Multiple Factors Affecting Cellular Redox Status and Energy Metabolism Modulate Hypoxia-Inducible Factor Prolyl Hydroxylase Activity In Vivo and In Vitro

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Received 6 July 2006/Returned for modification 4 August 2006/Accepted 2 November 2006

Prolyl hydroxylase of hypoxial-inducible factor alpha (HIF-α) proteins is essential for their recognition by pVHL containing ubiquitin ligase complexes and subsequent degradation in oxygen (O2)-replete cells. Therefore, HIF prolyl hydroxylase (PHD) enzymatic activity is critical for the regulation of cellular responses to O2 deprivation (hypoxia). Using a fusion protein containing the human HIF-1α O2-dependent degradation domain (ODD), we monitored PHD activity both in vivo and in cell-free systems. This novel assay allows the simultaneous detection of both hydroxylated and nonhydroxylated PHD substrates in cells and during in vitro reactions. Importantly, the ODD fusion protein is regulated with kinetics identical to endogenous HIF-1α during cellular hypoxia and reoxygenation. Using in vitro assays, we demonstrated that the levels of iron (Fe), ascorbate, and various tricarboxylic acid (TCA) cycle intermediates affect PHD activity. The intracellular levels of these factors also modulate PHD function and HIF-1α accumulation in vivo. Furthermore, cells treated with mitochondrial inhibitors, such as rotenone and myxothiazol, provided direct evidence that PHDs remain active in hypoxic cells lacking functional mitochondria. Our results suggest that multiple mitochondrial products, including TCA cycle intermediates and reactive oxygen species, can coordinate PHD activity, HIF stabilization, and cellular responses to O2 depletion.

A critical cellular adaptation to low oxygen (O2) levels is the stimulation of hypoxia-inducible factors (HIFs), which are heterodimeric transcription factors composed of α and β subunits (29, 55, 56). One β-subunit, the aryl hydrocarbon receptor nuclear translocator (ARNT), is primarily responsible for cellular HIF transcriptional activity (31, 39, 40, 56). In contrast, three α subunits play distinct roles in this “stress” response (6). HIF-1α is ubiquitously expressed and therefore responsible for a major component of HIF activity in O2-starved cells (3, 50). HIF-2α and HIF-3α expression is tissue restricted, implying alternative names are EglN2, EglN1, and EglN3, respectively. They share a highly conserved C-terminal domain carrying enzymatic activity, whereas each protein has a distinct N-terminal domain (16). Of note, PHD2 appears to be the predominant form that regulates HIF-1α protein stability in vivo (4).

The PHDs belong to an α-ketoglutarate-dependent dioxygenase superfamily (7, 16, 26), which uses O2 as a cosubstrate to add a hydroxyl group to specific proline residues within the oxygen-dependent degradation domains (ODDs) (23), allowing their association with von Hippel-Lindau protein (pVHL)-containing E3 ubiquitin ligase complexes (7, 16, 21, 27, 28, 44, 58). However, under hypoxic conditions (≤5% O2), HIF prolyl hydroxylation is largely diminished, causing dissociation from pVHL and HIF-α stabilization (27, 28, 58).

HIF-specific prolyl hydroxylases, or prolyl hydroxylation domain-containing proteins (referred to as PHDs here) catalyze HIF-α prolyl hydroxylation (16). PHDs and their involvement in HIF-α protein stability regulation are evolutionarily conserved. Only one PHD ortholog has been detected in Caenorhabditis elegans. In contrast, three PHDs have been identified in mammalian cells, namely, PHD1, PHD2, and PHD3, whose alternative names are EglN2, EglN1, and EglN3, respectively. They share a highly conserved C-terminal domain carrying enzymatic activity, whereas each protein has a distinct N-terminal domain (16). Of note, PHD2 appears to be the predominant form that regulates HIF-1α protein stability in vivo (4).

The PHDs belong to an α-ketoglutarate (2-oxoglutarate)-dependent dioxygenase superfamily (7, 16, 26), which uses O2 as a cosubstrate to add a hydroxyl group to specific proline residues within the HIF-α ODDS (20). Unlike some members of the dioxygenase family, the requirement for O2 as a substrate is absolute and cannot be substituted by an H2O molecule (20, 43). Ferrous iron (Fe2+) is also required for the enzyme to be assembled into its active conformation. In PHD2, the dominant prolyl hydroxylase for HIF-1α protein in vivo, the Fe2+ resides in a largely hydrophobic active site (42). During a complete reaction, the Fe2+ is transiently oxidized to Fe3+ and restored to the Fe2+ state (13, 46). However, when α-ketoglutarate is converted into succinate without hydroxylation of a peptide substrate, this Fe2+ is oxidized to Fe3+ during the reaction. During this process ascorbate is required to re-
duce the Fe$^{3+}$ back to Fe$^{2+}$ in order for the enzyme to be recycled (13, 46). Therefore, intracellular ascorbate and Fe$^{2+}$ levels can also affect PHD enzymatic activity and HIF-α protein accumulation (33, 34).

Other important factors that regulate PHD activity are its substrate and product, α-ketoglutarate and succinate, respectively. Not surprisingly, succinate functions as a competitive inhibitor which is reversible by α-ketoglutarate (35, 54). Both α-ketoglutarate and succinate are tricarboxylic acid (TCA) cycle intermediates, suggesting that cross talk between energy production and hypoxic transcriptional responses is an important consideration. Another TCA cycle intermediate, fumarate, has also been reported to function as a PHD inhibitor (25). Patients harboring germ line mutations within genes encoding succinate dehydrogenase or fumarase complex components develop tumors such as pheochromocytoma and paraganglioma (15, 49). Recent studies suggest that abnormal succinate and fumarate levels contribute to the highly vascular nature of such tumors due to perturbed PHD activity and elevated basal levels of HIF-α protein (25, 35, 54). The fact that VEGF is a HIF target gene likely explains their highly vascular nature. Although not currently linked to any human malignancies, several other glucose metabolites such as oxaloacetate have also been shown to affect PHD activity in multiple human cancer cell lines, such as U87 and U251 human glioma cells and human O22 head and neck cancer cells (36).

Reactive oxygen species (ROS) have been shown to regulate HIF-α protein levels in vivo and in vitro as well. We previously observed that increased mitochondrial ROS during hypoxia correlate with HIF-α stabilization (11, 12) and that exogenous ROS promote HIF accumulation under normoxia (12, 41). Tumors treated with ionizing irradiation exhibit elevated HIF-1α levels due to increased ROS (45). Furthermore, JunD-null mouse embryonic fibroblasts exhibit normoxic HIF-1α protein stabilization due to lower levels of ROS scavenging enzymes such as microsomal glutathione-S-transferase-1 and cysteine dioxygenase (17). Cells overexpressing mitochondrial superoxide dismutase exhibit higher baseline levels of HIF-1α, presumably due to increased levels of H$_2$O$_2$ (57), while macrophages produce ROS in response to bacterial infection and exhibit higher levels of HIF-1α protein due to enhanced transcription (5). Other nonhypoxic signals such as thrombin also stimulate HIF-1α transcription via ROS production (47). Taken together, these results suggest ROS are critical to HIF regulation in a variety of conditions. Paradoxically, ROS levels increase when cells encounter hypoxia. Therefore, we proposed that ROS contribute to HIF-α protein stabilization under low-O$_2$ conditions (8, 11, 12, 18, 41).

Cells lacking functional mitochondria are defective in HIF-α protein accumulation under low O$_2$ (8, 11, 12, 18, 41). Two possible mechanisms can explain these results. One is that ROS are required for hypoxic HIF-α protein stabilization; hypoxic cells lacking functional mitochondria fail to increase mitochondrial ROS levels or stabilize HIF-α protein (8, 18, 30, 41). An alternative interpretation is that mitochondria function as “O$_2$ sinks” to limit O$_2$ availability for other O$_2$-consuming cellular processes, including HIF hydroxylation during hypoxia (14, 19). Inhibiting mitochondria would allow enzymes such as PHDs to obtain enough O$_2$ to remain active even during O$_2$ deprivation. Unfortunately, cells deficient for functional mitochondria cannot distinguish between these two possibilities, since they do not separate the process of ROS production from O$_2$ consumption. However, published data using ROS scavengers to attenuate HIF accumulation under hypoxia support mitochondrial ROS regulation of HIF-α stability (8, 11, 12, 18, 52).

Although prolyl hydroxylation is a critical step during HIF regulation, there is currently no convenient way to measure PHD activity. Standard in vitro biochemical assays measure the conversion of radiolabeled α-ketoglutarate to CO$_2$ (20). Although quantitative, this method is limited to in vitro studies and often encounters high background signals due to uncoupled reactions. It also requires special equipment to handle radioactive gas. Another widely used method of measuring HIF prolyl hydroxylation is the pVHL capture assay (16). However, this technique involves multiple steps such as immunoprecipitation or far-Western assays. The most convenient way of measuring HIF prolyl hydroxylation is to use a hydroxyproline-specific HIF-1α antibody (10). Unfortunately, none of these methods allows the simultaneous detection of both hydroxylated and nonhydroxylated HIF-α, making them less amenable to data quantitation. Fusion proteins containing the human HIF-1α ODD exhibit differential migration upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when hydroxylated (22, 27). Taking advantage of this finding, we constructed a GAL4-HA-HIF1α ODD fusion protein and used its differential migration as a read-out of PHD activity in vivo and in vitro. Our studies demonstrate that multiple factors, such as Fe$^{2+}$, TCA cycle intermediates, and ROS levels, as well as mitochondrial respiration rates, modulate PHD activity. Importantly, all of these factors are affected by mitochondrial function, underscoring its role in coordinat-
Hydroxylation of GHO fusion protein leads to a mobility shift during SDS-PAGE. Prolyl hydroxylation of HIF-1α, HIF-2α, and HIF-3α is a key step in the regulation of their stability. During this process, an oxygen atom from dioxygen (O₂) is integrated into a hydroxyl group, covalently modifying two proline residues (Fig. 1A). α-Ketoglutarate is simultaneously converted to carbon dioxide (CO₂) and succinate, which contains the other oxygen atom from O₂. PHD binds Fe²⁺, and ascorbate is required to reduce any Fe²⁺ oxidized to Fe³⁺, maintaining enzymatic activity (Fig. 1A). In order to readily assess in vivo and in vitro PHD activity, we generated a fusion protein containing the HIF-1α ODD (amino acids 496 to 626) that changes its mobility on SDS-PAGE upon prolyl hydroxylation (Fig. 1B). The unhydroxylated peptide migrates more slowly relative to the hydroxylated form (27). Although two human HIF-1α proline residues can be hydroxylated (402 and 546), this fusion protein contains only proline 546. HIF-1α is stable under normoxic conditions when proline 546 is mutated to alanine (27, 28, 58). Since this protein is a fusion of a GAL4 (β-galactosidase) DNA-binding domain, an HA (hemagglutinin HA1 fragment) peptide, and human HIF-1α ODD domain, we called it the “GHO” reporter protein.

PHD2 appears to be the major prolyl hydroxylase regulating HIF-α protein stability in vivo (40). Therefore, we obtained bacterially produced GST-human PHD2 (wPHD2), as well as a mutant PHD2 with histine 313 in the catalytic domain mutated to a tyrosine (mPHD2). We used the wheat germ IVT system to produce unhydroxylated GHO fusion protein (27). In an in vitro hydroxylation assay, when Fe²⁺, ascorbate, and α-ketoglutarate were added to wPHD2, IVT-produced GHO proteins exhibited a substantial increase in the intensity of the lower hydroxylated species (Fig. 1C, lane 3). This indicates that a significant amount of GHO protein had been hydroxylated (Fig. 1C, left, upper panel). When mPHD2 was used, no GHO mobility shift was observed in the presence of all cofactors and cosubstrates (Fig. 1C, lane 1), indicating the effects on migration were the result of PHD enzymatic activity. Hydroxylation was further confirmed on duplicated blots containing samples subjected to identical conditions probed with both HA antibody and anti-hydroxy HIF-1α antibody, respectively (Fig. 1C, left, lower two panels). Importantly, when a mutant GHO protein in which the critical proline had been mutated to an alanine residue, GHO(P→A), was used as the substrate in identical reactions, no mobility shift was observed, demonstrating that proline hydroxylation was required (Fig. 1C, right).

We also used HEK 293T cell lysate as a source of PHD activity to perform in vitro hydroxylation assays. When Fe²⁺ or ascorbate was added, the percentage of the faster migrating band increased from 18% to 57 and 48%, respectively, indicating increased PHD enzymatic activity in these reactions (Fig. 1D). Desferrioxamine (DFX) inhibits PHD activity, presumably by acting as an Fe²⁺ chelator, while O₂ is a cosubstrate for the prolyl hydroxylation reaction (16). Indeed, adding DFX to the reaction or performing the same assay at 1.5% O₂ resulted in decreased intensity of the lower band (for DFX, from 57 to 40% in the presence of Fe²⁺ and from 48 to 26% in the presence of ascorbate; for hypoxia, from 57 to 42% in the presence of Fe²⁺ and from 48 to 32% in the presence of ascorbate). These results indicate that PHD activity decreased under these conditions (Fig. 1D). Importantly, similar experiments have been performed multiple times, giving comparable results, and representative assays are presented here.

In order to verify that the GHO peptide was useful as a measurement for PHD enzymatic activity in vivo, we transfected the GHO construct into HEK 293T cells along with either human PHD1, PHD2, or PHD3. Expression of Flag-tagged PHDs was confirmed by Western analysis with an anti-Flag antibody (data not shown). Without PHD cotransfection, ca. 50% of the GHO protein transiently expressed in HEK 293T cells migrated more slowly, indicating about half of the peptide was hydroxylated (Fig. 1D, lane 1). When each PHD was overexpressed, the amount of GHO migrating as the faster form increased to 75 to 92% (Fig. 1D, lanes 2, 3, and 4). This further confirms that the slower-migrating form is unhydroxylated GHO fusion protein and the faster-migrating species is hydroxylated. In addition, all GHO proteins migrated more slowly when cells were treated with the hypoxia mimetics DFX and CoCl₂ (Fig. 1D, lanes 5 and 6), further validating that the slower-migrating form is unhydroxylated.

Multiple factors affect HIF hydroxylation in vitro. The HIF prolyl hydroxylation reaction involves multiple factors that vary in concentration under different conditions in vivo. Therefore, it was important to investigate how different levels of either cofactors or potential inhibitors affect hydroxylation. We used HEK 293T cell lysate as a PHD source to hydroxylate IVT-produced GHO protein. Cell lysates in reactions lacking exogenous Fe²⁺ and ascorbate exhibited PHD activities that were insufficient to hydroxylate significant amounts of GHO protein (Fig. 2A). However, when Fe²⁺ and ascorbate were added, the abundance of the lower hydroxylated form increased from 17% to 52 and 46%, respectively (Fig. 2A). Addition of α-ketoglutarate, along with ascorbate and Fe²⁺, further increased the amount of the hydroxylated GHO protein to 77% (Fig. 2A).
FIG. 1. A fusion protein containing the human HIF-1α ODD exhibits differential mobility during SDS-PAGE when hydroxylated. (A) Schematic illustration of the prolyl hydroxylation reaction. (B) Proposed hydroxylation of the GHO fusion protein by PHDs. GHO contains a single proline residue that can be hydroxylated (red bold). (C) Bacterially produced GST-wtPHD2 was used to hydroxylate wheat germ IVT-produced GHO protein in vitro. HA antibody was used to detect both the hydroxylated and the unhydroxylated GHO species. Samples from identical experiments were also run on duplicate gels, probed with anti-HA and anti-HIF hydroxyproline antibody, respectively. In vitro-translated GHO(P→A) protein with the critical proline residue mutated to alanine was assayed in in vitro hydroxylation reactions and detected by anti-HA antibody. (D) HEK 293T cell lysates were prepared in HEB buffer and used to hydroxylate wheat germ IVT GHO protein (upper panel). Reactions included the indicated amounts of FeCl$_2$ and ascorbate under normoxic conditions with 1 mM DFX or inside a hypoxia workstation at 1.5% O$_2$. Densitometry analysis was performed to quantify relative GHO hydroxylation levels under each condition, and the results were plotted in a histogram. In the lower panel, GHO protein was transiently expressed in HEK 293T cells. In lanes 2 to 4, Flag-tagged human PHD1, PHD2, and PHD3 were individually coexpressed with the GHO protein. In lanes 5 to 7, transfected cells were treated with 100 μM DFX, 100 μM CoCl$_2$, and 1.5% O$_2$, respectively, for 4 h prior to harvest.
In order to test the sensitivity of this reaction to the addition of different cofactors and cosubstrates, we performed doseresponse experiments with Fe$^{2+}$, ascorbate and α-ketoglutarate. Interestingly, in vitro hydroxylation of GHO using HEK 293T cell lysate was extremely sensitive to exogenous Fe$^{2+}$ (Fig. 2B). The addition of as little as 1 μM FeCl$_2$ increased GHO hydroxylation from 45 to 53% (Fig. 2B). The addition of 10 to 1,000 μM ascorbate also enhanced GHO hydroxylation, presumably by reducing Fe$^{3+}$ to Fe$^{2+}$ in these assays (Fig. 2B). By lysing cells in a volume 10 to 20 times that of the original pellet, α-ketoglutarate concentrations in an in vitro reaction should be well below the reported $K_m$, which is ca. 50 μM (20). Surprisingly, exogenous α-ketoglutarate did not further increase GHO hydroxylation when added alone (Fig. 2B); in fact, α-ketoglutarate slightly inhibited the overall GHO hydroxylation at high concentrations (comparing 39% GHO hydroxylation with 1,000 μM α-ketoglutarate to 45% GHO hydroxylation in control samples). These results suggested that PHD activity is not only affected by the concentration of cofactors and cosubstrates but also by their relative levels.

Two TCA cycle intermediates are involved in HIF-α prolyl hydroxylation reactions: α-ketoglutarate as a substrate and succinate as a product. For many enzymes, the product can serve as a competitive inhibitor of the substrate. We have reported that intracellular succinate accumulation due to defective succinate dehydrogenase activity results in lower PHD activities and elevated HIF-α protein levels under normoxic conditions (54). Fumarate is another TCA cycle intermediate previously shown to negatively regulate intracellular PHD activity (25). In a dose-response analysis comparing the inhibitory effect of succinate and fumarate, fumarate appeared to be a more potent inhibitor of GHO protein hydroxylation (Fig. 2C, lanes 1 to 5). Adding 3 mM α-ketoglutarate reversed the inhibitory effect of 1 mM succinate but failed to influence the effect of 1 mM fumarate (Fig. 2C, lanes 6 to 9). Malate, which is downstream of fumarate in the TCA cycle, also inhibited the hydroxylation reaction in a manner that was not reversed by
α-ketoglutarate (Fig. 2C), suggesting that other TCA cycle intermediates modulate PHD activity by distinct mechanisms from those of succinate. Since Fe^{2+} levels significantly affect PHD activity in our in vitro assay, we speculate that malate may exert its inhibitory effect via an iron and ascorbate reversible mechanism. Indeed, we observed the loss of malate inhibition when 1 mM ascorbate or 50 μM FeCl_{2} was added (Fig. 2C, lower two panels). These results imply that malate may promote uncoupled catalytic reactions resulting in oxidation of the PHD bound Fe^{2+}. Interestingly, PHD inhibition by fumarate is much less sensitive to addition of ascorbate and Fe^{2+} (Fig. 2C, lower two panels, last lanes), further suggesting distinct inhibitory mechanisms by various TCA cycle intermediates.

Pyruvate, lactate, and oxaloacetate are all abundant in the cytosol. We examined their effect on PHD activity in our 293T cell lysate-based in vitro assay. However, we did not detect any obvious inhibitory activity of these three compounds (Fig. 2D). This suggests that PHD enzymes are not simply inhibited by any nonspecific glucose metabolites. In conclusion, our results indicate that the concentrations of multiple factors, including Fe^{2+}, ascorbate, and various TCA cycle intermediates, can alter PHD activity in vitro.

GHO mimics the stability of endogenous HIF-1α protein. To evaluate GHO hydroxylation within intact cells, we generated stable HEK 293T transformants expressing GHO peptide. At 21% O_{2}, GHO expression was barely detectable (Fig. 3A, left panel, lane 1; compare this also to the HIF-1α levels shown below); however, the proteasome inhibitor calpain inhibitor I (ALLN) increased GHO protein levels (Fig. 3A, lane 2). Upon DFX or 1.5% O_{2} treatment, GHO abundance increased dramatically (Fig. 3A, lanes 3 and 4). Moreover, lysates obtained from DFX-treated and hypoxic cells exhibited slower migration of GHO protein than in ALLN-treated cells (Fig. 3A), suggesting it was unhydroxylated. In sharp contrast, the GHO(P→A) protein, which cannot be hydroxylated, was readily detected by Western analysis under normoxic conditions (Fig. 3A, right panel, lane 1). Treatment with DFX or hypoxia failed to further increase the level of GHO(P→A) protein levels or alter its migration (Fig. 3A, right panel, lane 3 and 4). Taken together, these experiments strongly suggest that the GHO protein is degraded at 21% O_{2} as a consequence of its prolyl hydroxylation.

After exposure to 1.5% O_{2} for 6 or 24 h, GHO protein levels increased dramatically in HEK 293T cells (Fig. 3B, upper panel). When hypoxic cells were reexposed to 21% O_{2}, the GHO protein rapidly disappeared (Fig. 3B). The decrease in GHO protein level was accompanied by the appearance of a rapidly migrating species, indicating that hydroxylation resumed upon reoxygenation (Fig. 3B). We also performed Western analysis to assess endogenous HIF-1α protein abundance (Fig. 3B, lower panel). Impressively, the kinetics of HIF-1α accumulation and degradation were identical to the stably expressed GHO protein. In cells exposed to 1.5% O_{2} for 24 h, both HIF-1α and GHO protein were degraded more rapidly upon reoxygenation compared to cells that had been exposed for only 6 h (Fig. 3B). In order to more clearly measure the kinetics of GHO protein hydroxylation upon reoxygenation, we grew GHO-expressing cells at 1.5% O_{2} for 24 h and added the proteasome inhibitor ALLN to block protein degradation. When these cells were reexposed to 21% O_{2}, GHO hydroxylation instantly resumed (Fig. 3C). After 40 s of reoxygenation, GHO was 42% hydroxylated, compared to less than 10% hydroxylation before reoxygenation. GHO hydroxylation was completely restored to normoxic levels after only 6 min of reoxygenation (Fig. 3C). Since PHD2 and PHD3 expression is regulated by HIF-α proteins (2), our results are consistent with the concept that cells exposed to prolonged hypoxia have elevated PHD hydroxylase activities when O_{2} levels are restored. The synchronized expression pattern of endogenous HIF-1α and GHO proteins in this hypoxia-reoxygenation experiment further validates the use of GHO as a tool to assess PHD activities in tissue culture cells.

GHO protein is hydroxylated in hypoxic cells treated with mitochondrial respiration inhibitors. cyt c-null embryonic cells lack a functional respiratory electron transport chain and consume significantly less O_{2} than do wild-type cells (41). Since a functional electron transport chain is an important source of ROS, these cells also fail to generate mitochondrial ROS during hypoxia (18, 41). We confirmed our previous finding that cyt c-null cells cannot stabilize HIF-1α protein after growth at 1.5% O_{2} for 4 h and hypothesized that this is due to decreased mitochondria ROS (Fig. 4A). As shown previously (41), HIF-1α protein was stabilized in cyt c-null cells by the proteasome inhibitor ALLN under both normoxic and hypoxic conditions in cyt c-null cells (Fig. 4A). Using a HIF hydroxy proline specific antibody, we observed that HIF-1α prolyl hydroxylation levels in hypoxic cyt c-null cells were as high as in normoxic cells (Fig. 4A). This observation strongly suggests that without functional mitochondria, PHDs remain active under hypoxic conditions, causing HIF-1α instability.

Treatment with the mitochondrial inhibitors rotenone and myxothiazol eliminates hypoxic ROS production in addition to blocking mitochondrial O_{2} consumption (11). Furthermore, HIF-1α and HIF-2α fail to accumulate under hypoxic conditions in multiple cell lines treated with these mitochondrial inhibitors (11, 41). In order to demonstrate that diminished HIF-α stabilization upon mitochondrial inhibition is due to sustained PHD activity at 1.5% O_{2}, we used HEK 293T transformants expressing the GHO reporter protein. When normoxic cells were treated with ALLN, the GHO peptide was detected as a single band (Fig. 4B, lanes 1 to 3). Rotenone and myxothiazol treatment did not change GHO mobility (Fig. 4B), suggesting that under normoxic conditions all GHO peptides are hydroxylated and unaffected by these mitochondrial poisons. As expected, hypoxia inhibited GHO prolyl hydroxylation, reducing the quantity of the hydroxylated form (Fig. 4B, lane 4). In contrast, when treated with either rotenone or myxothiazol, GHO prolyl hydroxylation levels did not change in cells grown at 1.5% O_{2}, indicated by the maintenance of the hydroxylated form under various conditions (Fig. 4B, lanes 5 to 9). Rotenone and myxothiazol effects on GHO hydroxylation levels were also dose dependent (Fig. 4B), as confirmed by their ability to inhibit cellular respiration (Fig. 4C). DFX likely inhibits PHD function by chelating iron, therefore bypassing any mitochondrial signal. The addition of rotenone or myxothiazol to DFX-treated cells had no effect on its inhibitory effect on GHO prolyl hydroxylation (Fig. 4B, lanes 10 to 12). These results suggest that O_{2} deprivation inhibits PHD activity...
via a mechanism that requires functional mitochondria in hypoxic cells.

Mitochondrial inhibitors restore GHO prolyl hydroxylation in hypoxic VHL-deficient RCC cells. We performed similar experiments in 786-O cells, a human renal clear cell carcinoma (RCC) line lacking the HIF-α ubiquitin ligase component pVHL (24). In these cells, HIF-α protein is stabilized under normoxic conditions, while the hydroxylation is still maintained. The use of these cells allowed us to monitor GHO hydroxylation status without using proteasome inhibitors, which was toxic after prolonged treatment. As expected, GHO protein was hydroxylated but not degraded in normoxic 786-O cells (Fig. 5A, lane 1). Growing 786-O cells at 1.5% O₂ for 4 h reduced the fraction of hydroxylated GHO protein from 79 to 43% (Fig. 5A, lane 5). In contrast, hypoxic cells treated with rotenone or myxothiazol retained 61 to 66% of the hydroxylated GHO protein (Fig. 5A, lanes 6 and 7).

We determined whether PHD activity was restored upon inhibition of mitochondrial activity. We grew GHO expressing 786-O cells at 1.5% O₂ for 18 h to induce the accumulation of nonhydroxylated GHO protein. Compared to protein levels before treatment, the percentage of hydroxylated GHO decreased from 75 to 43% (Fig. 5B, upper panel, lanes 2 and 3). Upon adding 100 ng/ml of rotenone or myxothiazol, GHO protein was gradually rehydroxylated (Fig. 5B, lanes 4 to 7). After 2 h of treatment with these mitochondrial inhibitors, the

FIG. 3. GHO protein stability mimics that of endogenous HIF-1α protein in HEK 293T cells. (A) GHO and GHO(P ⃗A) protein were stably expressed in HEK 293T cells. Cells were treated with 100 μM ALLN, 100 μM DFX, and 1.5% O₂ for 4 h prior to harvest. (B) HEK 293T stable transformants expressing GHO protein were treated with 1.5% O₂ for 6 h or 24 h, respectively. Cells were removed from the hypoxia workstation and lysed at the indicated times after reoxygenation. Cells harvested inside the workstation were used as time zero. Untreated normoxic cells (N) and cells exposed to 100 μM DFX for 4 h (D) were used as controls. A nonspecific band (NS) serves as a loading control. (C) HEK 293T cells expressing GHO protein were exposed to 1.5% O₂ for 24 h. At 1 h prior to reoxygenation, 100 μM ALLN was added to the medium. Cells were harvested at the indicated times after reoxygenation. Normoxic cells treated with ALLN only (N) were used as a control. Four independent experiments were performed, and the results of GHO hydroxylation were quantified and are presented as a bar graph.
degree of GHO hydroxylation was largely recovered to normoxic levels (Fig. 5B). A more detailed study demonstrated that a resumption of PHD activity in hypoxic cells was observed as early as 5 min after the addition of myxothiazol (Fig. 5B, lower panel, lanes 2 to 5). We also confirmed that we effectively blocked the respiration of the cells we used in these experiments at the dose of rotenone and myxothiazol we administered to the cells (Fig. 5B). These results suggest that functional mitochondria are required for maintaining the inhibition of PHD in hypoxic cells.

It is not clear from our results whether mitochondrial poisons reverse hypoxic inhibition of PHD activities via increasing O₂ availability or by blocking ROS production. The use of antioxidants can potentially distinguish between these two mechanisms. However, previous antioxidant studies yielded inconsistent results (11, 12, 18, 19). Since most pharmacological and enzymatic antioxidants may possess nonspecific or secondary effects, we attempted to circumvent this problem by using mitoubiquinone (MitoQ). This compound was described as a mitochondrion-specific ROS scavenger with minimal effects on respiration that reverses hypoxic stabilization of HIF-1α protein in Hep3B cells (52). We added various concentrations of MitoQ to 293T GHO cell cultures and measured its effect on HIF-1α accumulation during hypoxia. MitoQ reversed hypoxic stabilization of HIF-1α protein with mitochondrial inhibitors rotenone or myxothiazol. (C) The inhibition of HEK 293T cell respiration by different doses of rotenone or myxothiazol was measured as cellular KCN-dependent O₂ consumption. All respiration rates were normalized to that of untreated cells.

FIG. 4. Cells lacking a functional mitochondrial respiratory chain retain PHD activity under low O₂. (A) Wild-type and cyt c-null embryonic cells were exposed to 1.5% O₂ or 100 μM DFX for 4 h in the presence or absence of 10 μM MG132 as indicated. Total HIF-1α protein and hydroxy HIF-1α protein levels were measured by Western blotting. Of note, samples assayed for HIF-1α accumulation demonstrated multiple alternatively phosphorylated species. Identical lysates were rerun on SDS-polyacrylamide gels to probe for hydroxylated HIF-1α so that HIF-1α appears as a single species at 100 kDa. (B) HEK 293T cells stably expressing the GHO protein were exposed to 1.5% O₂ or 100 μM DFX for 4 h in the presence of different concentrations of mitochondrial inhibitors rotenone or myxothiazol. (C) The inhibition of HEK 293T cell respiration by different doses of rotenone or myxothiazol was measured as cellular KCN-dependent O₂ consumption. All respiration rates were normalized to that of untreated cells.
further supports the hypothesis that functional mitochondria are required for HIF-1α/HIF-1α2 regulation. Exogenous ROS inhibit GHO hydroxylation in vivo and in vitro. Exogenous ROS (H₂O₂) have been shown to promote HIF-1α/HIF-1α2 protein accumulation under normoxic conditions (12, 18, 41). We therefore predicted that ROS treatment would abolish GHO protein hydroxylation in normoxic cells. Glucose oxidase is a continuous source of ROS when applied exogenously to cultured cells since it oxidizes glucose to yield H₂O₂ (Fig. 6A). The addition of catalase eliminates H₂O₂ by catalase. Exogenous hydrogen peroxide (H₂O₂) inhibits GHO prolyl hydroxylation in vivo and in vitro. (A) 786-O stable transfectants expressing GHO protein were exposed to 21% O₂ or 1.5% O₂ for 4 h in the presence or absence of DFX, rotenone, or myxothiazol as indicated. GHO protein was detected by Western analysis with HA antibody; densitometry analysis was used to measure GHO protein hydroxylation levels. (B) GHO-expressing 786-O cells were cultured at 1.5% O₂ overnight; rotenone or myxothiazol were added in the hypoxic workstation, and cells were harvested at different times after drug treatment as indicated. Normoxic and DFX-treated cells were used as controls. Levels of GHO protein hydroxylation were measured by Western blot and densitometry analyses. The inhibition of 786-O cellular respiration by different doses of rotenone or myxothiazol was measured as cellular KCN-dependent O₂ consumption. All respiration rates were normalized to that of untreated cells. (C) The indicated concentrations of MitoQ were added to culture medium for 4 h. After an additional 4 h of hypoxic and normoxic incubation, cells were harvested for analysis. Three parallel sets of samples were used. One set was harvested for HIF-1α and actin Western analysis. Another was treated with 100 μM ALLN to block protein degradation and harvested for GHO protein Western analysis. The third set was harvested at the same time for respiration measurements.
verting it to H₂O. To test the effect of exogenous ROS on GHO hydroxylation, glucose oxidase was added to the media of HEK 293T cells expressing the GHO fusion protein. Proteasome inhibitors were also used to stabilize GHO protein under all conditions. We observed that the addition of glucose oxidase to culture medium inhibited GHO prolyl hydroxylation in a dose-dependent manner, as indicated by the disappearance of the hydroxylated band (Fig. 6B, lanes 2 to 5). As expected, adding catalase to the medium reversed this inhibitory effect, demonstrating that the effect of glucose oxidase was due to the H₂O₂ production (Fig. 6B, lanes 6 to 10). Similar experiments using GHO expressing pVHL-deficient 786-O cells (Fig. 6C) and RCC4 cells (Fig. 6D) yielded identical results. In addition, tert-butyl-H₂O₂, a stabilized form of H₂O₂, also blocked GHO prolyl hydroxylation when added to the medium (Fig. 6D, lanes 5 and 6).

It is possible that exogenous ROS inactivate the PHDs by direct oxidation of the bound Fe²⁺. We hypothesized that adding H₂O₂ to in vitro hydroxylation reactions would also inhibit GHO hydroxylation. To test this, we used recombinant GST-PHD2 protein purified from bacteria and assessed its ability to hydroxylate IVT GHO protein in the presence of increasing amounts of glucose oxidase (Fig. 6E). In this experiment, the basal level of hydroxylation was 37%, and the addition of glucose oxidase or catalase alone did not have a significant impact on PHD2 enzyme activity (Fig. 6E, lanes 1 to 3). However, when glucose was added to reactions containing glucose oxidase to generate H₂O₂, the PHD2 was inhibited and hydroxylated GHO levels dropped to 14% (Fig. 6E, lane 4). Catalase addition, which reduces H₂O₂ levels, partially reversed the inhibitory effect in a dose-dependent manner (Fig. 6E, lanes 5 to 7). These results suggest that ROS in the form of H₂O₂ directly inhibit PHD2 enzymatic activity.

Exogenous H₂O₂ stabilizes HIF-1α protein but does not significantly increase HIF-1α transcript levels. Since exogenous ROS inhibit GHO prolyl hydroxylation, increased HIF-1α protein levels generated by exogenous ROS have been primarily attributed to protein stability. However, it has been reported that ROS can stimulate HIF-1α transcription as well (5, 47). To investigate whether increased HIF-1α transcription was observed upon exogenous H₂O₂ treatment, we treated Hep3B cells with different doses of either H₂O₂ or glucose oxidase for various time intervals. Quantitative RT-PCR and Western analysis demonstrated that although HIF-1α protein levels increased after H₂O₂ treatment, HIF-1α transcript levels did not (Fig. 7A). As expected, prolonged glucose oxidase treatment resulted in increased HIF-1α protein levels and elevated expression of the downstream target phosphoglycerate kinase gene (PGK-1) (Fig. 7A). We did not observe an increase in PGK-1 expression after 1 h of H₂O₂ or DFX treatment due to the time required for HIF downstream target gene stimulation (Fig. 7A). We conducted similar experiments in HEK 293T cells with brief H₂O₂ exposures and also failed to observe any increase in HIF-1α transcription (data not shown). In fact, we actually detected a decrease in HIF-1α transcript levels, which we attributed to possible toxicity effects from exogenous H₂O₂ (Fig. 7A). These data suggest that exogenous H₂O₂ treatment increases HIF-1α by stabilizing the protein and not by promoting transcription in our experiments. We also performed HIF-1α immunofluorescence experiments on H₂O₂ and glucose oxidase-treated Hep3B cells. As expected, these treatments resulted in nuclear accumulation of HIF-1α protein (Fig. 7B).

Since H₂O₂ did not elevate HIF-1α protein levels by affecting transcription, we investigated how H₂O₂ treatment inhibits PHD activity in vivo. It is possible that exogenous H₂O₂ inhibits PHD function in vivo by the same mechanisms shown in vitro: via Fe²⁺ oxidation. To test this hypothesis, we treated GHO-expressing Vhl-null ES cells (37) with 100 μM DFX or 1 mM H₂O₂ for 90 min in the presence or absence of 200 μM ascorbic acid. DFX is a specific chelator of Fe³⁺, and its inhibitory effect on PHD is probably due to deprivation of intracellular Fe pools. In the presence of ascorbate, all chelatable iron is in the reduced Fe²⁺ state. We therefore observed a reversal of the DFX-mediated inhibition of GHO prolyl hydroxylation in the presence of ascorbate (Fig. 7C, lanes 4 to 6). The addition of ascorbate also blocked the H₂O₂-mediated inhibition of GHO prolyl hydroxylation (Fig. 7C). The similar effect of ascorbate on DF-x- and H₂O₂-mediated hydroxylase inhibition suggests that exogenous H₂O₂ inhibits HIF PHD by oxidizing cellular Fe³⁺. Taken together, these data suggest that exogenous ROS induced HIF-1α protein accumulation by decreasing its prolyl hydroxylation.

**DISCUSSION**

Hydroxylation of key proline residues within HIF-α ODDs is a critical step regulating HIF transcriptional activity in O₂ replete cells. Monitoring these critical modifications enables us to evaluate factors affecting HIF-α protein stability. However, as stated in the introduction, widely used analyses of PHD activity are either inconvenient or not readily available. We therefore used a system based on a fusion protein containing the HIF-1α ODD domain, which we call GHO. When hydroxylated on the critical ODD proline residue (P⁶⁶₄), GHO protein exhibits differential mobility upon standard SDS-PAGE. As such, this method provides the opportunity to observe both hydroxylated and unhydroxylated forms of the GHO protein by standard Western analysis. When used in in vitro hydroxylation reactions, GHO protein mobility changed exactly as predicted in response to known regulators of the PHDs. We also demonstrated that when stably expressed, the GHO protein is stabilized and degraded with kinetics identical to endogenous HIF-1α protein. These data suggest that the GHO fusion protein is a useful tool for studying factors regulating HIF-α prolyl hydroxylation both in vitro and in vivo.

α-Ketoglutarate is required as a cosubstrate for the hydroxylation reaction, as demonstrated in our in vitro assay using bacterially purified PHD2. However, we were surprised to detect a mild but reproducible inhibition of GHO hydroxylation when α-ketoglutarate alone was added to the in vitro hydroxylation assays. It has been reported that without the peptide substrate, prolyl hydroxylases can undergo an uncoupled reaction to convert α-ketoglutarate to succinate (13). During this process, the Fe²⁺ is oxidized to Fe³⁺, rendering the enzyme inactive, an effect reversed by the addition of ascorbate to the reaction. Since the GHO peptide is the last component added to our in vitro assays and its availability can be limited in these reactions, this process could explain the inhibitory effect of high concentrations of α-ketoglutarate. A similar mechanism...
may operate in vivo as it has been reported that cellular PHD levels are in excess over available HIF-α subunits under normoxic conditions (1). Since the concentration of HIF-α molecules increases during hypoxia, overall intracellular hydroxylase activity may also increase because of fewer uncoupled reactions. This likely contributes to the rapid resumption (as little as less than 40 s) of HIF prolyl hydroxylation during hypoxia-reoxygenation events.

FIG. 7. Exogenous H₂O₂ does not stimulate HIF-1α transcription but stabilizes HIF-1α protein via PHD inhibition. (A) Hep3B cells were treated with 100 mM DFX and different doses of H₂O₂ for 60 min or various concentrations of glucose oxidase for 8 h. HIF-1α protein was detected by Western blotting, and a nonspecific band provided a loading control. RNA was extracted for quantitative RT-PCR analysis to measure HIF-1α and PGK-1 transcript levels. All data are presented as levels relative to untreated cells. (B) Hep3B cells were grown on coverslips and treated as indicated. Immunofluorescence analysis was performed with antibody recognizing human HIF-1α. DAPI containing mounting medium was used to stain for nuclei. (C) Vhl-null ES cells were treated with 100 mM DFX or 1 mM H₂O₂ for 90 min in the presence or absence of 100 μM ascorbate. GHO protein was assessed by Western blot analysis.
We found that when HEK 293T cell lysate was used as a source of PHD activity, in vitro reactions were extremely sensitive to addition of Fe$^{2+}$. This suggests that factors altering intracellular Fe$^{2+}$ levels may have profound effects on PHD activity in vivo. Since intracellular redox status is another factor that affects Fe$^{2+}$ levels, it is conceivable that the redox status of cells modulates the activities of the PHDs. It is interesting that the expression of transferrin, important for iron uptake, is induced by HIF transcriptional activity (51). Therefore, in cells whose iron levels change as a result of HIF activity, iron concentration can potentially function as a feedback signal regulating HIF-α subunit expression.

Other TCA cycle intermediates such as fumarate and succinate have been reported to inhibit the PHDs (25, 54). These effects were confirmed in our in vitro hydroxylation assays. The inhibitory effects of these two compounds were reversed by adding α-ketoglutarate to the system, suggesting they compete for a common binding site within PHD enzymes. It has been proposed that succinate dehydrogenase and fumarase deficiency cause intracellular accumulation of succinate and fumarate, which leads to compromised PHD activity in vivo. However, in most tissues, most of the succinate and fumarate are produced and consumed in the mitochondria. It is unclear how much of these compounds actually accumulate in the cytoplasm. Because 0.1 to 1 mM concentrations are required for succinate and fumarate to inhibit PHD activity in vitro, it may be possible that additional cellular factors sensitize PHDs to modest cytosolic increases of succinate and fumarate.

Unlike succinate and fumarate, several other glucose metabolites such as pyruvate, lactate, oxaloacetate, and malate are abundant in the cytoplasm. Therefore, they have the potential to modulate HIF-α levels when glucose metabolism fluctuates, such as during hypoxia. Using our cell lysate-based in vitro hydroxylation assay we did not detect any inhibitory effect of pyruvate, lactate, and oxaloacetate on PHD activity. However, malate, another TCA cycle intermediate, inhibited in vitro GHO hydroxylation. Interestingly, this inhibition is α-ketoglutarate independent. Since additional Fe$^{2+}$ and ascorbate reverse this inhibitory effect, we speculate that malate may promote uncoupled enzyme reactions to lock PHDs into the inactive Fe$^{3+}$ binding state. Finally, although our in vitro data suggest these glucose metabolites directly affect PHD enzymes, we cannot rule out the possibility that they affect GHO hydroxylation by altering other factors such as substrate conformation.

It is interesting that a previous study using a cell-based assay also suggests that multiple glucose metabolites such as pyruvate and oxaloacetate can inhibit PHD enzymes. Such effects were reversed by reducing agents such as ascorbate, cysteine, or histidine (36). Fumarate was not inhibitory to the PHDs in this previous study. Our in vitro assay differs from this previous study regarding which particular metabolites have inhibitory effects. These differences may stem from distinct experimental protocols. For example, in a cell-based assay, unknown cellular factors might affect the sensitivities of PHDs to different inhibitory agents. In that previous study, data obtained from an in vitro pVHL binding assay required very high levels (up to 10 mM) of pyruvate and oxaloacetate compared to a cellular assay. It will be important in future studies to investigate how different factors affect PHD activity under various in vivo conditions.

Previous studies have indicated that functional mitochondria are required for hypoxic HIF-α stabilization (8, 11, 12, 18, 41). Our results directly demonstrate that when cells are treated with mitochondrial inhibitors, PHDs remain active under hypoxic conditions. Since inhibiting mitochondrial activity can theoretically lead to both increased O$_2$ availability for the hydroxylation reaction (14, 19) and decreased ROS, it is difficult to conclude whether either or both consequences are responsible for inhibition of hypoxic HIF stabilization. We attempted to distinguish between these two mechanisms by using MitoQ, a mitochondrion-specific ROS scavenger. However, we determined that MitoQ inhibited both ROS production and respiration in our experimental system. It will be important in future studies to identify specific mitochondrial antioxidants that can clearly distinguish between ROS production and O$_2$ consumption to fully address the question of the relative contributions by them to HIF-α hypoxic accumulation.

Regardless of whether mitochondrial ROS play a significant role in hypoxic HIF prolyl hydroxylase inactivation, there is little doubt that H$_2$O$_2$ can function on its own to stabilize the HIF-1α protein. Although some data suggest that H$_2$O$_2$ causes an increase in HIF-1α gene transcripts (47), we observed that exogenous H$_2$O$_2$ primarily increased HIF-1α protein by inhibiting PHD activity, therefore blocking HIF-1α degradation. Our examination failed to detect any increase of HIF-1α transcripts even after prolonged H$_2$O$_2$ exposure. Moreover, ascorbate, which reduces Fe$^{3+}$ to Fe$^{2+}$, can reverse the effect of added H$_2$O$_2$. This suggests that exogenous H$_2$O$_2$ induces HIF-1α protein accumulation in a manner similar to that of cells with elevated levels of endogenous ROS due to impaired scavenging ability (17).

In conclusion, by using a fusion protein that migrates differentially upon prolyl hydroxylation, we studied factors modulating PHD activity in vivo and in vitro. Mitochondria are the major organelles required for cellular Fe storage and usage, the TCA cycle, most O$_2$ consumption, and ROS production. Our study demonstrates that a large number of these factors whose availability can be influenced by mitochondrial function and activity modulate intracellular PHD function. Considering the fact that HIF-mediated transcriptional activation is a critical regulator of cellular metabolism and mitochondrial activity (32, 48), our work delineates a complex interplay between mitochondrial function and cellular hypoxic responses.

**ACKNOWLEDGMENTS**

We thank Matthew Coleman for providing bacterially purified GST-wtPHD2 protein and GST-mPHD2(H313Y) and Michael Murphy for providing MitoQ. We thank Timothy Cash for critical review of the manuscript and members of the Simon lab for discussion and suggestions. This study was supported by the Howard Hughes Medical Institute, NIH grant CA104838, and the Abramson Family Cancer Research Institute. M.C.S. is an investigator of the Howard Hughes Medical Institute.

**REFERENCES**

1. Appelhoff, R. J., Y. M. Tian, R. R. Ravai, H. Turley, A. L. Harris, C. W. Pugh, P. J. Ratcliffe, and J. M. Gleadle. 2004. Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J. Biol. Chem. 279:38458–38465.
2. Aprélikova, O., G. V. Chandramouli, M. Wood, J. R. Vasselli, J. Riss, J. K. Maranich, W. M. Linehan, and J. C. Barrett. 2004. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J. Cell Biochem. 92:491–503.

23. Bardos, J. I., and M. Ashcroft. 2005. Negative and positive regulation of HIF-1: a complex network. Biochem. Biophys. Acta 1755:107–120.

24. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

25. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

26. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.

27. Bryant, C. P., and D. D. Steeves. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol. Life Sci. 60:1376–1393.

28. Bruck, R. K., and S. L. McKnight. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 291:1337–1340.

29. Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. 2005. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab. 1:569–577.

30. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

31. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

32. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.

33. Bryant, C. P., and D. D. Steeves. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol. Life Sci. 60:1376–1393.

34. Bruck, R. K., and S. L. McKnight. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 291:1337–1340.

35. Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. 2005. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab. 1:569–577.

36. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

37. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

38. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.

39. Bryant, C. P., and D. D. Steeves. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol. Life Sci. 60:1376–1393.

40. Bruck, R. K., and S. L. McKnight. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 291:1337–1340.

41. Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. 2005. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab. 1:569–577.

42. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

43. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

44. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.

45. Bryant, C. P., and D. D. Steeves. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol. Life Sci. 60:1376–1393.

46. Bruck, R. K., and S. L. McKnight. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 291:1337–1340.

47. Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. 2005. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab. 1:569–577.

48. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

49. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

50. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.

51. Bryant, C. P., and D. D. Steeves. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses. Cell Mol. Life Sci. 60:1376–1393.

52. Bruck, R. K., and S. L. McKnight. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 291:1337–1340.

53. Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla, and N. S. Chandel. 2005. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metab. 1:569–577.

54. Berra, E., E. Benizri, A. Ginouves, V. Volmat, D. Roux, and J. Pouyssegur. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J. 22:4082–4090.

55. Blouin, C. C., E. L. Page, G. M. Soucy, and D. E. Richard. 2004. Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1α. Blood 103:1124–1300.

56. Bruick, R. K., and S. L. McKnight. 2002. Role in normoxia. EMBO J. 21:4572–4582.
tumorigenesis: the examples of fumarate hydratase and succinate dehydrogenase. Ann. Med. 35:632–639.
50. Pugh, C. W., and P. J. Ratcliffe. 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. 9:677–684.
51. Rolfs, A., I. Kvietikova, M. Gassmann, and R. H. Wenger. 1997. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J. Biol. Chem. 272:20055–20062.
52. Sanjuan-Pla, A., A. M. Cervera, N. Apostolova, R. Garcia-Bou, V. M. Victor, M. P. Murphy, and K. J. McCreath. 2005. A targeted antioxidant reveals the importance of mitochondrial reactive oxygen species in the hypoxic signaling of HIF-1α. FEBS Lett. 579:2669–2674.
53. Schofield, C. J., and P. J. Ratcliffe. 2004. Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell. Biol. 5:343–354.
54. Selak, M. A., S. M. Armour, E. D. MacKenzie, H. Boulaibbel, D. G. Watson, K. D. Mansfield, Y. Pan, M. C. Simon, C. B. Thompson, and E. Gottlieb. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-α prolyl hydroxylase. Cancer Cell 7:77–85.
55. Tian, H., S. L. McKnight, and D. W. Russell. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev. 11:72–82.
56. Wang, G. L., B. H. Jiang, E. A. Rue, and G. L. Semenza. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. USA 92:5510–5514.
57. Wang, M., J. S. Kirk, S. Venkataraman, F. E. Domann, H. J. Zhang, F. Q. Schafer, S. W. Flanagan, C. J. Weydert, D. R. Spitz, G. R. Buettner, and L. W. Oberley. 2005. Manganese superoxide dismutase suppresses hypoxic induction of hypoxia-inducible factor-1α and vascular endothelial growth factor. Oncogene 24:8154–8166.
58. Yu, F., S. B. White, Q. Zhao, and F. S. Lee. 2001. HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc. Natl. Acad. Sci. USA 98:9630–9635.