Transcriptional regulation of $\alpha_{1H}$ T-type calcium channel under hypoxia

Hassan Sellak,1 Chun Zhou,2,3 Bainan Liu,2,3 Hairu Chen,1 Thomas M. Lincoln,4 and Songwei Wu1

1Department of Anesthesiology and Perioperative Medicine, Georgia Regents University, Augusta, Georgia; 2Center for Lung Biology, University of South Alabama, Mobile, Alabama; 3Department of Pharmacology, University of South Alabama, Mobile, Alabama; and 4Department of Physiology, University of South Alabama, Mobile, Alabama

Submitted 23 June 2014; accepted in final form 29 July 2014

Sellak H, Zhou C, Liu B, Chen H, Lincoln TM, Wu S. Transcriptional regulation of $\alpha_{1H}$ T-type calcium channel under hypoxia. Am J Physiol Cell Physiol 307: C648–C656, 2014. First published August 6, 2014; doi:10.1152/ajpcell.00210.2014.—The low-voltage-activated T-type Ca$^{2+}$ channels play an important role in mediating the cellular responses to altered oxygen tension. Among three T-type channel isoforms, $\alpha_{1G}$, $\alpha_{1H}$, and $\alpha_{1I}$, only $\alpha_{1H}$ is found to be upregulated under hypoxia. However, mechanisms underlying such hypoxia-dependent isoform-specific gene regulation remain incompletely understood. We, therefore, studied the hypoxia-dependent transcriptional regulation of $\alpha_{1H}$ and $\alpha_{1G}$ gene promoters with the aim to identify the functional hypoxia-response elements (HREs). In rat pulmonary artery smooth muscle cells (PASMCs) and pheochromocytoma (PC12) cells after hypoxia (3% O$_2$ exposure, we observed a prominent increase in $\alpha_{1H}$ mRNA at 12 h along with a significant rise in $\alpha_{1H}$-mediated T-type current at 24 and 48 h. We then cloned two promoter fragments from the 5′-flanking regions of rat $\alpha_{1G}$ and $\alpha_{1H}$ gene, 2,000 and 3,076 bp, respectively, and inserted these fragments into a luciferase reporter vector. Transient transfection of PASMCs and PC12 cells with these recombining constructs and subsequent luciferase assay revealed a significant increase in luciferase activity from the reporter containing the $\alpha_{1H}$ but not $\alpha_{1G}$, promoter fragment under hypoxia. Using serial deletion and point mutation analysis strategies, we identified a functional HRE at site 1,173CACGC within the $\alpha_{1H}$ promoter region. Furthermore, an electrophoretic mobility shift assay using this site as a DNA probe demonstrated an increased binding activity to nuclear protein extracts from the cells after hypoxia exposure. Taken together, these findings indicate that hypoxia-induced $\alpha_{1H}$ upregulation involves binding of hypoxia-inducible factor to an HRE within the $\alpha_{1H}$ promoter region.

T-type calcium channel; gene expression; hypoxia; hypoxia-response element

HYPOXIA IS AN ESSENTIAL and critical pathological component of many pulmonary disorders, including acute lung injury, chronic obstructive pulmonary disease, and pulmonary hypertension. One of the most important responses to hypoxia in the pulmonary vasculature is a change in intracellular calcium homeostasis. Smooth muscle cells in pulmonary arteries, i.e., pulmonary artery smooth muscle cells (PASMCs), and endothelial cell in the microvessels, i.e., pulmonary microvascular endothelial cells (PMVECs), are both equipped with voltage-gated Ca$^{2+}$ channels. The two major classes of voltage-gated Ca$^{2+}$ channels are differentiated by their electrophysiological properties: high-voltage-activated channels, which include the L-, N-, P/Q-, and R-subtypes, and low-voltage-activated channels, also known as T-type channels. In addition to their distinct voltage dependence of activation, T-type channels also exhibit a unique rapid inactivation and slow deactivation time course (29). Functionally, the L-type channel has long been considered the predominant source of Ca$^{2+}$ for pulmonary vasoconstriction, whereas the T-type channel is thought to be important in cell cycle progression and proliferation in PASMCs (32) and play an essential role in controlling exocytosis in PMVECs (43, 44).

T-type Ca$^{2+}$ channels comprise three isoforms, namely $\alpha_{1G}$ (CaV3.1), $\alpha_{1H}$ (CaV3.2), and $\alpha_{1I}$ (CaV3.3) (9, 23, 24, 30). They are found in many cell types and are involved in a variety of cellular mechanisms, activities, and physiological processes, such as cardiac pacemaking (21), hormone secretion (aldosterone, insulin) (3, 45), neuronal excitability (7), and cell differentiation and proliferation (19). Beside their involvement in cell physiological processes, T-type Ca$^{2+}$ channels have also been strongly implicated in pathological conditions, including cardiac hypertrophy (26), heart failure (8), vascular injury (33), and tumor growth (20, 22).

Changes in T-type Ca$^{2+}$ channel expression have been observed during organ development as well as under various pathological states (15, 27). However, little is known about the transcriptional regulation of the T-type Ca$^{2+}$ channel gene expression. In humans, the genes coding $\alpha_{1H}$ and $\alpha_{1G}$ are located on chromosomes 16 and 17, respectively. The previous cloned 5′-flanking region of $\alpha_{1G}$ lacks TATA and CAAT boxes (34), a characteristic commonly shared by so-called housekeeping genes. A recent cloning of the $\alpha_{1G}$ 5′-flanking region from differentiated and undifferentiated retinoblastoma cells revealed that two previously described alternative promoters could be used to generate short (differentiated cell) or long (undifferentiated cell) transcripts (5). Furthermore, this gene is subjected to extensive alternative splicing (11 sites), including two alternative promoters and two alternative polyadenylation sites (11, 16).

Hypoxia has been demonstrated to upregulate $\alpha_{1H}$ in pheochromocytoma (PC12) cells as well as in chromaffin cells (10, 18). Similarly, when cardiac ventricular myocytes were subjected to hypoxia/reoxygenation, both $\alpha_{1G}$ and $\alpha_{1H}$ were shown to be differentially regulated, with a decrease in $\alpha_{1G}$ mRNA and an increase in $\alpha_{1H}$ mRNA (31). Additionally, the observation that $\alpha_{1H}$ is upregulated under hypoxia is in agreement with the presence of several potential hypoxia-inducible factor (HIF) binding sites within the 5′-flanking region of the gene. However, mechanisms underlying such isoform-specific and hypoxia-dependent gene expression remain incompletely understood. Thus the present study was undertaken to examine hypoxia-dependent transcriptional regulation of $\alpha_{1G}$ and $\alpha_{1H}$ gene promoters with the aim to identify the functional hypoxia-response elements (HREs).

MATERIALS AND METHODS

Cell culture. Rat PASMCs and PMVECs were isolated, cultured, and characterized at the cell culture core facility of the Center for...
Lung Biology at the University of South Alabama using previously described methods (2, 14). Cells used in all experiments were below passage 12. The protocols were approved by the university’s Institutional Animal Care and Use Committee. Embryonic rat aortic smooth muscle cells (A7r5) and pheochromocytoma-derived cell line (PC12) were purchased from ATCC (Rockville, MD).

**PASMCs, PMVECs, and A7r5 cells were directly plated on regular 60-mm cell culture dishes or 12-well plates. PC12 cells were seeded on poly-L-lysine-coated dishes (cell density of 1 × 10^5 cells).** Once at 70–80% confluence, cells were either kept under normoxia (21% O2) or exposed to hypoxia (3% O2) in incubators that maintained a constant environment (5% CO2 balanced with N2) for indicated periods of time.

**Reverse-transcripase polymerase chain reaction.** Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed to detect α1H and α1G mRNAs expression in A7r5 and PC12 cells, PMVECs, and PASMCs exposed or not to hypoxia. Total RNA was prepared using Stat60 (Tel-Test, Friendswood, TX) following the manufacturer’s protocol. RT-PCR was performed using a one-step kit (Invitrogen, Carlsbad, CA) and specific primers as reported previously (10).

**Immunofluorescence labeling of α1G and α1H.** Subconfluent PC12 cells or PASMCs, grown on glass coverslips, were fixed with ice-cold methanol for 5 min, heated in antigen retrieval buffer (Dako, Glostrup, Denmark) at 95°C for 20 min. Cells were then permeabilized with 0.25% Triton X-100/PBS (PBST) for 10 min, and blocked with 10% normal goat serum in 1% bovine serum albumin (BSA). The samples were next incubated overnight at 4°C with a rabbit anti-α1G or anti-α1H antibody (Alomone Laboratories, Jerusalem, Israel) at 1:100 in 5% goat serum/PBST, washed with PBS, and then incubated with a secondary antibody conjugated with Alexa Fluor 488 or 594 (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in 5% goat serum/PBST for 2 h at room temperature in the dark and then washed with PBS. Lastly, the samples were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (1:10,000; Sigma, St. Louis, MO) for 1 min, washed, mounted with a fluorescent mounting medium (Vector Laboratories, Burlingame, CA), and examined with a Nikon A1 confocal laser microscope system. A ×60, 1.20 numerical aperture water immersion objective was used along with the following filter settings: for Alexa Fluor 488, 493-nm excitation and 518-nm emission; for cyanine 3, 590-nm excitation and 617-nm emission; and for DAPI, 345-nm excitation and 455-nm emission. A set of serial optical sections (z-stacks) was taken at 0.2-μm intervals.

**Electrophysiology, data acquisition, and analysis.** Patch-clamp recordings were performed in whole cell configuration as previously described (20, 44).

**Cloning of rat α1G and α1H 5’-flanking regions and generation of serial deletions constructs.** The 5’-upstream sequences of the rat, mouse, and human α1H genes were obtained from the Ensembl genome databases (available at www.ensembl.org). The rat α1G gene (GenBank accession number AF290213) is located in sequence NR0R01500654 on chromosome 10.

**PASMCs, PMVECs, and A7r5 cells were next incubated overnight at 4°C with a rabbit anti-α1G, forward (5’-GGCGTGGTGGTGGAGAACTT-3′) and reverse (5’-CATAGGTACCGTTCGCTTGCAGGAC-3′) were used as the internal control.

**Table 1.** The sequence of the primers used to clone the 2,000-bp α1G promoter and its serial deletion fragments

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5’-CATAGGTACCTCTGCGACCCACAGCAGCTTTG-3’</td>
</tr>
<tr>
<td>F2</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F3</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F4</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F5</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F6</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F7</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F8</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F9</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F10</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-CTATAGGTACCTCTGCGACCCACAGCAGCTTTG-3’</td>
</tr>
</tbody>
</table>

**Table 2.** The sequence of the primers used to clone the 3,076-bp α1H promoter and its serial deletion fragments

<table>
<thead>
<tr>
<th>Region</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F2</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F3</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F4</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F5</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F6</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F7</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F8</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F9</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>F10</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’-AAGGACACGCGGTTACCCCGAGCGTGTCATG-3’</td>
</tr>
</tbody>
</table>
HYPOXIA UPREGULATES $\alpha_{1H}$ T-TYPE CALCIUM CHANNEL

Ultracompetent Cells (Stratagene). All mutations were confirmed by DNA sequencing.

Electrophoretic mobility-shift assay. PC12 cells and PASMCs were subjected to hypoxia (3% $O_2$) for 6 h, and nuclear extract proteins were prepared using an NE-PER nuclear and cytosolic extraction reagent kit (Pierce Biotechnology, Rockford, IL) following the protocol provided by the manufacturer. The oligonucleotides were annealed, end-labeled with T4 polynucleotide kinase in the presence of [γ-32P]-ATP, and purified using MicroSpin G-25 columns (Amer sham Biosciences, Piscataway, NJ). Nuclear extract proteins (10 μg) from hypoxic or normoxic cells were incubated with the 32P-labeled probes (50,000 counts per minute) in a buffer BSA (10 μg/ml), Tris-HCl (10 mmol/l, pH 7.5), NaCl (50 mmol/l), dithiothreitol (1 mmol/l), EDTA (1 mmol/l), and glycerol (5%) in a total volume of 20 μl. To minimize nonspecific binding, 2 μg of poly(dI-dC) was added to the reaction. The binding was carried out on ice for 30 min. The DNA-protein complexes were resolved by 5% nondenaturing PAGE at 12 V/cm in low-ionic-strength buffer (0.25 X Tris/Borate/EDTA) at room temperature. The anti-HIF-1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was preincubated with nuclear extract proteins (PC12) for 30 min on ice before the addition of the radioactive probe (HRE-5), followed by an additional 30 min in the presence of the probe. Gels were then dried and exposed to autoradiography film. The probes used were as follows: 1) HRE-consensus sequence forward: 5'-TCCTGACTGACGTACACCTTCC-3'; reverse: 5'-GGTGTTGCTGGACGTCACGTCA-3'; 2) wild-type HRE at the end of fragment 5 (wtHRE-5) forward: 5'-CTCCCAAGGCTGCC-3'; reverse: 5'-GGGACGGTGGAGGAG-3'; and 3) mutated (mHRE-5) forward: 5'-CTCCCAACCTTGGC-3', reverse: 5'-GGGACGGTTGGAG-3' with the underlined mutated bases.

Data analysis. Numerical data are reported as means ± SE. A value of $P < 0.05$ was considered significant.

RESULTS

Hypoxia selectively increases $\alpha_{1H}$ expression. To test whether $\alpha_{1G}$ and $\alpha_{1H}$ T-type $Ca^{2+}$ channel expression is modulated under hypoxia, RT-PCR was first performed using $\alpha_{1G}$ and $\alpha_{1H}$ sequence-specific primers and the total RNA isolated from different types of cells (PASMCs, PMVECs, PC12 cells, and A7r5 cells) exposed to 3% $O_2$ for 12 h. We found that, under normoxic conditions, $\alpha_{1G}$ mRNA expression was detected in all analyzed cells, with a higher amount detected in PMVECs and marginal expression in PC12 cells (Fig. 1A), whereas $\alpha_{1H}$ mRNA was detected readily in PC12 cells and PASMCs, imperceptibly in PMVECs, but not at all in A7r5 cells. Under hypoxic conditions, $\alpha_{1H}$ mRNA expression was markedly elevated in PASMCs and PC12 cells but remained unchanged in A7r5 cells and PMVECs. In stark contrast, no change was appreciated in $\alpha_{1G}$ mRNA expression under hypoxia in any of the cell types examined. Changes in the $\alpha_{1G}$ and $\alpha_{1H}$ protein expression levels in PC12 cells and PASMCs were next assessed under hypoxia using immunofluorescence labeling and confocal microscopy (Fig. 1B). Consistent with mRNA expression findings, a significantly increased abundance of $\alpha_{1H}$, but not $\alpha_{1G}$, was observed in PASMCs and PC12 cells after 48 h of hypoxia (3% $O_2$) exposure. Thus, of the two T-type $Ca^{2+}$ channel $\alpha_{1}$-isoforms...
assessed, only α₁H, but not α₁G, demonstrates notable increases in mRNA as well as protein expression under hypoxia.

**Hypoxia increases the α₁H T-type Ca²⁺ channel activity.** Subsequently, conventional whole cell patch-clamp recordings were performed under a voltage-clamp configuration in PASMCs and PC12 cells to examine whether hypoxia-induced upregulation of α₁H mRNA and protein expression occur concomitantly with an increase in functional expression of the T-type Ca²⁺ channels. With Ca²⁺ (10 mmol/l) as a charge carrier and an inclusion of nifedipine (10 μmol/l) in the extracellular solution to eliminate potential interference of L-type currents, stepwise current-voltage (I-V) protocol as well as a two-step voltage protocol were utilized to assess the macroscopic T-type current in cells. Note that the two-step voltage protocol, through measuring the activating tail current (I_tail) following a strong depolarization pulse, is a well-established protocol allowing assessment of the maximally evoked T-type channel activity in a single cell insusceptible to the changes in activation or inactivation properties (20, 40).

We observed a marked increase in T-type tail current in both PASMCs and PC12 cells after 24-h and 48-h exposure to hypoxia (3% O₂) (Fig. 2). Compared with normoxia, the I_tail median at 48 h after hypoxia increased 77% in PASMCs and 88% in PC12 cells. It is of note that the deactivating tail current (I_tail) from a holding potential of −90 mV, as schematically illustrated. D: current-voltage (I-V) relationships of peak currents recorded by step depolarizations to varying test potentials (−70 to +50 mV with 10-mV increments) from a holding potential of −90 mV. Data represent the means ± SE, n = 12 each. Ca²⁺ (10 mmol/l) was used as a charge carrier. Nifedipine (10 μmol/l) was present in the extracellular solution. Temperature was 22−25°C.
the T-type Ca\(^{2+}\) current in A7r5 cells and PMVECs. No changes in the \(\alpha_{1G}\)-mediated current were observed in either cell type up to 48 h after hypoxia exposure (data not shown). Taken together, both molecular and functional analyses demonstrate that only the \(\alpha_{1H}\) T-type Ca\(^{2+}\) channel is specifically upregulated under hypoxia. This hypoxia-induced isoform-specific T-type Ca\(^{2+}\) channel upregulation was further analyzed at the gene level by cloning the 5' flanking regions of both \(\alpha_{1G}\) and \(\alpha_{1H}\) subunits.

**Hypoxia has no effect on \(\alpha_{1G}\) promoter activity.** To localize the putative HRE within \(\alpha_{1G}\) and \(\alpha_{1H}\) promoters, two promoter fragments from the 5' flanking regions of rat \(\alpha_{1G}\) and \(\alpha_{1H}\) gene, 2,000 and 3,076 bp upstream from ATG, respectively, were cloned using rat genomic DNA and inserted into a pGL3 luciferase reporter vector. Furthermore, serial deletions were generated using each of the full-length promoter fragments as a template and primers shown in Tables 1 and 2. Within the cloned full-length 2,000-bp \(\alpha_{1G}\) promoter, there are two potential HRE localized at \(-1,901\) to \(-1,895\) (GCCGTG) and \(-73\) to \(-69\) (GCCGTG) in the forward strand. PC12 cells were transfected with either full-length \(\alpha_{1G}\) promoter or various deletion constructs and then exposed to hypoxia for 48 h. As shown in Fig. 3, no increase in luciferase activities was observed when comparing different promoter fragments under hypoxia vs. normoxia. These data further demonstrate that \(\alpha_{1G}\) is insensitive to hypoxia and confirm the observations from the above-described mRNA analysis and electrophysiology studies.

**Serial deletions in \(\alpha_{1H}\) promoter revealed a functional HRE-binding site.** It has been previously reported that HIF is required for upregulation of \(\alpha_{1H}\) under hypoxia (11). However, to the best of our knowledge, functional assessment of the promoter, as well as the exact localization of the functional HRE on the 5' flanking region of \(\alpha_{1H}\), has not been addressed. Analysis of \(\alpha_{1H}\) 5'-flanking sequence revealed the presence of a number of potential HREs in both forward and reverse DNA strands. To determine which HRE site is mediating the upregulation of \(\alpha_{1H}\) under hypoxia, serial deletion constructs of the 3,076-bp \(\alpha_{1H}\) promoter were generated as described in **Materials and Methods**. The results in Fig. 4 show that the 3,076-bp \(\alpha_{1H}\) promoter fragment contains three HREs within the forward DNA strand and nine HREs on the reverse DNA strand. The functional luciferase assays performed following transfection of PC12 cells with different deletion constructs show that the luciferase activity of the reporter-containing fragment F-1, F-2, F-3, F-4, or F-5 increased under hypoxia. It is of note that fragments F-3, F-4, and F-5 undergo higher luciferase activity under hypoxia, suggesting the likelihood of a suppressor in the F-1 and F-2 promoter regions. Fragments F-6, F-7, F-8, F-9, and F-10 did not respond to hypoxia, suggesting that the HRE is localized at the 5' end of fragment F-5 (Fig. 4).

Hypoxia increased HIF-1\(\alpha\) binding to HRE-5 localized at site \(-1,173\)acaccc\(-1,169\) on \(\alpha_{1H}\) promoter. To confirm the involvement of HRE localized within construct F-5 (HRE-5) in mediating the increase of \(\alpha_{1H}\) promoter activity under hypoxia, PC12 cells and PASMCs were subjected to hypoxia (3% \(O_2\)) for 6 h. Nuclear proteins were prepared and incubated with indicated probes, and electrophoretic mobility-shift assay (EMSA) was performed as described in **Materials and Methods**. Results in Fig. 5 show that a band was retarded on the gel even under normoxia. However, the intensity of the retarded band was increased when both HRE consensus sequence (Fig. 5A, I) and HRE-5 (Fig. 5A, II) were used as probes, under hypoxia vs. normoxia. In addition, when mutated HRE-5 was used as a probe, no increase in the intensity of the band was obtained under hypoxia (Fig. 5A, III). These results reinforce the role of the HRE-5 site in mediating \(\alpha_{1H}\) response to hypoxia in both...
PC12 cells and PASMCs. In an antibody competition assay using HRE-5 as a probe and nuclear extracts from hypoxic PC12 cells, we next found that anti-HIF-1α antibody reduced the intensity of the retarded band. The anti-HIF-1α antibody is blocking the complex formation, without producing a super shift as shown in Fig. 5A (lane +), indicating that HIF-1α is the direct binding partner of the HRE-5 site. To confirm that the HRE-5 binding site is responsible for the hypoxia-induced increase of α1H promoter activity, site-directed mutagenesis was performed to generate reporter constructs harboring the 5′-flanking region of the genes, and performing EMSA. We identified a mutation of the HRE-5 binding site abolished both α1H-F1 and α1H-F5 promoter activities in transfected PC12 cells (Fig. 5B). Altogether, these data demonstrated that hypoxia-induced α1H gene upregulation involves an HRE site located at −1,173CACGC−1,169 within the α1H promoter region.

**DISCUSSION**

In this study, we have demonstrated that α1H, but not α1G, expression is markedly increased under hypoxia at the transcriptional, translational, and the functional level through examining mRNA and protein expressions, analyzing serial deletion constructs obtained from the 5′-flanking region of the genes, and performing EMSA. We identified the exact location of the HRE that mediates HIF binding on the 5′-flanking region of α1H. Our data indicate that cells express different mRNA levels of α1G (PMVEC > A7r5 > PASMC > PC12) and α1H (PASMC > PC12) mRNA. Among all analyzed cells, α1H mRNA expression increased under hypoxia in PC12 cells and PASMCs only. However, as previously reported (10), none of the analyzed cells showed any increase in α1G mRNA or protein expression. In addition, the increase in α1H mRNA and protein expression correlated very well with the increase in total α1H-mediated T-type Ca2+ channel activity in PC12 cells and PASMCs under hypoxia. The isoform-specific response of α1H T-type channel to hypoxia is in agreement with the HIF-1α binding to the 5′-flanking region of the gene and reinforces the earlier observations in PC12 and chromaffin cells (10, 18).

The sequence analysis of α1G and α1H 5′-flanking regions revealed, as previously reported (5, 10), the presence of potential HREs on both forward and reverse DNA strands. Although we found two potential HREs on the 2,000-bp α1G promoter, cells transfected with serial deletion constructs under hypoxia did not lead to any increase in α1G promoter activity. These data confirm previous reports of α1G insensitivity to hypoxia (6, 10). In contrast, exposing neonatal rat ventricular myocytes to hypoxia resulted in an HIF-dependent decrease in α1G mRNA and upregulation of α1H mRNA (31). This discrepancy in α1G expression may be due to the hypoxia method used, its severity (3% vs. 1% O2), in addition to the cell model used in the different studies. Future investigations exposing adult cardiomyocytes to hypoxia will certainly determine the precise effect of hypoxia on α1G expression. Thus differential regulation of α1G T-type channels may be truly occurring under hypoxia. It is also plausible that α1G channels may facilitate cell adaptation to lower oxygen instead of instigating various functions in response to hypoxia.

There are 12 potential HRE sites within the cloned 3,076-bp DNA fragment from the 5′-flanking region of α1H, three on the forward and nine on the reverse strand. To determine the precise localization of the HRE involved in elevated α1H expression under hypoxia, we used serial deletion strategy and found that HRE-5, located at −1,169 bp to −1,173 bp from the transcription start site ATG, is the main driver of the channel promoter activity under hypoxia. This was further confirmed...
PASMCs were subjected to hypoxia (3% O2) for 6 h. Nuclear protein extracts increases hypoxia-inducible factor (HIF)-1 to 24 h. The luciferase values (RLU) were normalized to PC12 cells and wild-type (F1, F5) or mutated HRE-5 (mF1, mF5) luciferase reporter constructs (250 ng) and β-galactosidase activity serving as internal control. Representative of 3 independent experiments performed in triplicate. CACGC, wild-type HRE-5; CAAAC, mutated HRE; F0, empty vector. *P < 0.05, **P < 0.005.

Fig. 5. HRE-5 within α1H promoter mediates hypoxia response. A: hypoxia increases hypoxia-inducible factor (HIF)-1α binding to HRE-5. PC12 cells and PASMCs were subjected to hypoxia (3% O2) for 6 h. Nuclear protein extracts were prepared as described in MATERIALS AND METHODS and incubated with different radio-labeled probes consensus sequence (I), HRE-5 (II) within α1H promoter, mutated HRE-5 (III) for 30 min before resolving DNA-protein complex on gel. In the immune competition assay, the anti-HIF-1α antibody was incubated for 30 min with nuclear extracts prepared from PC12 subjected to hypoxia (3% O2 for 6 h) before addition of the probe. F0, free probe; N, normoxia (21% O2); H, hypoxia (3% O2). B: HRE-5 mutation abolishes hypoxia response. Site-directed mutagenesis was performed as described in MATERIALS AND METHODS to generate constructs F1 and F5 harboring mutated HRE-5 named mF1 and mF5, respectively. PC12 cells were cotransfected with wild-type (F1, F5) or mutated HRE-5 (mF1, mF5) luciferase reporter constructs (250 ng) and β-galactosidase (50 ng). Cells were then exposed to hypoxia (3% O2 for 24 h). The luciferase values (RLU) were normalized to β-galactosidase activity serving as internal control. Representative of 3 independent experiments performed in triplicate. CACGC, wild-type HRE-5; CAAAC, mutated HRE; F0, empty vector. *P < 0.05, **P < 0.005.

In vascular smooth muscle cells, T-type Ca2+ channels are involved in excitation-contraction coupling and proliferation (32). Although, as with previous reports (6, 10, 18, 31), we did not observe any increase in α1G expression under hypoxia, a recent in vivo study has demonstrated that mice exposed to hypoxia exhibited an increased α1G mRNA expression in both pulmonary and mesenteric arteries (37). To the best of our knowledge, it is by far the first observation that hypoxia upregulates α1G. However, a recent study confirmed that, in rat neonatal ductus arteriosus, α1G mRNA and protein are not changed under hypoxia (1% O2) but rather upregulated significantly under oxygenation (1). Consequently, these seemingly conflicting data support the idea that hypoxia differentially modulates T-type Ca2+ channels depending on cells type as stated previously (31).

In summary, the present study has further advanced our understanding regarding the highly specialized hypoxia-dependent regulation of the T-type channels. We demonstrated that hypoxia specifically increased α1H expression at the transcriptional, translational, and functional level and that this upregulation involves HIF binding to an HRE site located at −1,173CACGC−1,169 of the α1H promoter region. Although the physiological relevance of these observations is not addressed in this study, the widespread and differential tissue expression of T-type channel α1-isoforms, as well as their distinct hypoxia regulation patterns, could impose different roles of the α1-isoforms in calcium homeostasis under various physiological and pathological conditions.

ACKNOWLEDGMENTS

We thank Anna Buford and Linn Ayers (Cell Culture Core Facility of the Center for Lung Biology at the University of South Alabama) for excellent technical assistance with cell culture study and Nadine Odo (Georgia Regents University) for editing the manuscript. Present address for B. Liu: Department of Immunology, Zunyi Medical College, Zunyi, Guizhou 563003, China.

GRANTS

This work was supported by the National Institutes of Health Grant HL66299 (to S. Wu).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.S. and S.W. conception and design of research; H.S., C.Z., and S.W. prepared figures; H.S. and S.W. drafted manuscript; H.S., T.M.L., and S.W. interpreted results of experiments; H.S., C.Z., and S.W. analyzed data; H.S., C.Z., B.L., and H.C. performed experiments; H.S., C.Z., and S.W. technical assistance with cell culture study and Nadine Odo (Georgia Regents Center for Lung Biology at the University of South Alabama) for excellent technical assistance with cell culture study and Nadine Odo (Georgia Regents University) for editing the manuscript. Present address for B. Liu: Department of Immunology, Zunyi Medical College, Zunyi, Guizhou 563003, China.

HYPOXIA UPREGULATES α1H T-TYPE CALCIUM CHANNEL

AJP-Cell Physiol • doi:10.1152/ajpcell.00210.2014 • www.ajpcell.org
REFERENCES


