

Differential Sensitivity of Hypoxia Inducible Factor Hydroxylation Sites to Hypoxia and Hydroxylase Inhibitors*^[5]

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Hypoxia inducible factor (HIF) is regulated by dual pathways involving oxygen-dependent prolyl and asparaginyl hydroxylation of its α -subunits. Prolyl hydroxylation at two sites within a central degradation domain promotes association of HIF- α with the von Hippel-Lindau ubiquitin E3 ligase and destruction by the ubiquitin-proteasome pathways. Asparaginyl hydroxylation blocks the recruitment of p300/CBP co-activators to a C-terminal activation domain in HIF- α . These hydroxylations are catalyzed by members of the Fe(II) and 2-oxoglutarate (2-OG) oxygenase family. Activity of the enzymes is suppressed by hypoxia, increasing both the abundance and activity of the HIF transcriptional complex. We have used hydroxy residue-specific antibodies to compare and contrast the regulation of each site of prolyl hydroxylation (Pro⁴⁰², Pro⁵⁶⁴) with that of asparaginyl hydroxylation (Asn⁸⁰³) in human HIF-1 α . Our findings reveal striking differences in the sensitivity of these hydroxylations to hypoxia and to different inhibitor types of 2-OG oxygenases. Hydroxylation at the three sites in endogenous human HIF-1 α proteins was suppressed by hypoxia in the order Pro⁴⁰² > Pro⁵⁶⁴ > Asn⁸⁰³. In contrast to some predictions from *in vitro* studies, prolyl hydroxylation was substantially more sensitive than asparaginyl hydroxylation to inhibition by iron chelators and transition metal ions; studies of a range of different small molecule 2-OG analogues demonstrated the feasibility of selectively inhibiting either prolyl or asparaginyl hydroxylation within cells.

including regulation of angiogenesis, erythropoiesis, energy metabolism, and cell growth and survival pathways (reviewed in Refs. 1–3). Regulation by oxygen availability is transduced by the HIF- α subunits through two distinct oxygen-dependent pathways; regulation of HIF- α protein stability and regulation of HIF- α transcriptional activation, mediated by prolyl and asparaginyl hydroxylation, respectively (for review, see Ref. 4).

Human HIF- α exists as three isoforms, the two best characterized of which (HIF-1 α and HIF-2 α) each contain two sites of prolyl hydroxylation and a single site of asparaginyl hydroxylation. HIF- α prolyl hydroxylation occurs at two sites lying in the N-terminal (NODD) and C-terminal (CODD) regions of a central oxygen-dependent degradation domain; residues Pro⁴⁰² and Pro⁵⁶⁴ in human HIF-1 α (5–7). HIF- α asparaginyl hydroxylation occurs in the C-terminal activation domain; residue Asn⁸⁰³ occurs in human HIF-1 α (8). HIF- α prolyl hydroxylation promotes interaction with the von Hippel-Lindau ubiquitin E3 ligase complex and targets HIF- α subunits for degradation by the ubiquitin-proteasome pathway (9, 10). HIF- α asparaginyl hydroxylation interferes with the binding of p300/CBP co-activators to the C-terminal activation domain and thus inhibits transcriptional activation, independently of effects on protein stability (11).

HIF prolyl hydroxylation is catalyzed by three closely related enzymes PHD (prolyl hydroxylase domain) 1, 2, and 3, otherwise known as EGLN2, -1, and -3 (12, 13), whereas HIF asparaginyl hydroxylation is catalyzed by FIH (factor inhibiting HIF) (14, 15). Both PHDs and FIH are Fe(II)- and 2-oxoglutarate-dependent dioxygenases (2-OG oxygenases) that utilize molecular oxygen as a co-substrate, and couple hydroxylation of HIF- α to the oxidative decarboxylation of 2-oxoglutarate (2-OG) and carbon dioxide (for review, see Refs. 16 and 17). This provides a dual control system whereby in the presence of oxygen HIF- α subunits are both inactivated and destroyed, whereas in hypoxia catalysis of these hydroxylations is suppressed, enabling HIF- α subunits to escape von Hippel-Lindau-mediated proteolysis, recruit co-activators, and form a productive transcriptional complex. This action of hypoxia can be partially mimicked by a number of agents that inhibit 2-OG oxygenases including iron chelators, transition metal ions, and small molecule 2-OG analogues. There is widespread interest in the possibility of developing HIF hydroxylase inhibitors that activate

Hypoxia inducible factor (HIF)² is the major transcriptional regulator of responses to hypoxia in metazoan cells. The HIF transcriptional complex binds DNA as an α/β heterodimer and is centrally involved in many homeostatic responses to hypoxia,

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S5.

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² The abbreviations used are: HIF, hypoxia inducible factor; 2-OG, 2-oxoglutarate; NODD and CODD, N- and C-terminal oxygen-dependent degradation domains; PHD, prolyl hydroxylase domain; FIH, factor inhibiting HIF; DFO, desferrioxamine; DMOG, dimethylxalylglycine; IVTT, *in vitro* transcription/translation; VHL, von Hippel-Lindau.

HIF Prolyl and Asparaginyl Hydroxylation

HIF pathways therapeutically in ischemic/hypoxic disease (18–20).

In understanding the regulation of the transcriptional response by hypoxia and its potential manipulation by such agents, it is therefore important to understand the extent to which prolyl and asparaginyl hydroxylation of HIF- α are differentially inhibited by hypoxia and hydroxylase inhibitors. Several studies have characterized the *in vitro* kinetic properties of the PHDs and FIH using full-length or truncated recombinant proteins and have reported differences in kinetics with respect to oxygen, iron, and small molecule inhibitors. In some studies, a lower apparent K_m for oxygen has been reported for FIH than the PHDs (21, 22). However extrapolation from these studies is not straightforward. For instance, it has been shown that the apparent K_m for oxygen can depend on the HIF- α polypeptide used as the prime substrate and no studies have used full-length HIF- α polypeptide (23, 24). Furthermore, the existence of many known and postulated alternative substrates for FIH and the PHDs, together with difficulty in measuring concentrations of the enzymes in the relevant cellular compartments make it extremely difficult to predict, from these *in vitro* measurements, how oxygen availability might restrict catalysis *in vivo*.

We have therefore developed methods employing hydroxy residue-specific antibodies to monitor the regulation of hydroxylation at all three sites in human HIF-1 α . Previous studies using hydroxy residue-specific antibodies have described important differences in the regulation of the two sites of prolyl hydroxylation in HIF-1 α (25) but to date there have been no studies of the differential regulation of prolyl and asparaginyl hydroxylation.

Here we report the results of utilizing highly specific antibodies recognizing all three sites of hydroxylation in human HIF-1 α to analyze patterns of hydroxylation of HIF-1 α in cells. Our results reveal major differences in the regulation of HIF prolyl and asparaginyl hydroxylation by hypoxia and different classes of chemical inhibitors of these hydroxylases.

EXPERIMENTAL PROCEDURES

Cells and Experimental Conditions—Cells were cultured in DMEM (MCF7, HeLa, HT1080, and human skin fibroblasts) or RPMI 1640 (PC3, OVCAR-3, HepG2, and K562), each supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml of penicillin, and 50 μ g/ml of streptomycin. Stable RCC4 transfectants expressing vector alone (RCC4/VA, referred to here as RCC4) or functional VHL (RCC4/VHL) have been described (26). U2OS cells expressing doxycycline-inducible HIF-1 α genes were derived from U2OS cells bearing the reverse tetracycline responsive transactivator and tetKRAB silencer transgenes (27), by transfection with pUHD10–3MCS expressing full-length C-terminal PK-tagged HIF-1 α sequences (HIF-1 α /pUHD) or a M561A + M568A mutant produced by site-directed mutagenesis (QuikChange, Stratagene) and pSV2-hph, containing the hygromycin resistance gene (ATCC). After selection of transfectants in hygromycin (200 μ g/ml) (Roche Applied Science), cells were maintained in DMEM supplemented with a 10% Tet system-approved FBS (Clontech), 5 μ g/ml of blasticidin S (Invitrogen), and 200 μ g/ml of hygromycin, in addition to the supplements detailed above.

Hypoxic incubations were performed in *In vivo*, 400 hypoxic workstations (Ruskin Technologies). Where specified, Lumox dishes (Greiner Bio-one) were used to achieve rapid gas equilibration between the atmosphere and the culture medium. Desferrioxamine (DFO), 2,2'-dipyridyl, and ethyl-3,4-dihydroxybenzoate (EDHB) were from Sigma. The following compounds were synthesized as reported: 4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid (28), bicyclic isoquinolinyl compounds A and B (29), and *N*-oxalyl phenylalanine derivatives C-F (30). Chemicals were dissolved in DMSO and added directly to culture medium at final DMSO concentrations of \leq 2%. Dimethyloxalylglycine (DMOG) was from Frontier Scientific and dissolved in PBS. MG132 was from Enzo Life Sciences. Exposure time was 4–5 h.

In Vitro Transcription/Translation (IVTT)—HIF- α proteins were expressed as GAL4 (residues 1–147) fusions using the IVTT kit (Promega). Plasmids expressing GAL4/HIF-1 α residues 28–826 (wild-type or Pro⁴⁰², Pro⁵⁶⁴, and Asn⁸⁰³ mutants), or GAL4/HIF-2 α residues 19–870 were constructed from pcDNA3 using standard recombinant methods.

Suppression of Gene Expression by siRNA—Cells were seeded at 40% confluence in antibiotic-free medium for 16 h prior to transfection with siRNAs directed against *Drosophila* SIMA (dHIF) (31), or human HIF-1 α or HIF-2 α (32). Oligofectamine (Invitrogen) was used for transfection of 20 nM siRNA duplex twice at 24-h intervals. Cells were harvested 24 h after the second transfection.

Antibodies—Rabbit anti-HIF-1 α Hyp402 was developed in collaboration with Millipore Biosciences (catalog number 07-1585). Mouse mAb against HIF-1 α hydroxy-Asn⁸⁰³ was as described (33). Other antibodies were as follows: anti-HIF-1 α Hyp564 (clone-D43B5, New England Biolabs); anti-GAL4 (clone-RK5C1, Santa Cruz Biotechnology), pan-anti-HIF-1 α (clone-54, BD Transduction Laboratories), anti-HIF-2 α (clone-190b; Wiesener *et al.* 51), and HRP-conjugated anti- β -actin (clone AC15, Abcam).

Immunoblotting and Densitometric Analyses—Cells were lysed in urea/SDS buffer (6.7 M urea, 10 mM Tris-Cl (pH 6.8), 10% glycerol, and 1% SDS) supplemented with 1 mM dithiothreitol and Complete Protease Inhibitor Mixture (Roche Applied Science). Extracts were resolved by SDS-PAGE, electroblotted onto PVDF membranes (Millipore), and probed with primary antibodies followed by HRP-conjugated secondary antibodies. SuperSignal Chemiluminescent Substrates (Pierce) were used to visualize immunoreactive species. Signal intensities were measured by densitometry using the AutoChemi System with Labworks 4.6 Image Acquisition and Analysis Software (UVP Inc. USA). Statistical significance was determined using a one-way analysis of variance Dunnett's post hoc test using SPSS statistics 17.0; differences were considered significant if *p* values were $<$ 0.05.

Mass Spectrometry—Doxycycline-inducible PK-tagged wild-type or mutant HIF-1 α transfectants were incubated with 1 μ g/ml of doxycycline for 24 h, either in the presence of the 2-OG oxygenase inhibitor DMOG (1 mM) for the same duration, or with the proteasome inhibitor MG132 (25 μ M) for the last 4 h. Following anti-PK immunoprecipitation, separation by SDS-PAGE and in-gel digestion of the isolated

bands with trypsin and Glu-C, samples were analyzed on a C18 nano-Acquity™ UPLC™ column coupled to a Waters Q-TOF Tandem mass spectrometer (Milford, MA) in positive ESI mode as described previously (34). Data acquisition was performed in high-low collision energy switching mode (MS^E). MS/MS data base searches were carried out using ProteinLynx Global Server (PLGS version 2.2.5) and MASCOT (version 2.2) on an in-house server (CBRG, University of Oxford).

Protein Extracts from Rat and Human Tissues—Studies of animal and human tissues were approved by the Universität Erlangen institutional review board for the care of animal subjects and the United Kingdom Ethical Committee (number C02.216), respectively.

Male Sprague-Dawley rats (Charles River; 200–300 g) were treated with carbon monoxide (CO, 0.1%) for 6 h to achieve a functional anemia. Animals were sacrificed and kidneys were harvested immediately and snap frozen in liquid nitrogen. Human tissues were obtained from renal and breast cancer patients at operation and snap frozen in liquid nitrogen.

Frozen tissues were mashed into a paste in a dry ice-chilled metal chamber and immediately homogenized in urea/SDS buffer. After centrifugation at $16,000 \times g$ for 10 min the supernatants were heat-denatured in sample buffer and used in immunoblotting. Protein contents were measured using the Detergent Compatible Assay kit (Bio-Rad).

RESULTS

Validation of Hydroxy Residue-specific Antibodies—Initially we carried out a series of experiments to determine the specificity of antibodies for the detection of different hydroxylated HIF- α species. We first prepared HIF- α polypeptides that were wild-type, or contained mutations at each of the prolyl and asparaginyl hydroxylation sites, as GAL4-HIF- α fusion proteins using an IVTT system. Reactions were performed in the presence of iron chelator DFO or iron supplement (FeCl₂) to inhibit or promote hydroxylation, respectively. Results are shown in Fig. 1. Antibodies directed against epitopes that encompass each of the hydroxyproline residues in HIF-1 α (Hyp402 and Hyp564) or the hydroxyasparaginyl residue (HyAsn⁸⁰³) showed strong reactivity to HIF-1 α that was produced in the presence of FeCl₂, but little or no reactivity to that produced in the presence of DFO, suggesting that each of these antibodies is highly specific for the hydroxylated form (Fig. 1, compare lanes 1 and 2). The antibodies were also highly specific for HIF-1 α versus HIF-2 α (Fig. 1, compare lanes 2 and 3, and supplemental Fig. S1). Substitution at each site of hydroxylation in HIF-1 α ablated reactivity of the relevant antibody, indicating that the antibodies were highly specific to each site; anti-Hyp402 did not react against Hyp564 and vice versa.

To further verify the specificity for hydroxylated forms of HIF-1 α in cells, we expressed HIF-1 α polypeptides in cells at levels that enabled direct analysis of hydroxylation by mass spectrometry (MS) and analyzed aliquots of cell extract in parallel by hydroxy residue-specific immunoblotting and MS. Under normoxic conditions, cells were exposed to DMOG to inhibit hydroxylation, or MG132 to inhibit the proteasome and cause accumulation of hydroxylated HIF-1 α . To simplify the

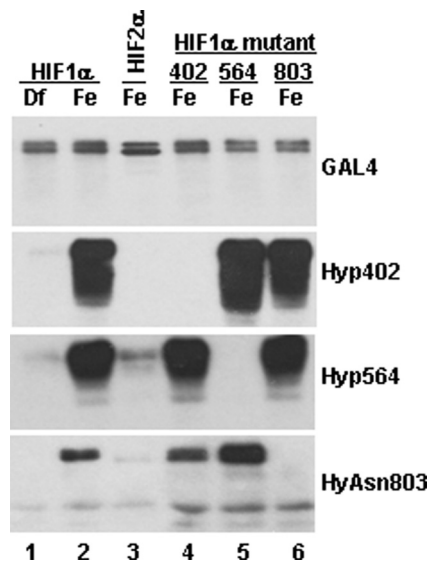


FIGURE 1. Reactivity of hydroxy residue-specific anti-HIF1 α antibodies. Immunoblots demonstrating the specificity of anti-hydroxy Pro⁴⁰² (Hyp402), anti-hydroxy Pro⁵⁶⁴ (Hyp564), and anti-hydroxy Asn⁸⁰³ (HyAsn803) antibodies for these hydroxylated residues in human HIF-1 α . HIF-1 α (wild-type and mutants P402A, P564G, or N803A) and HIF-2 α polypeptides were produced as GAL4 fusions in IVTT reactions in the presence of DFO (Df) or ferrous chloride (Fe). Anti-GAL4 signals provide an internal control for normalization. Note a low level of cross-activity with HIF-2 α observed for anti-hydroxy Pro⁵⁶⁴ and anti-hydroxy Asn⁸⁰³ in this immunoblot in lane 3.

semi-quantitative analysis of Hyp564 by MS, neighboring methionine residues (Met⁵⁶¹ and Met⁵⁶⁸) were mutated to alanines. The results of a typical set of experiments are shown in Fig. 2. Comparison with MS analysis confirmed that each antibody manifested a high degree of hydroxy residue specificity, demonstrating the complete absence of activity against the unhydroxylated epitope and enabling an estimate of the efficiency of detection of the hydroxylated form.

Status of Asparaginyl Hydroxylation of HIF-1 α in Hypoxic Cells—Because asparaginyl hydroxylation regulates the activity of HIF transcription, it is important to understand whether and to what extent HIF asparaginyl hydroxylation persists under conditions that induce HIF-1 α protein levels. We therefore examined a series of different cell types exposed to different levels of hypoxia or DMOG; in the first instance cultures were exposed in parallel to 1% oxygen, or DMOG, or maintained under normoxic conditions. As expected, strong up-regulation of the HIF-1 α protein levels was observed, reflecting inhibition of the HIF prolyl hydroxylation/degradation pathway. However, in 1% oxygen HIF-1 α asparaginyl hydroxylation persisted in all these cell types, although to a variable extent (Fig. 3A). Results obtained following exposure to DMOG were markedly different (Fig. 3B); despite similar levels of up-regulated HIF-1 α , exposure of cells to DMOG almost totally suppressed asparaginyl hydroxylation. To provide more quantitative data on the effect of 1% oxygen, different quantities of cell extracts from two cell types showing different responses (PC3 cells and HSF cells) were blotted for total and asparaginyl hydroxylated HIF-1 α and signals were compared with a calibration standard provided by extracts from the cells treated with proteasomal inhibitor MG132 and analyzed by MS (Fig. 3C). These experiments revealed that HIF-1 α induced by 1% oxygen in HSF cells

HIF Prolyl and Asparaginyl Hydroxylation

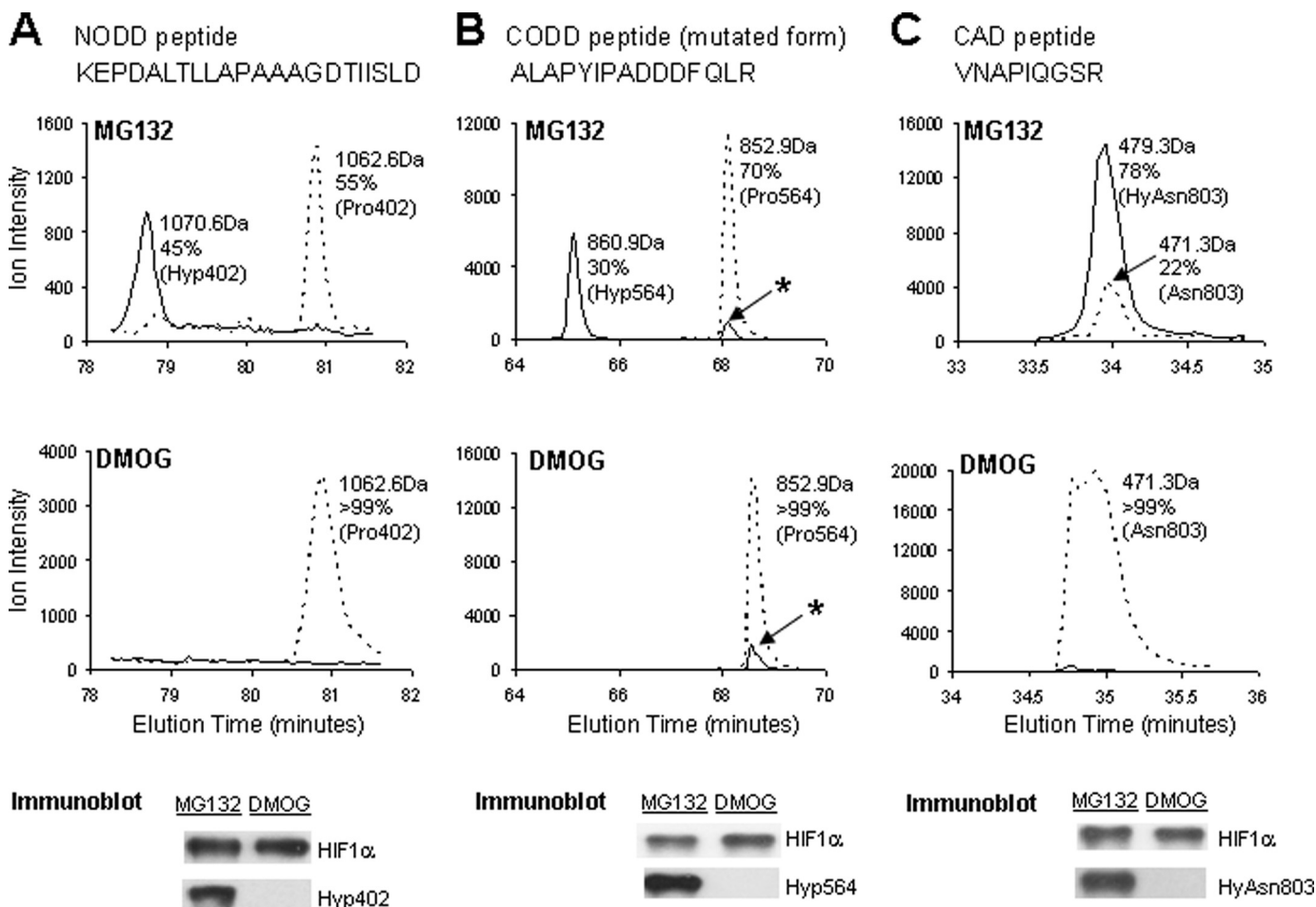


FIGURE 2. Validation of hydroxy residue-specific antibody performance by MS. A–C show data for sites in HIF-1 α NODD, CODD, and C-terminal activation domain (CAD) sequences, respectively. Cells expressing doxycycline-inducible PK-tagged wild-type or mutant (M561A + M568A) HIF-1 α were induced with doxycycline (1 μ g/ml) in the presence of 1 mM DMOG for 24 h, or 25 μ M MG132 for the last 4 h. For MS, PK immunoprecipitation was followed by in-gel digestion. The resulting peptides were analyzed by MS. The $[M+2H]^{2+}$ precursor ions corresponding to the unhydroxylated and hydroxylated peptides are given together with chromatographic output (extracted ion counts) at these masses. Percentage hydroxylation was calculated from peak areas. * indicates confounding ions with a similar mass to the hydroxylated CODD peptide. Aliquots of the immunoprecipitation samples were immunoblotted with the hydroxy residue-specific or pan-HIF-1 α antibodies as indicated. Note these hydroxyl residue-specific immunoblots were differentially exposed to allow secure ascertainment of the absence of signal in samples in which hydroxylation was demonstrated by MS to be suppressed.

was >90% hydroxylated, whereas in PC3 cells the region was 50% hydroxylated. To further investigate the regulation of asparaginyl hydroxylation by hypoxia, cultures of cells were exposed either to 1 or 0.2% oxygen (Fig. 3D). These experiments demonstrated that the asparaginyl hydroxylation that persisted at 1% oxygen was strongly suppressed at 0.2% oxygen.

Finally to determine whether asparaginyl hydroxylation might persist under conditions that induce HIF proteins in intact tissues, we studied HIF-1 α from kidneys of rats that had been exposed to 0.1% carbon monoxide to create functional anemia and from a range of human tumor samples (Fig. 3E). These studies demonstrate the existence of substantial levels of HIF asparaginyl hydroxylation under these pathophysiological conditions.

Differential Sensitivity of HIF-1 α Prolyl and Asparaginyl Hydroxylation to Hypoxia—The above experiments indicated that despite strong accumulation of HIF-1 α in cells cultured in 1% oxygen, high levels of asparaginyl hydroxylation persisted. Because accumulation of HIF-1 α in hypoxia is mediated by suppression of prolyl hydroxylation, these results suggest the

existence of differential sensitivity of prolyl and asparaginyl hydroxylation to hypoxia *in vivo*.

We therefore sought to compare the sensitivity of different prolyl and asparaginyl sites to graded hypoxia directly in their endogenous context. Prolyl-hydroxylated HIF is degraded by the VHL E3 ligase pathway, confounding comparison of prolyl and asparaginyl hydroxylation in VHL competent cells. We therefore conducted experiments in VHL-deficient RCC4 cells, in which this pathway is inactive. Immunoblotting of RCC4 extracts revealed strong signals with each of the hydroxy residue-specific HIF-1 α antibodies, indicating that both HIF prolyl and HIF asparaginyl hydroxylations proceed efficiently in the absence of VHL. To confirm this, we compared hydroxy residue-specific and total HIF-1 α signals in these cells with those obtained from MS-quantified samples. Although the accuracy of this comparison was limited by the need to introduce substitutions in the region of Pro⁵⁶⁴ to simplify MS analysis, which slightly impaired epitope recognition at that site (data not shown), HIF-1 α hydroxylation was estimated to be complete or close to complete at each of the three sites in normoxic RCC4

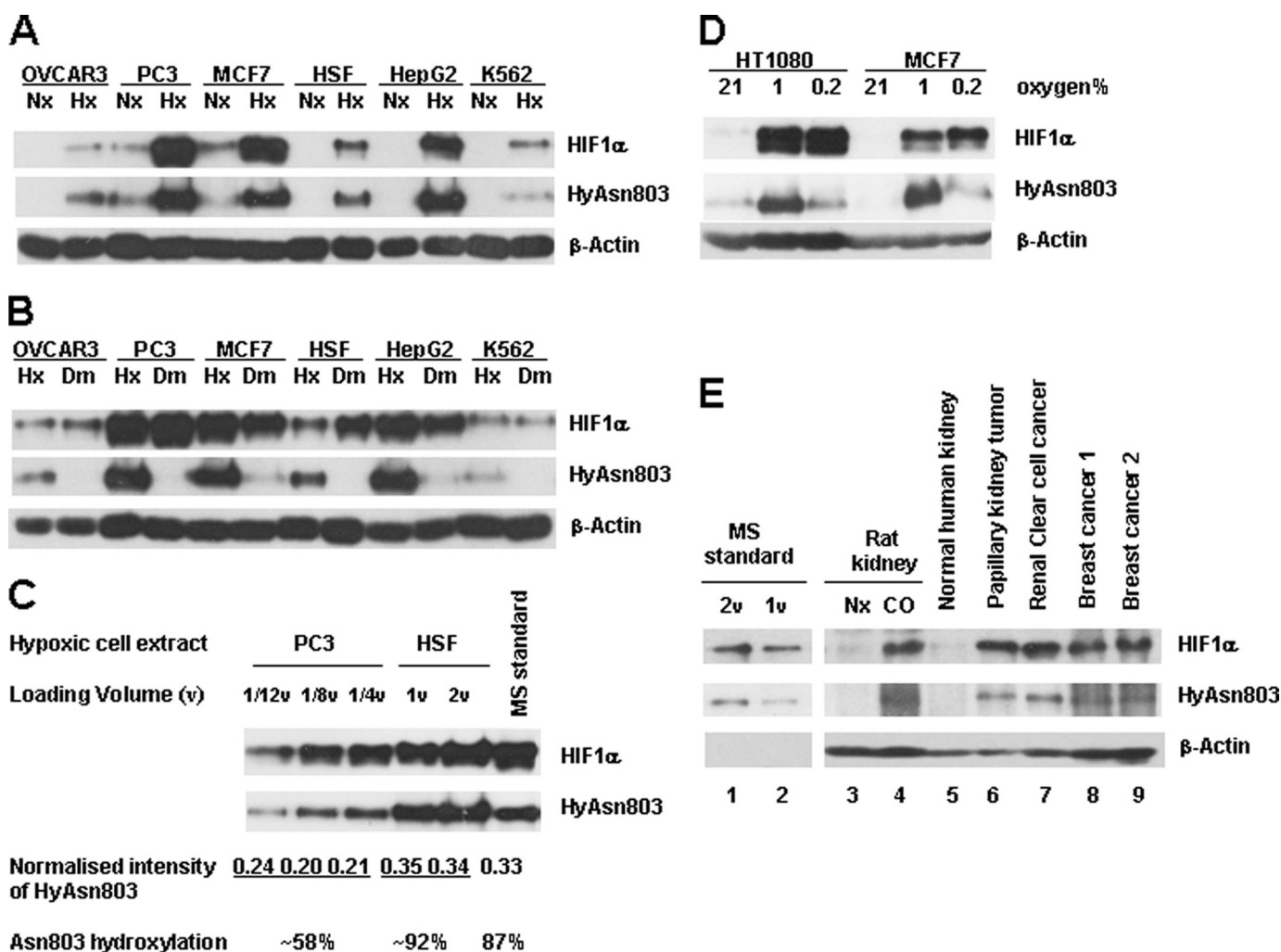


FIGURE 3. Levels of HIF asparaginyl hydroxylation in hypoxic cells and in rat and human tissues. *A* and *B* illustrate HIF-1 α protein levels and Asn⁸⁰³ hydroxylation status in different cell lines under normoxia (Nx), hypoxia (Hx, 1% oxygen), or exposed to 1 mM DMOG (Dm) for 5 h. HSF, primary human skin fibroblasts. *C* illustrates densitometric analyses of asparaginyl hydroxylation in two hypoxic cell extracts from *A*, with calibration against a standard assayed by MS. *D* illustrates the effect of more severe hypoxia (5 h) on HIF-1 α asparaginyl hydroxylation in HT1080 and MCF7 cells. *E*, immunoblots of rat kidney and human cancer tissues. Lanes 1 and 2, MS standard as in *C* loaded at different volumes (v). Lanes 3 and 4, kidney extracts of rats breathing air (Nx) or treated with carbon monoxide (CO, 0.1%) for 6 h. Lanes 5 and 6, extracts from normal kidney and papillary kidney tumor from the same patient. Lanes 7–9, tumor extracts from different patients.

cells (supplemental Fig. S2). Preliminary experiments established that the half-life of HIF-1 α in RCC4 cells was in the region of 60 min (supplemental Fig. S3); hence exposure to graded hypoxia for 4 h should be sufficient to allow HIF hydroxylation to achieve a new steady-state. In further pilot experiments we observed that suppression of hydroxylation occurred more rapidly in cells cultured in Lumox culture dishes (35) in which the cell monolayer is grown on a gas-permeable base, thereby minimizing gradients between the atmosphere and the monolayer and inaccuracies arising from release of oxygen dissolved in plasticware (supplemental Fig. S4). Thus, Lumox dishes were used for these and all other experiments involving short-term severe hypoxia ($\leq 0.1\%$ oxygen for ≤ 4 h).

Exposure of RCC4 cells to different levels of hypoxia had markedly different effects on each site of hydroxylation (Fig. 4, panels *A* and *B*). These experiments revealed that prolyl hydroxylation was more sensitive than asparaginyl hydroxylation to hypoxia and that Hyp402 was more sensitive than Hyp564. Whereas 0.1% oxygen almost completely suppressed

prolyl hydroxylation at both sites, asparaginyl hydroxylation remained in the region of 40%; at 0.5% oxygen, levels of hydroxylation were ~50, 75, and 100% at Pro⁴⁰², Pro⁵⁶⁴, and Asn⁸⁰³, respectively.

In understanding the physiological signaling of hypoxia, an important question is whether the specific activities of HIF hydroxylases alter in adaptation to chronic hypoxia. One possibility that has been considered is that increased specific activity in hypoxia might provide an adaptive response that would reset oxygen sensitivity in long-term hypoxia. Because two of the HIF prolyl hydroxylase enzymes, PHD2 and PHD3, are themselves transcriptional targets of HIF that are strikingly up-regulated in hypoxia (12, 36), assays of the effects of long-term hypoxia on the specific activity of the hydroxylases are complicated by large changes in enzyme abundance. However, in VHL-defective RCC4 cells HIF is constitutively activated, thus limiting changes in PHD abundance, and simplifying the interpretation of changes in HIF hydroxylation. We therefore exposed RCC4 cells for periods of hypoxia up to 7 days and

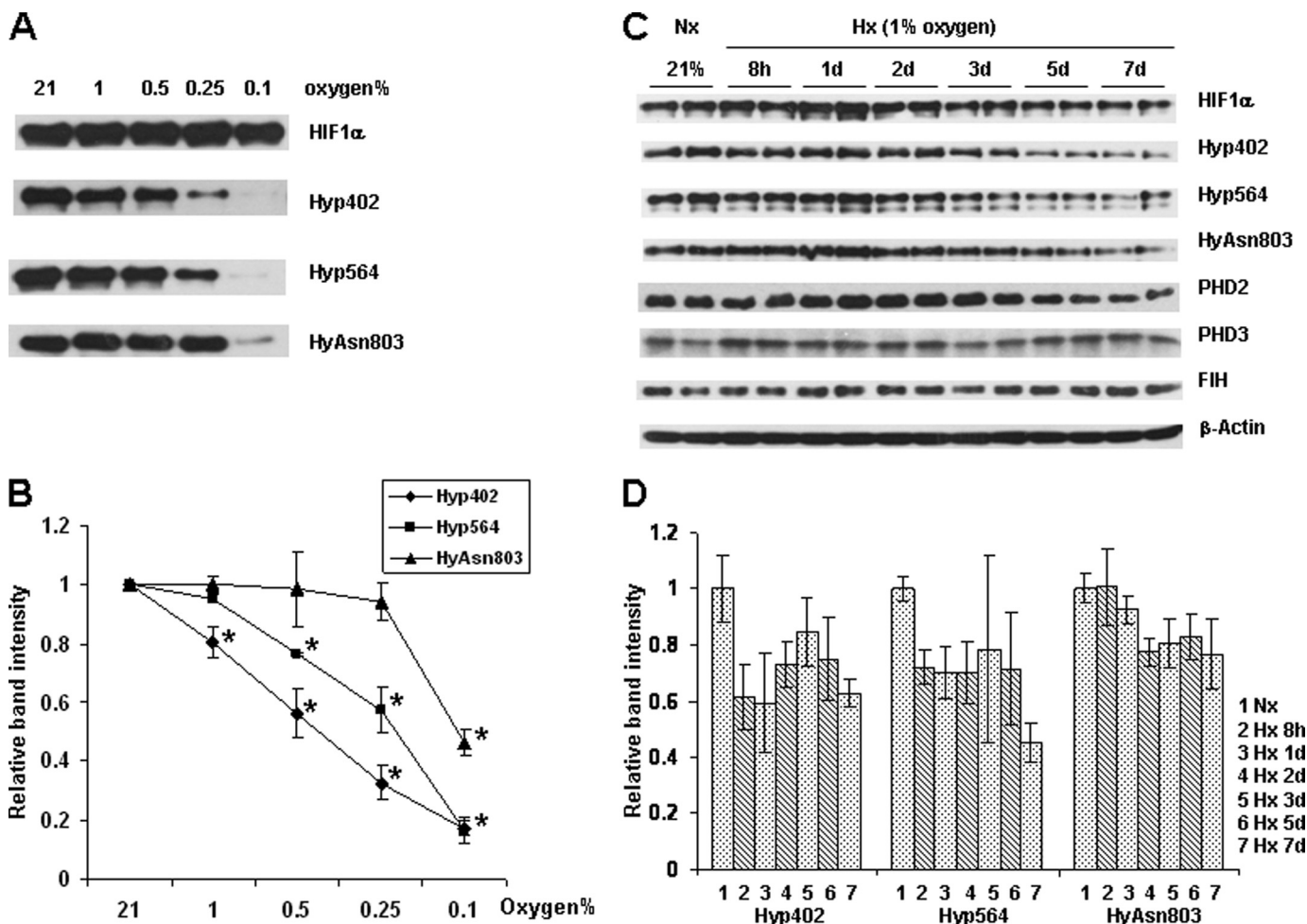


FIGURE 4. Effect of graded hypoxia and chronic hypoxia on HIF-1 α hydroxylation. *A* and *B*, graded hypoxia. RCC4 cells grown on Lumox dishes were incubated at the indicated oxygen concentrations for 4 h. The experiment was performed in triplicate. *A*, representative immunoblots; *B*, semi-quantitative analysis of relative band intensities (mean \pm S.E., $n = 3$). *, significantly different from normoxia (21%), $p < 0.05$. *C* and *D*, chronic hypoxia. RCC4 cells, seeded to achieve similar density at time of analysis, were incubated in normoxia (Nx) or hypoxia (Hx, 1% oxygen) for 8 h to 7 days. Two independent experiments were performed, each in duplicate. *C*, representative immunoblots. *D*, semi-quantitative analysis of relative band intensities (mean \pm S.E., $n = 4$). In *panels B* and *D*, the relative intensity is the ratio of the hydroxy residue-specific signal to total anti-HIF-1 α in that sample. Based on preliminary data indicating that hydroxylation at all sites were essentially 100% in normoxic RCC4 cells, these values were plotted with the ratio in normoxic cells set to one (equivalent to 100% hydroxylation). In *B*, normoxic S.E. values have been omitted for clarity.

monitored changes in hydroxylation at all three sites. Cells were plated so that levels of confluence were similar at the time of harvest. Results are shown in Fig. 4, *C* and *D*. Under these conditions, total HIF-1 α levels declined slightly in relationship to the β -actin normalization control during the course of the experiment. As predicted in VHL-deficient RCC4 cells, we did not observe induction of PHD protein levels that occurs normally as a consequence of HIF activation; over the 7-day period, levels of immunoreactive PHD and FIH enzymes were either unchanged or modestly reduced. After 8 h hypoxia (1% oxygen), there were clear reductions in the extent of prolyl hydroxylation but not asparaginyl hydroxylation. After continued exposure to hypoxia over 7 days, there was no evidence that suppression of prolyl hydroxylation was less, as might be expected with increased PHD activation; rather the extent of HIF hydroxylation was either unchanged or modestly reduced at all sites. These experiments therefore do not support the existence of major HIF-independent increases in the specific activity of the hydroxylases in response to chronic hypoxia over this period of time.

We next measured the effects of reoxygenation on each hydroxylation site. RCC4 cells were exposed to severe hypoxia (0.1% oxygen for 4 h) and then re-exposed to ambient (21%) oxygen. The results (Fig. 5*A*) reveal that although hydroxylation at Asn⁸⁰³ and Pro⁵⁶⁴ was essentially complete within 1 min but this was not the case for Pro⁴⁰² where a significant delay in hydroxylation was observed. To further analyze these differences, we returned to study the induction of HIF-1 α by hypoxia in VHL-competent cells in more detail. In preliminary experiments we observed differences between cell types in the behavior of Pro⁴⁰² and Pro⁵⁶⁴ during induction of HIF-1 α by hypoxia. In some cell types, but not others, during short periods of hypoxic exposure, substantial hydroxylation at Pro⁵⁶⁴ was observed in accumulating HIF-1 α protein; in contrast hydroxylation of Pro⁴⁰² remained low in all cell types studied. We therefore performed further experiments at different durations and severities of hypoxic exposure on two cell types (HT1080 and HeLa cells) that manifest contrasting behavior for hydroxylation at Pro⁵⁶⁴. Consistent and striking differences were observed. Accumulation of HIF-1 α that was hydroxylated at

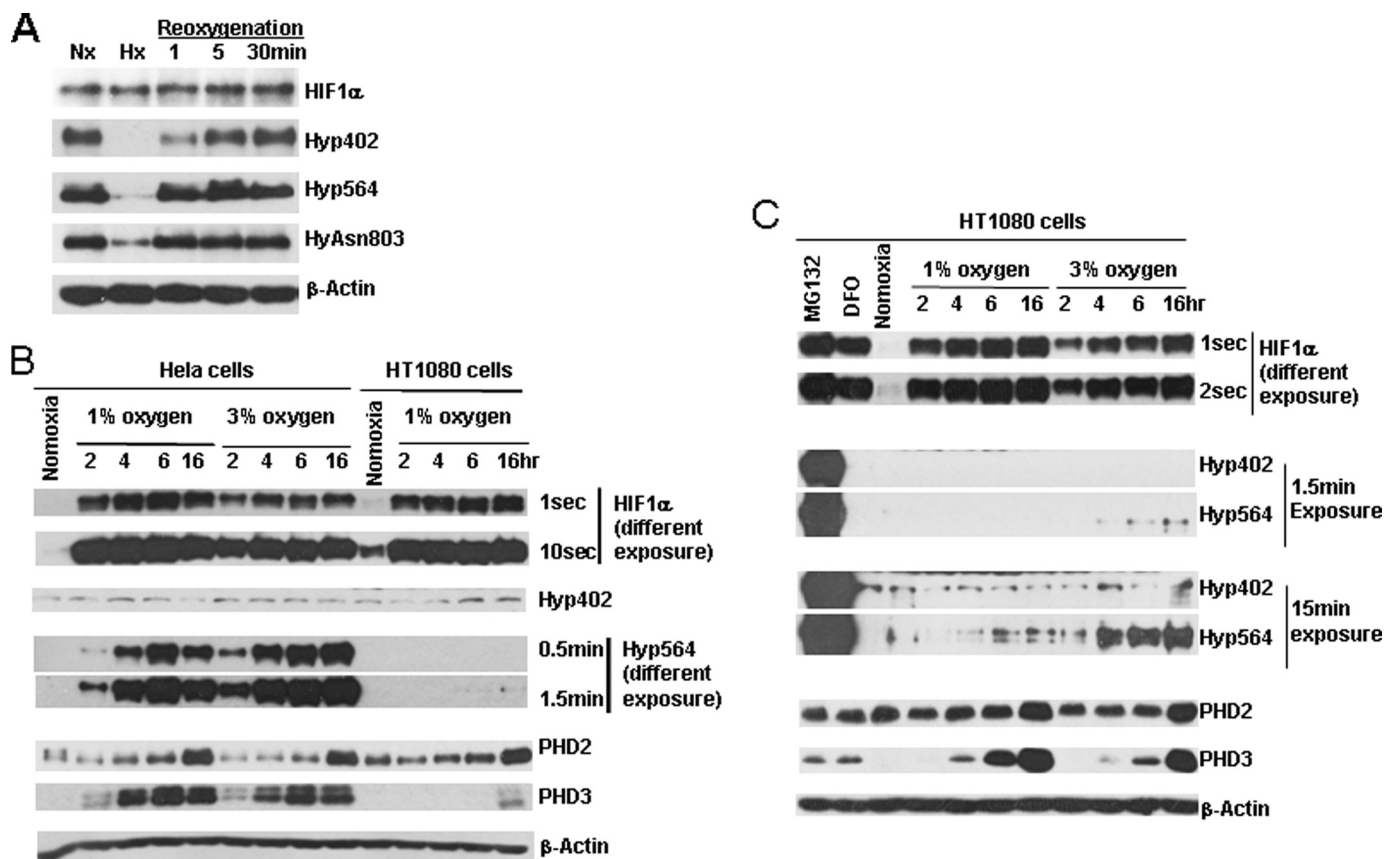


FIGURE 5. Differential regulation of HIF-1 α hydroxylation sites during hypoxic induction and during re-oxygenation. *A*, reoxygenation. RCC4 cells, incubated in Lumox dishes in normoxia (Nx) or hypoxia (Hx; 0.1% oxygen) for 4 h, were then exposed to normoxia for the indicated times. Hydroxylation at Hyp564 and HyAsn⁸⁰³ is complete within 1 min, but delayed at Hyp402. *B* and *C*, induction of HIF-1 α by hypoxia. HeLa or HT1080 cells were incubated in normoxia or hypoxia (1 or 3% oxygen) for the indicated times (h). Immunoblots were probed with the indicated antibodies. During the accumulation of HIF-1 α in hypoxic cells, substantial hydroxylation at Hyp564 but not Hyp402 is observed, particularly in HeLa cells.

Hyp564 was seen in HeLa cells at both 3 and 1% oxygen, whereas in HT1080 cells only minimal increases in hydroxylated Hyp564 were observed at 3% oxygen and none at 1% oxygen. In contrast, increases in hydroxylated Hyp402 were never observed during the accumulation of HIF-1 α in hypoxic cells of either type (Fig. 5, *B* and *C*). Immunoblotting for HIF prolyl hydroxylases PHD2 and PHD3 revealed that, as expected, both enzymes were strongly induced by hypoxia. Interestingly, however, induction of PHD3 was much more marked in HeLa than HT1080 cells, perhaps contributing to the differences in regulation of hydroxylation at Pro⁵⁶⁴. Taken together these results confirm that HIF-1 α prolyl hydroxylations at residues 402 and 564 are differentially regulated as a function of oxygen availability. Taken together with the other data, they are compatible with a model in which hydroxylation at Pro⁴⁰² is more readily suppressed in hypoxia than hydroxylation at Pro⁵⁶⁴ and in which hydroxylation at both sites is necessary for high rates of VHL-mediated degradation (25).

Differential Sensitivity of Prolyl and Asparaginyl Hydroxylation to Chemical Inhibition—We next sought to determine the specificity or otherwise of HIF hydroxylase inhibitors for the different sites of hydroxylation in HIF-1 α . Several supposedly nonspecific inhibitors of the HIF hydroxylases, including iron chelating agents, cobaltous ions, and the 2-oxoglutarate analogue DMOG have been commonly employed as “hypoxia

mimetics” in studies of the HIF system. First we tested the effects of these agents on HIF-1 α hydroxylations in RCC4 cells. Unexpectedly, these studies revealed considerable specificity in the response. Whereas asparaginyl hydroxylation was substantially more sensitive to DMOG than prolyl hydroxylation, the reverse was true for cobaltous ions, which inhibited prolyl but not asparaginyl hydroxylation. Both hydroxylations were sensitive to the iron chelators, DFO and 2,2'-dipyridyl, although prolyl hydroxylation was more sensitive than asparaginyl hydroxylation (Fig. 6, *A* and *B*). For comparison we also tested two bicyclic isoquinolinyl compounds (A and B) developed as HIF hydroxylase inhibitors in similar assays (Fig. 6*C*); these inhibitors were highly specific for prolyl over asparaginyl hydroxylation. These studies indicate that exposure of cells to these agents is not equivalent and that different agents might be predicted to have strikingly different effects on the asparaginyl hydroxylation status of HIF-1 α protein that is induced in VHL-competent cells.

To pursue this, we exposed RCC4/VHL (VHL-competent) cells to different types of HIF hydroxylase inhibitor, and compared levels of total and asparaginyl-hydroxylated HIF. Results are shown in Fig. 6, *D–F*. Cobaltous, nickel, manganese, and zinc ions all induced HIF-1 α protein that remained strongly hydroxylated on Asn⁸⁰³ in the 70–100% region (Fig. 6*D* and data not shown). Interestingly, the iron chelators, DFO and

HIF Prolyl and Asparaginyl Hydroxylation

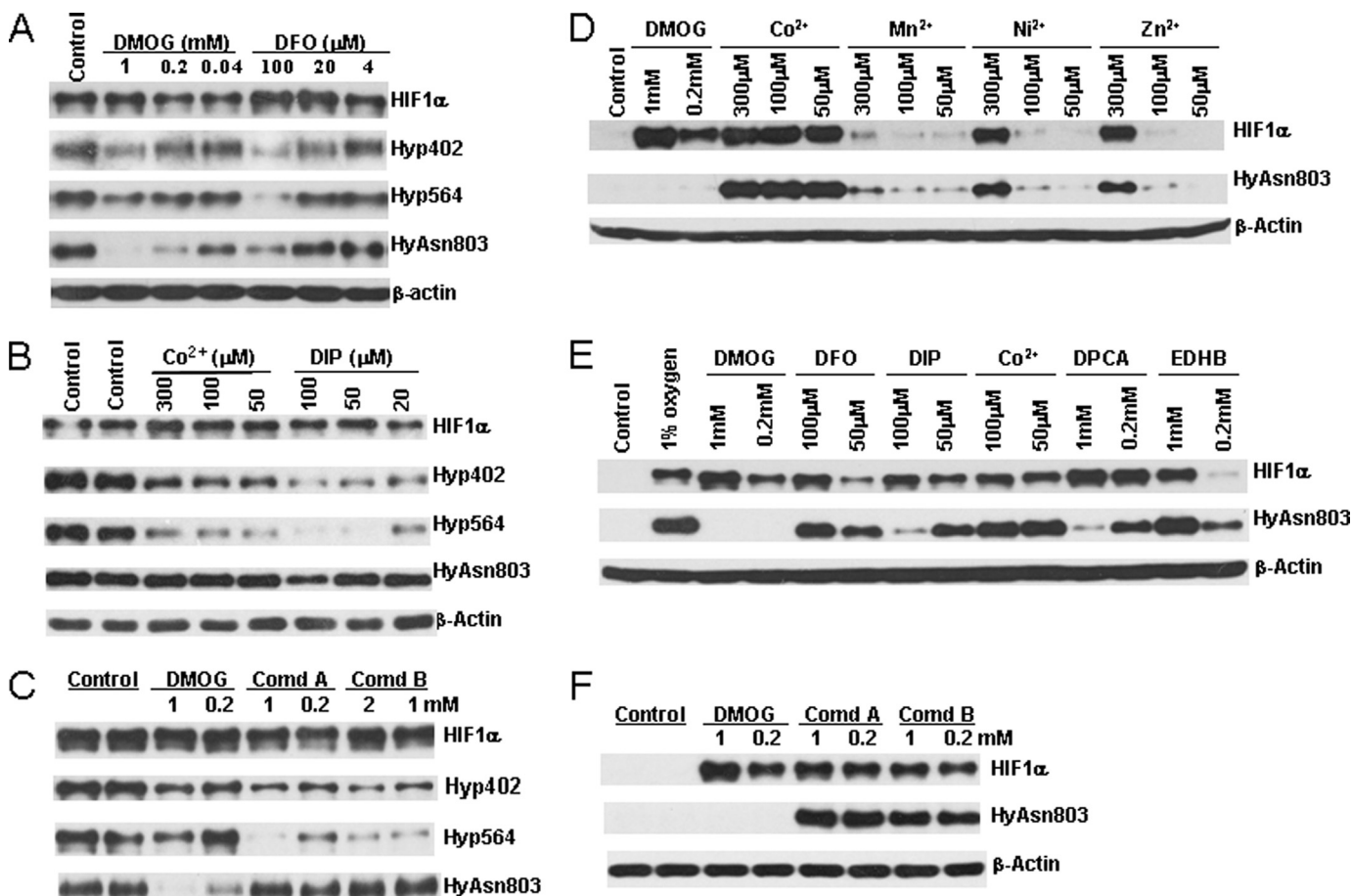


FIGURE 6. Differential inhibition of HIF prolyl and asparaginyl hydroxylation by HIF hydroxylase inhibitors. A–C, VHL-defective (RCC4) cells. D–F, VHL-competent (RCC4/VHL) cells. Cells were treated with inhibitors as indicated (see supplemental Fig. S5 for structures). Immunoblots were probed with the indicated antibodies. Controls were exposed to 0–2% DMSO in accordance with the diluent used for the test substance.

2,2'-dipyridyl, manifest different dose-dependent effects, with 2,2'-dipyridyl but not DFO restricting asparaginyl hydroxylation at high doses (Fig. 6E). Different small molecule inhibitors (see supplemental Fig. S5 for structures) had strikingly different effects; some inhibited asparaginyl hydroxylation strongly, whereas others had no apparent effect on asparaginyl hydroxylation even at high dosage (Fig. 6, E and F). Thus whereas all these substances induced HIF by inhibition of prolyl hydroxylation, they have highly variable effects on asparaginyl hydroxylation.

Finally, we sought to develop and test cell-permeable molecules that might inhibit HIF asparaginyl hydroxylation and not HIF prolyl hydroxylation. Based on the results of *in vitro* assays, which demonstrated that the *N*-oxalyl amino acid (*R*)-2-(carboxyformamido)-3-phenylpropanoic acid (compound F), but not the (*S*)-enantiomer (compound D) was a powerful inhibitor of FIH, we produced cell-penetrating esterified derivatives of these compounds (E and C, for structures, see supplemental Fig. S5). RCC4 cells were exposed to both the esterified derivatives and the free acids. Results are shown in Fig. 7A. (*R*)-Methyl-2-(2-methoxy-2-oxoacetamido)-3-phenylpropanoate (compound E) but not (*S*)-enantiomers (compounds C and D) specifically inhibited HIF asparaginyl but not prolyl hydroxylation. The free acid form of compound E (compound F) also showed the same effect but to a lesser extent.

When RCC4/VHL cells were exposed to compound E, no induction of HIF-1α was observed consistent with the absence of action on HIF-1α prolyl hydroxylation (Fig. 7B). However, when compound E was combined with hypoxia, inhibition of asparaginyl hydroxylation on induced HIF-1α was observed, demonstrating effective and specific inhibition of HIF-1α asparaginyl hydroxylation (Fig. 7C).

DISCUSSION

This work comprises the first direct analysis of the differential regulation of sites of HIF prolyl and asparaginyl hydroxylation in cells. Hydroxy residue-specific antibodies, whose performance was validated by MS, were used to analyze hydroxylation levels at each of the three known sites of hydroxylation in human HIF-1α (Pro⁴⁰², Pro⁵⁶⁴, and Asn⁸⁰³). The findings reveal major differences in the regulation of these hydroxylations by hypoxia and in their sensitivity to supposedly nonspecific hydroxylase inhibitors.

Using VHL-defective RCC4 cells to avoid confounding bias from selective degradation of prolyl-hydroxylated HIF-α polypeptides, we were able to directly compare changes in prolyl *versus* asparaginyl hydroxylation. These experiments demonstrated that in normoxic RCC4 cells HIF-1α is heavily or even completely hydroxylated at all three sites. They reveal that HIF-1α asparaginyl hydroxylation is substantially more resis-

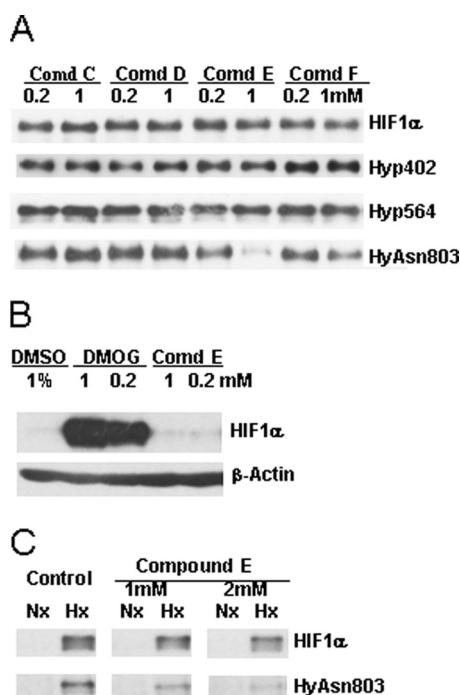


FIGURE 7. Selective chemical inhibition of HIF asparaginyl hydroxylation. A, VHL-defective (RCC4) cells were treated with inhibitors as indicated for 5 h. Immunoblots show inhibition of Asn⁸⁰³ hydroxylation by compounds E and F. B, compound E (5 h) did not up-regulate HIF-1 α in RCC4/VHL cells. C, RCC4/VHL cells were exposed to compound E (5 h) in the presence or absence of hypoxia (1% oxygen) for the last 2 h. Compound E inhibited Asn⁸⁰³ hydroxylation in the HIF-1 α induced by hypoxia.

tant to hypoxia than either site of prolyl hydroxylation. Similar differential sensitivity of prolyl and asparaginyl hydroxylation was observed over a range of short hypoxic exposures and maintained during chronic hypoxia.

Reported apparent K_m measurements for oxygen for the HIF hydroxylases (21, 22, 24) are all very much higher than the oxygen concentrations used in the graded hypoxia experiment in the current work and those previously measured in studies of human tissues (37, 38). Therefore, whereas differences in K_m values for oxygen have been reported for the PHDs versus FIH (21, 22), they cannot account for our *in vivo* findings, and the precise kinetic parameters underlying the differential sensitivity to hypoxia that we have observed *in vivo* are not yet clear. Such analyses are also likely to be complicated by the existence of alternative non-HIF substrates, particularly for FIH. In addition to HIF, FIH catalyzes hydroxylation of specific asparaginyl residues in a wide range of ankyrin repeat domain proteins (34, 39). Some of these, such as the Notch-1 receptor are very effective competitors of HIF hydroxylation *in vitro* (40–42). Given that many ankyrin repeat domain proteins that are subject to FIH-catalyzed hydroxylation such as cytoskeletal ankyrins (43) are also expressed at relatively high abundance across a wide range of cells, our finding of high levels of hydroxylation of HIF-1 α Asn⁸⁰³ except in cells exposed to severe hypoxia or chemical inhibitors was somewhat surprising and suggests that additional factors operate *in vivo* to direct FIH-mediated catalysis to particular molecular targets.

Our studies did not reveal any evidence for a compensatory increase in the specific activity of the HIF hydroxylases in

chronic hypoxia. Although an increased specific activity of the PHDs during chronic hypoxia has been reported in VHL-competent cells that manifest intact HIF transcriptional responses, this was potentially attributed to HIF-dependent reductions in mitochondrial oxygen consumption that could reduce the severity of cellular hypoxia (44). In our studies of chronic hypoxia in VHL-defective cells, such HIF-dependent effects would not be anticipated and our finding of no increase in specific activity of the HIF hydroxylases in this setting is therefore consistent with the authors' interpretation of the previous work.

Although the use of VHL-defective cells was necessary to enable direct comparison of interventions on prolyl and asparaginyl hydroxylation, up-regulation of HIF pathways in these cells leads to constitutive up-regulation of HIF prolyl hydroxylases, PHD2 and PHD3 (12, 36). In contrast, expression of FIH is unaffected by the VHL status. Thus it is likely that VHL-competent cells, which therefore express lower levels of PHD2 and PHD3 relative to FIH, effectively manifest even greater differential sensitivity of prolyl to asparaginyl hydroxylation at least during the acute phase of hypoxic exposure. Operationally, however, the key consideration in defining the response to hypoxia in VHL-competent cells is the extent to which HIF asparaginyl hydroxylation persists on HIF-1 α molecules that escape the prolyl hydroxylation/VHL degradation pathway. Our findings reveal that in moderately hypoxic cells, tissues from hypoxic animals, and human tumors, HIF-1 α remains substantially strongly hydroxylated on asparagine. The levels of persistent asparaginyl hydroxylation were cell type-specific and strongly dependent on the severity of hypoxic exposure. These findings are consistent with reports that suppression of FIH can increase HIF target gene expression in moderately hypoxic cells (27, 45) and they indicate that monitoring of HIF asparaginyl hydroxylation is likely to be important in future physiological analyses of the HIF transcriptional pathway (46).

Analysis of all three sites of HIF hydroxylation also permitted comparison of the two sites of prolyl hydroxylation. Consistent with previous work we observed that hydroxylation at Pro⁴⁰² apparently occurred after that at Pro⁵⁶⁴ and was more sensitivity to hypoxia. Whether this is due to intrinsic differences in the sensitivity of each site to hypoxia or competition between the two sites as has been demonstrated *in vitro* (47) is unclear. However, differences in the behavior of the two sites *in vivo* were marked. For instance, upon exposure to moderate hypoxia, striking accumulation of HIF-1 α that was hydroxylated at Pro⁵⁶⁴ but not Pro⁴⁰² was observed, which was again cell type-specific. Interestingly, accumulation of HIF-1 α that was hydroxylated on Pro⁵⁶⁴ correlated with increased expression of PHD3, the enzyme that preferentially hydroxylates this site (12, 25), suggesting that cell-type differences in expression of PHD3 may contribute to these differences. Mutational studies of HIF-1 α have indicated that each site of prolyl hydroxylation in HIF-1 α can independently mediate VHL-dependent degradation of HIF-1 α (7). Nevertheless, the current findings would suggest that the preferential hydroxylation of Pro⁵⁶⁴ that is observed in moderately hypoxic cells might not on its own be sufficient to cause VHL-mediated degradation of HIF-1 α at a rate that is adequate to overcome synthesis. It is consistent with

HIF Prolyl and Asparaginyl Hydroxylation

the proposal that efficient operation of the prolyl hydroxylation-VHL degradation pathway is dependent on some form of cooperative interaction between the 2 sites of prolyl hydroxylation that promotes more rapid, or more complete VHL-dependent degradation (25).

Dependence of rapid HIF-1 α degradation on hydroxylation at both sites would be predicted to further enhance the sensitivity of the prolyl hydroxylation-VHL degradation pathway to hypoxia relative to that of asparaginyl hydroxylation. Furthermore, because Pro⁵⁶⁴, like Asn⁸⁰³ is sited within a HIF- α transactivation domain, the hydroxylation status at Pro⁵⁶⁴ may have additional regulatory effects on HIF activity (48).

The use of hydroxy residue-specific antibodies also permitted comparative analysis of different modes of HIF hydroxylase inhibition on different sites of HIF hydroxylation. Striking differences were observed among agents that are often regarded as being nonspecific hydroxylase inhibitors and which are commonly applied to cells as hypoxia mimetics. In particular, at concentrations that had marked effects on HIF prolyl hydroxylation, iron chelators were much less effective, and transition metals ions such as Co(II) were almost completely ineffective in inhibiting asparaginyl hydroxylation of HIF-1 α . In contrast, the generic 2-OG analogue DMOG was found to be even more effective at suppressing HIF asparaginyl hydroxylation than HIF prolyl hydroxylation.

Interestingly, findings on the differential sensitivity of prolyl and asparaginyl hydroxylation to transition metals ions and iron chelators in cells are very different from effects of these inhibitors on purified or partially purified preparations of these enzymes *in vitro* (49). FIH has been reported to be much more sensitive than PHDs to inhibition by metal ions *in vitro* (49), whereas we observed the opposite *in vivo*. Similarly PHDs (particularly PHD2) bind Fe(II) tightly *in vitro* and are much less sensitive to inhibition by DFO than FIH (49, 50). However, *in vivo* we observed the opposite; greater sensitivity of prolyl *versus* asparaginyl hydroxylation to inhibition by iron chelators. The reasons for these differences are not clear and strongly suggest that the mechanism(s) of inhibition are more complex than facile exchange of iron at the catalytic center of the HIF hydroxylases with a chelatable iron pool.

Exposure of cells to several different small molecule HIF hydroxylase inhibitors revealed greatly differing levels of specificity for the two types of HIF hydroxylation. Some inhibitors, such as ethyl-3,4-dihydroxybenzoate and 4-oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid, inhibited both types of hydroxylation, in keeping with *in vitro* data on these compounds (21, 22, 28). In contrast, the bicyclic isoquinolinyl compounds A and B (29) revealed almost complete selectivity for inhibition of prolyl *versus* asparaginyl hydroxylation, whereas the *N*-oxalyl phenylalanine derivative, compound E (30), was strongly selective for HIF asparaginyl over HIF prolyl hydroxylation, clearly demonstrating the feasibility of specifically inhibiting each pathway in cells.

The HIF pathway plays a central role in directing cellular responses to hypoxia and is currently the focus of attempts at therapeutic manipulation in a range of disease settings that are associated with tissue hypoxia (18–20). Major differences in the regulation of HIF prolyl and asparaginyl hydroxylation

described above are therefore important in understanding biological responses to hypoxia. These insights, together with the existence of validated reagents for further study, should assist in guiding attempts at pharmacological modulation of hypoxia pathways.

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