety of cation-selective channels (10, 46). At present, proteins of the TRP family are the candidates for both store-operated and receptor-operated Ca2+ channels. Currently classified into three subfamilies (C-canonical, V-vanilloid/osm-9-like, M-melastatin) based on their structure and sequence, many TRP genes have been detected in mammalian pulmonary arterial smooth muscle and endothelial cells (20, 29, 43, 49, 67, 73, 75). In the current study, we identified four TRPC, six TRPM, and three TRPV genes in human PAEC. Initial studies of TRPC channels showed that expression of TRPC1 gives rise to nonselective cationic current activated by the depletion of intracellular Ca2+ stores (77). Later studies showed that a reduction of TRPC1 expression using antisense oligonucleotides led to the inhibition of endogenous SOC and CCE (39, 67). Similar results were obtained with TRPC3 (72) and TRPC4 (64) genes.

Knockout of TRPC4 genes in mice reduced (but did not abolish) an I\textsubscript{SOCE} in endothelial cells (16, 69). In addition to forming functional homotetrameric channels, TRPC4-encoded proteins may likely form heteromeric channels by associating with closely related TRPC1 and/or TRPC5 channels (66). Of the TRP genes we examined in human PAEC, only TRPC4 gene expression was altered (i.e., enhanced) by chronic hypoxic exposure (Figs. 6 and 7). In addition, we showed unequivocally that the increased TRPC4 mRNA (Fig. 8B) and protein (Fig. 9A) expression could be significantly attenuated by TRPC4-specific siRNA oligonucleotides. These data suggest that the TRPC4 gene encodes for hypoxia-enhanced SOC channels in human PAEC. The fact that the same siRNA could also decrease AP-1 binding activity (Fig. 9B) strongly suggests that Ca2+ influx through TRPC4 channels (homom- and/or heterotetrameric channels) underlies the increased Ca2+-dependent AP-1 binding we observed in hypoxic PAEC. In human PASM, we have recently reported that TRPC upregulation is linked to increased CCE and cell proliferation (20, 67, 75). It has long been known that Ca2+ in the cytosol, nucleus, and intracellular pools must be maintained to support vascular smooth muscle cell growth (26, 61). The results from this study suggest that the upregulated TRPC4 → enhanced CCE → increased resting [Ca2+]\textsubscript{cyt} → upregulated AP-1 expression → augmented AP-1 binding activity sequence of events in human PAEC during hypoxia may also stimulate PAEC growth and contribute to the pulmonary vascular intimal (or neointimal) hypertrophy in patients with hypoxia-induced pulmonary hypertension.
Role of Ca\(^{2+}\) in modulating endothelium-derived relaxing factors during hypoxia. Nitric oxide (NO) production can also modify the induction and transcription of vasoactive genes by hypoxia. For example, increased ET-1 and PDGF-B gene and protein expression (as observed during hypoxia) are suppressed by NO and NO donors (34). In endothelial cells, eNOS is constitutively expressed, but its maximal activation requires the activation of a Ca\(^{2+}\)-calmodulin pathway and is dependent on sufficient O\(_2\) (14, 48). Therefore, a rise in endothelial cell [Ca\(^{2+}\)]\(_{cyt}\) under normoxic conditions would stimulate eNOS activity, increase NO production, and inhibit vasoconstriction and vascular remodeling. However, an increase in [Ca\(^{2+}\)]\(_{cyt}\) in human PAEC under hypoxic conditions would predominantly increase the occurrence of O\(_2\)-independent events, such as upregulation of AP-1 transcription factors and AP-1-responsive gene expression, and promote pulmonary vascular remodeling. It is unclear whether chronic hypoxia-mediated increase in PAEC [Ca\(^{2+}\)]\(_{cyt}\) is sufficient, under hypoxic conditions, to increase NO production in lung tissues where NO expression is upregulated (36, 51, 57) or downregulated (17, 44, 53) during chronic hypoxia and in patients with pulmonary hypertension.

**Summary.** In human PAEC, chronic hypoxia upregulates the mRNA and protein expression of TRPC4, a cation channel subunit involved in forming heterotetrameric SOC, and increases the amplitude and current density of I\(_{SOC}\) (Fig. 10). The resultant increase in [Ca\(^{2+}\)]\(_{cyt}\) via enhanced CCE augments AP-1 binding activity and may ultimately increase the expression of AP-1-responsive genes (e.g., ET-1, PDGF, VEGF, and thromboxane genes) in PAEC. The increased endothelial synthesis and secretion of mitogenic and vasoactive factors may play an important role in the development of hypoxia-mediated pulmonary vascular remodeling.

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**DISCLOSURES**

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