THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 24, pp. 18217–18224, June 11, 2010
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Extended Ischemia Prevents HIF1α Degradation at Reoxygenation by Impairing Prolyl-hydroxylation

ROLE OF KREBS CYCLE METABOLITES[*][S]

Received for publication, January 7, 2010, and in revised form, March 22, 2010 Published, JBC Papers in Press, April 5, 2010, DOI 10.1074/jbc.M110.101048

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Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that activates the cellular response to hypoxia. The HIF1α subunit is constantly synthesized and degraded under normoxia, but degradation is rapidly inhibited when oxygen levels drop. Oxygen-dependent hydroxylation by prolyl-4-hydroxylases (PHD) mediates HIF1α proteasome degradation. Brain ischemia limits the availability not only of oxygen but also of glucose. We hypothesized that this circumstance could have a modulating effect on HIF. We assessed the separate involvement of oxygen and glucose in HIF1α regulation in differentiated neuroblastoma cells subjected to ischemia. We report higher transcriptional activity and HIF1α expression under oxygen deprivation in the presence of glucose (OD), than in its absence (oxygen and glucose deprivation, OGD). Unexpectedly, HIF1α was not degraded at reoxygenation after an episode of OGD. This was not due to impairment of proteasome function, but was associated with lower HIF1α hydroxylation. Krebs cycle metabolites fumarate and succinate are known inhibitors of PHD, while α-ketoglutarate is a co-substrate of the reaction. Lack of HIF1α degradation in the presence of oxygen was accompanied by a very low α-ketoglutarate/fumarate ratio. Furthermore, treatment with a fumarate analogue prevented HIF1α degradation under normoxia. In all, our data suggest that postischemic metabolic alterations in Krebs cycle metabolites impair HIF1α degradation in the presence of oxygen by decreasing its hydroxylation, and highlight the involvement of metabolic pathways in HIF1α regulation besides the well known effects of oxygen.

The hypoxia-inducible transcription factor (HIF)[5] is expressed at very low levels in cells under normal oxygen tension, but is rapidly induced upon exposure to hypoxia (1), triggering the activation of a genetic program that enables the metabolic adaptation of cells (2). HIF is a heterodimeric factor composed of a hypoxia-regulated α-subunit (HIF1α or HIF2α) and constitutively expressed HIF1β (also known as aryl hydrocarbon receptor nuclear translocator, ARNT) (2). Although the α-subunit is constantly transcribed and translated, it is also degraded in an oxygen-dependent mechanism. It is only with dwindling oxygen levels that HIF1α or HF2α expression is readily detected (3). In the presence of oxygen, HIF prolyl-hydroxylases (PHD) hydroxylate two proline residues (positions 402 and 564 in human HIF1α), in a reaction that requires molecular oxygen and α-ketoglutarate as co-substrates (4). These hydroxyproline residues are recognized by the Von Hippel-Lindau tumor suppressor protein (pVHL), one of the components of a E3 ubiquitin-ligase complex that also contains elongins B and C, cullin2, and Rbx, which conjugates ubiquitin to HIFα (4, 5). This results in the oxygen-dependent targeting of HIFα to the proteasome. Decreased oxygen concentration results in impaired prolyl-hydroxylation, reduced targeting of HIFα to the proteasome and the accumulation of HIF in the nucleus, where it activates a plethora of genes devoted to improving the delivery of oxygen and enhancing the production of ATP by glycolysis, among other actions (6).

HIF1 is a key component of the cellular response to brain ischemia (7) and may play role on survival. Thus, permanent MCA occlusion in rats induces a temporal and spatial co-induction of the mRNA of HIF1α and those of target genes, such as glucose transporter 1 (GLUT1) and glycolytic enzymes (8) in the penumbral area. This is further supported by the increased

* This work was funded by projects from MICINN (Projects SAF2005-05793-C02-02 and SAF2008-04515-C02-02).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

[1] Recipient of a predoctoral fellowship from the Generalitat de Catalunya, Spain.

[2] Supported by a FPI fellowship from the Ministerio de Educación y Ciencia.

[3] Funded by Grant SAF2007-64597, the ETORTEK Research Program, and the Bizkaia Xede Program from Bizkaia County.

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[5] The abbreviations used are: HIF, hypoxia-inducible factor; MCA, middle cerebral artery; HRE, hypoxia-response element; VEGF, vascular endothelial growth factor; EGFP, enhanced green fluorescent protein; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; PHD, prolyl-4-hydroxylase; ELISA, enzyme-linked immunosorbent assay; RA, all-trans retinoic acid; mTOR, mammalian target of rapamycin.

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damage in a model of transient MCA occlusion in mice with neuron-specific inactivation of HIF1α (9). In contrast, a truncated form of HIF1α with dominant-negative effects reduced delayed cell death in cultured neurons subjected to OGD (10), through effects on p53 expression (11, 12), and HIF1α-deficient mice were protected against ischemic brain damage (13). However, expression of the majority of hypoxia-dependent genes was unaffected in these mice whereas apoptotic genes were specifically down-regulated, suggesting a predominant pro-apoptotic role of HIF1α.

Much of the knowledge on the mechanisms that regulate the expression of HIF because of changing oxygen levels has been obtained from cultured cell models where the availability of glucose was not restricted, and less attention has been comparatively paid to the fact that under ischemia, a shortage of oxygen coincides with a limiting availability of glucose, particularly in cells with low glycogen storage such as neural cells. We hypothesized that the concurrent absence of glucose and oxygen could have a modulating effect on the expression of HIF.

EXPERIMENTAL PROCEDURES

Materials—Dimethyloxallyl glycine (DMOG) was purchased from Cayman Chemical. All-trans retinoic acid (RA) and MG132 were from Calbiochem. Diethyl ether was purchased from Merck, and ethyl acetate was purchased from J. T. Baker. All other chemicals were purchased from Sigma, while tissue culture reagents were ordered from Invitrogen.

Cell Culture—The culture and RA-induced differentiation of SH cells in our model has previously been described (14). The wild-type renal cell carcinoma (RCC4) line and a derivative clone stably transfected with an expression vector for pVHL were obtained from the European Collection of Cell Cultures (ECACC/Sigma). These cells were grown in low-glucose (5.6 mM) Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 500 μg/ml G418.

Anoxia plus Reoxygenation Experiments—SH cells seeded on 6-cm Petri dishes (14) were subjected to 15 h anoxia either in glucose-containing or glucose-free medium as described (14). This was followed by reoxygenation where indicated, with no intervening change in the media that had been used during the incubation in anoxia. For the experiments performed with RCC4 cells, 10^5 cells per 6 cm Petri dish were seeded 3 days before the experiment in 5 ml of the growth medium described above. Just before the experiment, cells were washed once in either growth medium with standard glucose concentration or growth medium prepared with glucose-free DMEM. This volume was aspirated, and cells were added another 3 ml of either of the media and assigned to the glucose-containing or glucose-free groups. Anoxia and reoxygenation were carried out in the same way as for SH cells.

Total Cell Extracts—At the end of the anoxia and reoxygenation experiments described above, total cell extracts were prepared for the analysis of protein expression by Western blotting. The procedure was carried out while keeping the cells on ice at all times. Because cells that had been subjected to OGD conditions were loosely attached to the surface, these were scraped off the dish in a small volume of medium, briefly spun at 4 °C, the cell pellet was resuspended in 1 ml of cold phosphate-buffered saline (PBS), spun again, and finally resuspended in 1.5× Laemmli sample buffer for SDS-PAGE (150 mM Tris-HCl pH 6.8, 1.5% SDS, 15% glycerol, no bromphenol blue) to lyse the cells. Cells that had been kept in control conditions or that had been subjected to anoxia in the presence of glucose were more firmly attached to the surface so cells were washed in PBS while still adhered to the Petri dish, and directly lysed with 200 μl of 1.5× Laemmli sample buffer. All lysates were briefly sonicated in order to reduce their viscosity, and incubated at 100 °C for 5 min. Protein concentration was quantitated by using the BCA protein assay (Thermo Scientific).

Western Blotting—20 μg of protein from total cellular extracts was loaded on SDS-PAGE gels. Western blotting was carried out as previously described (14), except for the detection of the hydroxylated Pro^{564}-HIF, where the membrane was blocked overnight at 4 °C in 5% skimmed milk in T-TBS, then incubated with the primary antibody (1:1000) for 2.5 h at room temperature in 1% skimmed milk in T-TBS. Detection of the signal in this case was carried out with the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and a Versadoc imaging system (Bio-Rad). Antibodies raised against the following proteins were used at the dilutions indicated: HIF1α (Novus Biologicals; NB100–449, 1:2000), HIF1β (Abcam; ab2771, 1:1000), GLUT1 (Abcam, ab652, 1:1000), Hexokinase II (Santa Cruz Biotechnology; sc-6521, 1:1000), α-actin (Assay Designs/Stressgene; CSA-400, 1: 5000), ATP synthase-β (BD Biosciences; 612518, 1:1000), hydroxy-Pro^{564}-HIF (Cell Signaling; 3434, 1:1000), PHD2 (Novus Biologicals; NB100–138, 1:1000), β-tubulin (Sigma; T-4026, 1:50000), GFP (Abcam, ab290, 1:5000).

Quantification of VEGF by ELISA—Secreted VEGF was quantitated in the culture medium of SH cells after incubation under anoxic conditions. This was performed by using the Quantikine Human VEGF Immunoassay (R&D Systems), and according to the manufacturer's instructions. Results were represented as fold activation over the control group.

Quantification of HIF1α Abundance—The Surveyor IC intracellular Human/Mouse total HIF1α immunoassay (R&D Systems) was used to quantitate HIF1α expression, according to the manufacturer's instructions. Extracts were prepared from RA-differentiated SH cells seeded at 3×10^5 cells per six cm Petri dish. A standard curve obtained with known amounts of HIF1α was run in parallel to the samples in every assay so that expression of HIF1α could be expressed as pg of HIF1α per μg of total cellular protein. Total cellular protein concentration in the lysates was quantitated with Bradford's assay (Bio-Rad).

HIF1 Binding Assays—Binding of HIF1 in nuclear extracts to an oligonucleotide containing an HRE was analyzed with the TransAM HIF-1 Transcription Factor Assay kit (Active Motif). SH cells were treated as above. At the end of the experiment, cells were placed on ice, and nuclear extracts were prepared according to the manufacturer's instructions with minor modifications. Cells were harvested by scraping and washed once in cold phosphate-buffered saline. The cell pellet was resuspended in 400 μl of a hypotonic solution (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA), kept on ice for 20 min, and Igepal CA-630 was then added to a 0.02% final concentration. The suspension

was briefly mixed by inversion and immediately centrifuged at 11,000 × *g* for 1 min at 4 °C. The nuclear pellet was resuspended in 50 μl of the lysis buffer provided in the kit (supplemented with dithiothreitol and protease inhibitors provided). Nuclei were incubated for 30 min on ice with occasional mixing by inversion, and centrifuged for 10 min at 14,000 × *g*, 4 °C. The supernatant was saved as nuclear extract, and stored in aliquots at −80 °C until assayed. Protein content of the extracts was assayed with Bradford's assay (Bio-Rad).

Transcriptional Reporter Assays—Relative HIF transcriptional activity was analyzed by performing transient transfections into SH cells with a plasmid that expressed firefly luciferase under the control of the basic c-*fos* promoter and a 29 bp enhancer from the *pfkfb3* gene that contains two HRE, cloned upstream (15). Control vectors included a vector that did not contain an HRE but only the c-*fos* basal promoter, and another one containing a mutated HRE that does not bind HIF (15). pRL-CMV (Promega), a constitutive expression vector for *Renilla reniformis* luciferase was used to normalize the efficiency of transfection. For transfection, cells were seeded on 24-well plates at a density of 40,000 cells per well, and allowed to differentiate with RA for 3 days. On the fourth day, a total of 1 μg of DNA (900 ng of firefly luciferase reporter vector plus 100 ng of pRL-CMV) per well was transfected with Superfect reagent (Qiagen), according to the manufacturer's instructions. The transfection mix was on the cells for 3 h, and at the end of the transfection, RA was added back to the medium to resume differentiation for the usual total of 5 days. Anoxia experiments were performed as described above, and lysates were prepared and analyzed by using the Dual-Glo kit (Promega), according to the manufacturer's instructions.

Stable Transfection—Expression vectors for the fusion proteins Ub-G76V-EGFP (Addgene plasmid 11941) and Ub-M-EGFP (Addgene plasmid 11938) were obtained from N. Dantuma (16) through Addgene. One million SH cells seeded on 10 cm Petri dishes were transfected as described in the preceding section with 10 μg of plasmid and selected with 400 μg/ml G418 to generate clones with stable expression of either of the vectors.

Metabolite Quantification—Analysis of α-ketoglutarate, succinate, and fumarate was carried out by gas chromatography-mass spectrometry (GC-MS) detection of ketoacids with a method adapted from Refs. 17, 18. Experiments were performed on SH cells seeded at 3×10^6 in 10 cm Petri dishes, which had been differentiated with 10 μM RA as described (14). At the end of the incubations, cells were washed with phosphate-buffered saline, and the cell pellet was resuspended in 500 μl of milli-Q water and frozen at −20 °C until assayed (a separate fraction was set aside for protein quantification). For the preparation of extracts, the 500-μl samples were taken to a volume of 2 ml with water, and further added 1 ml of 8 M NaOH and 1 ml of 25 mg/ml hydroxylamine. The sample was then heated at 60 °C for 30 min, and pH was adjusted by adding 1 ml of 6 N HCl. Sequential extractions were carried out as described (18) with the exception that samples were extracted twice with 2 ml of diethyl ether and twice with 2 ml of ethyl acetate. 6 μl of 5 mM undecanoic acid was added at the collection tube to serve as an internal standard of the procedure. Once completely evaporated with nitrogen gas, the final dry residue was resuspended in 75 μl of trimethylsilyl, incubated at 60 °C for 30 min to derivatize the keto acids, and kept at −20 °C until injected. 2-μl samples were injected into a 7890A-5975C GC-MS (Agilent Technologies), with an HP-5MS 60 × 0.25 × 0.25 capillary column using a splitless method and pressure ramp, and results were analyzed by using the ChemStation GC/MSD software. The ratio between the areas (area of the peak corresponding to each acid and the area of undecanoic acid) was normalized by the protein concentration of the sample (μg/μl).

Statistical Analysis—Data analysis was performed with GraphPad Prism software (GraphPadSoftware), by either one-way ANOVA, followed by post-hoc analysis with Bonferroni Multiple Comparison test; Mann-Whitney non-parametric test; or Kruskal-Wallis test for nonparametric data followed by a Dunn's multiple comparison test, as indicated in the figure legends.

RESULTS

The Availability of Glucose during Incubation of SH Cells under Anoxia Affects the Activation of HIF1α and Its Target Genes—We examined the expression of HIF1α protein in total cellular extracts obtained from RA-differentiated SH cells that were incubated for 15 h under anoxia in either the presence or the absence of glucose. HIF1α was not expressed under control conditions but was induced by anoxia (Fig. 1*A*). The induction was more intense in OD conditions than in OGD (Fig. 1*A*). On the other hand, the expression of HIF1β showed no differences under any of the conditions assayed (Fig. 1*A*). Densitometry of the Western blots showed a 4.5-fold higher expression of HIF1α in the OD than in the OGD group (Fig. 1*B*). Because Western blotting only offers semi-quantitative data, we quantitated HIF1α by ELISA. HIF1α increased from non-detectable levels under control conditions to 17.3 ± 4.8 pg of HIF1α per μg of total cellular protein (Fig. 1*C*) under OD. Cells under OGD displayed a much smaller increase (0.5 ± 0.1 pg of HIF1α per μg of total cellular protein, Fig. 1*C*). FACS analysis of cells incubated with pimonidazole during anoxia showed that oxygen availability was similarly reduced in the OD and OGD groups, showing that those differences in HIF1α expression were not caused by differences in the oxygen concentration available to the cells (supplementary Fig. S1). Because the transcriptional effects of HIF1 are dependent on the α/β heterodimer binding to DNA (19), we analyzed the binding of HIF1 from nuclear extracts to an oligonucleotide containing an HRE. Our data correlated with the abundance of HIF1α, with stronger binding to DNA in the presence than in the absence of glucose during anoxia (Fig. 1*D*). The expression of HIF1α in the same nuclear extracts paralleled the binding results (data not shown), and was in the range of the data presented in Figs. 1, *A* and *B*. Furthermore, HIF transcriptional activity behaved accordingly with the HIF1α expression data. Thus, in transient transfection experiments with a luciferase reporter vector under the control of the pfkfb3 HRE (wtHRE), the OD caused an ~74-fold increase in transcription (Fig. 2*A*). In contrast, cells under OGD conditions only reached 7-fold activation in transcription (Fig. 2*A*). The effects were specifically related to HIF activity since they were abolished when the HRE was mutated (mutHRE, Fig.

2*A*). In keeping with this, we tested the expression of known endogenous HIF transcriptional targets (20). First, we examined the secretion of VEGF into the culture media at the end of anoxia. OD brought about a 24-fold higher secretion of VEGF, and OGD just a 5-fold induction (Fig. 2*B*). Similarly to VEGF, GLUT1, and hexokinase II were induced after incubating SH cells under OD, but the absence of glucose in OGD dampened the increase in expression (Fig. 2*C*). Our data support that differences in the expression levels of HIF1α achieved under OD or OGD correlate with HIF activity and the expression of HIF target genes.

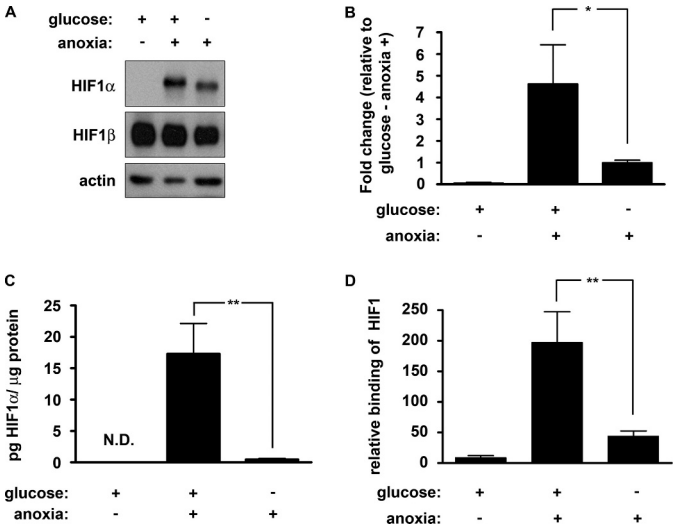

FIGURE 1. **The absence of glucose during anoxia blunts the increase in HIF1 expression and binding to DNA.** *A*, Western blot showing the relative expression of HIF1 subunits α and β in total extracts prepared from cells treated under anoxia for 15 h in either the presence (OD: glucose +, anoxia +) or absence of glucose (OGD: glucose −, anoxia +), or under control conditions (glucose +, anoxia −). The membrane was re-blotted with an antibody against α-actin to control equal loading. *B*, quantification of HIF1α by densitometry of Western blots. Data were expressed as fold change relative to the OGD group and are represented as the average ±S.E. of 10–17 independent experiments. Statistical analysis was by one-way ANOVA followed by Mann-Whitney post-test. OD *versus* OGD, *, $p < 0.05$. *C*, ELISA quantification of HIF1α in total cell extracts prepared from SH cells treated as above for the same period of time, as indicated. Data were expressed as pg of HIF1 per μg of total cellular protein and are represented as the average ± S.E. of seven independent experiments. Statistical analysis was by one-way ANOVA followed by Bonferroni post-test. *N.D.*: not detectable; OD *versus* OGD, **, $p < 0.01$. *D*, relative binding of HIF1 in nuclear extracts prepared from cells treated as above to an oligonucleotide containing an HRE. Data were normalized by the total protein content, expressed relative to the value of the control group and are represented as the average of four independent experiments ± S.E. Statistical analysis was by one-way ANOVA followed by Bonferroni post-test. OD *versus* OGD, **, $p < 0.01$.

HIF1α Expressed under OGD Conditions Is Not Readily Degraded after Reoxygenation—Because the availability of glucose affected the abundance of HIF1α during anoxia, we explored whether any differences may persist after an additional incubation under normoxic conditions (reoxygenation). Thus, we examined the expression of HIF1α protein at different times after reoxygenation. HIF1α was fully degraded 1 h after reoxygenation following OD (Fig. 3*A*). Unexpectedly, the HIF1α expressed after OGD was not degraded and was still detectable at least 6 h after reoxygenation (Fig. 3*A*). This effect was only observed after a long period of OGD (15 h), because SH cells subjected to a 6 h OGD completely degraded HIF1α within 30 min of reoxygenation (Fig. 3*B*). Furthermore, the persistence of HIF1α was strictly dependent on the induction of anoxia, since hypoxic conditions of 1.0% oxygen were not able to prevent HIF1α degradation after reoxygenation (supplementary Fig. S2).

The Persistence of HIF1α after Reoxygenation Is Not Because of Inhibition of Proteasome Activity—Because the ubiquitin-proteasome pathway is the HIF1α main and best characterized degradation system (21), we examined its functionality in our model to see if the stabilization of HIF1α could result from inhibition of the proteasome activity. To this end, we generated SH clones with stable expression of a proteasome activity reporter consisting of a ubiquitin-EGFP fusion protein (UbG76V-EGFP SH) (16). The ubiquitin moiety in this reporter contains a Gly to Val substitution at position 76 that prevents cleavage by ubiquitin hydrolases, so it commits the fusion protein to constitutive proteasomal degradation. Consequently, no EGFP fluorescence was observed under control conditions (Fig. 4*A*, *left*), but only upon inhibition of the proteasome with MG132 for 15 h (Fig. 4*A*, *left*). The UbG76V-EGFP SH clones were used in experiments such as those described above. The Western blotting showed that none of the conditions tested caused the accumulation of a 38 kDa band corresponding to the ubiquitin-EGFP fusion protein (Fig. 4*B*, *left*), except when the culture media was supplemented with MG132 (Fig. 4*B*, *left*). A stable SH clone expressing the Ub-M-EGFP fusion protein, which is not degraded in the proteasome because of rapid excision of the ubiquitin moiety by ubiquitin hydrolases (16, 22), was used to control that the expression of the reporter was not affected by the incubation conditions. This Ub-M-EGFP clone showed no differences in the expression of EGFP in any of the conditions tested (Fig. 4*B*, *right*).

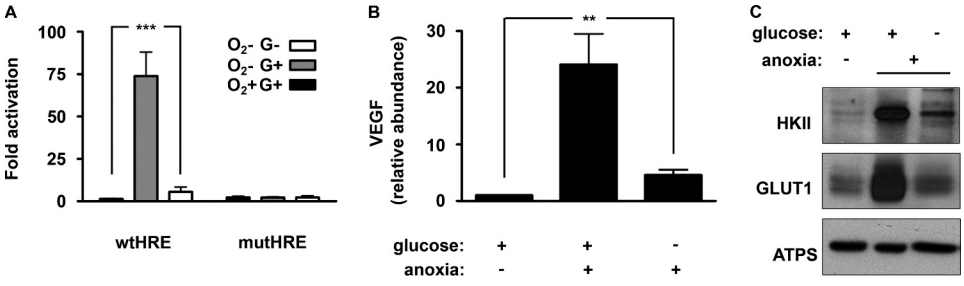

FIGURE 2. **OGD reduces HIF-mediated transcription and dampens the induction of HIF1 target genes.** *A*, quantification of the relative HIF transcriptional activity in transient transfection experiments with an HRE-dependent luciferase reporter (*wtHRE*) in SH cells. A vector containing a mutant HRE (*mutHRE*) was used as a negative control. Results were normalized by *Renilla* luciferase activity and expressed as fold activation relative to the control group. Data are represented as the average ± S.E. of five independent experiments, and were analyzed by one-way ANOVA followed by Bonferroni test. OD (O_2^-, G+) *versus* control (O_2^+, G+), or OD *versus* OGD (O_2^-, G-) ***, $p < 0.001$. *B* and *C*, induction of HIF targets (VEGF, hexokinase II, GLUT1) as a consequence of the treatments described above. Secreted VEGF was detected by ELISA, whereas hexokinase II and GLUT1 in total cellular extracts were detected by immunoblotting. VEGF data were normalized by the value of the control condition and were expressed as relative abundance. Data are represented as the average ± S.E. of four independent experiments, and were analyzed by one-way ANOVA followed by Bonferroni post-test (**, $p < 0.01$).

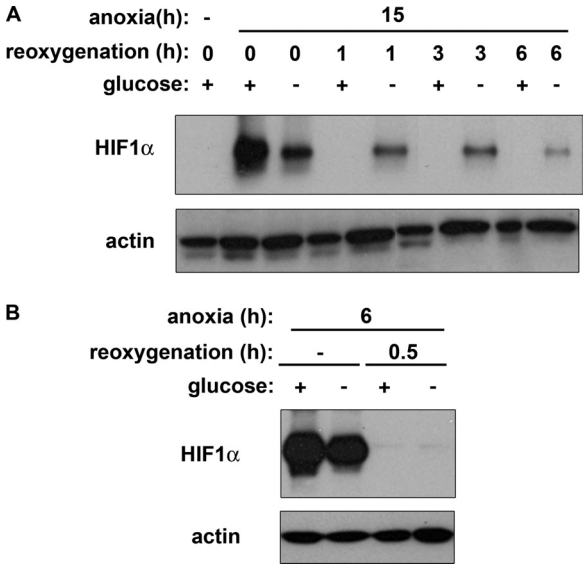

FIGURE 3. **HIF1α produced during OGD becomes resistant to degradation even after reoxygenation.** *A*, expression of HIF1α protein was assessed by immunoblotting in total cell extracts prepared from cells incubated either in control conditions (anoxia −, glucose +), or for 15 h in OD (15 h anoxia, glucose +) or OGD (15 h anoxia, glucose −) conditions, followed by 1, 3, or 6 h after reoxygenation, as indicated. *B*, expression of HIF1α in cells incubated under anoxia for 6 h in the presence or absence of glucose and 30 min after reoxygenation. Blots in both panels were also probed with the α-actin antibody to provide a control of the protein loading.

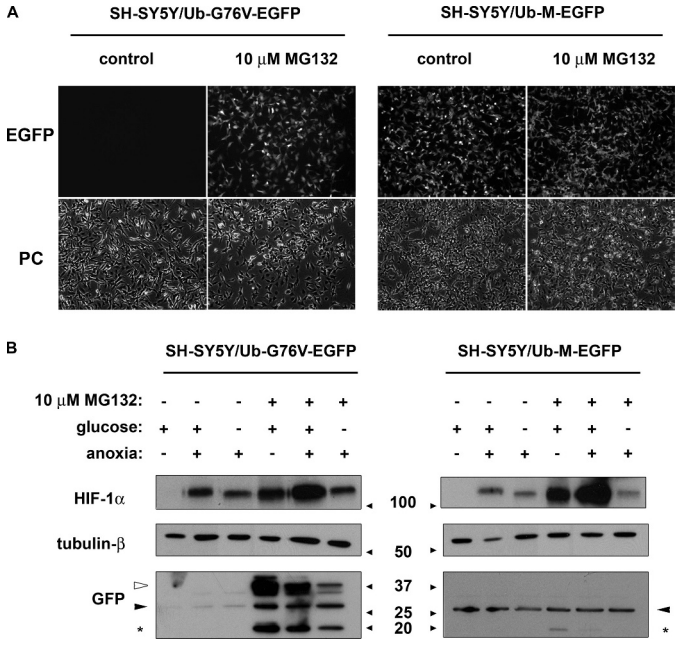

FIGURE 4. **The resistance of HIF1α to oxygen is not caused by impaired proteasome activity.** *A*, cells expressing either the proteasome activity reporter Ub-G76V-EGFP (*left panels*) or the control reporter Ub-M-EGFP (*right panels*) were incubated for 15 h in the presence or absence of proteasome inhibitor MG132 (10 μM), as indicated. Micrographs show fluorescence of EGFP (*EGFP*) or a phase-contrast image of the same field (*PC*). *B*, detection of the Ub-G76V-EGFP (*left panels*) or Ub-M-EGFP (*right panels*) reporters with an antibody against GFP by immunoblotting of total cellular extracts prepared in the conditions indicated. Media were supplemented with 10 μM MG132 as indicated. The *white arrowhead* (*left panels*) indicates the position of the Ub-G76V-EGFP fusion protein. Increased expression of the fusion protein because of inhibition of the proteasome allows low-efficiency cleavage of the ubiquitin moiety and detection of free EGFP (*black arrowhead*, (16)). An unidentified band of lower molecular weight was also detected (*asterisk*). The *right panel* shows constitutive expression of free EGFP in the clone expressing the Ub-M-EGFP fusion protein. As a control, HIF1α was detected on the same blots. They were also probed with an antibody against tubulin-β to provide a loading control.

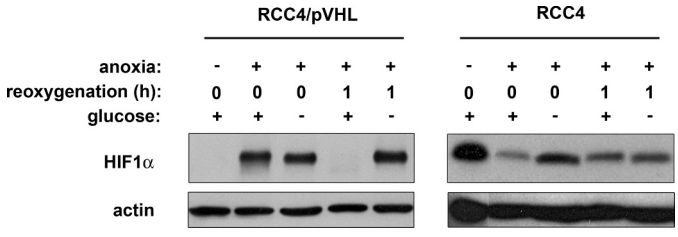

FIGURE 5. **The activity of pVHL is required for degradation of HIF1α after reoxygenation.** The expression of HIF1α protein was analyzed in wild-type RCC4 cells (which lack pVHL, *right panel*) and a clone transfected with a plasmid expressing pVHL to rescue the mutation (RCC4/pVHL, *left panel*). The two clones were subjected to control, OD, or OGD conditions for 15 h. The blots were probed with the α-actin antibody to provide a loading control.

The behavior of HIF1α in these cells was like that of the parental SH clone (Fig. 4*B*). Our results indicate that proteasome activity is preserved both under OD and OGD, and that the stability after reoxygenation of HIF1α induced by OGD cannot be explained by proteasomal inactivation.

The Activity of pVHL Is Required for Degradation of HIF1α after Reoxygenation—We next examined the involvement of pVHL in the differential stability of HIF1α protein after reoxygenation. We subjected RCC4 cells (which do not naturally express pVHL), and a derivative clone overexpressing pVHL from a plasmid (RCC4/pVHL), to OD or OGD, followed by 1 h of reoxygenation. The behavior of HIF1α expression in the RCC4/pVHL cells was similar to that seen in the SH cells (Fig. 5, *left panel*), whereas in the pVHL-deficient RCC4 cells, HIF1α was constitutively expressed under normoxia and did not experience the glucose-induced differences in stability after reoxygenation (Fig. 5, *right panel*). This suggests that (*a*) the behavior of HIF1α after reoxygenation of the SH cells is not exclusive to this cell line, and (*b*) pVHL is involved in the degradation of HIF1α after the reoxygenation following a period of anoxia.

The Hydroxylation of HIF1α at Reoxygenation after OGD Conditions Is Reduced—The proline hydroxylation catalyzed by PHD is crucial for the oxygen-dependent degradation of HIF1α (4). Thus, we examined whether OGD conditions could lead to impaired hydroxylation of HIF1α after reoxygenation. Analysis of total cellular extracts by Western blotting with an antibody specifically recognizing hydroxy-Pro^{564} in human HIF1α showed large differences in the proportion of hydroxylated-HIF1α. One hour of reoxygenation after OD in the presence of the proteasome inhibitor MG132 (to prevent the rapid degradation of HIF1α after reoxygenation) produced a higher proportion of hydroxylated HIF1α than after OGD (Fig. 6). When the PHD inhibitor DMOG was added instead of MG132, the oxygen-dependent hydroxylation of HIF1α after OD was inhibited and HIF1α was not degraded (Fig. 6). We hypothesized that the oxygen-dependent hydroxylation of HIF1α was impaired after a long period of OGD. This could be caused either by a reduction of PHD enzyme activity/abundance or by changes in the availability of substrates or inhibitors affecting this reaction. We first tested whether the abundance of PHD2, the main isoform involved on HIF1α hydroxylation (23) was affected by OGD, and we found it was not the case (Fig. 6).

Under our conditions, the physical diffusion of atmospheric oxygen into the medium at reoxygenation seems to be a rapid process, based on the fast disappearance of HIF1α during reoxygenation of the glucose-treated group (Fig. 3 and supplemental Fig. S3, as well as previous data (1)). Thus, it is unlikely that oxygen availability could be limiting during reoxygenation after OGD.

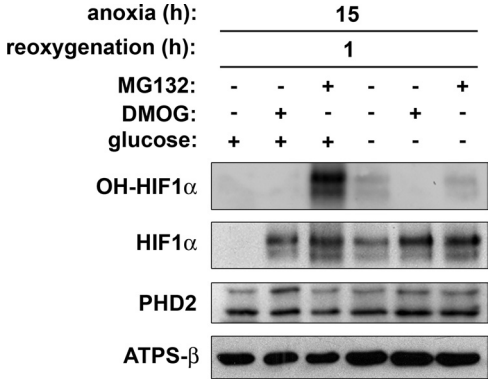

FIGURE 6. **The hydroxylation of HIF1α is reduced after OGD followed by 1 h of reoxygenation.** Detection of hydroxy-Pro^{564}-HIF1α by immunoblotting of total cellular extracts prepared from SH cells 1 h after reoxygenation following OD (glucose +) or OGD (glucose −) for 15 h, as indicated. Supplementation of the medium with 10 μM MG132 prior to anoxia prevented HIF1α degradation following reoxygenation after OD treatment and allowed detection of hydroxylation under this condition. PHD inhibitor DMOG (1 mM) was added prior to anoxia to a separate dish to control for the specificity of the signal. Total HIF1α and PHD2 protein levels were subsequently measured on the same blot. ATP synthase-β (*ATPS-β*) was used to control for equal loading.

Altered Levels of Krebs Cycle Metabolites after OGD Might Limit HIF1α Hydroxylation in the Presence of Oxygen—The hydroxylation reaction catalyzed by PHD2 also depends on α-ketoglutarate as co-substrate, and its activity has been shown to be inhibited by metabolites of the tricarboxylic cycle such as succinate (which is also the product of the hydroxylation reaction) and fumarate, the latter being the most effective as an inhibitor (24, 25). Thus, we carried out GC-MS analysis on extracts prepared from SH cells to quantitate the relative abundance of α-ketoglutarate, succinate, and fumarate at 1 h of reoxygenation after a 15 h treatment under OD or OGD. Our data showed that the abundance of α-ketoglutarate and succinate was significantly reduced in the OGD group relative to control (Fig. 7, *A* and *B*). While OD reduced the hydroxylation inhibitor fumarate, the abundance of fumarate after OGD tended to be even higher than in controls (Fig. 7*C*). The abundance of α-ketoglutarate or succinate in the OD group was not significantly different from the control group. The ratio (mean ± S.E.) α-ketoglutarate/fumarate was 0.0065 ± 0.0015 in controls. This value was significantly reduced (one way ANOVA, $p < 0.05$) in OGD (0.0015 ± 0.0005), while it was not statistically different from control in OD (0.0097 ± 0.0030). The lower ratio between α-ketoglutarate (reaction substrate) and fumarate (inhibitor) in the OGD group prompted us to speculate whether these metabolites would play a role in the stabilization of HIF1α by inhibiting HIF1α hydroxylation. Thus, we treated SH cells for 6 h with 20 mM monoethyl fumarate (*me-f*, Fig. 7*D*) in the presence of glucose under normoxia, to increase the intracellular availability of fumarate (26). Western blot analysis showed that the treatment with me-f prevented HIF1α degradation under normoxic conditions (Fig. 7*D*). In all, our data indicate that alterations in the abundance of metabolites could play a regulatory role in the expression of HIF1α, and could partly contribute to the glucose-dependent differences in HIF1α stability at reoxygenation.

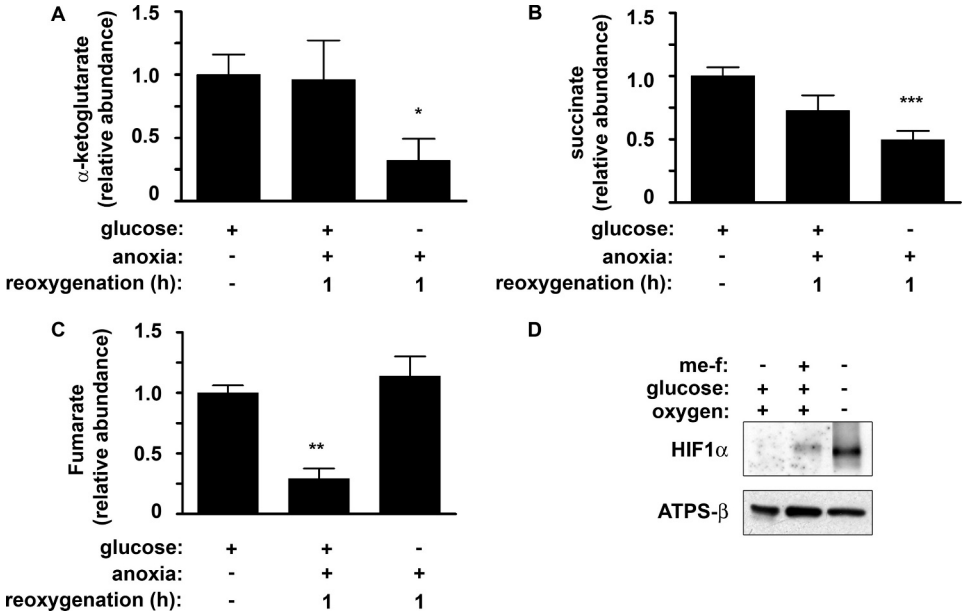

FIGURE 7. **OGD induces alterations in tricarboxylic acid (*TCA*) cycle metabolites that may affect HIF1α hydroxylation and expression.** Intracellular levels of some TCA cycle metabolites that may affect HIF1α expression: α-ketoglutarate (*A*), succinate (*B*), and fumarate (*C*), were detected by GC-MS (see "Experimental Procedures") in the conditions indicated. Normalized data were expressed relative to the abundance of the control group, and are shown as the average ± S.E. of seven independent experiments. Data on α-ketoglutarate (*A*, OGD *versus* control, *, $p < 0.05$) were analyzed by Kruskal-Wallis test for non-parametric data followed by a Dunn's multiple comparison test, while succinate (*B*, OGD *versus* control, ***, $p < 0.005$) and fumarate levels (*C*, OD *versus* control, **, $p < 0.01$) were analyzed by one-way ANOVA followed by Bonferroni post-test. *D*, supplementation of the medium with 20 mM monoethyl fumaric acid was enough to increase HIF1α expression in cells under control conditions. An OGD sample was included as a positive control. ATP synthase-β was used as a loading control.

DISCUSSION

Our study shows that the induction of HIF1α protein during anoxia is strongly affected by the prolonged absence of glucose in neuroblastoma cells. The effects of the absence of glucose are manifested in two ways: (*a*) by diminished induction and reduced transcriptional effects of HIF1α, and (*b*) by the acquired resistance of HIF1α against the oxygen-induced degradation. According to these results, severe ischemia is expected to induce a lower transcriptional activity of HIF1α than mild ischemia or hypoxia. Moreover, oxygen avail-

ability at reperfusion after severe ischemia might not induce rapid HIF1α degradation, whereas oxygen will readily induce full degradation of HIF1α after a period of mild ischemia or hypoxia. Our results confirm and extend previous observations by Vordermark *et al.* (27) who described a full activation of HIF1α expression in tumor cell lines only in the presence of glucose during hypoxia, which helped to explain the previously observed uncoupling between HIF1α expression and hypoxic areas in solid tumors. It stands to reason there may be a feedback mechanism to prevent the HIF-mediated up-regulation of glycolytic enzymes when no glucose is available, because no immediate benefit would derive from it and may otherwise contribute to deplete the already strained cellular energy stores.

Several lines of evidence suggest that the serine-threonine kinase mammalian target of rapamycin (mTOR) may bridge glycolytic flux and the expression of HIF1α. This kinase is part of a multiprotein complex named mTORC1 that receives input from mitogenic as well as energy-sensing pathways and, in turn, promotes protein synthesis and cell growth (28). This complex has also been put forward as a positive regulator of HIF1α function (29, 30). Our results agree with a previous report that also described decreased HIF1α expression during OGD (31) and suggested this might be due to inhibition of mTOR signaling by increased AMPK activity. Other evidences support a negative role of AMPK for HIF1α expression (32). We know that in our experimental model, ATP reduction is more severe after OGD than after OD (14), so it is plausible that AMPK activity could be higher under OGD conditions, as described by Laderoute *et al.* (33). Nevertheless, there may also be mTORC1 inhibition in the absence of glucose that is independent of AMPK (34). Thus, whether an AMPK-dependent or -independent mechanism lies behind the reduced levels of expression of HIF1α in our OGD model should deserve separate investigation.

We report how the HIF1α protein that is expressed under conditions of OGD displays resistance against the reoxygenation-induced degradation that otherwise occurs in the presence of glucose. This phenomenon was not due to inhibition of the general process of protein degradation in the proteasome but seemed to be due to decreased prolyl-hydroxylation of HIF1α. This effect occurred alongside alterations in the abundance of tricarboxylic cycle metabolites (low α-ketoglutarate/fumarate ratio) that are directly involved in the regulation of PHDs (25). Despite the effects of such metabolites have been studied in heritable alterations of tricarboxylic acid cycle enzyme expression and in cancer (24, 26), we are not aware of other studies like ours where metabolic regulation induced by environmental changes correlated with HIF1α stability through control of prolyl-hydroxylation. Thus, in our study, OGD conditions caused significantly diminished abundance of α-ketoglutarate and succinate relative to control cells. The α-ketoglutarate data coincide with a study by the Robert Vanucci group in the early 90's that reported decreased abundance of α-ketoglutarate after ischemia in rat brain (35). On the other hand, we found in our model that fumarate abundance was higher at reoxygenation after OGD than after OD, and that the α-ketoglutarate/fumarate ratio was greatly reduced after OGD *versus* OD or control conditions (Fig. 7*C*). In our cells, an increase in fumarate intracellular abundance by supplementation of the medium with monoethyl fumarate caused the up-regulation of HIF1α under normoxic conditions (Fig. 7*D*), which, according to the literature, would be caused by inhibition of hydroxylases targeting HIF1α (25, 26). The observed changes in metabolite abundance induced by OGD, results in a substrate/inhibitor ratio that would be less favorable for HIF1α hydroxylation. In keeping with this, previous data indicate that induction of HIF1α expression in normoxia by monoethyl-fumarate can be reverted by supplementation with a membrane-permeable form of α-ketoglutarate (26).

Regarding the possible role of the extended expression of HIF1α beyond reoxygenation after OGD, a recent report by Filiano *et al.* (36) has shown how transglutaminase 2, which is increased after subjecting RA-differentiated SH cells to OGD and is protective against OGD-induced cell death, binds HIF1 β and dampens HIF1 transcriptional effects (36). The evidence provided in that report suggests that HIF1 expression induced by OGD would play a detrimental role in SH cell survival. This is controversial, because HIF1α inhibition caused negative effects on the survival of SH cells to an ischemic insult followed by reoxygenation, because of a shift in the cellular redox environment toward a more oxidizing state (37). Building on those reports and in concordance with data presented in our previous publication (14), it is tempting to speculate that the low-key sustained expression of HIF1α after OGD and reoxygenation may partly account for the activation of the genetic program that leads to OGD-induced delayed cell death in our model. Nevertheless, proper demonstration of this hypothesis lies beyond the scope of this report, and shall await further research.

In conclusion, we present data suggesting that HIF1α protein expression could be regulated by changes in cellular metabolism that reflect environmental cues. In one hand, HIF1α would be regulated by a mechanism purely dependent on the presence of oxygen, which in the presence of glucose, would result in a high level of activation of HIF1α and enhancement of ATP production by glycolysis with rapid clearance of HIF1α after reoxygenation when aerobic metabolism is resumed. On the other hand, our results support the involvement of another mechanism driven by alterations in the abundance of tricarboxylic acid cycle metabolites that would restrain the expression of HIF1α in the absence of both oxygen and glucose, thus preventing unnecessary activation of glycolysis. This mechanism would maintain HIF1 activity at reoxygenation while the metabolic profile remains altered. Our findings lend further support to the idea that it might be possible to regulate HIF1α expression up to a certain extent independently of oxygen levels by coupling metabolism and prolyl-4-hydroxylase activity (38, 39), in metabolic contexts that may extend beyond the range of situations where the effects of metabolites on HIF1α expression have been successfully explored so far (24, 26, 40).

Acknowledgments—We thank Ramon Bartrons (University of Barcelona) and Nico Dantuma (Karolinska Institute) for providing the pfkfb3HRE-Luc and Ub-GFP reporter constructs, respectively. We are grateful to Jaume Comas (Serveis Científics i Tècnics, Universitat de Barcelona) for help with flow cytometry experiments.

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