Endogenous 2-oxoacids differentially regulate expression of oxygen sensors

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Adaptations to change in oxygen availability are crucial for survival of multi-cellular organisms and are also implicated in several disease states. Such adaptations rely upon gene expression regulated by the heterodimeric transcription factors HIFs (hypoxia-inducible factors). Enzymes that link changes in oxygen tensions with the stability and transcriptional activity of HIFs are considered as oxygen sensors. These enzymes are oxygen-, iron- and 2-oxoglutarate-dependent dioxygenases that hydroxylate key proline and asparagine residues in HIFα subunits. The constitutive inhibitory action of these enzymes on HIFs is relieved by hypoxia and by agents that displace iron or 2-oxoglutarate. Two of the enzymes, HPH (HIF prolyl hydroxylase)-1 and HPH-2, are known to be inducible by hypoxia in a HIF-dependent manner. This suggests the existence of a novel feedback loop for adjusting hypoxia-regulated gene expression. We have recently shown that HIF-1α stability, HIF-1 nuclear translocation and HIF-mediated gene expression in human glioma cell lines can be stimulated by pyruvate independently of hypoxia. In the present study we show that the endogenous 2-oxoacid oxaloacetate can also activate HIF-mediated gene expression. Pyruvate and oxaloacetate treatment of cells also up-regulates HPH-1 and HPH-2, but not HPH-3 or the HIF asparaginyl hydroxylase FIH-1 (factor inhibiting HIF). Regulation of HIF-1 and the expression of HPH homologue genes can thus be influenced by specific glycolytic and tricarboxylic acid cycle metabolites. These findings may underlie important interactions between oxygen homoeostasis, glycolysis, the tricarboxylic acid cycle and gluconeogenesis.

Key words: hypoxia, hypoxia-inducible factor (HIF), oxaloacetate, oxygen sensor, prolyl hydroxylase, pyruvate.

INTRODUCTION

Hypoxia induces adaptive responses in many organisms via the hypoxia-inducible factor family of transcriptional factors (HIF-1, HIF-2 and HIF-3), which up-regulate genes involved in glycolytic energy metabolism, cell growth and survival, angiogenesis and erythropoiesis [1]. Members of the HIF family are heterodimers comprising HIFα and HIFβ subunits [2], both of which are constitutively expressed in mammalian cells. Regulation of the HIF-1 complex is mainly dependent upon degradation of the HIF-1α subunit. Under normoxic conditions, HIF-1α is targeted for ubiquitinylation and proteasomal degradation via the E3 ligase component pVHL (von Hippel–Lindau protein), a tumour suppressor [3,4]. This process requires the binding of pVHL to an ODD (oxygen-dependent degradation domain) on the HIF-1α protein [5]. Enzymic incorporation of oxygen into the HIF-1α ODD is required to generate the binding site for pVHL [6], thus subjecting this process to regulation by oxygen availability.

Three distinct HIF prolyl hydroxylases (HPH-1, HPH-2 and HPH-3) have been identified, which regulate the binding of pVHL to HIF-1α by using molecular oxygen to hydroxylate proline residues 402 and 564 on the HIF-1α ODD [7–9]. HIF-1α is also hydroxylated at Asn^{803} by another hydroxylase known as FIH-1 (factor inhibiting HIF) [10–12]. Hydroxylation of Asn^{803} prevents HIF from interacting with the transcriptional co-activator p300 and therefore blocks HIF-mediated transcriptional activation [10–12]. By virtue of their ability to regulate HIF, the HPH homologues and FIH-1 are together referred to as oxygen sensors. These enzymes require oxygen, iron and 2-oxoglutarate for their activity. This explains why the hypoxic stimulation of HIF-1α protein stabilization and HIF-mediated gene transcription is mimicked by the iron chelator DFO (desferrioxamine), the iron-displacing metal cobalt or synthetic 2-oxoglutarate antagonists, such as DMOG (dimethyloxalylglycine) [7–12]. In addition to these regulatory factors, forced over-expression of each HPH homologue and FIH-1 has been shown to blunt HIF activation by hypoxia in cultured cells [13]. This suggests that the ability of cells to make transcriptional responses to hypoxia may be modified via altered expression of the HIF hydroxylases. Differential expression of the three HPH homologues and FIH-1 amongst various mammalian organs supports this notion [14,15].

The *hph-1* and *hph-2* genes have been shown to be inducible by hypoxia [7,13,16,17]. This up-regulation appears to underlie previous observations that the rate of HIF-1α decay following hypoxic induction depends upon the duration of the hypoxic period, with longer hypoxic periods promoting a more rapid degradation of HIF-1 upon re-oxygenation [18]. HIF activation is itself required for the hypoxic induction of HPH homologues, since this induction is blocked by HIF-1α small interfering RNA [19] and is absent in *hif1*$\alpha^{-/-}$ cells [17]. HIF-dependent HPH induction thus appears to represent a novel feedback mechanism for blunting HIF-induced gene expression during and after prolonged periods of hypoxia.

Although hypoxia is the ubiquitous inducer of HIF-1α in all cells tested, other stimuli, such as cytokines, growth factors, reactive oxygen species and nitric oxide, can also activate HIF under normoxic conditions (for review, see [20]). It is not known whether these stimuli also regulate expression of HPH homologues or of FIH-1. We have recently identified the glucose metabolite pyruvate (2-oxopropanoate) as a normoxic regulator of HIF [21]. Pyruvate was shown to increase HIF-1α levels, promote HIF-1 DNA binding and increase mRNA levels of several genes known to be regulated by HIF in human gliomas and other cell types, even in the presence of oxygen [21]. In the present study we

Abbreviations used: CA9, carbonic anhydrase IX; DFO, desferrioxamine; DMOG, dimethyloxalylglycine; HIF, hypoxia-inducible factor; FIH-1, factor inhibiting HIF; HPH, HIF prolyl hydroxylase; HRE, hypoxia-response element; IAA, iodoacetate; ODD, oxygen-dependent degradation domain; pVHL, von Hippel–Lindau protein, TCA cycle, tricarboxylic acid cycle; RT, reverse transcriptase.

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demonstrate direct activation of a HIF-regulated promoter by the glycolytic metabolite pyruvate, as well as the TCA (tricarboxylic acid) cycle intermediate oxaloacetate, in human glioblastoma cells. We show that these endogenously occurring 2-oxoacid analogues of 2-oxoglutarate can effectively stabilize HIF-1α and activate HIF-dependent gene expression. Pyruvate and oxaloacetate also prominently increase mRNA and protein levels of HPH-1 and HPH-2 independently of hypoxia. Glycolytic and TCA cycle metabolites may thus regulate a feedback loop featuring HIF and HPH homologues independently of hypoxia.

EXPERIMENTAL

Cell culture and hypoxia treatments

The human glioblastoma U87, U251 and U373 cell lines were cultured in Eagle's minimum essential medium with Earle's balanced salt solution without L-glutamine, supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. Cell lines were maintained in 5 % CO_2/95 % air atmosphere at 37 °C. For hypoxia treatments, the culture dishes were sealed in an incubator chamber and flushed with a gas mixture of 1 % O_2/5 % CO_2/94 % N_2 for 5 min, and incubated under this environment at 37 °C for the indicated time.

Materials

Culture media and fetal bovine serum were from Mediatech. DMOG was from Frontier Scientific. All remaining chemicals were from Sigma. The pcDNA3.1/V5-HIS human VHL vector was a kind gift from Dr Richard Bruick and Dr Steven McKnight (Graduate School of Biomedical Sciences, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.).

Antibodies

Mouse monoclonal anti-HIF-1α (610959) antibody was obtained from Transduction Laboratories. Polyclonal antibodies against HPH-1 (NB 100-139) and HPH-2 (NB 100-137) were purchased from Novus Biologicals. Mouse monoclonal antibody against β-actin (ab6276) was purchased from Abcam. Horseradish-peroxidase-coupled sheep anti-mouse antibody and mouse anti-rabbit antibody were obtained from Amersham Biosciences.

HIF-1α immunocytochemisty

Cells were seeded in 48-well plates and treatments were performed in glucose-free Krebs–Henseleit buffer (1.3 mM $CaCl_2$, 1.3 mM $MgCl_2$, 124 mM NaCl, 3.5 mM KCl, 1.25 mM K_2HPO_4 and 26.3 mM $NaHCO_3$, pH 7.5). All drugs were dissolved in glucose-free Krebs–Henseleit buffer. Treatments were performed as indicated and then cells were washed in cold PBS, fixed (10 % formalin for 20 min) and permeabilized (0.2 % Triton X-100). Endogenous peroxidase activity was blocked with 0.3 % H_2O_2 and 0.5 % normal horse serum in PBS. Cells were incubated overnight at 4 °C with anti-HIF-1α antibody at 1:200 in PBS with 0.3 % Triton X-100 and 1.5 % normal horse serum. After washing with cold PBS, primary antibody was detected using ABC elite kit with biotinylated horse anti-mouse secondary antibody at 1:200 (Vector Laboratories). Immunoreactions were visualized with 3,3-diaminobenzidine.

Western blot analysis of HIF-1α, HPH-1 and HPH-2

For HPH-1 and HPH-2, whole cell extracts were prepared by lysing pelleted cells in RIPA buffer (0.1 % SDS, 1 % Nonidet P40, 5 mM EDTA, 0.5 % sodium deoxycholate, 150 mM NaCl, 50 mM Tris/HCl, freshly supplemented with 2 mM dithiothreitol and protease inhibitors) for 30–60 min on ice. Lysates were centrifuged at 16 000 ***g*** for 10 min (4 °C), the supernatant was subjected to electrophoresis in Novex 4–12 % Tris/glycine precast gels (Invitrogen), transferred on to nitrocellulose, and blotted using the polyclonal antibodies as described above. Nuclear extracts and Western blotting for HIF-1α was performed as previously described [21].

RT (reverse transcriptase)-PCR and quantificative RT-PCR analysis of oxygen sensors in various tissues and human glioma cell lines

Total RNA from cell lines were prepared using the RNeasy Mini Kit (Qiagen). Total RNA from human brain, heart and testis tissue was purchased (ClonTech). Total RNA (1.8 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The following forward/reverse primers were used: for human HPRT1 (hypoxanthine phosphoribosyltransferase 1) (GenBank® accession no. NM_000194), 5′-TGACACTGGCAAAACAATGCA-3′/5′-GGTCCTTTTCACCAGCAAGCT-3′; for human HPH-1/EGLN3 (see the Results section for an explanation of the EGLN nomenclature) (GenBank® accession no. NM_022073), 5′-AGATGTGGAGCCCATTTTTG-3′/5′-CAGATTTCAGAGCACGGTCA-3′; for human HPH-2/EGLN1 (GenBank® accession no. NM_022051), 5′-AAACCATTGGGCTGCTCAT-3′/5′-CGTACATAACCCGTTCCATTG-3′; for human HPH-3/EGLN2 (GenBank® accession no. NM_053046), 5′-CTGCTGCAGATCTTCCCTGAG-3′/5′-TAGGCGGCTGTGATACAGGT-3′; for human FIH-1 (GenBank® accession no. NM_017902), 5′-AACTGGGCCTACAAGCTCAA-3′/5′-CTTGCACCCCTAGTGTGGAT-3′; for human CA9 (carbonic anhydrase IX) (GenBank® accession no. NM_001216): 5′-CACTCCTGCCCTCTGACTTC-3′/5′-AGAGGGTGTGGAGCTGCTTA-3′. HotStarTaq DNA Polymerase (Qiagen) was used for RT-PCR amplifications. For quantificative real-time PCR analysis, the SYBR Green PCR Master Mix (PerkinElmer) and ABI Prism 7700 Detection System was used. Single-band amplification was verified through multicomponent analysis.

HIF peptide hydroxylation assay

U251 cell cytoplasmic extract was made by harvesting cells in lysis buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$ and 1 mM dithiothreitol) at 4 °C using a Dounce homogenizer. The lysate was centrifuged at 20 000 ***g*** for 15 min. The supernatant (200 μg) was used as the source of enzyme in the ^{35}S-labelled pVHL pulldown assay, as described previously by Bruick et al. [8].

U251 cell HRE (hypoxia-response element)–luciferase assay

U251 cells stably expressing a luciferase reporter gene under the control of three copies of HRE (a gift from Dr Giovanni Melillo, National Cancer Institute-Frederick, Frederick, MD, U.S.A.) were cultured in 12-well plates. Luciferase assays were performed in a 96-well microtiter plate luminometer (Dynex Technologies) using the Luciferase Assay System (Promega). Each treatment was carried out in triplicate.

RESULTS

Human glioma cells express HIF hydroxylases and degrade HIF-1α more rapidly after prolonged hypoxia

Three different nomenclatures are currently used for the three identified human homologues of HIF prolyl hydroxylases: the HPH nomenclature reflects the unique HIF prolyl hydroxylase

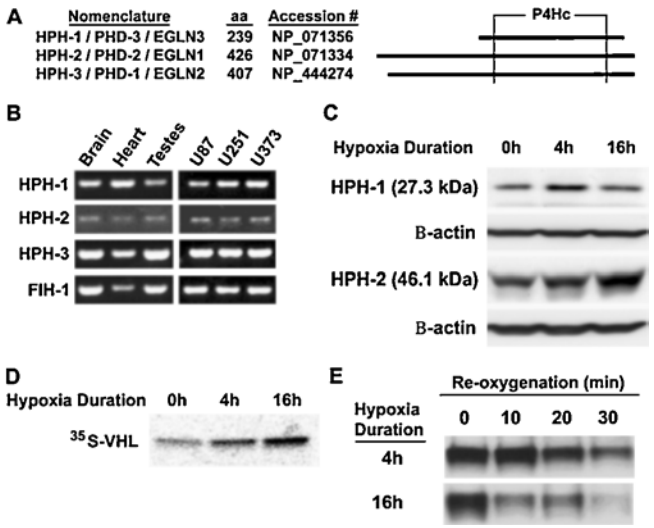

Figure 1 Human glioma cells express inducible oxygen sensors

The expression of oxygen sensors in human glioma cells was studied by PCR analysis, HIF-1α protein decay, HIF-1α ODD hydroxylation activity and Western blotting. (**A**) Schematic depiction of HPH homologues with their respective nomenclatures, amino acid content, GenBank® accession numbers and relative position of the consensus prolyl 4-hydroxylase domain (P4Hc). (**B**) PCR amplification of HPH homologues and FIH-1 from human glioma cDNA as compared with cDNA from human brain, heart and testis. (**C**) Hypoxia increases HPH protein levels. Cell extracts were prepared from control cells grown in 21 % O_2 (0 h) or from cells pretreated for 4 h and 16 h with 1 % O_2. Western blot analysis of the U251 extracts shows increased immunoreactivity for HPH-1 and HPH-2. β-Actin was used as a loading control. (**D**) Induction of HIF prolyl hydroxylase activity by hypoxia in U251 cells. Cell extract (200 μg) prepared in (**C**) was used for proline hydroxylation of an immobilized HIF-1α peptide, and was assayed by the ^{35}S-labelled pVHL pull-down assay, as described in the Experimental section. Extracts from cells exposed to increasing duration of hypoxia displayed increasing HIF-1α-peptide-hydroxylating activity. (**E**) Enhancement of HIF-1α decay rate following prolonged periods of hypoxia. U251 cell nuclear extracts were analysed by Western blotting for HIF-1α protein at different re-oxygenation times, as indicated, following either 4 h or 16 h exposure to 1 % O_2.

activity of these enzymes and is the nomenclature used here. The PHD nomenclature reflects the presence in these enzymes of a consensus prolyl hydroxylase domain that is found in other prolyl hydroxylases, such as those involved in collagen synthesis [7]. The EGLN nomenclature reflects the homology of these enzymes to the *Caenorhabditis elegans* egg-laying defect gene, EGL-nine [7]. The relationship between these nomenclatures is such that HPH-1, HPH-2, HPH-3 $\equiv$ PHD-3, PHD-2, PHD-1 $\equiv$ EGLN-3, EGLN-1, EGLN-2 respectively (Figure 1A).

Mammalian tissues display unique expression profiles for HPH mRNAs. Using specific PCR primers we identified mRNA for all 3 HPH homologues, as well as FIH-1, in each of the U87, U251 and U373 human glioma cells lines (Figure 1B). The relative expression profile of these genes was similar among the glioma cell lines, and more similar to that of human brain than of heart or testis [15] (Figure 1B). Both HPH-1 and HPH-2 have recently been shown to be up-regulated by hypoxia [7,13,16,17]. Using polyclonal antibodies against HPH-1 and HPH-2, we also observed enhanced expression of the HPH-1 and HPH-2 proteins following hypoxia (1 % O_2) for 4 h and 16 h, as compared with normoxic (21 % O_2) controls (Figure 1C). Using the same glioma cell extracts that displayed enhanced HPH-1 and HPH-2 protein expression, we also observed a greater enhancement of HIF-1α ODD peptide hydroxylating activity in extracts from U251 cell exposed to 16 h of hypoxia compared with those exposed to only 4 h of hypoxia (Figure 1D). HPHs promote proteolytic decay of HIFα subunits. HeLa cervical carcinoma cells have been shown to display an acceleration of HIF-1α decay following prolonged hypoxia [18]. Similarly, we found that in human U251 glioma cells, the rate of HIF-1α decay upon re-oxygenation was faster following 16 h of exposure to hypoxia (1 % O2) than following 4 h of hypoxia (Figure 1E). Thus human glioma cells express inducible HPH homologues.

2-Oxoacid metabolites of glycolysis and the TCA cycle can activate HIF

The HIF pathway can also be activated by several stimuli under normoxia [22]. We recently employed short-term culture of human glioma cells in Krebs buffer to identify pyruvate as a key glucose metabolite that could increase HIF-1α protein levels [21]. To distinguish the contribution of glucose metabolites from other stimuli in HIF activation, we cultured glioma cells for several hours in glucose-free Krebs buffer with selective additions (Figure 2). The glioma cell lines grown in this manner were found to be viable for at least 24 h and were capable of robust HIF activation. This was demonstrated using Western blotting to assess HIF-1α protein stabilization in both U87 and U251 cells following 4 h exposure to hypoxia, DFO, cobalt or DMOG (Figure 2A). Similar to these well-known stimulators of HIF-1, we found that pyruvate and oxaloacetate, two endogenous 2-oxoacid analogues of 2-oxoglutarate that are derived from glycolysis and the TCA cycle respectively, were also efficient inducers of HIF-1α protein stability (Figure 2A). Other TCA cycle intermediates that occur between pyruvate and oxaloacetate were without effect ([21], and results not shown). Similar to hypoxia and the other hypoxia-mimicking agents, pyruvate and oxaloacetate also clearly promoted HIF-1α nuclear translocation (Figure 2B), as well as the expression of a luciferase construct under the control of a HIF-regulated promoter (Figure 2C). 2-Oxoglutarate itself did not promote HIF-1α accumulation (Figure 2B). Thus, similar to pyruvate, the TCA cycle intermediate oxaloacetate can specifically promote HIF-1α stability and HIF-1 transactivational activity independently of hypoxia.

Pyruvate and oxaloacetate differentially induce expression of oxygen sensors in glioma cells

To evaluate the contribution of hypoxic and normoxic HIF-1α activators in regulating the expression of HIF-1α hydroxylating enzymes, we cultured U251 glioma cells in glucose-free Krebs for 8 h and individually determined the effects of hypoxia, hypoxia-mimicking agents, pyruvate and oxaloacetate on the expression of HPH-1, HPH-2, HPH-3 and FIH-1 mRNA by using quantificative real-time RT-PCR analysis. As shown in Figure 3(A), hypoxia, DFO, cobalt, DMOG, pyruvate and oxaloacetate all markedly enhanced the expression of HPH-1 and HPH-2, but not HPH-3 or FIH-1. As expected the expression of CA9, a well-known HIF-1 regulated gene, was also upregulated by these stimuli. The enhancement of HPH-1 and HPH-2 by all stimuli, as shown for pyruvate in Figure 3(B), was inhibited by actinomycin D. Moreover, protein levels of both HPH-1 and HPH-2 were upregulated by each of these treatments (Figure 3C). These studies were performed in glucose-free buffers in order to eliminate substantial contribution to HIF-1 activation via a build-up of glycolytic metabolites. To confirm that glucose metabolism could indeed influence expression of HPH homologues, we also employed an alternate pharmacological strategy. U251 cells were transferred from medium into Krebs buffer with or without 20 mM glucose. A significantly higher expression level of HPH-1 and HPH-2 was seen after 8 h culture in the glucose-containing buffer, which was blocked by the presence of 5 μM iodoacetate (Figure 4). The inhibitory effect of iodoacetate could, however, be bypassed

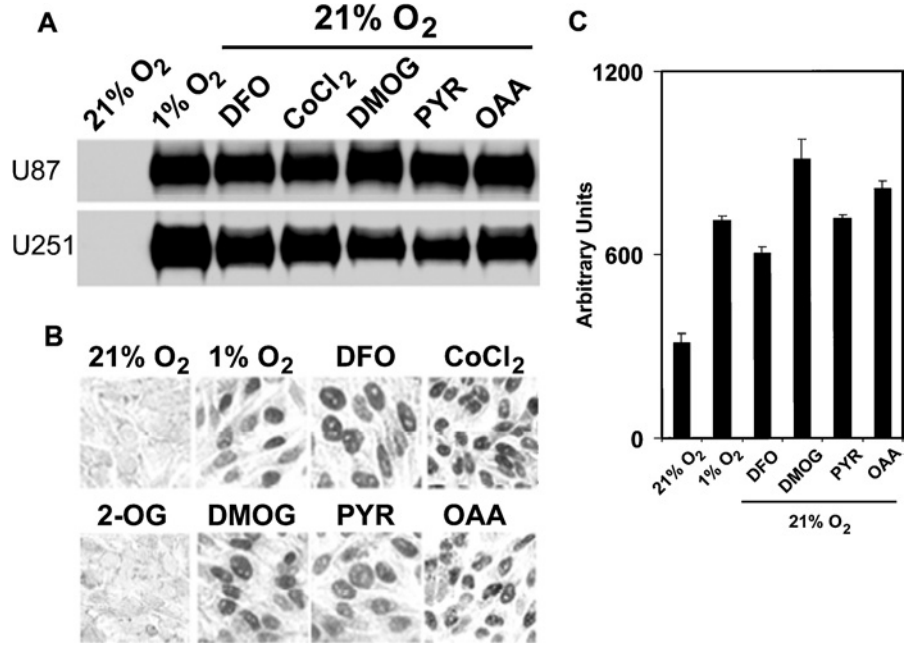

Figure 2 Normoxic activation of HIF-1 by endogenous 2-oxoacids

In order to independently examine HIF-1 activation by hypoxia, hypoxia mimetics and glucose-derived metabolites, U87 and U251 cells were switched from their culture media to glucose-free Krebs–Henseleit buffer. (**A**) Cells were treated with either hypoxia (1 % O_2) or the indicated stimuli under normoxia (21 % O_2). Following 4 h treatment, cells were evaluated for nuclear HIF-1α levels by Western blot analysis. PYR, pyruvate; OAA, oxaloacetate. (**B**) Direct nuclear accumulation of HIF-1α levels was monitored in U251 cells by immunocytochemsitry after treating cells with 1 % O_2, or the indicated stimuli under normoxia in glucose-free Krebs–Henseleit buffer for 4 h. 2-OG, 2-oxoglutarate. (**C**) Increases in HIF-1-mediated gene expression was monitored in U251 cells stably transfected with HRE–lucerifase reporter construct. Cells in 12-well plates were switched to glucose-free Krebs–Henseleit buffer, and treated with 1 % O_2 or the indicated stimuli under normoxia for 4 h. Doses used: DFO, 150 μM; $CoCl_2$, 100 μM; DMOG, 1 mM in (**A**) and (**B**), and 0.5 mM in (**C**); pyruvate, 2 mM; oxaloacetate, 2 mM. Experiments were performed in triplicate and repeated at least twice. Results from all treatments were statistically significant with respect to 21 % O_2 ($P < 0.01$, Student's t test).

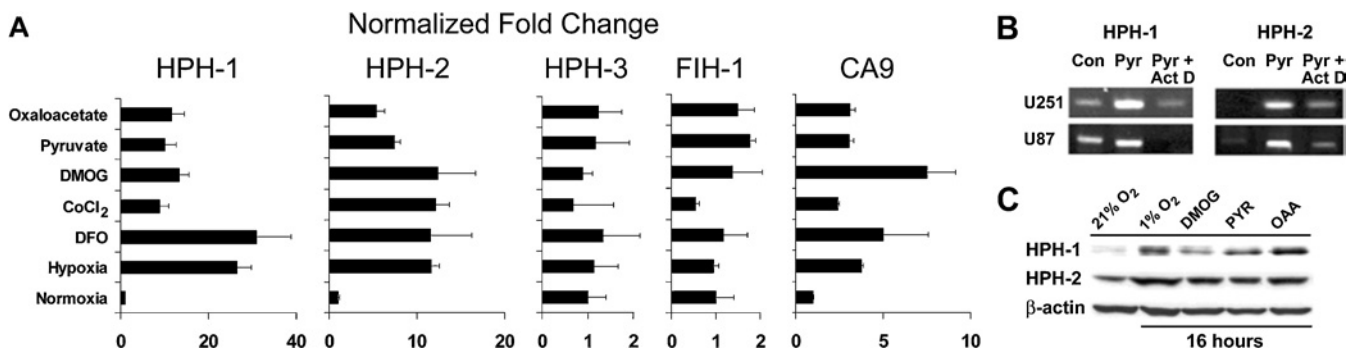

Figure 3 Differential regulation of oxygen sensor mRNA expression by hypoxia, hypoxia mimetics and endogenous 2-oxoacids

(**A**) Cells were cultured as described earlier, switched to glucose-free Krebs–Henseleit buffer, and treated with 1 % O_2, or the following agents in normoxia for 8 h: 150 μM DFO, 100 μM $CoCl_2$, 1 mM DMOG, 2 mM pyruvate or 2 mM oxaloacetate. The level of mRNA was determined by quantificative RT-PCR using the SYBR green dye, as described in the Experimental section. The amount of each mRNA in samples was normalized to the HPRT1 mRNA in the same sample. Genomic amplification was minimized by using primers that span exons and by verifying single amplicons through multicomponent analysis. Results are represented as the fold change of normalized level of mRNA in the treatment over the value obtained in normoxic controls. The mean from at least four independent experiments is shown. For HPH-1, HPH-2 and CA9, results from all treatments were statistically significant with respect to normoxia ($P < 0.01$, Student's t test). (**B**) U251 and U87 cells were switched to Krebs–Henseleit buffer and treated with 2 mM pyruvate (Pyr) for 8 h in the presence or absence of 0.5 μg/ml of actinomycin D (Act D), as indicated. mRNA levels of HPH-1 and HPH-2 were analysed by RT-PCR and compared with untreated controls (Con). A representative experiment, out of two, is shown. (**C**) U251 cells were switched to Krebs–Henseleit buffer and treated with 1 % O_2, or the following agents in normoxia for 16 h: 1 mM DMOG, 2 mM pyruvate (PYR) or 2 mM oxaloacetate (OAA). Western blot analysis was performed as described in the Experimental section.

by the inclusion of 2 mM pyruvate or oxaloacetate in the culture buffer.

DISCUSSION

The main novel data presented in the present study are that oxaloacetate, a TCA cycle metabolite, can activate HIF, and that both pyruvate and oxaloacetate can also induce expression of HPH-1 and HPH-2 genes. HIF prolyl and asparaginyl hydroxylases are now recognized as mammalian oxygen sensors that belong to the large family of oxygen-, iron- and 2-oxoglutarate-dependent dioxygenases [7,8,11]. HIF hydroxylase activity can be inhibited by lack of oxygen and displacement of iron or 2-oxoglutarate. This observation explains the ability of several

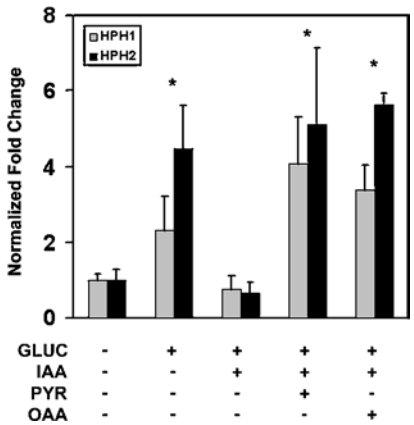

Figure 4 Regulation of oxygen sensor mRNA expression by glycolysis is inhibited by the glycolysis inhibitor, iodoacetate, and restored by endogenous 2-oxoacids

Cells were cultured as described earlier, switched to glucose-free Krebs–Henseleit buffer, and were treated with the following agents in normoxia for 8 h, as indicated: 20 mM glucose (GLUC), 5 μM iodoacetate (IAA), 2 mM pyruvate (PYR) and/or 2 mM oxaloacetate (OAA). The level of mRNA was determined by quantificative RT-PCR using the SYBR green dye, as described in the Experimental section. The amount of each mRNA in samples was normalized to the HPRT1 mRNA in the same sample. Genomic amplification was minimized by using primers that span exons and by verifying single amplicons through multicomponent analysis. Results are represented as the fold change of normalized level of mRNA in the treatment over the value obtained in normoxic controls. The means $\pm$ S.D. for three independent experiments are shown. For all treatments, results were statistically significant with respect to the normoxia and glucose plus iodoacetate (IAA) treatments ($P < 0.05$, Student's t test).

mimickers of hypoxia to activate HIF. Altered gene expression of specific HPH homologues is a newly appreciated means of regulating hypoxic transcriptional responses. So far, the HPH-1 and HPH-2 homologues are known to be inducible genes with hypoxia being the only known physiological stimulus capable of their induction [7,13,16,17]. HIF-1 is strongly implicated in the hypoxic induction of HPH homologues, thus suggesting a novel feedback loop for blunting hypoxic gene expression. In the present work, we have confirmed the existence of such a feedback mechanism in human glioma cells. We have demonstrated that hypoxia up-regulates mRNA, protein and enzymic activity of HPH-1 and HPH-2. Increased HIF prolyl hydroxylation activity with increasing duration of hypoxia may underlie the faster rate of HIF-1α proteolysis seen upon re-oxygenation (Figure 1). This may represent a mechanism for limiting hypoxia-inducible gene expression and also for adapting to re-oxygenation. Additionally, changes in the relative expression profile of oxygen sensors may be of physiological relevance, since the three HPHs have differing affinity for HIFα subunits [23,24] and individual HPH homologues may have different activities toward the two target proline residues on various HIFα subunits [7,23]. Differential induction of HPH homologues may thus alter the relative cellular ratios of HIFα subunit isoforms.

Although hypoxia is the ubiquitous inducer of HIF-1α in all cells tested, other physiological stimuli, such as cytokines, growth factors, reactive oxygen species, nitric oxide and pyruvate, can also activate HIF under normoxic conditions [20,21]. The mechanism of action for most of these normoxic HIF activators remains unclear and only nitric oxide has been shown to directly inhibit HIF prolyl hydroxylases [25]. We have shown here that the endogenous 2-oxoacids pyruvate and oxaloacetate can both effectively stabilize HIF-1α and activate HIF-dependent gene expression (Figure 2). These results provide the first evidence for regulation of the HIF-1 pathway by oxaloacetate. Although similar to pyruvate, oxaloacetate is a key intermediate at the crossroads of several metabolic pathways, it plays unique biochemical roles in transferring reducing equivalents between the mitochondrial and cytoplasmic compartments, and also serves as the key intermediate in gluconeogenesis. Both pyruvate and oxaloacetate have been shown to inhibit some 2-oxoglutarate-dependent dioxygenases [26], but not others [27]. By acting as endogenous antagonists for HIF hydroxylases, pyruvate and oxaloacetate may constitute a hypoxia-independent regulation of HIF-mediated gene expression, which responds to changes in cellular energy metabolism and nutritional status. Moreover, inhibition of mitochondrial oxidative metabolism during hypoxia could lead to elevated levels of these 2-oxoacids, thus suggesting a collaborative role for these metabolites in hypoxic signalling.

Anaerobic energy metabolism is prominently stimulated by HIF activation via the induction of several genes for glucose transporters and glycolytic enzymes [28–30]. The hypoxia-induced up-regulation of HIF which elevates levels of anaerobic metabolites, such as pyruvate and oxaloacetate, could act in a feedforward manner to sustain HIF activation, even upon re-oxygenation. We postulated that, as with hypoxia, normoxic HIF activators may also enhance the expression of HPH homologues to prevent such a feedforward cycle. Like hypoxia, both pyruvate and oxaloacetate were found to up-regulate HPH-1 and HPH-2 mRNA levels in cell culture (Figure 3). Furthermore, inhibition of ongoing glycolytic metabolism by iodoacetate inhibited the expression of HPH-1 and HPH-2. This provides the first evidence for transcriptional regulation of HPH homologues by physiological stimuli other than oxygen. Regulation of HPH gene expression by glycolytic and TCA cycle metabolites could independently modify the oxygen-sensing abilities of cells, and may underlie important interactions between oxygen homoeostasis and glucose metabolism [29,30]. Intermediates of cellular energy metabolism may also influence the newly identified actions of HPH homologues in regulating cell growth control [31,32].

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REFERENCES

1 Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer **3**, 721–732
2 Wang, G. L. and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. **270**, 1230–1237
3 Huang, L. E., Gu, J., Schau, M. and Bunn, H. F. (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin–proteasome pathway. Proc. Natl. Acad. Sci. U.S.A. **95**, 7987–7992
4 Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L. (1999) Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin–proteasome pathway. J. Biol. Chem. **274**, 6519–6525
5 Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (London) **399**, 271–275
6 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-α to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science (Washington, D.C.) **292**, 468–472
7 Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhanda, A. et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell (Cambridge, Mass.) **107**, 43–54
8 Bruick, R. K. and McKnight, S. L. (2001) A conserved family of prolyl-4-hydroxylases that modify HIF. Science (Washington, D.C.) **294**, 1337–1340

9 Sang, N., Fang, J., Srinivas, V., Leshchinsky, I. and Caro, J. (2002) Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1α is governed by a von Hippel–Lindau protein-independent, hydroxylation-regulated association with p300/CBP. Mol. Cell. Biol. **22**, 2984–2992
10 Mahon, P. C., Hirota, K. and Semenza, G. L. (2001) FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. **15**, 2675–2686
11 Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. **16**, 1466–1471
12 McNeill, L. A., Hewitson, K. S., Claridge, T. D., Seibel, J. F., Horsfall, L. E. and Schofield, C. J. (2002) Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the β-carbon of asparagine-803. Biochem. J. **367**, 571–575
13 Metzen, E., Berchner-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W. et al. (2003) Intracellular localisation of human HIF-1α hydroxylases: implications for oxygen sensing. J. Cell Sci. **116**, 1319–1326
14 Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R. and Taubman, M. B. (2002) Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. Biochem. Cell Biol. **80**, 421–426
15 Oehme, F., Ellinghaus, P., Kolkhof, P., Smith, T. J., Ramakrishnan, S., Hutter, J., Schramm, M. and Flamme, I. (2002) Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem. Biophys. Res. Commun. **296**, 343–349
16 Naranjo-Suarez, S., Castellanos, M. C., Alvarez-Tejado, M., Vara, A., Landazuri, M. O. and del Peso, L. (2003) Down-regulation of hypoxia-inducible factor-2 in PC12 cells by nerve growth factor stimulation. J. Biol. Chem. **278**, 31895–31901
17 del Peso, L., Castellanos, M. C., Temes, E., Martin-Puig, S., Cuevas, Y., Olmos, G. and Landazuri, M. O. (2003) The von Hippel–Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. J. Biol. Chem. **278**, 48690–48695
18 Berra, E., Richard, D. E., Gothie, E. and Pouyssegur, J. (2001) HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1α degradation. FEBS Lett. **491**, 85–90
19 Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D. and Pouyssegur, J. (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
20 Bilton, R. L. and Booker, G. W. (2003) The subtle side to hypoxia inducible factor (HIFα) regulation. Eur. J. Biochem. **270**, 791–798
21 Lu, H., Forbes, R. A. and Verma, A. (2002) Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J. Biol. Chem. **277**, 23111–23115
22 Chun, Y. S., Kim, M. S. and Park, J. W. (2002) Oxygen-dependent and -independent regulation of HIF-1α. J. Korean Med. Sci. **17**, 581–588
23 Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I. and Myllyharju, J. (2003) Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J. Biol. Chem. **278**, 30772–30780
24 Huang, J., Zhao, Q., Mooney, S. M. and Lee, F. S. (2002) Sequence determinants in hypoxia-inducible factor-1α for hydroxylation by the prolyl hydroxylases PHD1, PHD2, and PHD3. J. Biol. Chem. **277**, 39792–39800
25 Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J. and Brune, B. (2003) Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. Mol. Biol. Cell **14**, 3470–3481
26 Ng, S. F., Hanauske-Abel, H. M. and Englard, S. (1991) Cosubstrate binding site of *Pseudomonas* sp. AK1 γ-butyrobetaine hydroxylase: interactions with structural analogs of α-ketoglutarate. J. Biol. Chem. **266**, 1526–1533
27 Kaule, G., Timpl, R., Gaill, F. and Gunzler, V. (1998) Prolyl hydroxylase activity in tissue homogenates of annelids from deep sea hydrothermal vents. Matrix Biol. **17**, 205–212
28 Semenza, G. L., Roth, P. H., Fang, H. M. and Wang, G. L. (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem. **269**, 23757–23763
29 Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K. and Johnson, R. S. (2001) Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. Mol. Cell. Biol. **21**, 3436–3444
30 Minchenko, A., Leshchinsky, I., Opentanova, I., Sang, N., Srinivas, V., Armstead, V. and Caro, J. (2002) Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene: its possible role in the Warburg effect. J. Biol. Chem. **277**, 6183–6187
31 Erez, N., Milyavsky, M., Eilam, R., Shats, I., Goldfinger, N. and Rotter, V. (2003) Expression of prolyl-hydroxylase-1 (PHD1/EGLN2) suppresses hypoxia inducible factor-1α activation and inhibits tumor growth. Cancer Res. **63**, 8777–8783
32 Frei, C. and Edgar, B. A. (2004) *Drosophila* cyclin d/cdk4 requires hif-1 prolyl hydroxylase to drive cell growth. Dev. Cell **6**, 241–251

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