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Integrity of the prolyl hydroxylase domain protein 2:erythropoietin pathway in aging mice

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ABSTRACT

The central transcriptional response to hypoxia is mediated by the prolyl hydroxylase domain protein (PHD):hypoxia inducible factor (HIF) pathway. In this pathway, PHD prolyl hydroxylates and thereby negatively regulates the α-subunit of the transcription factor HIF (HIF-α). An important HIF target gene is that for erythropoietin (EPO), which controls red cell mass. Recent studies have identified PHD2 as the critical PHD isoform regulating the *EPO* gene. Other studies have shown that the inducibility of the HIF pathway diminishes as a function of age. Thus, an important question is whether the PHD2:EPO pathway is altered in the aging. Here, we employed a mouse line with a globally-inducible *Phd2* conditional knockout allele to examine the integrity of the Phd2:Epo axis in young (six to eight months old) and aging (sixteen to twenty months old) mice. We find that acute global deletion of *Phd2* results in a robust erythrocytosis in both young and aging mice, with both age groups showing marked extramedullary hematopoiesis in the spleen. *Epo* mRNA is dramatically upregulated in the kidney, but not in the liver, in both age groups. Conversely, other *Hif* targets, including *Vegf*, *Pgk1*, and *Phd3* are upregulated in the liver but not in the kidney in both age groups. These findings have implications for targeting this pathway in the aging.

Introduction

The HIF pathway plays a central role in coordinating cellular, local, and systemic responses to hypoxia [1,2]. In this pathway, HIF-α (of which there are two main isoforms) is site-specifically prolyl hydroxylated by a family of three PHDs (also known as HIF prolyl hydroxylases and egg laying defective nine proteins) [3]. The three PHD isoforms are PHD1, PHD2, and PHD3. This modification provides a recognition motif for the von Hippel Lindau tumor suppressor protein (VHL), a component of an E3 ubiquitin ligase complex that specifically targets hydroxylated HIF for degradation by the ubiquitin–proteasome pathway [4]. Under normoxic conditions, this modification occurs constitutively and hence, HIF-α is maintained at very low steady state levels. This modification is inherently oxygen dependent [5] and is inhibited by hypoxia-generated reactive oxygen species [6–8]. Therefore, under hypoxic conditions the modification is inhibited, allowing the stabilization of HIF-α. HIF-α then heterodimerizes with a distinct protein, HIF-β, and binds to hypoxia response elements (HREs) in the promoters and enhancers of HIF target genes, allowing the transactivation of these genes.

The prototypical HIF target gene is that encoding for EPO [9–12]. EPO is a glycoprotein that is the central growth factor controlling red cell mass. In situations such as anemia, *EPO* gene transcription is induced, leading to increased circulating levels of EPO. EPO then binds to the EPO receptor on red cell progenitors in the bone marrow, initiating signaling through the JAK:STAT pathway, and leading to expansion of red cell mass. The expanded red cell mass increases delivery of oxygen to the tissues of the body, and this in turn downregulates HIF and hence *EPO* gene transcription. This negative feedback loop thus ensures that HIF maintains EPO at a level that is appropriate for a given tissue oxygenation. *EPO* gene transcription is also regulated in a developmental and tissue specific manner. In the mammalian fetus, the liver is the predominant source of EPO [13]; postpartum, the site of EPO production shifts from the liver to the kidney.

The multiplicity of PHD isoforms raises the question of which, if any, are involved in EPO regulation. Studies of patients with idiopathic erythrocytosis, an uncommon condition characterized by elevated red cell mass, have revealed that a subset of these patients harbor heterozygous mutations in the *PHD2* gene [14–17]. These mutations comprise missense, nonsense, and frameshift mutations. PHD2 consists of an N-terminal non-catalytic domain, and a C-terminal catalytic domain that is homologous to a larger family of 2-oxoglutarate dependent prolyl hydroxylases that include the collagen 4-prolyl hydroxylases [3]. All reported missense mutations studied to date affect the catalytic domain and severely impair enzymatic activity [16–18]. The frameshift mutations occur within the N-terminal domain and thereby delete the catalytic domain, and the single nonsense mutation reported is predicted to delete the C-terminal 50 amino acids [14].

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Studies of genetically engineered mice have demonstrated a critical role for Phd2 in red blood cell control. Conventional knockout of *Phd2* leads to embryonic lethality between day E12.5 to E14.5 due to placental and cardiac defects [19], necessitating a conditional knockout (CKO) approach to studying its role in adult red cell control. Employing a global, tamoxifen-inducible system, we and others have found that acute deletion of *Phd2* in the mouse results in marked erythrocytosis [20,21]. In contrast, neither *Phd1*−/− nor *Phd3*−/− mice display erythrocytosis, but interestingly, *Phd1*−/−; *Phd3*−/− double knockout mice exhibit a mild erythrocytosis [20]. Collectively, the human and mouse studies point to PHD2 as being the central PHD isoform regulating red cell mass.

These and other findings have stimulated great interest in targeting this pathway for therapeutic benefit [22]. Based on available evidence, inhibition of PHD2 would be predicted to increase EPO and thereby stimulate erythropoiesis, which could be of benefit in situations such as anemia. However, an important caveat to the mouse studies mentioned above is that they were performed on young mice (two to three months of age). Therapeutic targeting of this pathway, in contrast, would likely have its greatest impact in the aging population, which is the population that is preferentially affected by anemia due to causes that include end stage renal disease and chemotherapy. It should also be noted that a substantial segment of the aging population has anemia of unknown cause [23,24]. Anemia in the aging is associated with increased mortality risk, with the degree of risk correlated with the degree of anemia [25].

This issue is all the more relevant because there is substantial evidence that there are aging-associated changes in the HIF pathway. Thus, there is impaired hypoxia-induced HRE-binding activity in senescent mice [26], a defect in hypoxia-induced HIF-1α activation in aging aortic smooth muscle cells from rabbits [27], decreased hypoxia-induced activation of HIF-1α in rat carotid bodies as a function of age [28], decreased hypoxia-induced HRE-binding activity in aging rat lungs [29], impaired induction of HIF-1α protein by ischemia in hindlimb skeletal muscle of aging mice [30], decreased hypoxia-induced HIF-1α expression in the aging mouse brain [31], and diminished ischemia-induced HIF-1α activation in tissue flaps of aging mice [32]. Collectively, these studies suggest an impairment of the HIF activation pathway as a function of aging.

This then raises the important question of whether there is a fundamental defect in the HIF pathway in aging mammals. The PHD2:EPO pathway, with its high level of inducibility and its ability to be assessed quantitatively by indices that include hematocrit (Hct) and hemoglobin (Hb), provides an ideal and clinically relevant system for examining this. To pursue this, we employed a genetic model in which *Phd2* can be temporally knocked out in order to examine young and aging mice. Contrary to what might have been expected based on previously published reports on aging and the HIF pathway, we find that loss of *Phd2* in both age groups induces dramatic induction of erythrocytosis. The kidney continues to be the source of Epo in these aging mice. Furthermore, other *Hif* target genes are inducibly upregulated in the liver in both age groups. These findings indicate that the PHD2:EPO pathway is robustly inducible in aging mammals.

Materials and methods

Mice

The generation of mice in which exon 2 of the *Phd2* gene has been flanked by loxP sites (floxed) has been described previously [19]. These mice were maintained in a mixed CD1/129/BL6 background. Mice expressing either a constitutively active Cre recombinase or a tamoxifen-inducible CreER^{T2} fusion protein from the Rosa26 locus were obtained from Taconic [the strain designations are C57BL/6-*Gt(ROSA)26Sor*$^{tm16(cre)Arte}$ (which we hereafter refer to as *Rosa26*-Cre) and C57BL/6-*Gt(ROSA)26Sor*$^{tm9(cre/Esr1)Arte}$ (which we hereafter refer to as *Rosa26*-CreER^{T2}), respectively]. *Phd2*+/− mice were generated by crossing *Phd2* f/f (where f = flox) mice with the constitutive active *Rosa26*-Cre deleter mouse. *Phd2* f/−; *Rosa26*-CreER^{T2} and *Phd2* f/+ mice were generated by crossing *Phd2* f/f mice with *Phd2*+/−; *Rosa26*-CreER^{T2} mice. For genotyping or determination of *Phd2* exon 2 deletion efficiency, DNA was isolated from tails or various organs using a DNeasy tissue kit (Qiagen). For genotyping the *Phd2* allele, we employed the following three primers: PHD2rec55: 5′-AGG GCT TCT GGC ATT AGT TGA CC-3′; PHD2mouseR: 5-TCA ACT CGA GCT GGA AAC C-3′; and Pint1-4 5′: 5′-ATG AAT CAG AGT TCC CCG TG-3′. The wild type *Phd2* allele produces a 1.09 kb band with the PHD2rec55 and PHD2mouseR primers, the floxed *Phd2* allele produces a 0.95 kb band with the Pint1-4 5′ and PHD2mouseR primers, and the knockout *Phd2* allele produces a 0.8 kb band with the PHD2rec55 and PHD2mouseR primers. For genotyping the Cre allele, we employed the following three primers: Rosa26-1: 5′-TGG AGG CAG GAA GCA CTT GCT CTC-3′; Rosa26-2: 5′-CAT ACT GTA GTA AGG ATC TCA AGC-3′; and Cre-1(R): 5′-GCA TGT TTA GCT GGC CCA AAT G-3′. The wild type *Rosa26* locus produces a 0.55 kb band with the Rosa26-1 and Rosa26-2 primers. The *Rosa26* locus containing either the Cre or CreER^{T2} transgene produces a 0.69 kb band with the Rosa26-1 and Cre-1(R) primers. All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania in compliance with Animal Welfare Assurance.

Tamoxifen administration

A 10 mg/ml stock solution of tamoxifen free base (MP Biomedicals) was prepared in corn oil with shaking at 37 °C, and then aliquoted and stored at −20 °C. Tamoxifen was administered by oral gavage for five consecutive days (2 mg doses for young mice; 1 mg doses for aging mice).

Hematologic analysis

Peripheral blood samples were obtained from the retroorbital cavity and collected in Microvette 100 lithium heparin tubes (Sarstedt). Hematocrit was measured using a Critspin hematocrit reader (Iris). Hemoglobin measurements and complete blood counts were determined using a Hemavet FS950 hematology analyzer (Drew Scientific).

Epo assay

Plasma was obtained by centrifuging the retroorbital blood samples at 20 min at 2000 ×*g* for 20 min at room temperature. Plasma Epo levels were then determined using a rodent Quantikine Epo Immunoassay kit (MEP00, R&D Systems). Enzyme linked immunoabsorbent assay (ELISA) readings were made on a Tecan Sunrise microplate reader.

Erythroid burst forming unit (BFU-E) assay

Cells were obtained from either bone marrow or spleen, and red blood cells were lysed in a solution containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA for 5 min at room temperature. The

Fig. 1. Analysis of peripheral blood in young and aging mice. (A, E) Hematocrit (Hct), (B, F) hemoglobin (Hb), (C, G) white blood cell count (WBC), and (D, H) platelets were measured. For all panels, $n = 6$–8. For (A–D), genotypes are as follows. f/+ = *Phd2* f/+; f/− = *Phd2* f/−; *Rosa26*-CreER^{T2}. * indicates $p<0.05$ and ** indicates $p<0.01$ in comparing f/+ and f/− groups at a given age. For (E–H), genotypes are as follows. Controls (Con): *Phd2* f/+; CKO: *Phd2* f/−; *Rosa26*-CreER^{T2}. Control and *Phd2* CKO mice were administered tamoxifen for five consecutive days. Four weeks after the initial tamoxifen dose, peripheral blood was collected. * indicates $p<0.05$ and ** indicates $p<0.01$ in comparing control and CKO groups at a given age.