Hypoxia-dependent regulation of PHD1: cloning and characterization of the human PHD1/EGLN2 gene promoter

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Abstract The recent identification of hypoxia-inducible-factor (HIF) prolyl hydroxylases (PHD1, 2, and 3), which modify HIF-1α in an oxygen-dependent manner, provided an important link between oxygen availability and hypoxia-induced gene expression. However, little is known about the regulation of the PHDs. To investigate the transcriptional regulation of PHD1, we cloned the PHD1 gene promoter. Here, we report that the expression of PHD1 is reduced under hypoxic conditions. Furthermore, we identified binding sites for aryl hydrocarbon nuclear translocator (ARNT/HIF-1β) within the PHD1 promoter, and showed that ARNT is associated in vivo with the PHD1 promoter following hypoxia, which implies a role for ARNT in the hypoxia-dependent regulation of PHD1. Taken together, our findings suggest a hypoxia-induced regulatory loop of PHD1 expression, mediated by ARNT.

Keywords: Prolyl hydroxylases; HIF-1α; Hypoxia; Aryl hydrocarbon nuclear translocator

1. Introduction

Maintaining oxygen homeostasis is essential for the survival of all organisms, and the ability of cells to sense and respond to changes in oxygen availability is critical for many physiological and pathological processes. Conserved oxygen responsive pathways are present in most mammalian cells and result in changes in gene expression [1]. The hypoxia-inducible-factor-1 (HIF-1) is a central transcription factor complex driving the cellular response to lack of oxygen. Following induction by hypoxia, HIF-1 binds to a hypoxia responsive element (HRE) within the promoters of a rapidly increasing number of target genes and drives the cellular adaptive response to reduced oxygen availability. HIF target genes mediate changes in cellular metabolism such as increased glucose uptake and glycolysis, red blood cell maturation, the production of several angiogenic factors and their receptors [2,3], and, under severe hypoxia, apoptosis [4,5].

HIF-1 is a heterodimeric complex consisting of two subunits: HIF-1β (also termed ARNT: aryl hydrocarbon nuclear translocator), which is expressed constitutively in the nucleus and also participates in other transcriptional pathways, and HIF-1α, whose stable expression is tightly regulated in a hypoxia-dependent manner. In normoxia, HIF-1α is rapidly degraded by the ubiquitin–proteasome pathway, and its low levels do not allow heterodimer formation and transcriptional activation [6,7]. When hypoxia occurs, this degradation is suppressed and HIF-1α is rapidly stabilized. The degradation of HIF-1α is mediated by the product of the Von Hippel-Lindau (VHL) tumor suppressor gene, which acts as the recognition component of a ubiquitin E3 ligase complex. VHL interacts specifically with an oxygen-dependent degradation domain (ODDD) within HIF-1α. VHL recognition requires enzymatic hydroxylation of specific prolyl residues within the HIF-1α ODDD, which depends on the availability of molecular oxygen (reviewed in [8]). The recent cloning of the mammalian HIF prolyl hydroxylases provided an important link between oxygen availability and HIF-1α activation, since the activity of the PHDs was shown to depend on oxygen [9–11]. The three prolyl hydroxylases were termed PHDs, HPHs or EGLNs by various groups, and we shall hereafter use the PHD nomenclature. We cloned the mouse homolog of PHD1, mPHD1 [12], which we initially designated Falkor [13].

Under normoxic conditions, mPHD1 is expressed in all the tissues we examined, most abundantly in testis [13]. The other PHDs were also shown to be ubiquitously expressed [9,14]. The concomitant expression of all three PHDs raises the possibility that they might have distinctive roles. Indeed, PHD2 was suggested recently to be the key oxygen sensor of the three family members [15]. Additionally, it was shown that when cells are exposed to hypoxic conditions, or to hypoxia mimetic drugs, there is an increase in mRNA levels of PHD2 and PHD3, but not of PHD1 [9,14,16]. Thus, the PHDs seem to also differ in their regulation.

In this study, we set out to learn about the transcriptional regulation of PHD1. We cloned the human PHD1 gene promoter and found putative binding sites for several transcription factors that might participate in its regulation. We show here that the mRNA levels of PHD1 decrease in response to the hypoxia-mimetic drug desferrioxamine (DFO) or to hypoxia, suggesting hypoxia-dependent regulation of PHD1. Furthermore, we found that HIF-1β (ARNT) is associated in vivo with the PHD1 gene promoter following hypoxia, which implies a role for ARNT in the hypoxia-dependent regulation of PHD1.

2. Materials and methods

2.1. Cloning of the PHD1 genomic 5′ region

Genomic DNA was prepared from primary human smooth muscle cells and the 5′ flanking region of the PHD1 gene was amplified using the primers: Forward: 5′-CTTTTCTCAAGGGCAACCCGCAC-3′

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Reverse: 5′-GTCCTCTGCGGTCCCTAAGGGC-3′. The PCR product was cloned using the pcDNA3.1/V5-His-TOPO TA expression kit (Invitrogen). PHD1promoter-luc reporter plasmid was constructed by subcloning the PHD1 promoter into the pGL3-Basic luciferase plasmid (Promega).

2.2. Transient transfection and luciferase assay

Cells were seeded in 24-well culture dishes. Triplicate wells were transfected with 50 ng of a reporter plasmid expressing the firefly luciferase gene under the transcriptional control of the PHD1 gene promoter, together with a control β-gal plasmid. DFO was added 24 h post-transfection and luciferase activity was assayed 48 h post-transfection. Luciferase assays were performed using (D)-luciferin (Roche). Luminescence was determined using a Rosys-Anthos Lucy 3 luminometer. The luciferase values were normalized to β-gal activity.

2.3. Chromatin immunoprecipitation (ChIP) analysis

ChIP experiments were performed according to the Farenham laboratory protocol [17]. HCT116 cells, either non-treated or treated with 200 μM DFO for 6 h, were fixed by adding Formaldehyde (Merck), in a final concentration of 1% at room temperature for 10 min. Fixation was stopped by the addition of glycine to a final concentration of 0.125 M. Plates containing HCT116 cells were rinsed with cold PBS, incubated with 5 ml of 20% trypsin–EDTA and then scraped off plates. Nuclei were collected by suspending the cells for 20 min in cell lysis buffer and microcentrifugation at 4000 rpm. The nuclei were resuspended in nuclei lysis buffer. Samples were sonicated on ice to an average length of 600–1000 bp and then microfuged at 14 000 rpm. The chromatin solution was precleared with the addition of protein A beads (blocked with BSA) for 2 h at 4 °C. Precleared chromatin from 50×10^6 cells was diluted 1:5 in dilution buffer and incubated with 2 μg of anti-ARNT antibody (Alexis Biochemicals), or anti-HA antibody (Santa-Cruz Biotechnology) for 12 h. 30 μl Agarose beads coated with anti-mouse antibody (Amersham) was added for two additional hours. Immunoprecipitants were washed twice with dilution buffer, twice with wash buffer and once with TE buffer (composition of buffers is detailed in [17]). Crosslinks were reversed by addition of NaCl to a final concentration of 200 mM, proteins and RNA were removed by addition of 10 μg of RNase A per sample for 30 min followed by addition of 30 μg proteinase K for 2 h at 42 °C. Cross-links were reversed by incubation at 65 °C overnight. DNA was extracted using Qiagen PCR extraction kit. 5% of total input samples was collected by microcentrifugation. Immunoprecipitated samples were resuspended in 50 μl of H_2O, input samples were resuspended in 50 μl of H_2O and then diluted 1:10. All samples were analyzed by PCR. PCRs contained 2 μl of immunoprecipitated or diluted total input, 50 ng of each primer, 10% DMSO and ReadyMix PCR master mix (Promega) in a total volume of 50 μl. After 35 cycles of amplification, PCR products were run on a 2% agarose gel and analyzed by ethidium bromide staining.

Primers used are:

semi-quantitative PCR: ARNT1+2 sites:
Forward: 5′-GTAGGCCACGGACCGCTGTTTCC-3′
Reverse: 5′-CGGCGCCGCCGTTTGTGCC-3′
ARNT3 site: Forward: 5′-GTGCCCTGGATGGGGGCACC-3′
Reverse: 5′-CTGCCTCGGCGTTCCAGAAAG-3′

Real-time PCR: ARNT1+2 sites:
Forward: 5′-GCCATTTCTCCGTCTCTCACC-3′
Reverse: 5′-TCAGCTCCTCCTCCTTCTCG-3′
ARNT3 site: Forward: 5′-GGCACCTTTATCTCTCGTCCTGT-3′
Reverse: 5′-CAGAGGAAAGGACGGAGGAG-3′

3. Results and discussion

3.1. Cloning of human genomic DNA fragment containing the PHD1 promoter

To facilitate studies on transcriptional regulation of PHD1, we cloned a genomic DNA fragment containing the human PHD1 promoter. The promoter containing region from the 5′ flanking sequence of human PHD1 was predicted by using the PromoterInspector software at the Genomatix site (http://www.genomatix.de). Primers designed according to the software's prediction were used to obtain a 2 kb fragment upstream of the PHD1 gene from genomic DNA prepared from human primary smooth muscle cells.

The PCR product was purified, cloned as described in Section 2 and sequenced. The nucleotide sequence of the PHD1 promoter region is shown in Fig. 1.

3.2. Sequence analysis of the human PHD1 promoter

To find out if the promoter contains potential regulatory elements, we used the web-based program MatInspector (http://www.gsf.de/biodv/matinspector.html). This program predicts putative binding sites for transcription factors within a

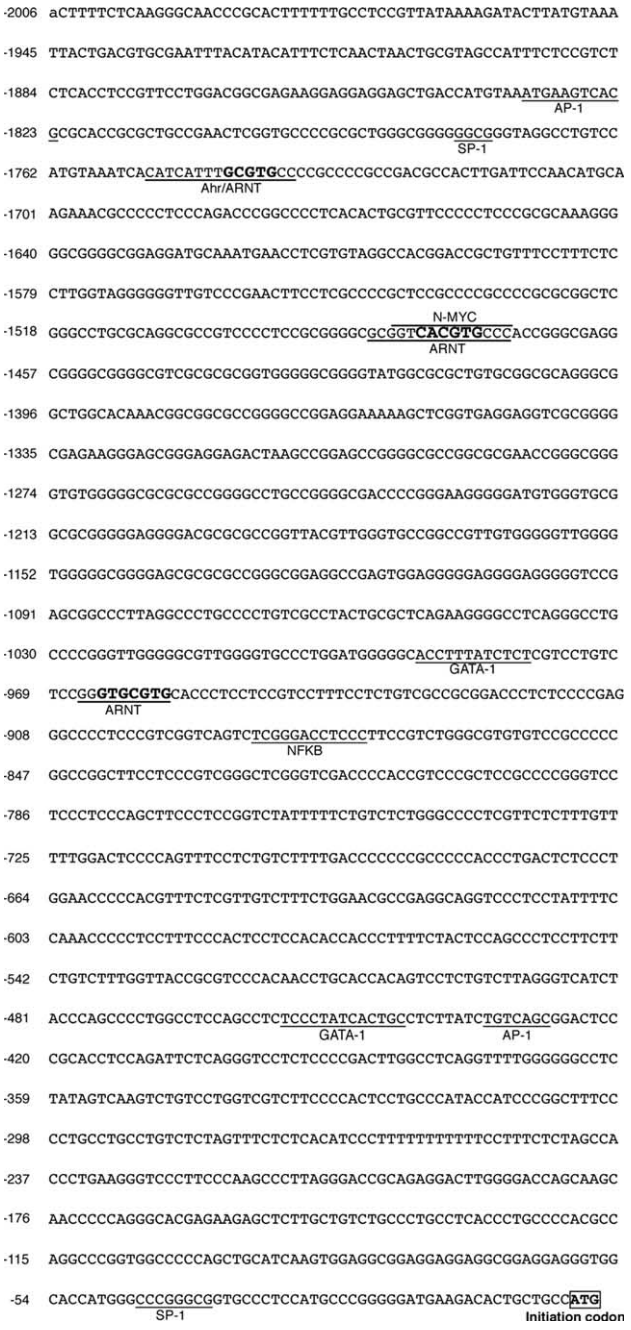

Fig. 1. Nucleotide sequence of the 5′-flanking region of the human PHD1 gene. The various potential transcriptional factor binding sites are indicated. The nucleotide number was counted from the first base of the initiation codon.

given promoter sequence. We found several binding sites for known transcription factors which might transcriptionally regulate the expression of PHD1 such as SP1, GATA-1, NF-κB, and N-Myc. Their putative binding sites are indicated in Fig. 1. Additionally, the results of the analysis of the PHD1 promoter sequence predicted three binding sites for HIF-1β/ARNT.

3.3. Promoter activity and hypoxia-dependent regulation of PHD1 expression

The 2 kb PCR product containing the 5′ genomic region of the human PHD1 gene was cloned into the luciferase reporter plasmid pGL3-basic.

To test for transcriptional activity of the putative promoter, HCT116 cells were transfected with the putative PHD1 promoter in pGL3-basic or with the empty vector. 48 h following transfection, cells were harvested and luciferase activity driven by the PHD1 promoter was measured. As seen in Fig. 2A, there was a dose-dependent, strong transcriptional activity from the plasmid containing the cloned genomic region, confirming that it includes the promoter of PHD1. To test whether the expression of PHD1 is affected by hypoxia, HCT116 cells were transfected as in Fig. 2A, treated with the hypoxia mimetic drug DFO 24 h following transfection, and then analyzed for luciferase activity. Fig. 2B shows that the hypoxic conditions caused by DFO resulted in suppression of transcription from the PHD1 promoter.

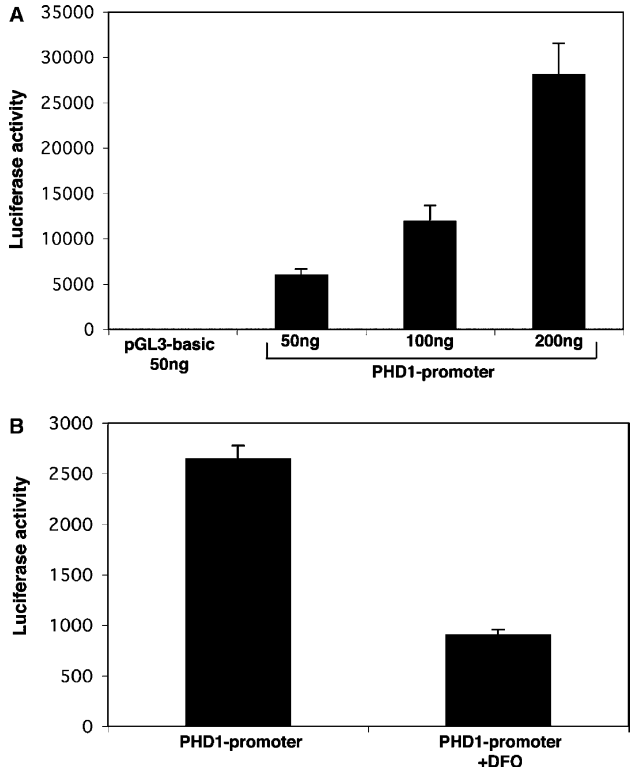

Fig. 2. Transcription activity and response to DFO of the PHD1 promoter. (A) Luciferase assay in HCT116 cells transfected with either 50 ng pGL3-basic or with increasing amounts of the PHD1 promoter cloned into pGL3-basic as indicated. Cells were plated at 3×10^5 cells/well in a 24-well plate. Results shown are the average of triplicate wells of a representative experiment, normalized to β-gal activity. (B) Luciferase assay as in (A). Cells were transfected with 50 ng PHD1 promoter-pGL3 basic and were left untreated or were treated overnight with 200 μM DFO.

We next wanted to know whether this hypoxia-induced suppression of PHD1 expression is evident in expression analysis in cells. To that end, we performed a semi-quantitative RT-PCR of both mouse (C2C12 skeletal myoblast cell line) and human (HCT116) derived cells. We performed our experiments in HCT116 cells, which are p53 deficient, since we previously showed that the expression and the activity of mPHD1 are p53 independent [12,13]. Both cell lines showed a decrease in PHD1 mRNA following treatment of cells with DFO, starting from 16 h following treatment. PCR with primers for the house keeping gene GPDH is shown as a loading control (Fig. 3A). To confirm this observation, we also performed a Northern blot analysis on RNA prepared from HCT116 cells treated with DFO, as compared to RNA prepared from non-treated cells. Upon treatment with DFO, a reduction in PHD1 mRNA levels was detected in cells. A probe for GPDH was hybridized with the membrane as a loading control (Fig. 3B).

This observation was further supported by a quantitative real-time PCR analysis performed on cDNA from HCT116 or from the breast carcinoma cell line MCF-7 cells treated with DFO. The same experiment was performed with cDNA of HCT116 cells incubated in 1% O_2 hypoxia incubator. There was about two folds reduction in PHD1 expression (Fig. 3C and D). The results were normalized to those of GPDH or to β-tubulin, as indicated, and show a representative experiment out of four such experiments performed.

These findings suggested that there might be a regulatory pathway in cells following hypoxic stress to downregulate PHD1. However, it was recently suggested that PHD2, and not PHD1 or PHD3, is the relevant hydroxylase controlling HIF-1α levels in vivo; silencing of PHD2, but not of PHD1 or PHD3 by siRNA was sufficient to stabilize and activate HIF-1α in normoxia [15]. Interestingly, PHD3 and PHD1 were both identified in contexts other than hypoxia and were suggested to be cell growth regulators [13,18–20]. Thus, their regulation may be connected to their other targets. The significance of PHD1 downregulation following hypoxia may become clearer as more target proteins are identified.

3.4. Transcriptional regulation of PHD1 by ARNT

Hypoxic-dependent regulation of the prolyl hydroxylases mRNA was recently suggested to depend on the HIF transcription factor itself [15,16]. We were therefore most interested in the possible regulation of PHD1 by ARNT, as implied by the ARNT binding site we found in the PHD1 promoter.

We next asked whether ARNT might be the transrepressor responsible for the downregulation of PHD1 following hypoxia. To confirm binding of ARNT to its consensus sites within the PHD1 promoter, we performed ChIP analysis, as described in Section 2. HCT116 cells were treated with DFO for 6 h or were left untreated. As seen in Fig. 4, PHD1 promoter sequences containing the putative ARNT consensus sites were selectively immunoprecipitated with anti-ARNT antibodies following treatment with DFO. Fragments immunoprecipitated with anti-HA tag antibodies are shown as control for non-specific binding. Thus, ARNT is associated in vivo with the PHD1 promoter under hypoxic conditions.

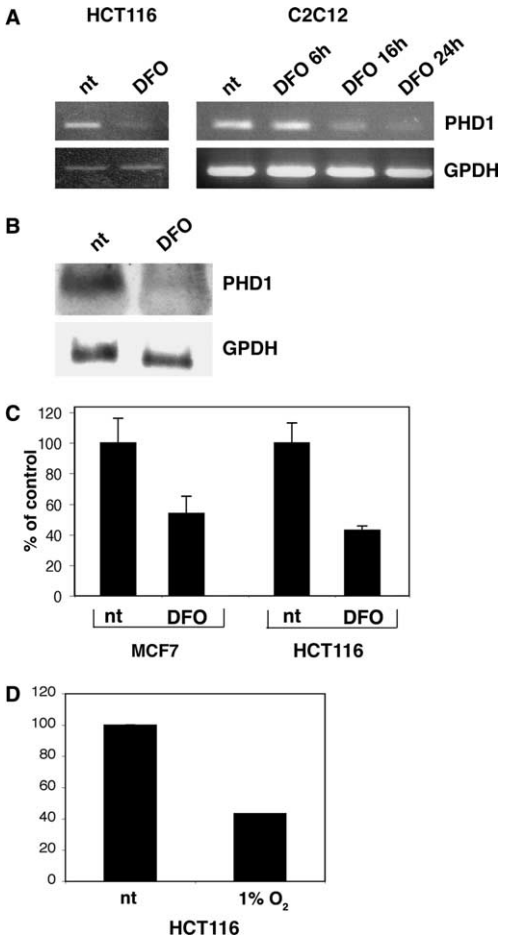

Fig. 3. Transcription of PHD1 is suppressed under hypoxia. (A) RT-PCR of cDNA prepared from HCT116 cells. Cells were left non-treated (nt) or were treated with 200 μM DFO overnight (left panel). The right panel shows RT-PCR of cDNA from C2C12 cells for the indicated time periods. PCR with GPDH primers is shown as loading control. (B) Northern blot of RNA from HCT116 cells, either non-treated (nt) or treated with 200 μM DFO overnight. RNA was probed with PHD1 cDNA specific probe or with GPDH probe as loading control. (C) cDNA from HCT116 or MCF-7 cells was prepared as in (A) and analyzed by real-time PCR. The results shown are the average of duplicate wells of a representative experiment, normalized to those of GPDH (HCT116) or β-tubulin (MCF-7), with error bars. (D) HCT116 cells were incubated overnight in a 1% O_2 incubator, collected and cDNA was analyzed by real-time PCR as in (C). The results were normalized to those of β-tubulin.

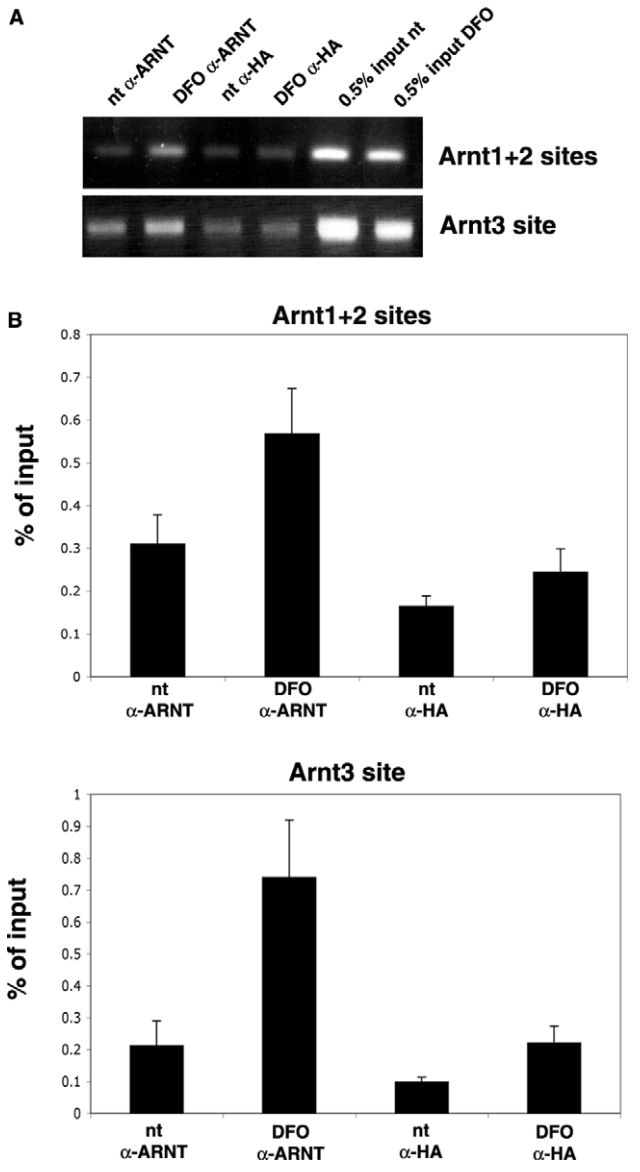

Fig. 4. ARNT is associated in vivo with the PHD1 promoter. (A) PCR analysis of ChIP products. The consensus sites for ARNT found at −1944 and at −1439 were designated ARNT1 and ARNT2 sites, respectively. These two sites are too closely located to be efficiently separated by ChIP, and were thus analyzed together (ARNT 1+2 sites, upper panel). The site found at −939 was designated ARNT3 site (lower panel). nt: non-treated. (B) Real-time PCR analysis of ChIP products. PCR was done in duplicates. Results are shown as % of input, with error bars.

3.5. Future aspects of transcriptional regulation of the PHD1 gene

The ubiquitously expressed basic helix-loop-helix (bHLH)-PAS protein ARNT forms transcriptionally active heterodimers with a variety of other bHLH-PAS proteins, including HIF-1α and AHR (arylhydrocarbon receptor), as well as ARNT homodimers. These complexes regulate gene expression in response to hypoxia and xenobiotics, respectively [21,22]. Interestingly, ARNT was shown to heterodimerize with the murine protein Single Minded (SIM), another member of the bHLH-PAS family, and trans-repress HIF-1α targets under hypoxic conditions [23,24]. This suggests that ARNT may be involved in both induction and repression of genes under hypoxia, depending on its heterodimer partner. Interestingly, the binding site for ARNT, found at −939 (ARNT3) in the PHD1 promoter (5′-GTGCGTG-3′), is typical for heterodimers of ARNT with SIM and not to ARNT homodimers or ARNT-HIF-1α heterodimers, which bind the HRE [25]. Thus, ARNT may heterodimerize with SIM to repress PHD1 expression.

On the other hand, the binding site at −1439 (ARNT2) (5′-CACGTG-3′) is the DNA recognition site specified for ARNT homodimers and the binding site at −1944 (ARNT1) (5′-GCGTG-3′) is an AHR/ARNT consensus site [26]. Further experiments are needed to decipher the molecular mechanism underlying the regulation of PHD1 by ARNT following hypoxic stress, and to identify the relevant partners of ARNT in this regulation.

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