Prolyl Hydroxylase Domain 2 Protein Suppresses Hypoxia-Induced Endothelial Cell Proliferation

Kotaro Takeda, Guo-Hua Fong

Abstract—Prolyl hydroxylase domain 2 protein (PHD2) signals the degradation of hypoxia-inducible factor (HIF)-1α by hydroxylating specific prolyl residues located within oxygen-dependent degradation domains. As expected, endothelial cells (ECs) overexpressing PHD2 had reduced HIF-1α and vascular endothelial growth factor-A expression and failed to accelerate their proliferation in response to hypoxia. Surprisingly, although these cells displayed further reductions in HIF-1α and vascular endothelial growth factor-A expression when cultured under normoxia, there was no further reduction in EC proliferation. Thus, there seemed to be no consistent correlation between PHD2 hydroxylase–mediated suppression of HIF-1α expression and inhibition of EC growth. Indeed, overexpression of a mutant PHD2 lacking hydroxylase activity also greatly diminished EC response to hypoxia-induced increase in proliferation, in spite of the fact that hypoxia-induced HIF-1α accumulation was not affected by mutant PHD2. These data strongly suggest the existence of a hydroxylase-independent mechanism for PHD2-mediated inhibition of EC proliferation under hypoxia. In support of a physiological relevance of PHD2 overexpression, we found that endogenous PHD2 expression was significantly upregulated by hypoxia and that silencing of the Phd2 gene by RNA interference significantly enhanced hypoxia-induced EC proliferation. In conclusion, this study demonstrates that PHD2 may act as a negative feedback regulator to antagonize hypoxia-induced EC proliferation. (Hypertension. 2007;49:178-184.)

Key Words: prolyl hydroxylase domain 2 protein ■ hypoxia-inducible factor ■ vascular endothelial growth factor ■ vascular endothelial cells and cell proliferation

Hypoxia-inducible factor (HIF)-1 plays a central role in cellular responses to hypoxia by regulating the transcription of a large number of genes important for adaptation to hypoxic conditions, including those encoding vascular endothelial growth factor (VEGF)-A, erythropoietin, and phosphoglycerate kinase-1. HIF-1 is a heterodimeric transcription factor of α and β subunits. Although HIF-β expression is constitutive, HIF-1α levels fluctuate dramatically depending on oxygen tension and are very low under normoxia but significantly induced by hypoxia. Hydroxylation at the 4-position of Pro402 and Pro564 within the oxygen-dependent degradation domains of HIF-1α is responsible for its destabilization under normoxia. Hydroxylated HIF-1α binds to von Hippel–Lindau protein, which targets it for polyubiquitination and proteasomal degradation. The hydroxylation reaction is catalyzed by at least 3 prolyl hydroxylase domain proteins (PHDs), including PHD1, PHD2, and PHD3, all of which are members of the 2-oxoglutarate/iron-dependent dioxygenase superfamily. Because PHD hydroxylase activity depends on molecular oxygen and their Michaelis constant values for oxygen are near the normoxic oxygen level, PHDs are suited for oxygen sensing. Among them, PHD2 is considered to function as a major oxygen sensor under normoxia, because Phd2 small interfering RNA (siRNA), but not Phd1 or Phd3 siRNAs, specifically induces HIF-1α protein under normoxia.

PHDs have diverse functions in regulating cell properties. In Drosophila, deletion of its Phd gene decreased cell sizes, whereas PHD overexpression resulted in cell hypertrophy. In mammalian cells, overexpression of a C-terminal fragment of PHD1, which includes only half of the hydroxylase catalytic domain, promoted proliferation of mouse embryonic fibroblasts cultured under normoxia. However, overexpression of full-length PHD1 does not have a similar effect. PHD3, also known as SM-20, promotes apoptosis in neuronal cells. These diverse functions of PHD members underscore the complexity of their functional mechanisms and may not be explained only by the PHD/HIF-1α/HIF-target gene pathway.

One important target for hypoxia regulation is the vascular system. Vascular endothelial cells (ECs) are subject to hypoxia in a variety of pathophysiological processes, such as embryonic development, cardiac ischemia, and tumor growth. Hypoxic conditions are created by increased metabolic activities in rapidly expanding...
tissues or by decreased blood supply in ischemic tissues, leading to the accumulation of hypoxia-induced gene products, notably, VEGF-A. In general, proliferation of ECs increases in response to hypoxia, and such a response is a part of the fundamental mechanism that underlies the process of angiogenesis. As a result of angiogenic growth and, hence, increased blood supply, local oxygen tension increases, leading to the activation of PHDs and degradation of HIF-1α. These chain events regulating the HIF-1α/VEGF system predict that PHDs may be critical regulators of EC functions. However, to date, there have been no reported investigations focused on the role of PHD2 in vascular EC proliferation. In this article, we report our surprising finding that a hydroxylase-deficient PHD2 mutant also negatively regulated hypoxia-induced EC proliferation. The existence of a hydroxylase-independent mechanism may provide cells with an additional option to adapt to complex environmental changes.

**Methods**

Detailed description of materials and methods are posted in the online section (available at http://hyper.ahajournals.org), but a summary of procedures is provided below.

PHD2 overexpression plasmid (pPHD2) was constructed by placing the full-length Phd2 cDNA under the control of CMV enhancer/chicken α-actin promoter, and pPHD2 was introduced into MS1 ECs by transfection. For Phd2 siRNA knockdown, a short hairpin RNA expression vector was built using pSilencer 3.1-H1/Hygro as parental plasmid and nucleotide positions 1113 to 1131 of Phd2 (5’-TGAGCGAGCAGGCTAAA-3’) as the target. His351 to Phe351 (H351F) mutation was introduced by PCR. To quantify MS1 EC proliferation, [3H]thymidine incorporation assays were performed. For cell counting, cultures were treated with trypsin and counted with a hemocytometer.

Anti-PHD2 antibody was raised against a PHD2 peptide (EKGVRVELKPNSVSKDV) and used in conjunction with Alexa-488–conjugated donkey anti-rat IgG to fluorescently label PHD2 for confocal analysis. For Western blotting analyses by anti-PHD2 and anti-HIF-1α antibodies, both nuclear fraction and total cell lysates were prepared from mock-transfected (MOCK) or PHD2-overexpressing (OE#5, OE#9, OE#12) ECs after anti-PHD2 immunofluorescence staining. MOCK or OE#5 cell lysates were incubated with HIF-1α peptide (residues 569 to 587), and the amount of [35S]methionine-labeled VHL protein bound to hydroxylated peptide was determined by autoradiography. N indicates normoxia; H, hypoxia; Mut#3, H351F mutant under normoxia; “–”, no lysate; Input, 1% of the total addition of [35S]methionine-labeled VHL protein. Anti-HIF-1α Western blot of nuclear extracts from cells cultured under normoxia or hypoxia for 6 hours. The HIF-1α doublet may represent posttranslational modifications. However, they are unlikely to represent hydroxylated forms, which are rapidly degraded. E and F, PHD2 overexpression (OE#5, OE#9, OE#12) or MOCK (OE#5) ECs were cultured under indicated conditions and subject to [3H]thymidine incorporation assays (E) and direct cell counting (F). n=4 to 6. Data were shown as the mean±SE. *P<0.05, **P<0.01, ##P<0.05 vs MOCK. G, Anti-PHD2 Western blot of MOCK and OE#5 cells cultured under conditions indicated in the figure. H, Anti-HIF-1α Western blot of nuclear extracts from OE#5 cultured under hypoxia for 72 hours.
overexpressing MS1 ECs. The hydroxylase activity of PHD2 was evaluated based on the ability of PHD2 to hydroxylate a specific proline residue in a murine HIF-1α peptide derived from an oxygen-dependent degradation domain (residues 569 to 587), and hydroxylation was revealed based on the binding interaction between hydroxylated peptide and [35S]methionine-labeled von Hippel–Lindau protein. Expression of mRNAs encoding PHD2, VEGF-A, and VEGF receptors was determined by Northern blotting and real-time quantitative PCR (Q-PCR).

Statistical analyses were performed using 2-way ANOVA or 1-way analysis of variance and Fisher’s test where appropriate. Data were shown as mean±SEM. *P<0.05 was considered to be statistically significant.

**Results**

**PHD2 Overexpression Suppressed HIF-1α Expression in ECs**

To uncover the roles of PHD2 in EC growth, we stably transfected MS1 ECs with pPHD2 or the parental plasmid without Phd2 cDNA (pMOCK). Western blotting analysis indicated that 3 independent clones, referred to as OE#5, OE#9, and OE#12, overexpressed PHD2 (Figure 1A). Immunofluorescence staining showed that PHD2 protein was expressed much more abundantly in OE#5 than in MOCK control (Figure 1B). Cytoplasmic localization of overexpressed PHD2 agreed with literature.24

Next, we examined the hydroxylase activity of overexpressed PHD2. As shown in Figure 1C, OE#5 lysates contained much higher hydroxylase activity than in lysates from MOCK ECs, especially when the assay was performed under normoxia. From the same experiment, it was also clear that hypoxia greatly suppressed PHD2 hydroxylase activity in OE#5 lysates. In agreement with reduced hydroxylase activity under hypoxia, HIF-1α protein levels in OE#5, OE#9, and OE#12 cultured under hypoxia were at 1.9, 3.1, and 4.6 folds of the corresponding values under normoxia (Figure 1D).

**Figure 2. Phd2 knock-down promoted hypoxia-induced EC proliferation.** A and B, Northern blots of ECs stably transfected with expression vectors for scrambled siRNA or Phd2 siRNA. A, normoxia; B, hypoxia, except for normoxia controls in lanes labeled as “C.” Phd2 probe detects 2 isoforms of endogenous Phd2 mRNA. C and D, ECs transfected with expression vectors for scramble () or Phd2 siRNA (■) were cultured under hypoxia in 0.5% serum and subjected to [3H]thymidine incorporation assays (C) and direct cell counting (D). n=4. Data were shown as the mean±SE. **P<0.01.
under normoxia (Figure 1F, left), under hypoxia, MOCK cultures expanded at ~1.6-fold of OE#5 cultures (Figure 1F, right). In both assays, PHD2 overexpression essentially prevented EC cultures from accelerating their proliferation in response to hypoxia but did not further reduce their proliferation to slower than normoxia cultures.

To correlate altered proliferation of OE#5 cells with PHD2 expression, we compared PHD2 levels under normoxia and hypoxia. A shift from normoxia to hypoxia increased endogenously expressed PHD2 in MOCK, consistent with a previous report describing hypoxia-induced increase in PHD2 expression, but PHD2 levels in OE#5 were much higher than in MOCK under both normoxia and hypoxia (Figure 1G). In Figure 1H, we also demonstrate that PHD2 overexpression had a relatively long-term effect (72 hours) on suppressing HIF-1α accumulation.

Silencing of the Endogenous Phd2 Gene Enhances Hypoxia-Induced EC Proliferation

Because the endogenous PHD2 is upregulated under hypoxia, we asked if it could negatively regulate EC proliferation under hypoxia. Therefore, we knocked down Phd2 gene expression in MS1 cell by Phd2 siRNA. Northern blotting revealed that Phd2 specific siRNA, but not a scrambled sequence, significantly reduced Phd2 mRNA level (Figure 2A) and inhibited hypoxia-induced upregulation of Phd2 expression (Figure 2B). Phd2 siRNA did not affect the expression of other genes, such as Phd1 (data not shown) or β-actin, suggesting that Phd2 siRNA worked specifically to inhibit PHD2 transcription.

Next, we investigated the effect of Phd2 silencing on hypoxia-induced EC growth. Both [3H]thymidine incorporation assays (Figure 2C) and direct cell counting (Figure 2D)
indicated that Phd2 knockdown allowed faster expansion of EC cultures. These results suggest that the endogenous PHD2 induced by hypoxia also negatively regulates hypoxia-induced EC proliferation.

Contribution of a Hydroxylase-Independent Mechanism to PHD2-Mediated Suppression of EC Proliferation

Because VEGF-A plays a crucial role in hypoxia-induced EC proliferation, we determined the effect of PHD2 on Vegfa gene expression. In MS1 cells, Vegfa mRNA expression was induced by hypoxia in a time-dependent manner and inversely correlated with oxygen concentration (Figure 3A and 3B). Although MOCK cells behaved essentially identically to MS1 cells, PHD2 overexpression inhibited Vegfa expression (Figure 3C). On the other hand, whereas Vegfa expression in PHD2-overexpressing cells was undetectable under normoxia, it was easily detectable after culturing the cells under hypoxia for ≥6 hours, albeit at lower levels than in MOCK cells (Figure 3C). These data suggested that the ability of PHD2 to suppress Vegfa expression was partially compromised under hypoxia.

We tested whether the addition of exogenous VEGF-A would overcome the negative effect of PHD2 on proliferation. When supplemental VEGF-A was added up to 10 ng/mL, no significant rescue of proliferation was achieved in cultures overexpressing PHD2 (Figure 3D). In a control experiment, VEGF-A significantly stimulated the growth of MS1 ECs in a dose-dependent manner (Figure 3E). These data suggested that a reduced level of endogenous VEGF-A expression was not mainly responsible for reduced EC proliferation. Consistent with this notion, MS1 cultures secreted negligible amounts of VEGF-A (analyzed by ELISA, data not shown); hence, their proliferation most likely depended on serum-derived VEGF-A rather than endogenous secretion. To see whether PHD2 inhibited EC proliferation by downregulating VEGF receptors, we also analyzed Vegfr-1 and Vegfr-2 mRNA levels by Q-PCR. Although Vegfr-1 mRNA levels were very low in both OE#5 or MOCK, Vegfr-2 mRNA was much more abundant (Figure 3F). However, Vegfr-2 levels were similar between OE#5 and MOCK under hypoxia.

Because hydroxylase-dependent alterations, such as inhibition of HIF-1 and Vegfa expression, did not consistently correlate with the negative effect of PHD2 overexpression on EC proliferation, we hypothesized that PHD2 inhibited EC proliferation by a hydroxylase-independent pathway. To test this hypothesis, we mutated His351, which is essential for Fe2+ binding and, hence, PHD2 hydroxylase activity, to Phe (H351F mutation). The approximate location of His351 is indicated in Figure 4A. MS1 ECs were transfected with the expression plasmid for mutant PHD2 (pPHD2mut), and 2 independent clones (Mut#3 and Mut#11) were isolated that overexpressed mutant PHD2 at comparable levels to wild-type PHD2 in OE#5 (Figure 4B). As expected, hydroxylase assay confirmed that the H351F mutant lacked hydroxylase activity (Figure 1C). Consistent with this finding, overexpression of mutant PHD2 did not reduce HIF-1α protein or Vegfa mRNA levels (Figure 4C and 4D).

We analyzed the effect of mutant PHD2 on EC proliferation by [3H]thymidine incorporation assays. EC proliferation...
was not affected under normoxia (Figure 4E, left), but cells overexpressing mutant PHD2 had significantly reduced incorporation of [3H]thyidine under hypoxia (Figure 4E, right). These data convincingly demonstrate that PHD2 hydroxylase activity is not essential for the suppression of hypoxia-induced EC proliferation.

**Discussion**

PHD2 is one of the key regulatory proteins in the HIF-1α degradation pathway.7–9 Under hypoxia, the expression of the Phd2 gene is upregulated, presumably to act as a feedback mechanism to quickly extinguish hypoxia response on reoxygenation.25 We hypothesized that even before reoxygenation, a feedback mechanism may be also necessary so that hypoxia responses do not escalate to disastrous levels.

Indeed, PHD2 overexpression suppressed EC proliferation under hypoxia but not under normoxia. Because PHD2 hydroxylase activity is known to be reduced under hypoxia,12 which is also confirmed in this work, its ability to selectively suppress EC proliferation under hypoxia suggests that hydroxylase activity may not be required for this particular function. This premise was strongly supported by our finding that a mutant PHD2 lacking hydroxylase activity also suppressed hypoxia-induced EC proliferation.

Although details for the hydroxylase-independent mechanism remain to be elucidated, one possibility is that PHD2 may work by forming complexes with hypoxia-induced molecules without hydroxylating the binding partner. For example, PHD2 binds to OS-9 or inhibitor of growth family member 4 to regulate HIF-1α stability or transcriptional activity.26,27 However, it should be emphasized that our findings do not dispute the importance of the PHD2/HIF-1α/VEGF-A pathway in regulating EC proliferation. More likely, both hydroxylase-dependent and -independent mechanisms are important, but one mechanism may be more readily manifested than the other depending on experimental conditions.

The possible negative feedback role for the endogenous PHD2 is suggested by siRNA-mediated knockdown of endogenous PHD2. The effects of reduced PHD2 expression nearly perfectly mirrored those of overexpression, resulting in enhanced EC proliferation under hypoxia. These observations demonstrate that the negative feedback role is not limited to PHD2 overexpression from a plasmid. Indeed, endogenous PHD2 seems to be involved in pathological processes. For example, treatment by Phd2 siRNA ameliorates myocardial damage caused by ischemic insult,30 presumably by increasing EC proliferation and angiogenesis in damaged cardiac muscles.

**Perspectives**

This study demonstrates that hypoxia-induced EC proliferation is under negative feedback control by PHD2 in a hydroxylase-independent manner. Such a function may be important for optimal adaptation of ECs to the hypoxic environment in normal physiology, as well as pathological processes. Although the mechanism underlying this function is not yet defined, we speculate that hydroxylase-independent interactions between PHD2 and other proteins involved in regulating HIF activity may be potentially important. Further investigations should focus on identifying proteins that interact with PHD2 and demonstrating the pathophysiological significance of PHD2-mediated negative regulation of EC proliferation.

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**Disclosures**

None.

**References**


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