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The zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine, increases the level of nonfunctional HIF-1α protein in normoxic cells ☆

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Abstract

The hypoxia-inducible factor-1α (HIF-1α) subunit is activated in response to lack of oxygen. HIF-1α-specific prolyl hydroxylase and factor inhibiting HIF-1α (FIH-1) catalyze hydroxylation of the proline and asparagine residues of HIF-1α, respectively. The hydroxyproline then interacts with ubiquitin E3 ligase, the von Hippel–Lindau protein, leading to degradation of HIF-1α by ubiquitin-dependent proteasomes, while the hydroxylation of the asparagine residue prevents recruitment of the coactivator, cAMP-response element-binding protein (CBP), thereby decreasing the transactivation ability of HIF-1α. We found that the Zn-specific chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), enhances the activity of HIF-1α-proline hydroxylase 2 but the level of HIF-1α protein does not fall because TPEN also inhibits ubiquitination. Since the Zn chelator does not prevent FIH-1 from hydroxylating the asparagine residue of HIF-1α, its presence leads to the accumulation of HIF-1α that is both prolyl and asparaginyl hydroxylated and is therefore nonfunctional. In hypoxic cells, TPEN also prevents HIF-1α from interacting with CBP, so reducing expression of HIF-1α target genes. As a result, Zn chelation causes the accumulation of nonfunctional HIF-1α protein in both normoxia and hypoxia.

Keywords: Hypoxia; HIF-1α; TPEN; Ubiquitination; PHD2; FIH-1

Hypoxia-inducible factor 1 (HIF-1) acts as a master regulator of oxygen homeostasis by activating the transcription of several genes involved in angiogenesis, erythropoiesis, and glycolysis [1,2]. It consists of HIF-1α and HIF-1β subunits. HIF-1α is rapidly degraded under normoxic condition by the ubiquitin–proteasome system, whereas the level of HIF-1β is constant [3,4]. Under normoxic condition, the proline-564 and/or 402 residues of HIF-1α are hydroxylated by HIF-1α-specific prolyl-4 hydroxylases (PHDs) which require O_2, 2-oxoglutarate, vitamin C, and Fe^{2+} [5–9]. The hydroxylated prolines interact with von Hippel–Lindau (VHL) protein, a component of E3 ubiquitin ligase, and the HIF-1α is ubiquitinated by the VCB E3 ubiquitin–ligase complex, containing VHL protein, ElonginB, ElonginC, Cul2, and Rbx1 [10,11]. In hypoxic conditions, proline hydroxylation decreases so that HIF-1α protein accumulates. In mammalian cells, a family of HIF-1α-specific prolyl-4 hydroxylases has been identified and given the acronyms PHD1 (HPH3, EGLN2), PHD2 (HPH2, EGLN1), and PHD3 (HPH1, EGLN3) [12,13]. Experiments employing short interfering

☆ *Abbreviations:* Arnt, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix–loop–helix; CA9, carbonic anhydrase 9; CBP, CREB-binding protein; FIH-1, factor inhibiting HIF-1α; HDAC, histone deacetylase; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia-responsive element; MYND, myeloid translocation protein 8, Nervy, and DEAF1; ODD, oxygen-dependent degradation domain of HIF-1α; PAS, period, aryl hydrocarbon receptor, single minded; PHD, HIF-1α-specific prolyl hydroxylase; TPEN, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau protein.

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RNAs revealed that silencing of PHD2 is enough to stabilize and activate HIF-1α in normoxic cells, suggesting that PHD2 is the major form responsible for hydroxylating HIF-1α [14,22]. Oxygen molecules inhibit not only the stabilization of HIF-1α, but also its transactivation activity since the protein, factor inhibiting HIF-1α (FIH-1), catalyzes hydroxylation of its asparagine residue using O_2, 2-oxoglutarate, vitamin C, and Fe^{2+}. Hydroxylation of the asparagine residue in the transactivation domain of HIF-1α prevents it from recruiting its coactivator, cAMP-response element-binding protein (CBP). Our previous findings showed that PHD2 has a catalytic domain at C-terminal 2-oxoglutarate-dependent hydroxylase consensus motif, and that it has an inhibitory domain located at N-terminal MYND-type zinc-finger motif [15]. We showed that treatment of zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), increases the activity of PHD2, by suppressing the inhibitory activity of MYND-type zinc-finger motif. In order to evaluate the potential use of TPEN as a regulator of HIF-1α, we investigate any changes in stability and functions of HIF-1α in TPEN-treated human neuronal cells and liver cells.

Here we report that the zinc chelator, TPEN, inhibits several steps in activation of HIF-1α. Its net effect is to cause the accumulation of nonfunctional HIF-1α protein in both normoxic and hypoxic cells. This work provides insight into the mechanism of regulation of HIF-1α by many Zn-finger-containing proteins.

Materials and methods

Cells, cDNAs, and reagents. Human HeLa cervical epithelium cells (ATCC CCL-2), human HepG2 hepatoma cells (ATCC HB-8065), human DU145 prostate cancer cells (ATCC HTB-81), and human SH-SH5Y neuroblastoma cells were purchased from the American Type Culture Collection (ATCC CRL-2266) and maintained as recommended by the ATCC. The cells were made hypoxic by incubation in an anaerobic incubator (Model 1029, Forma Scientific) in 5% CO_2, 10% H_2, and 85% N_2 at 37 °C. We used the following human cDNAs in expression vectors, transfection assays, and in vitro transcription and translation experiments; PHD2 (GenBank Accession No. AJ310543), PHD3 (AJ310545), HIF-1α (U22431), VHL (AF010238), and FIH-1(AF395830). The p(HRE)$_4$-luc reporter plasmid contained four copies of the erythropoietin hypoxia-responsive element (nucleotides 3449–3470) [16]. Anti-HIF-1α was obtained from BD Pharmingen. We obtained *N*,*N*,*N*′,*N*′-*tetrakis* (2-pyridylmethyl) ethylenediamine (TPEN) from Calbiochem, and all other chemicals from Sigma Chemical. Culture media were purchased from Gibco-BRL, fetal bovine serum from Bio Whittaker.

Expression and purification of FIH-1. The human FIH-1 gene (AF395830) was cloned into pET28a vector (Novagen), and FIH-1 was overexpressed in *Escherichia coli* as a histidine-tagged fusion protein and purified by Ni-affinity chromatography. The fusion protein was further purified by gel-filtration chromatography (Hi-Load Superdex 200) and concentrated by ultrafiltration.

Measurement of PHD activity by a VHL pull-down assay. An in vitro VHL interaction assay was performed as described by Jaakkola et al. [8]. Briefly, [^{35}S]methionine-labeled VHL protein was synthesized by in vitro transcription and translation using plasmid pcDNA3.1/hygro-VHL, according to the instructions of the suppliers (Promega, Cat# L1170). Unlabeled PHD2 and PHD3 proteins were synthesized by in vitro transcription and translation. Seven micrograms of biotinylated HIF-1α peptide (Biotin-DLDLEMLAPYIPMDDDFQLR: residues 556–575 of human HIF-1α) was preincubated with PHD-programmed rabbit reticulolysate (10 μl) in a final volume of 100 μl in NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 1 mM PMSF] containing 2 mM ascorbic acid, 100 μM of $FeCl_2$, and 5 mM α-ketoglutarate at 30 °C for 90 min. ImmunoPure Immobilized Monomeric Avidin (Pierce, Cat# 20227) (30 μl of a 50% slurry) was pretreated with 3 mg of bovine serum albumin for 5 min at room temperature and added to the above hydroxylation reaction mixture, which was incubated with mild agitation for 60 min at 22 °C. Avidin-associated peptide was washed three times with 1 ml NETN buffer and then mixed with 10 μl of [^{35}S]-labeled VHL in 100 μl of EBC buffer [120 mM NaCl, 50 mM Tris–HCl (pH 8.0), and 0.5% (v/v) nonidet P-40] with mild agitation at 4 °C for 2 h. The resin was washed four times with 1 ml NETN buffer, and proteins were eluted in elution buffer (0.1 M $NaHPO_4$, 0.15 M NaCl, and 2 mM D-biotin). Eluted VHL was analyzed by 12% SDS–PAGE and autoradiographed.

Measurement of FIH-1 activity by a GST-CBP pull-down assay. Twenty-five microliters of [^{35}S]-labeled human HIF-1α protein was incubated for 1 h at 30 °C with the indicated amounts of purified FIH-1 protein in reaction buffer [200 μl; 20 mM Tris–HCl (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM ascorbic acid, 2 mM α-ketoglutarate, and 250 μM $FeCl_2$]. One microgram of immobilized GST-CBP N domain (amino acids 1–450) [17,33] was added to each mixture in 500 μl of binding buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 μM $ZnCl_2$, and 0.5 mM DTT] and incubated for 1 h at 4 °C. Protein bound to the glutathione-uniflow resin was washed four times with 1 ml of binding buffer containing 0.1% Nonidet P-40, eluted by boiling in SDS sample buffer, and analyzed by SDS/PAGE [18].

In vitro ubiquitination assay. HeLa cells were washed twice with cold hypotonic extraction buffer [20 mM Tris–HCl (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.2 mM PMSF]. After removing the buffer, the cells were disrupted in a Dounce homogenizer and the crude extract was centrifuged at 10,000*g* for 10 min at 4 °C to remove cell debris and nuclei;the supernatant (S-10 fraction) was stored in aliquots at −70 °C. Ubiquitination assays were carried out at 30 °C for 270 min in a total volume of 40 μl, containing 2 μl of [^{35}S]-labeled human HIF-1α-programmed reticulocyte lysate, 27 μl of S-10 extract, 4 μl of 10× ATP-regenerating system [20 mM Tris (pH 7.5), 10 mM ATP, 10 mM magnesium acetate, 300 mM creatine phosphate, and 0.5 mg/ml creatine phosphokinase], 4 μl of 5 mg/ml ubiquitin (Sigma), and 0.83 μl of 150 μM ubiquitin aldehyde (Sigma). SDS sample buffer was added and the reaction products were analyzed by 6% SDS–PAGE and autoradiography [19].

Co-immunoprecipitation. HepG2 cells were grown to 80% confluence on 100-mm tissue culture plates and treated with TPEN (5 or 10 μM) for 6 h in normoxic conditions, or pre-treated with TPEN (5 μM) for 1 h in hypoxic conditions. Whole cell extracts were prepared as previously described [8]. For immunoprecipitation, 200 μg samples of whole cell lysates were pre-cleared by incubating with 1 μg of anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 20 μl of 0.5% ImmunoPure immobilized protein A/G gel (Pierce, Rockford, IL) for 30 min at 4 °C. The cleared extracts were mixed with 1 μg of anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After addition of 15 μl of 0.5% ImmunoPure immobilized protein A/G gel, the mixtures were rotated overnight at 4 °C. The immunoprecipitates were pelleted and washed four times with phosphate-buffered saline and resuspended in SDS sample buffer. The samples were then boiled for 5 min and run on 8% SDS–polyacrylamide gels, and the proteins transferred to nitrocellulose membranes by semi-dry transfer (Trans-Blot SD; Bio-Rad, Hercules, CA). Co-immunoprecipitated proteins were reacted with anti-human HIF-1α antibody (BD Pharmingen) and/or anti-CBP antibody, and visualized by enhanced chemiluminescence, according to the manufacturer's instructions (Pierce, Rockford, IL), with anti-mouse or rabbit Ig conjugated with horseradish peroxidase (HRP) as secondary antibody. The weak signal of protein bands on Western blots was visualized with a luminescence image analyzer (Model LAS-3000, Fuji, Japan).