Prolyl hydroxylase 2 deficiency limits proliferation of vascular smooth muscle cells by hypoxia-inducible factor-1α-dependent mechanisms

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Schultz K, Murthy V, Tatro JB, Beasley D. Prolyl hydroxylase 2 deficiency limits proliferation of vascular smooth muscle cells by hypoxia-inducible factor-1α-dependent mechanisms. Am J Physiol Lung Cell Mol Physiol 296: L921–L927, 2009. First published March 20, 2009; doi:10.1152/ajplung.90393.2008.—Arterial O2 levels are subject to complex regulation, details of which are still coming to light. A fundamental unanswered question in this connection is whether intracellular O2-sensing systems are positioned to modulate proliferative control in VSMC.

In VSMC, as in other cell types, reductions in O2 tension trigger a hypoxic transcriptional response program that is activated largely by hypoxia-inducible factors (HIFs). HIF-1 and HIF-2 are heterodimeric transcription factors, each consisting of a regulated α-subunit and a constitutively expressed β-subunit. Together, HIF-1 and HIF-2 orchestrate multiple responses that promote cellular adaptation to hypoxia, inducing expression of genes encoding angiogenic factors, glycolytic enzymes, erythropoietin, and inducible nitric oxide synthase (38). A link between HIF-1 and VSMC proliferation is supported by findings that growth factors and cytokines are potent inducers of HIF-1α expression in normoxic and moderately hypoxic VSMC (34) and that HIF-1α production in the context of stimulation by growth factors can promote proliferative responses in VSMC (9, 36).

A prominent control point in the regulation of HIF-1 activity is the O2-dependent hydroxylation of Pro402 and Pro564 within its α-subunit, in a region termed the O2-dependent degradation domain (11, 21). Prolyl hydroxylation at these sites permits binding of the recognition unit of an E3 ubiquitin ligase (DMOG) inhibited proliferation and cyclin A expression, as expected, but we also found that HIF-1α knockdown abolished the inhibitory effect of PHD2 knockdown on PDGF-induced cyclin A expression. Therefore, we conclude that PHD2 promotes growth factor-induced responses of human VSMC, acting by HIF-1α-dependent mechanisms. Given the role of PHD2 as an oxygen sensor in mammalian cells, these results raise the possibility that PHD2 links VSMC proliferation to O2 availability.

platelet-derived growth factor; fibroblast growth factor; cyclin A; oxygen

OXYGEN SUPPLY IS A KEY DETERMINANT of cellular metabolic activity, and appropriate cellular responses to hypoxia have critical roles in homeostasis and human disease. A key outcome of adaptive cellular responses to inadequate oxygen supply is vascular remodeling, including angiogenesis and arteriogenesis, which when insufficient can contribute to ischemic vascular diseases. In the pulmonary circulation, hypoxia can elicit detrimental vascular remodeling, characterized by thickening and muscularization of the small arteries, and reduced blood flow to the lung (15, 32). Vascular remodeling in this context results from inappropriate vascular smooth muscle cell (VSMC) proliferation, perhaps influenced by direct effects of reduced O2 levels on the cell cycle (7, 8, 36). However, the systems that have evolved to modulate cellular activities in accordance with O2 availability are subject to complex regulation, details of which is still coming to light. A fundamental unanswered question in this connection is whether intracellular O2-sensing systems are positioned to modulate proliferative control in VSMC.

In VSMC, as in other cell types, reductions in O2 tension trigger a hypoxic transcriptional response program that is activated largely by hypoxia-inducible factors (HIFs). HIF-1 and HIF-2 are heterodimeric transcription factors, each consisting of a regulated α-subunit and a constitutively expressed β-subunit. Together, HIF-1 and HIF-2 orchestrate multiple responses that promote cellular adaptation to hypoxia, inducing expression of genes encoding angiogenic factors, glycolytic enzymes, erythropoietin, and inducible nitric oxide synthase (38). A link between HIF-1 and VSMC proliferation is supported by findings that growth factors and cytokines are potent inducers of HIF-1α expression in normoxic and moderately hypoxic VSMC (34) and that HIF-1α produced in the context of stimulation by growth factors can promote proliferative responses in VSMC (9, 36).

A prominent control point in the regulation of HIF-1 activity is the O2-dependent hydroxylation of Pro402 and Pro564 within its α-subunit, in a region termed the O2-dependent degradation domain (11, 21). Prolyl hydroxylation at these sites permits binding of the recognition unit of an E3 ubiquitin ligase complex, known as von Hippel-Lindau tumor suppressor protein (pVHL), to HIF-1α, leading to rapid proteasomal degradation of HIF-1α. This posttranslational hydroxylation of carbon 4 of proline is catalyzed by O2-, 2-oxoglutarate (OG)-, and Fe2+-dependent dioxygenases, known as prolyl-4-hydroxylases. Prolyl hydroxylation is intrinsically O2-dependent because the hydroxyl group is derived from molecular O2. Prolyl hydroxylases are well-suited to serve as intracellular O2 sensors and mediators of O2-dependent functions in mammalian cells because their Km values for O2 appear to range between 67 and 250 μM (10, 16, 23), within the range of cellular O2 levels. The human genome encodes three potential HIF-1α prolyl-4-hydroxylases, designated prolyl hydroxylase domain-containing proteins 1, 2, and 3 (PHD1, PHD2, and PHD3), each of which is capable of hydroxylating two proline residues within a HIF-1α peptide substrate in vitro (5) and suppressing hypoxia response element (HRE)-dependent reporter gene activity when overexpressed in cell lines (17, 27). PHD2 regulates HIF-1α expression by this pathway in many transformed cell lines, but it is unknown whether it does so in human VSMC nor whether it could thereby modulate O2-dependent functions including cell proliferation.

Here, we used small interfering RNA (siRNA)-mediated gene silencing to test the hypothesis that PHD2 modulates HIF-1α levels, and hence growth factor-induced proliferation (36), in human VSMC. We found that PHD2 inhibition or
knockdown inhibits PDGF- and FGF-2-induced proliferation and expression of the cell cycle-regulatory protein cyclin A via a mechanism that involves modulation of HIF-1α activity. The results raise the possibility that HIF-1α-regulating PHD2 activity is required for normal VSMC proliferative responses.

METHODS

Human pulmonary artery SMC. SMC derived by enzymatic digestion of human pulmonary arteries (HPASMC; Clonetics) were cultured in SMGM-2 supplemented with 5% FCS, basic FGF (2 ng/ml), epidermal growth factor (0.5 ng/ml), insulin (5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 ng/ml) and used at passages 4-9. For each experimental protocol, HPASMC were serum-deprived and incubated in basal media (SMBM; Clonetics) that was supplemented with 1% FCS.

siRNA transfections. HPASMC were plated at subconfluent density (25,000 per square centimeter) and transfected the following day with PHD2-specific or nonspecific siRNAs complexed with Lipofectamine 2000 (Invitrogen). After 4 h, RNA-lipid complexes were removed, and SMC were incubated overnight in complete media. SMC were then trypsinized, replated, and serum-deprived for analysis of proliferation and protein expression, as described below. To verify specificity of the observed effects, experiments employed 2 distinct sets of nonspecific control and PHD2-specific siRNAs. One set consisted of 4 pooled nonspecific siRNAs (cat. no. M-004276; Dharmacon) and 4 pooled PHD2-specific siRNAs (cat. no. D-001206; Dharmacon), whereas the second set consisted of a single nonspecific (CU-UACGCUGAGUACUUGGAtdTdT) control and a single PHD2-specific siRNA (CAAGGUAAGUGGAGGUAUU). The total concentration of siRNA used was 50 nM.

Hypoxia. HPASMC were exposed to moderate (5% O2) hypoxia in a water-jacketed CO2 incubator that maintains a subambient O2 level by the regulated injection of N2 (Forma Scientific). Control cells were placed in a similar incubator maintained at the ambient room air O2 level (20%). In all studies, cells remained at the lower specified O2 level for the entire experimental protocol without any transient periods of reoxygenation that might affect VSMC proliferation. In each experiment, HPASMC were incubated in basal media with or without PDGF-AB (20 ng/ml; R&D Systems) or FGF-2 (4 ng/ml; Clonetics).

Each experiment included controls incubated 72–96 h in 20 or 5% O2 and between growth factor-induced analyte levels or proliferation in 5% O2 in HPASMC transfected with nonspecific or PHD2-specific siRNAs were analyzed by two-way analysis of variance with paired measurements followed by Bonferroni post hoc tests where indicated using Prism 4.0 software. Values of P < 0.05 were taken as significant.

RESULTS

HPASMC express PHD1 and PHD2 isoenzymes constitutively. We used Western blotting to analyze the expression of PHD isoenzymes in HPASMC. Both PHD1 (~45 kDa) and PHD2 (~48 kDa) were expressed constitutively in unstimulated normoxic HPASMC and were readily detected in whole cell extracts. Their levels did not appear to be affected by exposure for 6 or 48 h to either moderate hypoxia (5% O2) or severe hypoxia (1% O2) or by stimulation with PDGF or FGF-2 (Fig. 1). PD3 was not detectable in HPASMC by Western blot analysis (data not shown).

Inhibiting prolyl hydroxylase activity in HPASMC reduces FGF-2-induced proliferation and cyclin A expression. To determine whether proliferative responses of VSMC are modulated by O2-sensing prolyl hydroxylases, we first determined the effect of pharmacological blockade using DMOG, a compound that is taken up by cells and then converted to the 2-OG analog N-oxalylglycine, a competitive inhibitor of 2-OG-dependent dioxygenases. DMOG reduced the enhancement of FGF-2-induced proliferation seen in the presence of 5% O2.

![Fig. 1. Normoxic human pulmonary artery smooth muscle cells (HPASMC) express prolyl hydroxylase domain-containing proteins 1 (PHD1) and 2 (PHD2). Shown is a Western blot analysis of PHD1 and PHD2 in whole cell extracts of HPASMC incubated 6 h (top) or 48 h (bottom) with or without FGF-2 (4 ng/ml) or PDGF-AB (20 ng/ml) in the indicated ambient O2 concentrations. PHD1 and PHD2 levels were unaffected by hypoxia or growth factors (GF) (representative of 2 experiments).](http://ajplung.physiology.org/)
PHD2 ROLE IN VSMC PROLIFERATION

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Cell cycle progression is regulated by the formation of complexes between preexisting cyclin-dependent kinases and multiple cyclins, which undergo cyclical changes in protein abundance, exhibit distinct temporal expression patterns, and promote distinct stages of mitosis. In VSMC, growth factors strongly stimulate cyclin A gene transcription (14, 45). Thus we tested whether inhibiting prolyl hydroxylase activity influences growth factor-induced cyclin A expression and found that DMOG reduced FGF-2- and PDGF-AB-induced cyclin A expression in normoxic HPASMC (Fig. 2B). This finding suggests a potential role of PHD activity in the intracellular pathways that control proliferative responses of VSMC.

PHD2 knockdown inhibits HPASMC proliferative responses. Because DMOG is a nonselective inhibitor of 2-OG-dependent dioxygenases that inhibits multiple PHD isoenzymes, collagen prolyl hydroxylases, and asparagine hydroxylases, we developed a specific gene-targeting approach to test the roles of specific PHD isoenzymes. Transfection with PHD2-directed siRNA markedly reduced cellular PHD2 content in HPASMC. The effect was highly selective, as PHD1 content was unaffected, whereas nonspecific siRNA had no effects on PHD1 or PHD2 protein expression (Fig. 3A). These tests documented the efficacy and specificity of siRNA-mediated PHD2 knockdown, validating it as a tool for assessing the role of PHD2 in HPASMC proliferative responses.

Knockdown of PHD2 by RNA interference inhibited FGF-2- and PDGF-induced proliferation of HPASMC, under either normoxic or proliferation-enhancing moderately hypoxic conditions (Fig. 3B). Similar results were observed using a single-duplex PHD2-specific siRNA (data not shown), which likewise reproduced the effect seen with the PHD inhibitor DMOG (Fig. 2). Together, the results support a role of PHD2 in growth factor-stimulated proliferative responses.

PHD2 knockdown inhibits growth factor-induced cyclin A expression in HPASMC. To explore the mechanisms by which PHD2 knockdown may inhibit growth factor-induced proliferative responses, we tested its influence on the expression of cyclins A/A1 and D1/D2. Exposure of HPASMC to FGF-2 or PDGF-AB markedly increased the expression of cyclin A, and this induction was markedly reduced by PHD2 knockdown (Fig. 4A). Densitometric analysis of PHD2 and cyclin A Western blots confirmed that PHD2-directed siRNA produced efficacious knockdown of PHD2 expression and inhibited PDGF-induced cyclin A expression (Fig. 4B), in agreement with the inhibitory effect of DMOG on cyclin A expression. PHD2 knockdown had no effect on PDGF and FGF-induced expression of cyclin D1/D2 (Fig. 4A), cyclins for which levels increase during early G1 phase in VSMC and other cell types and are thought to be important for G1 progression (35, 39, 40).

To confirm the specificity of the observed knockdown of PHD2 expression and resulting inhibition of growth factor-induced cyclin A expression seen using pooled siRNAs, in separate experiments we tested the effects of PHD2 knockdown using an additional set of single PHD2-directed and nonspecific siRNAs. Both single and pooled PHD2 siRNAs caused similar and significant PHD2 knockdown without affecting β-actin or β-tubulin protein levels and virtually abolished PDGF-AB-induced cyclin A expression in HPASMC.

Fig. 2. Dimethyloxalylglycine (DMOG), a prolyl hydroxylase inhibitor, reduces FGF-induced proliferation (A) in HPASMC incubated in moderate hypoxia and reduces FGF-2- and PDGF-AB-induced cyclin A expression (B) in normoxic HPASMC. A: cell proliferation. SMC were plated (5,000 per well), serum-deprived, and then incubated 3 days in 20 or 5% O2 in basal media with or without FGF-2 (4 ng/ml) or PDGF-AB (20 ng/ml). Differences in cell counts between days 3 and 0 are shown (n = 6 experiments). *P < 0.05, **P < 0.01 vs. 20% O2 with corresponding growth factor and siRNA; +P < 0.05 vs. ns siRNA.

Fig. 3. PHD2 knockdown inhibits FGF- and PDGF-induced HPASMC proliferation and the costimulatory effect of hypoxia. SMC were transfected with nonspecific (ns) or PHD2-specific small interfering RNA (siRNA) as indicated. A: specificity of PHD2-directed siRNA. Marked reduction of PHD2, but not PHD1, occurred in HPASMC transfected with PHD2-specific siRNA, shown by Western analysis of whole cell extracts prepared 48 h after transfection. B: antiproliferative effects of PHD2 knockdown. Shown are the proliferative responses of transfected HPASMC after replating, serum deprivation, and incubation for 3 days in 20 or 5% O2 in basal media with or without FGF-2 (4 ng/ml) or PDGF-AB (20 ng/ml). Differences in cell counts between days 3 and 0 are shown (n = 6 experiments). *P < 0.05, **P < 0.01 vs. 20% O2 with corresponding growth factor and siRNA; +P < 0.05 vs. ns siRNA.
PHD2 knockdown selectively inhibits FGF- and PDGF-induced expression of cyclin A in normoxic HPASMC. A: HPASMC were transfected with pooled nonspecific or PHD2-specific siRNAs. After 48 h, cells were incubated with or without FGF-2 (4 ng/ml) or PDGF-AB (20 ng/ml) as indicated, and whole cell extracts were prepared after 24 h for Western blot analysis of PHD2 and cyclin D1/D2 (36 and 35 kDa, respectively) and cyclin A/A1 (60 and 50 kDa, respectively). B: densitometric analysis of PHD2 and cyclin A expression in HPASMC transfected with nontargeting and PHD2-targeting pooled siRNAs (n = 3 experiments). *P < 0.05 vs. ns siRNA.

DISCUSSION

These findings indicate that endogenous PHD2 promotes growth factor-induced proliferation in human VSMC. Reducing PHD2 activity, using either a nonselective competitive inhibitor or selective siRNA-mediated gene silencing, increased HIF-1α levels and reduced the proliferative responses of human VSMC to growth factors including PDGF and FGF-2. Together, the results suggest that during conditions of reduced PHD2 activity, VSMC proliferation is restricted by HIF-1α-dependent mechanisms. The results raise the possibility of previously unrecognized mechanisms through which O2 availability or metabolic factors might influence vascular remodeling.

The present finding that PHD2 may play a mechanistic role in determining the proliferative responses of vertebrate cells is novel, but a growth-promoting role of HIF-linked prolyl hydroxylases may be phylogenetically ancient. This idea is based on findings in *Drosophila*, which express a single O2-sensing PHD homolog known as HIF prolyl hydroxylase (Hph). Ecotropic expression of Hph was sufficient to promote cell growth in *Drosophila* larvae (12), wherein Hph appears to mediate the hypertrophic effects of cyclin D1 rather than its proliferative effects, as loss-of-function mutations in Hph in larval cells...
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Hph may function as an integrating link that can modulate cell
growth, but cell growth can proceed normally in the absence of
HIF-1\(\alpha\)/H9251
expression. It remains
controversial which of these agents act as endogenous PHD
inhibitors (24, 26), perhaps owing to cell type specificity
issues, but this general mechanism provides a potential basis
for metabolically linked regulation of HIF-1\(\alpha\) activity, and
thence VSMC proliferation as shown presently, via modulation
of PHD function.

suppressed the increased cell size seen in flies overexpressing
cyclin D/cdk4 (12). Loss-of-function mutations in Drosophila
Hph also cause delayed larval development and growth de-
fects, and these growth impairments were completely reversed
when combined with loss-of-function mutations of Sima, the
endogenous HIF-1\(\alpha\) homolog expressed by Drosophila (6).
Therefore, in Drosophila, Hph is required for normal cell
growth, but cell growth can proceed normally in the absence of
Hph when the HIF-1\(\alpha\) homolog is not expressed. Similarly,
here we found that endogenous PHD2 is required for normal
growth factor-induced cyclin A expression in HPASMC but
also found that growth factors can stimulate cyclin A expres-
sion in the absence of PHD2 provided that HIF-1\(\alpha\) expression
is inhibited. These findings thus raise the possibility of a
HIF-dependent role of O2-sensing prolyl hydroxylases in the
regulation of cyclins that is phylogenetically conserved and
expressed in HPASMC.

Because both O2 and the citric acid cycle intermediate 2-OG
are cosubstrates of Hph, it was suggested that in Drosophila,
Hph may function as an integrating link that can modulate cell
growth in accordance with both O2 availability and cellular
metabolic activity as indicated by intracellular 2-OG levels
(12). In human cells, other tricarboxylic acid (TCA) cycle-
derived metabolic intermediates can bind to the 2-OG binding
site of PHD2 and other PHDs, thereby inhibiting PHD2 activity,
stabilizing HIF-1\(\alpha\), and providing a mechanism for meta-
bolic regulation of its activity (26). Such intermediates include
the 2-oxoacids pyruvate and oxaloacetate (26), fumarate, and
also succinate, which is not only a TCA cycle intermediate, but
also a product of HIF-1\(\alpha\) hydroxylation and thus a potential
feedback inhibitor product of PHD2 activity, which, in turn,
stimulates HIF-1\(\alpha\) expression (20, 24, 31, 37). It remains
controversial which of these agents act as endogenous PHD
inhibitors (24, 26), perhaps owing to cell type specificity
issues, but this general mechanism provides a potential basis
for metabolically linked regulation of HIF-1\(\alpha\) activity, and
thence VSMC proliferation as shown presently, via modulation
of PHD function.

The HIF-1\(\alpha\)-dependent influence of PHD2 knockdown on
HPASMC proliferation suggests that endogenous HIF-1\(\alpha\)
produced in the context of PHD2 deficiency may suppress prolif-
erative responses by inhibiting PDGF-induced cyclin A expres-
sion. The effects of PHD2 knockdown were highly consistent,
inhibiting growth factor-induced proliferation and cyclin A
expression in HPASMC cell lines derived from four different
donors. PHD2 knockdown abolished PDGF-induced
cyclin A expression but did not affect \(\beta\)-actin levels,
indicating its specificity. Concurrent HIF-1\(\alpha\) knock-
down abolished the suppression of cyclin A seen with
PHD2 knockdown.

A J P - L u n g C e l l M o l P h y s i o l • V O L 2 9 6 • J U N E 2 0 0 9 • w w w . aj pl ung.o r g
strated in VSMC. Our previous study showed that 5% O₂ increased HIF-1α levels moderately, whereas 1% O₂ increased it further, consistent with generally accepted working models predicting that, in cells exposed to this ambient O₂ concentration range, PHD2 catalyzes the prolyl hydroxylation of HIF-1α, leading to VHL binding that targets HIF-1α for polyubiquitination and proteasomal degradation (1, 3, 21, 22).

Although available in vivo evidence indicates that PHD2 is the principal regulator of hydroxylation and thence the abundance of HIF-1α (30, 43, 44), in vitro evidence supports potential roles of PHD1 and PHD3 as well. These roles may be manifested in a cell type-, HIF isofrom-, O₂ level-, and stimulus-specific manner, based in part on their relative abundance (1). For example, PHD1 and PHD3 preferentially hydroxylate HIF-2α in vitro and preferentially modulate HIF-2α expression in vivo. Also, in mice, PHD1 deficiency increased HIF-2α, but not HIF-1α, levels in skeletal muscle (2), and combined deficiency of PHD1 and PHD2 led to accumulation of HIF-2α but not HIF-1α in the liver (42). Also, PHD2 deficiency in mouse liver and kidney is associated with increased accumulation of HIF-1α but not HIF-2α (43). Here, we found that HPASMC constitutively express PHD1, which, like PHD2, is inhibited by DMOG. Therefore, a question remaining for future research is whether PHD1 contributes to the regulation of HIF-1α or HIF-2α levels and proliferation in HPASMC.

In conclusion, we found that PHD2 participates in proliferative control in VSMC via mechanisms that are HIF-1α-dependent. As a goal for future research, elucidating the functional relationships between the PHD-HIF axis and vascular cell proliferation would help to illuminate whether restricted O₂ availability acts in concert with regulatory metabolic intermediates to modulate proliferation in such cells, thereby contributing to pathogenesis of vascular and pulmonary disorders. The present results provide conceptual support for a potential therapeutic benefit of small-molecule PHD inhibitors designed to limit excessive VSMC proliferation, which occurs in human diseases including pulmonary hypertension and atherosclerosis. This possibility is attractive because specific PHD inhibitors are currently being developed for the treatment of ischemic diseases, based on their potential to promote angiogenesis and tolerance for hypoxia (28, 29).

GRANTS

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REFERENCES


