Essential Role for Prolyl Hydroxylase Domain Protein 2 in Oxygen Homeostasis of the Adult Vascular System

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Background—Prolyl hydroxylase domain (PHD) proteins, including PHD1, PHD2, and PHD3, mediate oxygen-dependent degradation of hypoxia-inducible factor (HIF)-α subunits. Although angiogenic roles of hypoxia-inducible factors are well known, the roles of PHDs in the vascular system remain to be established.

Methods and Results—We evaluated angiogenic phenotypes in mice carrying targeted disruptions in genes encoding different PHD isoforms. Although Phdl−/− and Phd3−/− mice did not display apparent angiogenic defects, broad-spectrum conditional knockout of Phd2 led to hyperactive angiogenesis and angiectasia. Blood vessels in PHD2-deficient mice were highly perfusible. Furthermore, examination of medium-sized vessels in subendocardial layer in the heart demonstrated successful recruitment of vascular smooth muscle cells. Surprisingly, increased vascular growth was independent of local efficiency of Phd2 disruption. Mice carrying significant Phd2 disruption in multiple organs, including the liver, heart, kidney, and lung, displayed excessive vascular growth not only in these organs but also in the brain, where Phd2 disruption was very inefficient. More surprisingly, increased accumulation of hypoxia-inducible factor-1α and angiectasia in the liver were not accompanied by corresponding increases in hepatic expression of Vegfa or angiotropin-1. However, the serum vascular endothelial growth factor-A level was significantly increased in PHD2-deficient mice.

Conclusions—PHD2, but not PHD1 and PHD3, is a major negative regulator for vascular growth in adult mice. Increased angiogenesis in PHD2-deficient mice may be mediated by a novel systemic mechanism. (Circulation. 2007;116:774-781.)

Key Words: angiogenesis • hypoxia • cardiovascular system

Angiogenesis in pathophysiological processes such as embryonic development and tumorigenesis is triggered by tissue hypoxia3–4 mediated by hypoxia-inducible factors (HIFs).5 HIFs are heterodimeric transcription factors consisting of α and β subunits. The α subunits are rapidly degraded by the polyubiquitination/proteosomal degradation pathway in an oxygen-dependent manner, whereas the β subunit, which is a common heterodimerization partner for all α subunits, is stable.6,7 Rapid degradation of α subunits is initiated by HIF–prolyl hydroxylases (PHFs)/prolyl hydroxylase domain (PHD)–containing proteins,8–11 which are 2-oxoglutarate/iron-dependent dioxygenases and use molecular oxygen as a substrate to hydroxylate specific prolyl residues within oxygen-dependent degradation domains of α subunits.11,12 Hydroxylated HIF-α subunits are recognized by von Hippel-Lindau protein for polyubiquitination and proteosomal degradation.8–11

There are at least 3 PHD isoforms in mammals, including PHD1/EGLN2/HPH3, PHD2/EGLN1/HPH2, and PHD3/EGLN3/HPH1,11–14 each displaying similar catalytic properties in vitro.15 However, different PHDs have differential expression profiles15,16 and subcellular localization patterns.17

Thus, despite similar in vitro enzymatic properties, it is not surprising that different PHDs are not equally required in vivo.18

Targeted disruption of HIF-1α, HIF-2α, or HIF-β leads to defective angiogenesis in mouse embryos.3,19–22 Subcutaneous and muscular HIF-1α overexpression in mice promotes angiogenesis, resulting in the formation of healthy blood vessels.23–25 In addition, several PHD inhibitors promoted angiogenesis by stabilizing HIF–1α.26,27 However, specific roles of different PHD isoforms in adult angiogenesis remain unknown. Although PHD2 is a main isoform for the control of HIF levels under normoxia,28 partial hypoxia typically is present in normal tissues. Thus, possible roles for other PHD isoforms in angiogenesis also should be examined. Indeed, PHD1 was reported to have an ability to regulate angiogenesis in tumors.29
We previously reported the generation of mice carrying floxed Phd1, Phd2, and Phd3 alleles and demonstrated that germ-line disruption of Phd2, but not Phd1 or Phd3, resulted in developmental defects and embryonic lethality. However, significant vascular defects were not found before embryonic death. We have now used tamoxifen-inducible Cre (CreERT2) under the control of the ubiquitously expressed Rosa26 locus to inactivate the Phd2 gene in adult mice and found significant vascular and blood phenotypes. Here, we focus on the characterization of vascular phenotypes and demonstrate that PHD2, but not PHD1 or PHD3, is the essential isoform to regulate angiogenesis in adult mice.

Methods

Mice

All animal procedures were approved by the Animal Care Committee at the University of Connecticut Health Center in compliance with Animal Welfare Assurance. Phd1<sup>−/−</sup>, Phd3<sup>−/−</sup>, and Phd2<sup>−/−</sup> mice were generated previously (f denotes floxed allele). To delete floxed Phd2 exon 2, tamoxifen (Sigma, St. Louis, Mo) was fed to 6-week-old Phd2<sup>−/−</sup>Rosa26<sup>creERT2</sup> mice by forced gavages (20 mg/mL in corn oil, 1 mg/d for 7 consecutive days). Rosa26<sup>creERT2</sup> mice used to establish Phd2<sup>−/−</sup>Rosa26<sup>creERT2</sup> colony were provided by A.L. Joyner (S.H. Cheng and A.L. Joyner, manuscript in preparation). Three days after the last dose, ear or tail DNA samples were prepared and used for polymerase chain reaction (PCR) to monitor the deletion of Phd2 exon 2 and the presence of the Rosa26 CreERT2 allele. Primers for these and all other PCR reactions are listed in Table I of the online Data Supplement. Genotypes at the Phd1 and Phd3 loci were determined as described before.

Northern Blotting, Reverse-Transcription PCR, and Quantitative Real-Time Reverse-Transcription PCR Analyses

Total RNA samples were used for Northern blotting with Vegfa or Vegfr-2 cDNA probes or reverse transcribed (RT) for RT-PCR and quantitative real-time RT-PCR (QPCR) analyses. QPCR was specific for wild-type Phd2 mRNA because one of the primers was derived from the exon 2 sequence, which was deleted in the mutant. QPCR was performed with the SYBR green PCR master mix (Applied Biosciences, Foster City, Calif) and the ABI PRISM 7900HT Sequence Detection System (Applied Biosciences). Phd2 mRNA abundance was determined by the counting blood vessels intersecting a line drawn along the midline of each cartilage ring, and branching was quantified by counting the number of branching points per 0.1-mm<sup>2</sup> area overlying cartilage rings.

Statistical Analyses

Statistical analyses were performed by Student t test. Data are shown as mean±SEM. Values of P<0.05 were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Phd2 Expression in Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> Mice Is Efficiently Reduced by Tamoxifen Treatment

To delineate the role of PHD2 at the adult stage, we generated Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> mice. The administration of tamoxifen at ~6 weeks of age activated Cre recombinase and resulted in conditional knockout (CKO) mice as a result of the excision of the floxed exon 2 (Figure 1A and 1B). We also performed RT-PCR for tail RNA from Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> or Phd2<sup>−/−</sup> mice before and after tamoxifen treatment using primers flanking the exon 2—encoded sequence (Figure 1C, arrows a and b). Three days after the last dose of tamoxifen, wild-type Phd2 mRNA (387-bp band) was almost completely converted to exon 2—deleted mRNA (267-bp band) in Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> mice (Figure 1D, left). These findings were further confirmed by anti-PHD2 Western blot of total lysates from tail specimens, which demonstrated that the abundance of PHD2 protein was reduced only in tamoxifen-treated Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> mice (Figure 1D, right).

Next, we performed QPCR to determine deletion efficiencies (defined as percent reduction in Phd2 mRNA abundance relative to Phd2<sup>−/−</sup> controls) in various organs from tamoxifen-treated Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> mice (Figure 1C, arrows c and d, the latter of which anneals only to exon 2). As shown in Figure 1E, the highest deletion efficiency was seen in the liver, followed by heart, lung, and kidney, whereas deletion was inefficient in the brain. These findings were confirmed by anti-PHD2 Western blotting (Figure 1F). Overall, our data establish that tamoxifen-treated Phd2<sup>−/−</sup>/Rosa26<sup>creERT2</sup> mice were Phd2 CKO mice.
Hyperactive Angiogenesis in Adult Phd2 CKO Mice

At \( \approx 6 \) weeks after tamoxifen treatment, major vascular branches in the ear of Phd2 CKO mice became significantly dilated and reddened (Figure 2A, top right); however, there were no signs of edema, inflammation, or ulceration. In contrast, tamoxifen-treated Phd2f/f mice were not affected (Figure 2A, top left), confirming that this phenotype was related to PHD2 deficiency but was not due to nonspecific pharmacological effects of tamoxifen. The development of dilated blood vessels took a relatively long time course. At 2 weeks after treatment, ear vasculature in Phd2 CKO mice (Figure 2A, bottom right) was rather similar to that in Phd2f/f mice (Figure 2A, bottom left).

In hematoxylin and eosin (H&E)–stained ear sections (Figure 2B and 2C), an increased number of blood vessels was observed in the dermal and subcutaneous layers in Phd2 CKO mice (Figure 2C, arrows). To more clearly identify blood vessels and to investigate whether the extra blood vessels formed as a result of PHD2 deficiency were perfusable, we labeled the vasculature by injecting biotinylated Lycopersicon esculentum (tomato) lectin into the circulation. As shown in Figure 2D and 2E, vascular density in Phd2 CKO ears was obviously higher than that in Phd2f/f controls. Similarly, tracheal vasculature from Phd2 CKO mice was more densely packed with capillaries, which were more branched and enlarged compared with those in Phd2f/f controls (Figure 2F and 2G). A quantitative analysis confirmed that trachea in Phd2 CKO mice had statistically significant increases in capillary density and vessel branching (capillary density: Phd2 CKO, 14.5 ± 0.6/mm; Phd2f/f, 11.6 ± 0.6/mm; \( P < 0.01 \); vessel branching: Phd2 CKO, 25.8 ± 2.4/0.1 mm²; Phd2f/f, 18.1 ± 1.1/0.1 mm²; \( P < 0.05 \); \( n = 12 \)). These findings not only confirmed increased angiogenesis and angiectasia in Phd2 CKO mice but, more importantly, demonstrated that the resulting blood vessels had functional perfusion properties.

Increased Angiogenesis and Angiectasia in Internal Organs

To examine whether PHD2 deficiency also led to increased angiogenesis in internal organs, we examined histological sections from the heart, lung, kidney, and liver. As revealed by anti–PECAM-1 IHC staining, assemblies of medium-sized blood vessels were scattered in the subendocardial layer of Phd2 CKO mice (Figure 3B, arrow, and 3D), whereas such blood vessels were rarely seen in control mice (Figure 3A and 3C). To assess the recruitment of vascular smooth muscle cells (VSMCs), heart sections were double labeled with...
isolectin and anti–α-smooth muscle actin to visualize both endothelial cells and VSMCs (Figure 3E through 3H). Enlarged blood vessels covered with VSMCs were readily found in the subendocardial layer in \( \text{Phd2} \) CKO specimens (Figure 3F, arrowheads) but were rarely seen in controls (Figure 3E). Higher-magnification images further confirmed these findings and revealed that capillaries amid cardiac fibers were noticeably enlarged in \( \text{Phd2} \) CKO hearts (Figure 3G and 3H).

Taken together, \( \text{Phd2} \) CKO in the heart promoted not only vessel growth but also maturation by VSMC recruitment. The size and number of blood vessels also were increased in the lung of \( \text{Phd2} \) CKO mice (Figure 4A and 4B). Lung angiectasia was accompanied by packed erythrocytes in dilated blood vessels, which could be seen more clearly under higher magnification (Figure 4C and 4D). As in the lung or heart, angiectasis is apparent in the kidney cortex of \( \text{Phd2} \) CKO mice (Figure 4F, asterisk). In addition, glomeruli in the CKO mice were significantly enlarged (glomerulomegaly) (Figure 4E and 4F, arrow indicates glomeruli), and their capillaries were dilated relative to normal controls (Figure 4G and 4H).

In H&E-stained liver sections of \( \text{Phd2} \) CKO mice, an apparent increase in large vessels (depleted of blood contents by perfusion) was observed under low magnification (Figure 5A and 5B). In individual lobules, the central vein and hepatic sinusoids were enlarged (Figure 5C and 5D). To visualize hepatic vasculature more clearly, liver sections were processed for anti–PECAM-1 IHC staining. The central veins in \( \text{Phd2} \) CKO mice typically were \( \approx 4 \) times larger than their counterparts in \( \text{Phd2}^{+/+} \) mice (Figure 5E and 5F). Furthermore, sinusoids also were dilated in \( \text{Phd2} \) CKO mice (Figure 5E and 5F). We also observed increased angiogenesis in the brain from \( \text{Phd2} \) CKO mice (Figure 5G and 5H). Because the \( \text{Phd2} \) disruption was inefficient in the brain (Figure 1E), increased angiogenesis might be mediated by systemic effects such as circulatory angiogenic factors rather than locally produced factors. Taken together, these data demonstrate that...
Phd2 CKO mice exhibited hyperactive angiogenesis and angiectasia in multiple tissues.

Molecular Analysis of Phd2 CKO Mice

Because PHD2 regulates the stability of HIF-α subunits, we isolated nuclear extracts from the liver as a representative organ and performed anti–HIF-1α Western blotting to determine whether HIF-1α protein was upregulated in Phd2 CKO mice. As shown in Figure 6A, the HIF-1α level was increased in Phd2 CKO mice compared with Phd2f/f mice. Because the Hif-1α mRNA level was similar between both mice (data not shown), HIF-1α upregulation more likely reflected its increased stability. On the other hand, HIF-2α in both Phd2f/f and Phd2 CKO livers was below the detectable level in our hands, even though the same Western blots easily identified HIF-2α in similar extracts from von Hippel-Lindau protein–deficient mice. In addition, HIF-β was easily detectable in the same extracts from Phd2 CKO livers, and its expression level was unaffected by PHD2 deficiency.

To identify angiogenic factors that might mediate increased angiogenesis and angiectasia in Phd2 CKO mice, we determined the level of serum vascular endothelial growth factor (VEGF)-A. As expected, serum VEGF-A was significantly upregulated in Phd2 CKO mice (Figure 6B). Surprisingly, Northern blotting for various organs, including liver, kidney, heart, and lung, showed no statistically significant upregulation of Vegfa mRNA, although slightly upward trends were observed (Figure 6C and data not shown). We also determined the Vegfr-2 expression after 1 week of tamoxifen treatment but found no significant upregulation in Phd2 CKO mice (Figure 6D). Because overexpression of the stabilized form of HIF-1α in skin tissues or skeletal muscles promoted angiogenesis and VEGF-A upregulation, we also determined Vegfa expression in these tissues of PHD2-deficient mice. Again, however, no significant upregulation was observed (Figure 6E). Similarly, despite reported roles of angiopoietin (Ang)-1 and Ang2 in angiogenesis, no statistically significant upregulation was detected in the liver, kidney, heart, and lung from Phd2 CKO mice (Figure 6F).
No Significant Vascular Phenotypes in Phd1<sup>-/-</sup> and Phd3<sup>-/-</sup> Mice

We investigated Phd1<sup>-/-</sup> and Phd3<sup>-/-</sup> mice to see whether there were any nonlethal abnormalities in angiogenesis. In contrast to Phd2 CKO mice, these mice did not display reddish ears at any age examined; one such example at 10 to 12 weeks of age is shown in Figure 7A through 7C. In the trachea, labeling with biotinylated Lycopersicon esculentum lectin did not reveal noticeable differences among wild-type, Phd1<sup>-/-</sup>, and Phd3<sup>-/-</sup> mice (Figure 7D through 7F). Histological analyses for internal organs were performed to determine whether any detectable changes existed in Phd1<sup>-/-</sup> and Phd3<sup>-/-</sup> mice. In the liver, the vessel sizes of central veins or hepatic sinusoids from Phd1<sup>-/-</sup> and Phd3<sup>-/-</sup> mice were equivalent to those in wild-type livers (Figure 7G through 7I). In the lung, the vasculature or structure of bronchus appeared normal, and no hemorrhage was observed (Figure 7J through 7L). Similarly, no increased angiogenesis was seen in the ventricular walls of Phd1<sup>-/-</sup> or Phd3<sup>-/-</sup> mice (Figure 7M through 7O). Consistent with these findings, serum VEGF-A levels were not increased in Phd1<sup>-/-</sup> or Phd3<sup>-/-</sup> mice (wild-type, 38.3 ± 3.6 pg/mL; Phd1<sup>-/-</sup>, 40.5 ± 5.8 pg/mL; Phd3<sup>-/-</sup>, 32.3 ± 3.3 pg/mL; n = 10 to 12; P > 0.05 for all pairs of t tests).

Discussion

Animals adapt to chronic hypoxia by activating mechanisms that increase the efficiency of oxygen delivery and reduce the consumption of oxygen, including angiogenesis, erythropoiesis, and increased glycolysis.6 2 These responses are thought to originate from the suppression of oxygen-dependent PHD hydroxylase activities, which leads to the accumulation of HIF-α subunits and activation of HIF-target genes such as Vegfa and erythropoietin.

Although it may be reasonable to expect PHD deficiency to have consequences similar to hypoxia, the existence of at least 3 PHD isoforms presents uncertainty with regard to the importance of individual PHDs. By individually disrupting genes encoding PHD1, PHD2, and PHD3, we previously demonstrated that PHD2 was a major essential isoform during embryogenesis. Here, we present evidence that deficiency of PHD2, but not PHD1 or PHD3, leads to hyperactive angiogenesis and angiectasia in multiple organs in adult mice.
Blood vessels in PHD2-deficient mice were perfusable and recruited VSMCs. These findings were consistent with observations in transgenic mice overexpressing stabilized HIF-1α or HIF-2α under the control of the K14 promoter or carrying conditional inactivation of the Vhl gene in dermal tissues. In contrast, blood vessels induced by VEGF-A overexpression alone are immature (poor VSMC recruitment) and very leaky. Because HIF-α, PHD2, and von Hippel-Lindau protein all function upstream of VEGF-A and control the expression of multiple targets, it is plausible that manipulations leading to the overexpression of HIF-1α under systemic hypoxia (ischemia). Known modes of adaptation to systemic hypoxia include an increase in ventilation and the oxygen supply. More closely mimic physiological hypoxia and stimulate the formation of essentially naked and leaky blood vessels. Such a discrepancy may reflect differential responses between normal and malignant tissues to the activation of the hypoxia signaling activities. Understanding the cellular and molecular bases for such differential responses to the activation of the hypoxia pathway will be of significant interest.

At present, hypoxia-induced angiogenesis is typically thought to be a local adaptive response when increased expression of angiogenic factors such as VEGF-A act by a paracrine mechanism to stimulate local angiogenesis. Surprisingly, increased angiogenesis in the brain of Phd2 CKO mice did not depend on local Phd2 disruption. In our hands, tamoxifen failed to efficiently induce Phd2 disruption in the brain, but hyperactive angiogenesis occurred in the brain in a manner similar to that in other organs in which Phd2 was efficiently disrupted. The existence of such a phenomenon suggests that angiogenic stimuli were somehow delivered from other sources to the brain to promote angiogenesis. In agreement with this possibility, increased serum levels of VEGF-A, rather than a tissue-specific increase in VEGF-A, were associated with hyperactive angiogenesis. Taken together, our findings indicate that a novel systemic mechanism may exist to mediate hypoxia-induced angiogenesis. Such a mechanism may complement the paracrine mechanism of hypoxia-induced angiogenesis and might be mildly operational when animals are exposed to chronic hypoxia such as in geographically high-altitude areas, in which case moderately increased angiogenesis may ensure sufficient blood supply.

It is not immediately clear why Phd2 CKO mice did not display localized Vegfa mRNA upregulation, especially because such upregulation was observed in Vhl CKO mice or HIF-1α transgenic mice. Nonetheless, several possibilities may be considered. For example, a noticeable difference between Phd2 CKO and Vhl CKO mice is systemic versus localized induction of HIF-1α expression. In general, mammals respond in different ways to systemic hypoxia or local hypoxia (ischemia). Known modes of adaptation to systemic hypoxia include an increase in ventilation and the oxygen transport capacity by increased erythropoesis. Phd2 CKO mice were generated by nearly global (systemic) disruption of the Phd2 gene, whereas Vhl CKO and HIF-1α overexpression was restricted to specific tissues. Future studies will address whether specific Phd2 disruption in different tissues will result in a tissue-specific increase in Vegfa expression. Another possibility is potential compensation by other PHDs. Although Phd1+/− and Phd3+/− mice did not show any apparent phenotype, we cannot exclude possible compensation by PHD1 or PHD3.

Overall, our data are consistent with a role of PHD2 in regulating angiogenesis via the hypoxia pathway. On the other hand, we have not ruled out potential contributions of hydroxylase-independent mechanisms, and a causative role for increased HIF-1α accumulation to hyperactive angiogenesis remains to be confirmed by future studies. Although increased HIF-α abundance may conceivably raise total capacity of HIF-mediated transcription, it is unlikely to alter the proportion of active HIF because HIF-1α hydroxylation by factor inhibiting HIF, a negative regulator for HIF transcription activity, is expected to remain intact in Phd2 CKO mice. Furthermore, transcription-independent events such as modified HIF-1α interaction with other regulatory molecules like p53 are also among potential mechanisms that are not excluded by the present study.

Our work complements earlier studies that showed protective effects of siRNA-mediated Phd2 knockdown in a cardiac ischemia/reperfusion injury model or PHD inhibitors on brain ischemia induced by middle cerebral artery occlusion. Because of the short-term nature (≈24 hours) of these studies, it was not possible to address whether increased angiogenesis resulting from PHD2 deficiency would offer further protection against chronic ischemic insults. Our finding that PHD2 deficiency promotes the formation of mature and perfusable blood vessels raises the possibility that PHD2 may be a candidate therapeutic target for longer-term protective effects.

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Disclosures
None.

References


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