Reversible Inactivation of HIF-1 Prolyl Hydroxylases Allows Cell Metabolism to Control Basal HIF-1*8

Huasheng Lu1, Clifton L. Dalgard1,2, Ahmed Mohyeldin1, Thomas McFate2, A. Sasha Tait, and Ajay Verma3
From the Department of Neurology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Continuous hydroxylation of the HIF-1 transcription factor α subunit by oxygen and 2-oxoglutarate-dependent dioxygenases promotes decay of this protein and thus prevents the transcriptional activation of many genes involved in energy metabolism, angiogenesis, cell survival, and matrix modification. Hypoxia blocks HIF-1α hydroxylation and thus activates HIF-1α-mediated gene expression. Several nonhypoxic stimuli can also activate HIF-1, although the mechanisms involved are not well known. Here we show that the glucose metabolites pyruvate and oxaloacetate inactivate HIF-1α decay in a manner selectively reversible by ascorbate, cysteine, histidine, and ferrous iron but not by 2-oxoglutarate or oxygen. Pyruvate and oxaloacetate bind to the 2-oxoglutarate site of HIF-1α prolyl hydroxylases, but their effects on HIF-1 are not mimicked by other Krebs cycle intermediates, including succinate and fumarate. We show that inactivation of HIF-1 hydroxylation by glucose-derived 2-oxoacids underlies the prominent basal HIF-1 activity commonly seen in many highly glycolytic cancer cells. Since HIF-1 itself promotes glycolytic metabolism, enhancement of HIF-1 by glucose metabolites may constitute a novel feed-forward signaling mechanism involved in malignant progression.

Mammalian cells adapt to hypoxia through the action of the heterodimeric transcription factor HIF-1. Such adaptations can also promote carcinogenesis by inducing angiogenesis, treatment resistance, and invasiveness in hypoxic cancer cells within tumors (1). In the presence of oxygen, the HIF-1α subunit undergoes rapid decay via a ubiquitin-proteasome degradation pathway involving the von Hippel-Lindau tumor suppressor gene product pVHL (2–4). The binding of pVHL to HIF-1α requires the post-translational hydroxylation of proline residues (Pro402 and Pro564) within the HIF-1α oxygen-dependent degradation (ODD)4 domain (5, 6). This modification is prevented during hypoxia, thus allowing HIF-1α to escape proteolysis, dimerize with HIF-1β, and translocate to the nucleus. A separately controlled, O2-dependent hydroxylation of asparagine 803 in the HIF-1α C-terminal transactivation domain inhibits HIF-1 interaction with the p300/CBP coactivator, thereby blocking HIF-1 transcriptional activity in the presence of oxygen (7, 8). Three HIF-1α prolyl hydroxylases (HPH1 to -3; also referred to as PHD3 to -1, respectively) and one O2-dependent HIF-1α asparaginyl hydroxylase (factor inhibiting HIF, or FIH) have been clearly identified so far (9–11). These enzymes are all members of the 2-oxoglutarate-dependent family of dioxygenases and have an absolute requirement for oxygen, ferrous iron, and 2-oxoglutarate (2-OG).

This explains how hypoxia, iron chelators such as desferrioxamine (DFO), and artificial 2-OG analogs such as N-oxalylglycine or its cell-permeable precursor dimethylglyoxaliglycine (DMOG) can all prevent HIF-1α proteolysis and activate HIF-mediated gene expression. Ascorbate is also required for the sustained activity of many 2-OG-dependent dioxygenases (12, 13).

Hypoxia-independent mechanisms also regulate HIF-1 and are highly relevant to the progression of human cancers. This is because high levels of HIF-1α protein are seen not only in hypoxic tumor zones, but also in well oxygenated tumor areas and metastatic nodules (1). In fact, many cancer cell lines cultured in room air (21% O2) routinely display significant basal levels of HIF-1α protein as well as high basal expression of genes and biological activities controlled by HIF-1 (14). The mechanisms underlying basal HIF-1 expression in cancer have been highly pursued recently. Growth factors (e.g. insulin, insulin-like growth factor-1, EGF, and heregulin) that stimulate the phosphatidylinositol 3-kinase-AktmTOR pathways can activate HIF-1 in the presence of O2, probably through the stimulation of HIF-1α translation (15). Reactive oxygen species (ROS) (16) and nitric oxide (NO) may promote HIF-1 activation by direct actions on HPHs (17). We recently discovered that accumulation of the glycolytic metabolites lactate and pyruvate could also promote hypoxia-independent HIF-1 activation (18). Further support for a distinct oxygen-independent mechanism for HIF-1 regulation was recently provided by the observation that ascorbate selectively reverses basal but not hypoxia-induced HIF-1α accumulation (19). Despite these clues, the main factors underlying high basal HIF-1 levels in cancer cells remain unclear. We therefore set out to identify the major determinants of hypoxia-independent HIF-1 regulation in cancer cell lines.

MATERIALS AND METHODS

Cell Culture and Hypoxia Treatment—Human U87, U251, U251-HRE (20), and U373 glioma cells were cultured in Eagle’s MEM (Mediatech). DU145 human prostate cancer cells and O22 human head and neck cancer cells were cultured in RPMI 1640 medium (Sigma). 22B human head and neck cancer cells were cultured in high glucose DMEM (Invitrogen) containing 2 mM glutamine. C6 ODD-GFP rat glioma cells (21) were cultured in high glucose DMEM with 1.5 mg/ml G418. All culture media were supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. Rat C6 glioma cells were cultured in high glucose DMEM. For cell hypoxia treatment, the culture dishes were sealed in a modular incubator chamber, flushed with gas containing 1%
O₂, 5% CO₂, and 94% N₂ for 5 min, and incubated in this environment at 37 °C for the indicated times.

**Antibodies and Chemical Reagents—**Protein extraction. Western blot analysis, and immunocytochemistry were performed as described (18, 22). Mouse monoclonal anti-HIF-1α antibodies were 610958 (BD Biosciences) and NB100-123 (Novus). Mouse monoclonal anti-GFP antibody was 1814660 (Roche Applied Science). Mouse monoclonal anti β-actin antibody was ab6276—100 (Abcam). Dimethylsulfoxide/glycine was D1070 (Frontier Scientific). All other chemicals were from Sigma.

Antibodies recognizing HPH homologues were from Novus Biologicals, and the antiserum recognizing hydroxyproline 654 of HIF-1α was kindly provided by R. Freeman.

**In Vitro Translation and HPH Prolyl Hydroxylation Assay—**Human HPH1, -2, and -3 and VHL pcDNA3.1/V5-HIS vectors (9) (kindly provided by R. Bruick) were used for TNT coupled reticulocyte lysate in vitro transcription/translation (Promega). Cytoplasmic extract for HPH activity was made by lysing cell pellets in assay buffer (20 mM Tris, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, and 1 mM dithiothreitol) at 4 °C using a Dounce homogenizer, centrifuging at 20,000 × g for 15 min, and collecting supernatant for the assay. HPH enzymatic reactions were performed as described using either IVT products or cell extracts (23). This assay, chosen for its specificity for the HPH reaction, was linear over 30 min. For each reaction, either 7.5 μl of IVT products or 100 μg of cell extract protein were used as a source of the enzyme. Activity was measured by either quantitative scintillation counting or autoradiography after gel electrophoresis on a 10–20% Novex Tris/glycine gradient gel (Invitrogen).

**2-Oxoglutarate-HPH Binding Assay—**Epoxy-activated Sepharose (Amersham Biosciences) coupled to 2-oxoglutarate was produced using the manufacturer’s recommended protocol (24). 25,000 cpm of 35S-labeled human HPH protein was added to 100 μl of 2-oxoglutarate-Sepharose gel. The gel was washed four times with the indicated buffer. Analysis of HPH binding was measured by scintillation counting.

**VHL-ODD Binding Assay—**35,000 cpm of 35S-labeled human VHL protein was added to 1 μl of hydroxylated HIF peptide bound to Ultra-Link ImmunoPure immobilized Streptavidin beads (Pierce) in the indicated buffer. The beads were washed three times with cold NTEN buffer, and bound 35S-labeled VHL was measured by scintillation counting.

**HIF-1 Reporter Assays—**HIF-1 luciferase reporter assay was performed using U-251-HRE cells as described (20).

**RT-PCR and Quantitative RT-PCR Analysis—**Total RNA was isolated using the RNeasy kit (Qiagen). For RT-PCR, 1 μg of total RNA was used with the SUPERSCRIPT One-Step System (Invitrogen). VEGF, GLUT3, and β-actin primers were described previously (18). For quantitative PCR analysis, the iQ SYBR Green Master Mix (Applied Biosystems) was used with the SUPERScript One-Step System (Invitrogen). VEGF, GLUT3, and β-actin primers were described previously (18). For quantitative PCR analysis, the iQ SYBR Green Master Mix (Applied Biosystems) was used with the SUPERScript One-Step System (Invitrogen). VEGF, GLUT3, and β-actin primers were described previously (18). For quantitative PCR analysis, the iQ SYBR Green Master Mix (Applied Biosystems) was used with the SUPERScript One-Step System (Invitrogen). VEGF, GLUT3, and β-actin primers were described previously (18).

**RESULTS**

**Specific Metabolic Fuels Support Hypoxia-independent HIF-1α Levels—**Serum is a major source of growth factors and the main source of iron in cell culture, whereas medium provides metabolic fuels. When we examined basal HIF-1α levels in several cancer cell lines cultured in medium with widely varying glucose concentrations but identical serum supplementation, we observed that basal HIF-1α accumulation, which is known to be ascorbate-reversible (19), was typically higher in cell lines grown in high glucose DMEM (25 mM) than in RPMI (11.1 mM) or MEM (5.5 mM) (Fig. 1A). The time-dependent accumulation of basal HIF-1α in 22B cells required glucose but was not affected by the presence or absence of 10% fetal bovine serum (Fig. 1B). These cells are cultured in DMEM along with the nonglycolytic energy source glutamine. With or without glucose, the 22B cells were still able to prominently induce HIF-1 under hypoxia (Fig. 1B). This not only showed that the cells could maintain prolonged viability in the presence of glutamine as the sole energy source, but also clearly

**Cell Invasion Assay—**Cell invasion experiments were performed using 24-well BioCoat Matrigel™ invasion chambers with an 8-μm pore polycarbonate filter according to the manufacturer’s instructions (catalog no. 35-4480; BD Biosciences). Cells in the growing phase were trypsinized and resuspended at a concentration of 1 × 10⁵ cells/ml in medium with 0.5% fetal bovine serum. The lower compartment of the plate received 750 μl of serum-free or serum medium. All drug or serum treatments were added to the lower compartment of the plate prior to cell plating. 5 × 10⁴ cells were plated in each insert and allowed to invade for 48 h at 37 °C in a humidified incubator with 21% O₂. Cells that remained inside the insert after 48 h were thoroughly washed with a cotton swab, and invading cells were fixed and stained using Diff-Quick Stain Solution (Dade Bering). Stained invading cells were quantified by counting in 10 predetermined fields at ×20 magnification. All treatment groups were studied in triplicate. Difference in invasion was statistically analyzed using a two-tailed Student’s t test.

**Cellular Reactive Oxygen Species and Nitrite Measurements—**Cells (80% confluent) were loaded with 5 μM CM-H2DCFDA (Molecular Probes, Inc., Eugene, OR) in Krebs buffer for 1 h and then treated with 2 mM pyruvate or oxaloacetate for 2 h. Positive controls were treated with 40 μM H₂O₂ for 2 h. Cells were then collected, washed twice with phosphate-buffered saline, and resuspended in phosphate-buffered saline plus 0.1% (w/v) bovine serum albumin before measuring fluorescence in a Coulter flow cytometer. Nitrite concentration in culture medium was measured as described (25).

**VEGF Enzyme-linked Immunosorbent Assay, Glucose Metabolites, and Cellular ATP Assays—**Culture medium VEGF was measured via an enzyme-linked immunosorbent assay (Quantikine). Glucose, lactate, and pyruvate were measured in phenol red-free DMEM using a CMA 600/microdialysis analyzer (CMA Microdialysis AB). Cellular ATP levels were measured using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science).

**Calcein Fluorescence Assay for Cellular Iron—**The ability of 2-oxoacids to chelate the labile intracellular iron pool was measured using the calcein fluorescence assay as described by Epsztejn et al. (26). The iron chelators desferrioxamine and bipyridyl were used as positive controls.

**EF-5 Assay for Cellular Hypoxia—**Cellular hypoxia was monitored via immunofluorescence detection of EF-5 cellular adducts as described (27).
distinguished glycolytic and hypoxic regulation of HIF-1α as separate mechanisms.

The U87 and U251 human glioblastoma cells (cultured in MEM) were used to further explore the relationship between glucose metabolism and basal HIF-1α. When switched from their native MEM to Krebs buffer for 4 h, U251 cells showed no basal HIF-1α accumulation if glucose was replaced by the nonglycolytic energy source glutamine (Fig. 1C). Despite the presence of 10 mM glutamine, basal HIF-1α accumulation in U251 cells was dose-dependently increased by glucose.

2-Oxoacid Functional Groups Distinguish Endogenous HIF-1α-stabilizing Metabolites—Since glucose feeds into many metabolic pathways, we determined which cellular intermediary metabolites were capable of promoting HIF-1α accumulation when substituted for glucose in Krebs buffer. An extensive analysis of all glycolytic, all tricarboxylic acid cycle, and many amino acid metabolic intermediates revealed that most metabolic substrate substitutions did not significantly alter cellular ATP levels over 4 h of cell culture (supplemental Fig. S1A). Moreover, only a select group of 2-oxoacids was found to promote HIF-1α accumulation. Of these, only pyruvate and

![FIGURE 1](image-url)

**A.** Human cancer cell lines in culture media

| Glucose (25 mM) | Serum (10% FBS) | Time (h) |
|-----------------|-----------------|----------|
| 22B             | MCF-7           | U145     | U87     | U251     |
| 2% O2           | +              | +        | +       | +        |
| 1% O2           | +              | +        | +       | +        |
| Asc (100 mM)    | +              | +        | +       | +        |

**B.** Glucose (25 mM) + Serum (10% FBS) + 1% O2 (4 h)

| Glucose (25 mM) | Serum (10% FBS) | Time (h) |
|-----------------|-----------------|----------|
| 22B             | MCF-7           | U145     | U87     | U251     |
| +              | +              | +        | +       | +        |

**C.** U251 cells in modified Krebs buffer

| Glc (mM) | Gin (10 mM) |
|----------|-------------|
| 0        | 0.25        |
| 1        | 0.25        |
| 2        | 0.25        |
| 5        | 0.25        |
| 10       | 0.25        |
| 20       | 0.25        |

**D.** 2-Oxoglutarate

- Pyruvate
- Oxaloacetate
- α-ketoglutarate
- α-ketocaproate
- N-α-oxalylglycine
- Lactate
- Malate
- α-ketoisocaproate
- α-ketoisovalerate
- Ketoisovalerate
- Ketoisocaproate
- Fumarate
- Pyruvatedehyde
- 3-Hydroxybutyrate
- α-ketocaprate

**E.** U75 Cells

- 1% O2
- 2-OG
- Asc

- DMOG
- Pyruvate
- Oxaloacetate
- Methylpyruvate
- AcCoA
- Citrate
- DMF
- Succinyl
- Kic
- Kiv
- Kmv
- M-Pyr
- Oaa
- Pyruvate
- Succinate

**U251 Cells**

- 2-OG
- Asc

- HRE luciferase activity was determined at 8 h. Cultures also contained 2-OG (10 mM) or ascorbate (100 μM) where indicated. AcCoA, acetyl-CoA; Cit, citrate; DMS, dimethylsulfonate; E-Pyr, ethyl-pyruvate; Fum, fumarate; Kic, ketoisocaproate; Kiv, ketoisovalerate; Kmv, ketomethylvalerate; M-Pyr, methylpyruvate; Oaa, oxaloacetate; Pyr, pyruvate; Succ, succinate.
oxaloacetate were found to be active in all cancer cell lines used in this study. The threshold dose for inducing HIF-1α accumulation by these 2-oxoacids over a 4-h period of cell culture was determined to be ~300 μM. However, doses as low as 100 μM induced HIF-1α with culture times longer than 8 h (supplemental Fig. S1B). The branched chain 2-oxoacids α-ketoisocaproate and α-keto-β-methylvalerate were also active in some but not all cell lines, and α-ketoisovalerate showed minor activity (Fig. 1C). The 1-carboxylate and 2-oxo functional groups common to 2-OG and to the artificial HIF-1α inducers N-oxalylglycine and DMOG (Fig. 1D) were also features of naturally occurring metabolic intermediates that induced HIF-1α. Succinate, fumarate, alanine, pyruvaldehyde, malate, acetoadipate, and β-hydroxybutyrate, all of which lack a 2-oxo group, were thus unable to induce HIF-1α accumulation in intact or digitonin-permeabilized cells (Fig. 1C and supplemental Fig. S1C). However, several 2-oxoacids such as ketomalonate, α-ketobutyrate, and α-ketoadipate, which contained the 1-carboxylate and 2-oxo groups, did not induce HIF-1α accumulation in intact cells, suggesting that molecular features opposite from the 2-oxoend of the molecule were also crucial for inducing HIF-1α. Phenylpyruvate and fluoropyruvate, which are modified on the end opposite the 2-oxoacid group, were not able to induce HIF-1α accumulation, whereas ethyl and methyl esters of pyruvate at the 1-position were effective. Although lactate can induce HIF-1α accumulation, this requires its conversion to pyruvate via lactate dehydrogenase (18).

**HIF-1 Activation by 2-Oxoacids Is Selectively Reversed by Ascorbate**—As with the basal HIF-1α expression of U87 and U251 glioma cells cultured in medium, induction of HIF-1α by 3 mM pyruvate or oxaloacetate in glucose-free Krebs buffer was also completely reversed by 100 μM ascorbate (Fig. 1E). This effect was not reversed by 10 mM 2-OG, and neither ascorbate or 2-OG could reverse HIF-1α induction by hypoxia or DMOG. This selective ascorbate blockade of pyruvate and oxaloacetate-inducible HIF-1α accumulation was observed in all cells used in this study (data not shown). Using U251 cells stably expressing a hypoxia regulatory element (HRE)-luciferase construct (U251-HRE cells; Fig. 1E), we found that pyruvate and oxaloacetate induced the HIF-1α-regulated reporter gene as effectively as hypoxia or DMOG. This selective ascorbate blockade of pyruvate and oxaloacetate-inducible HIF-1α accumulation was observed in all cells used in this study (data not shown). Using U251 cells stably expressing a hypoxia regulatory element (HRE)-luciferase construct (U251-HRE cells; Fig. 1E), we found that pyruvate and oxaloacetate induced the HIF-1α-regulated reporter gene as effectively as hypoxia or DMOG. A similar effect was seen using glioma cells transfected with HRE-green fluorescent protein (HRE-GFP; supplemental Fig. S1D). Induction of the HIF-1 reporter gene by the 2-oxoacids was also selectively reversed by ascorbate but not by 2-OG. Ascorbate did not reverse HRE-luciferase activation by hypoxia or by DMOG, again suggesting a unique mode of HIF-1α regulation by pyruvate and oxaloacetate.

**Cysteine, Histidine, and Glutathione Antagonize Glycolytic HIF Induction**—At an equivalent time in culture, we noted that HIF-1α expression in human glioma cells tended to be higher in Krebs buffer than in complete culture medium, even when the glucose concentration was identical. Thus, when U87 glioma cells cultured in MEM were changed either to Krebs buffer or to fresh MEM with identical (5.5 mM) glucose concentrations for 4 h, a much higher induction of HIF-1α was always seen in Krebs buffer (Fig. 2A). Since neither Krebs buffer nor the complete MEM contained ascorbate, this suggested to us that complete MEM contained some inhibitory activity that antagonized glycolytic activation of HIF-1α.

MEM differs from Krebs buffer by its vitamin and amino acid additives. Upon reconstitution of Krebs buffer with these additives, we discovered that the amino acid mixture, and not the vitamin mixture, specifically blocked basal HIF-1α accumulation (Fig. 2A). The amino acid mixture also blocked the buildup of HIF-1α induced by pyruvate in cells cultured for 4 h in glucose-free Krebs. By reconstituting Krebs buffer with one essential amino acid at a time at the concentration normally present in MEM, we determined that cysteine and histidine were the key amino acids responsible for inhibiting basal HIF-1α accumulation (Fig. 2B). This determination was reached following evaluation of all naturally occurring amino acids, only some of which are presented. Increasing the levels of each amino acid to 5 times its normal level in MEM also revealed that cysteine and histidine were the likely endogenous inhibitors of basal HIF-1α accumulation in complete culture medium. Both cysteine and its dimer cystine (Cys5) were effective, whereas D-cysteine and D-histidine were not (Fig. 2C). Both amino acids lowered HIF-1α induction by glucose, pyruvate, and oxaloacetate in all cell lines used in this study (data not shown) but did not affect HIF-1α accumulation by hypoxia or the iron chelator DFO (Fig. 2C). Cysteine is involved in GSH synthesis. As with ascorbate, cysteine, and histidine, GSH also selectively inhibited HIF-1α accumulation induced by pyruvate and oxaloacetate but not by hypoxia or DFO (Fig. 2C). Increasing the levels of cysteine and histidine, but not other amino acids, to 5 times the level normally found in MEM also lowered the basal expression of the HIF-1-regulated genes VEGF and GLUT3 as determined by RT-PCR (Fig. 2D). Cysteine and histidine also lowered HRE-luciferase activity induced by pyruvate or oxaloacetate in the U251-HRE cells (Fig. 2D). These data provide evidence for both positive and negative oxygen-independent regulation of basal HIF-1α expression by cell metabolites.

**Pyruvate and Oxaloacetate Impede Oxygen-dependent Protein Decay**—To confirm that the metabolite effects on HIF-1α were indeed oxygen-independent, we sought to rule out the possibility that 2-oxoacids such as pyruvate might induce local hypoxia in cell culture through enhancement of cell respiration. Changes in mitochondrial electron transport chain activity have indeed been shown to alter local oxygen concentrations in cell culture (28). To address this, we used the hypoxia-localizing nitroimidazole compound EF-5, which forms cellular adducts only upon reductive metabolism to reactive intermediates in anaerobic environments (27). Whereas EF-5 adducts were clearly detected by immunofluorescence in hypoxic cells, no adducts were found under normoxia, with or without added pyruvate, despite the accumulation of HIF-1α by pyruvate (Fig. 3A) or oxaloacetate (not shown). Some reactive oxygen species can also induce HIF-1α. Since ascorbate, cysteine, histidine, and glutathione all have antioxidant and/or iron-reducing properties, we also wanted to rule out enhanced production of oxidants such as H2O2 or NO by pyruvate or oxaloacetate. Despite confirming the ability of H2O2 and NO to induce HIF-1α accumulation in U251 cells, we did not detect an increase in H2O2 or NO levels upon treatment of U251 cells with pyruvate or oxaloacetate (Fig. 3B). Pharmacological inhibition of other signaling pathways implicated in HIF-1α stabilization, such as protein acetylation, phosphatidylinositol 3-kinase, mTOR, and hsp70, also failed to alter HIF-1α induction by pyruvate and oxaloacetate (Fig. 3C). We next focused our attention on HIF-1α proline hydroxylation as a target for 2-oxoacids. To explore this, we first examined whether HIF-1α induced by the 2-oxoacids contained hydroxyproline in the ODD domain. This would be expected if the 2-oxoacids interfered with oxygen-independent, we sought to rule out the possibility that 2-oxoacids such as pyruvate might induce local hypoxia in cell culture through enhancement of cell respiration. Changes in mitochondrial electron transport chain activity have indeed been shown to alter local oxygen concentrations in cell culture (28). To address this, we used the hypoxia-localizing nitroimidazole compound EF-5, which forms cellular adducts only upon reductive metabolism to reactive intermediates in anaerobic environments (27). Whereas EF-5 adducts were clearly detected by immunofluorescence in hypoxic cells, no adducts were found under normoxia, with or without added pyruvate, despite the accumulation of HIF-1α by pyruvate (Fig. 3A) or oxaloacetate (not shown). Some reactive oxygen species can also induce HIF-1α. Since ascorbate, cysteine, histidine, and glutathione all have antioxidant and/or iron-reducing properties, we also wanted to rule out enhanced production of oxidants such as H2O2 or NO by pyruvate or oxaloacetate. Despite confirming the ability of H2O2 and NO to induce HIF-1α accumulation in U251 cells, we did not detect an increase in H2O2 or NO levels upon treatment of U251 cells with pyruvate or oxaloacetate (Fig. 3B). Pharmacological inhibition of other signaling pathways implicated in HIF-1α stabilization, such as protein acetylation, phosphatidylinositol 3-kinase, mTOR, and hsp70, also failed to alter HIF-1α induction by pyruvate and oxaloacetate (Fig. 3C). We next focused our attention on HIF-1α proline hydroxylation as a target for 2-oxoacids. To explore this, we first examined whether HIF-1α induced by the 2-oxoacids contained hydroxyproline in the ODD domain. This would be expected if the 2-oxoacids interfered with steps subsequent to proline hydroxylation, such as pVHL binding or proteasome activation. Using an antibody that detects hydroxylated Pro564 in the HIF-1α C-terminal ODD region, we detected hydroxyproline only in HIF-1α induced by the proteasome inhibitor MG132. HIF-1α induced by pyruvate, oxaloacetate, or the HPH inhibitor DMOG did not contain hydroxyproline (Fig. 3D).

We next employed a live cell assay for oxygen-dependent HIF-1α degradation using C6 rat glioma cells that had been stably transfectected
with a vector encoding the HIF-1α ODD region fused to GFP (ODD-GFP). The ODD-GFP protein is hydroxylated and degraded in an oxygen-dependent manner similar to HIF-1α. As with HIF-1α, a basal as well as hypoxia-inducible accumulation of ODD-GFP has been reported (21). We found that basal ODD-GFP accumulation in C6 cells, cultured in DMEM for 24 h, was selectively inhibited by ascorbate, whereas the hypoxia-inducible ODD-GFP expression was not (Fig. 3E). In Krebs buffer, basal ODD-GFP accumulation was also stimulated by glucose, pyruvate, and oxaloacetate but not by succinate. The ineffectiveness of succinate versus pyruvate or oxaloacetate in these cells was again not due to a relative difference in cell permeability, since the same pattern was seen in digitonin-permeabilized C6 cells (supplemental Fig. S1C). Moreover, histidine and cysteine also reduced ODD-GFP induction by pyruvate or oxaloacetate. These results suggested that endogenous 2-oxoacids resembling 2-oxoglutarate could selectively block O2-dependent protein degradation.

Pyruvate and Oxaloacetate Directly Interact with HPHs—Pyruvate does not inhibit proteasomal activity (18), and neither pyruvate nor oxaloacetate was found to inhibit 35S-pVHL binding to hydroxylated ODD (Fig. 4A). Artificial 2-OG analogs such as N-oxalylglycine promote HIF-1α accumulation by strongly competing for the 2-OG binding site in HPHs (10). To determine whether pyruvate and oxaloacetate were recognized by the 2-OG binding site of HPH enzymes, we developed a binding assay using immobilized 2-OG and in vitro translated 35S-HPH homologues. The observed binding of each homologue to the 2-OG column under our assay conditions was doubled by the addition of 200 μM ferrous iron (Fig. 4B). Moreover, the iron-dependent binding of 35S-HPHs to immobilized 2-OG was also readily displaced by the
addition of free 2-OG but not by succinate (Fig. 4C). Pyruvate and oxaloacetate also reversed the iron-dependent binding of 35S-HPHs to immobilized 2-OG (Fig. 4D), suggesting that these 2-oxoacids can interact with the HPH enzyme active site.

2-Oxoacid Inhibition of HPH Activity Is Ascorbate-sensitive—To determine whether HPH activity was directly modulated by pyruvate or oxaloacetate, we studied Pro564 hydroxylation in a biotinylated HIF-1α/H9251 ODD peptide by in vitro translated HPH1, HPH2, and HPH3. The ability of streptavidin beads to pull down 35S-pVHL bound to the biotinylated peptide was used as evidence of HIF-1α/H9251 ODD peptide hydroxylation. This is the most specific and sensitive assay for HPH activity (23). Other assays, such as the [14C]CO2 release assay from 1-[14C]2-oxoglutarate, which are suitable for highly overexpressed HPH enzymes (29), can give rise to signal even in uncoupled 2-oxoglutarate decarboxylation reactions where effective substrate hydroxylation does not take place (30). Such assays can also give high background values in cell extracts (discussed in Ref. 23). As exemplified by HPH1, the 35S-pVHL pull-down enzyme assay activity of each HPH homologue was dependent on 2-OG, Fe(II), and ascorbate (Fig. 4E). In the absence of ascorbate or other reducing agents, cysteine and histidine were also found to replace ascorbate in the assay and could dose-dependently stimulate HIF-1α/H9251 ODD hydroxylation (Fig. 4F). Some 2-OG-dependent dioxygenases are able to accept pyruvate and oxaloacetate as substitutes for 2-OG (31), whereas others are known to be inhibited by these 2-oxoacids (32). When we replaced 2-OG in the reaction mix with identical concentrations of pyruvate or oxaloacetate, no enzyme activity was detected for any of the three HPH homologues (Fig. 4G). The sustained activity of the

FIGURE 3. Pyruvate and oxaloacetate block oxygen-dependent protein degradation. A, EF-5 staining in U251 glioma cells. EF-5 (500 μM) was added to the culture medium, and cells were then incubated under the indicated conditions for 4 h followed by fixation and immunofluorescence staining for EF-5 adducts. B, nuclear HIF-1α in U251 cells treated with the indicated doses of H2O2 or DETA-NO for 4 h. ROS and nitrite in cells were determined after 4 h of culture in glucose-free Krebs (control) supplemented with either 40 μM H2O2, 3 mM pyruvate, 3 mM oxaloacetate, or 300 μM DETA-NO. C, nuclear HIF-1α levels in U87 grown for 4 h in glucose-free media alone (lanes 1, 7, and 11) or with added pyruvate or oxaloacetate in the presence of butyrate (Butyr; 10 mM), trichostatin A (TSA; 300 ng/ml), wortmannin (Wort; 1 μM), LY294002 (LY; 20 μM), rapamycin (Rapa; 100 nM), or geldanamycin (Gelda; 3 μM). D, HIF-1α hydroxyproline detection. U251 cells were cultured for 4 h in MEM (lanes 1 and 2) or glucose-free Krebs supplemented with the indicated agents. Whole cell extracts were then stained with antibodies recognizing either HIF-1α or the HIF-1α ODD region containing hydroxyproline at P546. E, GFP expression in ODD-GFP-transfected C6 glioma cells was measured by fluorescence microscopy or Western blotting. F, ODD-GFP transfected C6 glioma cells were grown for 24 h in Krebs buffer modified to contain the indicated amounts of glucose or 2 mM oxaloacetate, succinate, or pyruvate. Ascorbate (100 μM) and the amino acids histidine, cysteine, and asparagine (0.5 mM each) were added where indicated.
isolated recombinant HPH enzymes required ascorbate, and, as shown above, ascorbate also selectively reversed the effect of 2-oxoaids on HIF-1α stabilization. Therefore, we examined the influence of pyruvate and oxaloacetate on HPH activity over a range of ascorbate concentrations. We detected inhibition of the 2-OG-supported enzymatic activity by pyruvate and oxaloacetate for all three homologues over a window of ascorbate concentrations. Examples of raw data showing the effect of these 2-oxoaids are shown in supplemental Fig. S1, C and D, and quantitative evaluation using
scintillation counting of the bound $^{35}$S-pVHL is shown in Fig. 4H. Inhibition of HPH2 and HPH3 by the 2-oxoacids was more potently reversed by ascorbate than HPH1. Ascorbate at 1 mM reversed the 2-oxoacid inhibition of all HPHs.

**2-Oxoacids Reversibly Inactivate Cellular HPHs**—Ascorbate is required for preventing the syncatalytic inactivation of 2-OG-dependent dioxygenases. The ascorbate reversibility of pyruvate- and oxaloacetate-induced HIF-1α/hydroxylation thus suggested that these 2-oxoacids could somehow inactivate cellular HPHs. To test this notion, we exposed U251 cells in glucose-free Krebs buffer to either hypoxia (1% O$_2$) or with 3 mM pyruvate or oxaloacetate. After 4 h, the cell extracts were washed in glucose-free Krebs buffer for various times with or without 100 μM ascorbate, 5 mM GSH, or the MEM amino acid mixture being included in the wash. C, U251 cells were cultured for 4 h in glucose-free Krebs buffer with or without the indicated additions of 2 mM pyruvate, 2 mM oxaloacetate, 1 mM DMOG, or 100 μM ascorbate. After washing, whole cell extracts were prepared and used as the source of HPH enzyme in $^{35}$S-pVHL pull-down assays with or without exogenous addition of iron or ascorbate.

D, experiments similar to C were performed, except ascorbate or FeSO$_4$ was added to the extract at 100 μM each.

E, nuclear HIF-1α accumulation was monitored in U251 grown for 4 h in MEM supplemented with either DETA-NO (300 μM) or for 4 h in glucose-free Krebs supplemented with pyruvate (3 mM) or oxaloacetate (3 mM). Cells were co-incubated with ascorbate (100 μM), Fe(II) (100 μM), or GSH (5 mM) as indicated. Decay of NO-induced HIF-1α was followed after washing with glucose-free Krebs with or without Fe(II) or Fe(III) supplementation (100 μM each). F, decay of HIF-1α induced by either pyruvate, oxaloacetate, or DFO was followed after washing with glucose-free Krebs with or without Fe(II) or Fe(III) supplementation (100 μM each). Unlabeled lanes indicate cells cultured in glucose-free Krebs buffer alone.

G, intracellular iron levels were measured via fluorescence in calcein-loaded U251 cells exposed to the indicated agents.
reduction was not seen in cells co-treated with ascorbate (Fig. 5C). Moreover, no loss of HPH activity was seen in extracts from cells treated for 4 h with hypoxia or DMOG, with or without ascorbate. The minimal HPH activity of extracts from pyruvate- or oxaloacetate-treated cells could also be restored by supplementation of the extracts with ascorbate or with exogenous ferrous iron (FeSO₄) during the assay (Fig. 5D).

Oxidation of HPH-associated iron from the Fe(II) to the Fe(III) state has recently been proposed as a mechanism for H₂O₂ regulation of HIF-1 in cells mutated for JunD (16). We hypothesized that NO, another potent iron oxidizer, may also work this way and observed a complete inhibition of NO-induced HIF-1/α accumulation by ascorbate, Fe(II), and GSH (Fig. 5E). The addition of Fe(II) to cultured cells also prevented HIF-1/α induction by pyruvate and oxaloacetate. Moreover, NO-treated cells also showed sluggish HIF-1/α decay, even after extensive washing (Fig. 5E) with MEM. The addition of Fe(II) but not Fe(III) to the wash accelerated the decay of NO-induced HIF-1/α. A similar enhancement of HIF-1/α decay by a wash containing Fe(II) but not Fe(III) was seen following induction with pyruvate or oxaloacetate (Fig. 5F). The need for ferrous iron for the decay of HIF-1/α during the postinduction wash was also demonstrated using DFO. Chelation of cellular iron by this agent inhibits cellular HPH activity and prevents HIF-1/α decay. After washing out the DFO, the addition of Fe(II) but not Fe(III) promoted decay of the accumulated HIF-1/α protein. To rule out the possibility that pyruvate and oxaloacetate chelated intracellular iron, we used a live cell fluorescence-based assay for labile iron. Interaction of calcein trapped inside cells with iron specifically lowers its fluorescence.
Iron chelators in turn increase fluorescence (25). Calcein-loaded U251 cells displayed an increase in fluorescence when cells were challenged with the iron chelators DFO or bipyridyl and a decrease in fluorescence when Fe(II) was added (Fig. 5G). The addition of pyruvate or oxaloacetate (not shown) did not produce an increase in intracellular calcein fluorescence.

Aerobic Glycolysis Regulates Basal HIF-1 Activity and Invasive Phenotype in Human Cancer Cells—Even in the presence of oxygen, most cancer cells accumulate high levels of pyruvate and lactate (33). In head and neck carcinomas and other human cancers, high tumor lactate levels are in fact predictive of the likelihood of metastases and poor clinical outcome (34). Given the strong role of HIF-1 in cancer cell invasiveness (1, 15), we hypothesized that the accumulation of 2-oxoacids via aerobic glycolysis could contribute to cancer cell invasiveness by inducing basal HIF-1α. To explore this notion, we examined two head and neck squamous carcinoma cell lines known to have disparate basal HIF-1α levels (15). The JHU-SCC-022 cell line (022) was grown in RPMI containing 11.1 mM glucose, and the UM-SCC-22B cell line (22B) was grown in DMEM containing 25 mM glucose. Media of both cell lines were supplemented equally with 10% fetal bovine serum and the non-glucose-lactate energy source glutamine (2 mM). Both cell lines demonstrated HIF-1α accumulation under hypoxia (Fig. 6A). However, the 22B cells displayed much higher basal HIF-1α, elaborated higher basal levels of pyruvate and lactate (33). To determine whether the endogenous accumulation of pyruvate led to inactivation of HPH activity in the 22B cells, we prepared cell extracts in the presence and absence of exogenous ascorbate (Fig. 6B). This means that as a result of catalyzing iron-mediated oxidations, the progressive ascorbate-reversible HPH inactivation in 22B cells was paralleled by the accumulation of ascorbate-reversible basal HIF-1α, and as in other cells, ascorbate did not reverse hypoxia- or DMOG-stimulated HIF-1α in these cells (Fig. 6F). Furthermore, the addition of excess pyruvate (10 mM) to DMEM markedly enhanced the progressive accumulation of HIF-1α (Fig. 6K). After the 24-h HIF-1α accumulation period, we supplemented the medium with either ferric or ferrous iron or ascorbate (100 μM each) and incubated the cultures for another 30 min. We found that the accumulated HIF-1α was diminished to a greater extent by ferrous iron than by ferric iron, whereas ascorbate abolished immunoreactivity. Ascorbate also lowered the basal expression of several HIF-1-regulated genes in 22B cells, including carbonic anhydrase IX, GLUT3, and matrix metalloprotease-2 (MMP-2) (Fig. 6L). Furthermore, ascorbate lowered the basal invasiveness of 22B cells through Matrigel without affecting serum-induced invasiveness (Fig. 6M). These results implicate ascorbate-reversible, glycolysis-dependent basal HIF-1α activity in cancer progression.

**DISCUSSION**

**HIF-1 Hydroxylation Is Physiologically Regulated by Multiple Mechanisms**—HIF-1α hydroxylases are 2-OG-utilizing dioxygenases that also require Fe(II), molecular oxygen, and ascorbate. The proposed sequential molecular mechanism for activity of 2-OG dioxygenases (Fig. 7, A–C) first involves bidentate ligation of the apoenzyme Fe(II) complex by the 1-carboxylate and 2-oxoacid functional groups of 2-OG. Binding of the 5-carboxylate to a distinct subsite, as well as binding of the primary substrate (e.g. HIF-1α), may be also required for allosterically opening access of molecular oxygen to the axial coordination site of the ferrous iron. By properly assembling these components, the dioxygenases allow molecular oxygen to catalyze the insertion of one oxygen atom into the 2-carbon of 2-OG to form succinate and CO2, with the other oxygen atom probably forming a ferryl intermediate that subsequently produces substrate hydroxylation (reviewed in Refs. 12 and 13). Reduced availability of the co-substrates oxygen (by hypoxia), iron (by DFO), or 2-OG (by artificial 2-OG analogs such as N-oxalylglycine or DMOG) is known to inhibit this family of enzymes. 2-OG-dependent dioxygenases can also be synthetically inactive (Fig. 7D). This means that as a result of catalyzing iron-mediated oxidations,
either these enzymes become oxidized at critical amino acid residues over time or the redox state of the iron becomes useless in carrying out sustained reaction cycles. In most cases, this syn-catalytic inactivation can be blocked or reversed by ascorbate (Fig. 7E; reviewed in Ref. 13). The routine observation of ascorbate and Fe(II) reversed basal HIF-1α in cancer cells suggests that the inactivated state of HPHs is either a prominent feature of malignancy or a result of the cell culture and tumor microenvironment.

HPH inactivation via oxidation of Fe(II) to Fe(III) may underlie the ability of ROS and NO to promote HIF-1α buildup under physiological conditions and in cancer (35). For example, as a result of JunD mutation, some cancer cells generate excessive ROS and accumulate HIF-1α in an ascorbate- and cysteine-reversible manner (16). A similar mechanism may underlie HIF-1 activation by ROS produced during cytokine signaling (36) or radiation therapy (37). Although NO has been proposed to form inhibitory nitrosothiols on the HPH proteins (17), our data with ascorbate- and Fe(II)-mediated reversal suggest that NO also oxidizes HPH-associated iron (Fig. 5). Like ascorbate, cysteine and histidine can also modulate iron redox status (16, 38, 39), and the relative cellular levels of these substances or of glutathione could thus play a significant role in determining the basal HIF-1α degradation rate of many cells. Deficiencies in iron reducing capacity may conceivably produce greater HPH inactivation in neoplastic cells than in normal cells (40).

HPH Inactivation Mediates HIF-1 Regulation by Anaerobic Glucose Metabolites—We have shown that, in many cancer cells in culture, medium glucose makes a much greater contribution to basal HIF-1α than serum-derived factors. We also identified 2-oxoacid glucose metabolites as the likely mediators of this effect. Despite recent reports suggesting a link between succinate or fumarate buildup and HIF-1 activity (41–43), our method of analysis did not find a significant effect of succinate or fumarate on HIF-1α accumulation, 2-OG binding to HPHs, regulation of ODD-GFP, or HRE-Luc expression. Although we ruled out differential cell permeability as a basis for this observation, we cannot rule out a cell-specific sensitivity to succinate and fumarate or the interconversion of these metabolites into other metabolites. Fumarate and succinate inhibition of HPHs is reversed by 2-OG (41). Selective reversal of the actions of pyruvate and oxaloacetate by ascorbate, but not 2-OG, supports the possibility of unique but overlapping recognition sites for ascorbate and 2-OG on the dioxygenases (32). This also suggests that different mechanisms may exist for the interaction of glycolytic and TCA cycle metabolites with HPHs.

We also could not detect significant ROS or NO production upon treatment of cells with glucose or with pyruvate and oxaloacetate. However, we did find that ascorbate, cysteine, histidine, and Fe(II) reversed HIF-1 activation by these stimuli as well as the 2-oxoacids. This is surprising, since 2-oxoacids do not chelate or oxidize free iron but are instead well known as potent antioxidants (44, 45). These 2-oxoacids bound to the 2-OG site of HPHs and suspended HPH activity without irreversible, suicidal inactivation. It is possible that the absence of a C5 carboxyl group allows pyruvate and oxaloacetate binding to interfere with allosteric enzyme mechanisms for the scheduled sequential binding of other reactants. This may prevent HIF-1α hydroxylation while allowing oxidation of HPH-bound iron in the active site (Fig. 7D), thus explaining reactivation by ascorbate and Fe(II). Inhibition of HIF-1 hydroxylation activity by pyruvate and oxaloacetate was more readily seen with extracts from live cells exposed to these agents than with translated enzymes in vitro. This may be due to the fact that Fe(II) and ascorbate, which reverse the effect of pyruvate and oxaloacetate, need to be added to the in vitro assay to get any signal. However, we cannot rule out the role of additional factors in live cells. The lowering of basal HIF-1α, basal expression of HIF-1-regulated genes, and basal invasive-

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