Copper-dependent activation of hypoxia-inducible factor (HIF)–1: implications for ceruloplasmin regulation

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Cellular oxygen partial pressure is sensed by a family of prolyl-4-hydroxylase domain (PHD) enzymes that modify hypoxia-inducible factor (HIF)α subunits. Upon hydroxylation under normoxic conditions, HIFα is bound by the von Hippel–Lindau tumor suppressor protein and targeted for proteasomal destruction. Since PHD activity is dependent on oxygen and ferrous iron, HIF-1 mediates not only oxygen- but also iron-regulated transcriptional gene expression. Here we show that copper (CuCl2) stabilizes nuclear HIF-1α under normoxic conditions, resulting in hypoxia-response element (HRE)-dependent reporter gene expression. In vitro hydroxylation assays CuCl2 inhibited prolyl-4-hydroxylation independently of the iron concentration. Ceruloplasmin, the main copper transport protein in the plasma and a known HIF-1 target in vitro, was also induced in vivo in the liver of hypoxic mice. Both hypoxia and CuCl2 increased ceruloplasmin (as well as vascular endothelial growth factor [VEGF] and glucose transporter 1 [Glut-1]) mRNA levels in hepatoma cells, which was due to transcriptional induction of the ceruloplasmin gene (CP) promoter. In conclusion, our data suggest that PHD/HIF/HRE-dependent gene regulation can serve as a sensory system not only for oxygen and iron but also for copper metabolism, regulating the oxygen-, iron- and copper-binding transport proteins hemoglobin, transferrin, and ceruloplasmin, respectively. (Blood. 2005;105:4613-4619) © 2005 by The American Society of Hematology
sensors but also display iron-sensing properties. Whereas transition metal ions such as Co$^{2+}$ and Ni$^{2+}$ stabilize HIF-1α by inhibiting PHD function, it is unknown whether copper salts also interfere with PHD function. In a screen for agents that modulate HIF-1 transcriptional activity, we found that Cu$^{2+}$ can induce HIF-1–dependent reporter gene induction. Detailed analysis of this finding suggests that free Cu$^{2+}$ can induce ceruloplasmin synthesis in a HIF-1–dependent way.

Materials and methods

Cell lines and cell culture

All cell lines were cultured in Dulbecco modified Eagle medium (high glucose) as described previously. Oxygen partial pressures in the hypoxic workstation (InVivoX-400; Ruskin Technology, Leeds, United Kingdom) or in the incubator (Model 3319; Forma Scientific, IL, Illkirch, France) were either 140 mmHg (20% O$^2$ vol/vol, normoxia) or 7 mmHg (1% O$^2$ vol/vol, hypoxia). Stable transfection with the plasmid pHSV29 led to HIF-1α-dependent luciferase reporter cell lines designated HRCHO5 (derived from CHO Chinese hamster ovary cells) or HRB5 (derived from Hep3B human hepatoma cells), respectively, as described before. The luciferase reporter gene pHHSV29 is driven by a simian virus 40 (SV40) promoter and contains a total of 6 HIF-1 DNA-binding sites derived from the transferrin gene.

Cell proliferation/viability assays

Cell proliferation/viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay as described before. Absorbances at 570 nm were determined in a 96-well photometer and noncellular background was subtracted. Data were normalized to the MTT conversion activity of solvent-treated normoxic control cells, which were arbitrarily defined as 1.

Reporter gene assays

Stably transfected hypoxia reporter cell lines were seeded in triplicates into 96-well dishes at a density of 10$^4$/well. After incubation overnight, freshly dissolved CuCl$_2$ or CuCl$_2$ in phosphate-buffered saline (PBS) was added at the concentrations indicated and the cells were exposed to normoxic or hypoxic conditions for 24 hours. In some experiments, the kinase inhibitors LY294002, PD98059, or PD169316 (Calbiochem, VWR International, Darmstadt, Germany) were added in addition to 100 μM of the kinase inhibitors.

Transient transfections

Hep3B cells were transiently transfected with the CP promoter firefly reporter construct pGL-Cp4774$^{20}$ by the calcium phosphate coprecipitation method. CP enhancer constructs, either wild-type or a mutant HRE, were cloned into pGL3Prom (Promega) as described previously. Semi-quantitative mRNA from a 10-cm dish was cotransfected with 20 μg of pGL-Cp4774 together with 0.2 μg of the renilla luciferase control construct pRL-SV40 (Promega) for 16 hours. Cells were transfected with 2 mM EDTA (ethylenediaminetetraacetic acid; pH 8.0) in PBS, distributed onto 24-well dishes, and allowed to recover for 8 hours. Freshly prepared CuCl$_2$ in PBS or PBS alone was added and the cells were incubated for 24 hours under normoxic or hypoxic conditions. Luciferase activities were determined using the dual-luciferase kit (Promega) as described previously.

Protein extraction and immunoblot analyses

Cells were treated with CuCl$_2$ or the iron chelator cyclcoproxi oamine (CPX)$^{32}$ at the concentrations indicated for 6 or 24 hours. For stability experiments, 20 μg/mL cycloheximide was added following 6 hours hypoxic induction with or without CuCl$_2$ and the cells were allowed to reoxyg enate. Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 M NaCl and 0.1% Nonidet P-40 (NP-40) in extraction buffer as described previously. Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as a standard. For immunoblot analysis, cellular protein (100 μg or 50 μg in stability experiments) was electrophoresed through 5% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoresed onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semidyey blotting (BioRad, München, Germany). Membranes were stained with Ponceau S (Sigma, Buchs, Switzerland) to confirm equal protein loading and transfer. HIF-1α was detected using a mouse monoclonal anti–HIF-1α immunoglobulin G1 (IgG1; Transduction Laboratories, Heidelberg, Germany) followed by a goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Anti–β-actin antibodies were from Sigma. Chemiluminescence detection of horseradish peroxidase–coupled secondary antibodies was performed by incubation of the membranes with 100 mM Tris (tris(hydroxymethyl)aminomethane)–HCl (pH 8.5), 2.65 mM H$_2$O$_2$, 0.45 mM luminol, and 0.625 mM coomaric acid (all purchased from Sigma) for 1 minute followed by exposure to X-ray films (Fuji, Düsseldorf, Germany).

HIF-1α immunofluorescence

Immunofluorescence analysis was performed as described before. Briefly, cells were fixed with formaldehyde for 10 minutes, washed with PBS, permeabilized with Triton X-100, and rinsed again with PBS. After blocking nonspecific binding sites with 3% BSA in PBS for 30 minutes, the cells were incubated for 1 hour with mouse monoclonal anti–HIF-1α IgG1 (Transduction Laboratories) diluted 1:10 in 3% BSA in PBS, followed by a fluorescein isothiocyanate (FITC)–coupled secondary anti-mouse (Dako, Copenhagen, Denmark) antibody diluted 1:100 with 3% BSA in PBS. After extensive washings with PBS, the slides were mounted in mowiol and analyzed by fluorescence microscopy (Axioplan 2000, equipped with an Axiovision digital camera and Axiovision software; Carl Zeiss Vision, Mannheim, Germany).

In vitro prolyl-4-hydroxylation assay

Prolyl-4-hydroxylation activity was determined as recently described in detail elsewhere. Briefly, a biotinylated peptide containing Pro564 of HIF-1α was bound to NeutrAvidin-coated 96-well plates and incubated with partially purified PHD1, PHD2, or PHD3 in the presence of 2-oxoglutarate, FeSO$_4$, and ascorbate for 1 hour. After washing, thio redoxin–tagged pVHL in complex with elongins B and C was allowed to bind to the hydroxylated peptide, followed by detection of the thio redoxin tag by primary antithioredoxin antibodies and secondary horseradish peroxidase–coupled anti-mouse antibodies (Sigma) using the TMB (3,3′,5,5′- tetramethylbenzidine) substrate kit (Pierce, Bonn, Germany). MBP-PHD2 (amino acids 196-426) was expressed and purified by using the pMAL system according to the instructions provided by the manufacturer (New England Biolabs, Frankfurt, Germany). PHD1 and PHD3 were expressed with an N-terminal 6His-Tag in S9 insect cells as described elsewhere.

mRNA quantification

Expression of mice in triplicate at 7.5% oxygen for 0, 24, 48, and 72 hours and excision of their livers was described elsewhere. Total RNA from frozen mouse liver tissue or cultured Hep3B cells was isolated and analyzed by Northern blotting as described previously. Hybridization probes were obtained by restriction digestion and gel isolation followed by labeling with the random-primed method. The mouse ceruloplasmin cDNA was a kind gift of J. Gitlin (St Louis, MO) and the ribosomal protein L28 cDNA was cloned from a HepG2 cDNA library. Radioactive signals were detected by phosphorimaging and quantitated using QuantityOne software (Bio-Rad). Human ceruloplasmin, vascular endothelial growth factor (VEGF), glucose transporter-1 (Glut-1) and L28 were determined by real-time polymerase chain reaction (PCR). Briefly, 2 μg of total RNA was reverse transcribed with Superscript III according to the manufacturer’s
Results

CuCl₂ can activate a hypoxia-response element-containing reporter gene

We previously established a Chinese hamster ovary (CHO) cell line, called HRCHO5, which was stably transfected with an HRE-containing luciferase reporter gene containing multiple hypoxia-response elements (HREs).30 Apart from hypoxia, reporter activity in these cells could also be induced by cobalt and nickel salts as well as by iron chelation.30,32 Unexpectedly, we found that CuCl₂ dose-dependently induced reporter gene induction approximately 4.1-fold at 500 μM CuCl₂ in CHO cells (Figure 1A). The human hepatoma Hep3B cell line is known to synthesize the plasma proteins transferrin and ceruloplasmin, both of which are induced in a HIF-dependent manner upon iron depletion of these cells.15,20 We thus stably transfected Hep3B cells with the HIF-dependent reporter gene like previously used to generate the HRCHO5 cell line, called HRCHO5, which was stably transfected with a HIF-1-dependent luciferase reporter gene containing multiple HSREs.

Instructions (Invitrogen, Basel, Switzerland). Real-time PCR was performed in duplicates with 1% of the cDNA reaction mixture using a SybrGreen Q-PCR reagent kit on a MX3000P PCR cycler according to the manufacturer’s instructions (Stratagene, Amsterdam, the Netherlands). Dilution series of the corresponding plasmids were used to obtain standard curves. Primers: ceruloplasmin, hCpfwd 5'-gattaattggccccctgatt-3’ and hCpRev 5'-tgcattgtgaggccttgtag-3’; VEGF, hVEGF165fwd 5’-gaggagggcaagaatcatcac-3’ and hVEGFrev 5’-aggcccacagggattttcttgtc-3’; Glut1, hGlut1fwd 5’-tcactgtgctcctggttctg-3’ and hGlut1rev 5’-cctgtgctcctgagagagagtcgctcctggttctg-3’; and L28, hL28fwd 5’-gccataagggcagtcaatggatggt-3’ and hL28rev 5’-tcactgtgctcctggttctg-3’.

The most likely explanation for the induction of reporter gene expression by CuCl₂ is the activation of the HIF system. We therefore analyzed whether CuCl₂ treatment leads to the stabilization of the HIF-1α protein. As shown by immunoblotting (Figure 2A), CuCl₂ treatment strongly induced HIF-1α protein stability in Hep3B cells after 6 and 24 hours. Both the range of CuCl₂ concentrations and the combined effects with hypoxia are consistent with the reporter gene data, especially after 24 hours of induction. To examine whether Cu²⁺ also stabilizes HIF-1α in a nonhepatic cell line, human cervical carcinoma HeLa cells were treated with the CuCl₂ concentrations indicated for 6 and 24 hours. HIF-1α protein. As shown by immunoblotting (Figure 2A), CuCl₂ treatment strongly induced HIF-1α protein stability in Hep3B cells after 6 and 24 hours. Both the range of CuCl₂ concentrations and the combined effects with hypoxia are consistent with the reporter gene data, especially after 24 hours of induction. To examine whether Cu²⁺ also stabilizes HIF-1α in a nonhepatic cell line, human cervical carcinoma HeLa cells were treated with the CuCl₂ concentrations indicated for 6 and 24 hours. HIF-1α protein activity was assessed by immunoblotting using a monoclonal anti–HIF-1α antibody. Hypoxia (A) and the iron chelator CPX (B) were used as positive controls. (C) Hep3B hepatoma cells (subline HRB5) were exposed to normoxic (20% O₂) or hypoxic (1% O₂) conditions or to 125 μM CuCl₂ for 24 hours. The cells were prepared for indirect immunofluorescence analysis as described in “Materials and methods.” Original magnification, × 630.
CuCl2 stabilizes HIF-1α under normoxic conditions

To gain more insight into the molecular mechanisms by which CuCl2 induces and activates HIF-1α, the phosphatidylinositol 3 (PI3)-kinase and mitogen-activated protein kinase (MAPK)-kinase signaling pathways, which are known to be responsible for normoxic HIF-1α induction by a large number of stimuli, were investigated. Therefore, the PI3-kinase inhibitor LY294002, the MAPK kinase-1 (MEK-1) inhibitor PD98059, and the p38 kinase inhibitor PD169316 were used to specifically block these pathways. However, as shown in Figure 3A, these kinase inhibitors had no significant impact on reporter gene induction by 100 μM CuCl2, suggesting that other mechanisms are responsible for Cu2+ -dependent HIF-1α induction.

In contrast to the kinase pathways that induce HIF-1α protein by translational up-regulation, hypoxia is known to induce HIF-1α by preventing its normoxic degradation. Thus, we induced HIF-1α accumulation by hypoxia in Hep3B cells for 6 hours in the presence or absence of 150 μM CuCl2. Thereafter, cycloheximide was added to block de novo translation of HIF-1α and reoxygenation was allowed for up to 135 minutes. As shown in Figure 3B, the continued presence of CuCl2 clearly delayed HIF-1α degradation, suggesting that CuCl2 stabilizes HIF-1α protein rather than inducing its production. In contrast, β-actin protein levels remained unaffected by this treatment, demonstrating a specific inhibition of HIF-1α degradation.

CuCl2 inhibits HIF-1α prolyl-4-hydroxylation independent of Fe2+ concentration

CuCl2-dependent stabilization of HIF-1α might be due to inhibition of HIF-1α prolyl-4-hydroxylation by PHD isoenzymes. Indeed, using a recently developed cell-free in vitro assay, we found that CuCl2 strongly inhibited hydroxylation of a peptide containing HIF-1α Pro564 by PHD1, PHD2, and PHD3, respectively (Figure 4A).

To gain more insight into the mechanism by which CuCl2 inhibits PHD activity, we repeated the prolyl-4-hydroxylation assays in the presence of limited concentrations (1 μM) or a vast excess (100 μM) of FeSO4. As shown in Figure 4B, PHD3 activity was inhibited by CuCl2 with an IC50 value of approximately 2.3 μM. However, the inhibitory concentration at 50% (IC50) value did not significantly change when Fe2+ was present in excess, suggesting that iron oxidation and/or competitive replacement from the active center is not the cause for CuCl2-dependent inhibition of PHD activity.

Induction of ceruloplasmin mRNA by hypoxia and Cu2+

Cu2+-dependent induction of HIF-1α offers the intriguing possibility that the Cu2+-binding protein ceruloplasmin itself is regulated by Cu2+ in a HIF-1α-dependent manner. Thus, we further analyzed ceruloplasmin regulation, a known HIF-1 target gene inducible by hypoxia and iron depletion in vitro cultured cells.20,28 First, we determined ceruloplasmin mRNA levels in livers of mice exposed to 7.5% O2 for up to 3 days by Northern blotting (Figure 5A). Liver ceruloplasmin mRNA levels significantly (t test, P < .05) increased 1.7-fold and 1.5-fold from days 1 and 2 to day 3, respectively. Only nonsignificant changes occurred during the first 2 days of exposure (Figure 5B). These data provide the first in vivo evidence that prolonged hypoxia can induce the mouse Cp gene.
We next determined mRNA concentrations in cultured Hep3B cells (subline HRB5) exposed to hypoxic conditions for up to 3 days by real-time reverse transcription (RT)–PCR. As shown in Figure 6, ceruloplasmin as well as the prototype oxygen-regulated VEGF and Glut-1, but not L28 control mRNA, are induced by hypoxia at all time points examined, confirming that ceruloplasmin is hypoxia-inducible in Hep3B cells. Of note, 150 μM CuCl2 also induced ceruloplasmin, VEGF, and Glut-1, but not L28 mRNA, in Hep3B cells and the combination with hypoxia further enhanced these mRNA levels (Figure 6). Maximal ceruloplasmin induction by CuCl2 was 5.5-fold after 72 hours of treatment under normoxic conditions.

The ceruloplasmin gene promoter is activated by Cu2+

Cu2+-dependent HIF-1α protein and ceruloplasmin mRNA induction suggested that ceruloplasmin gene expression is regulated by HIF-1–dependent promoter activation. We thus transiently transfected Hep3B cells with a ceruloplasmin promoter–firefly luciferase reporter gene containing a functional HRE within a 4774 base pair (bp) fragment upstream of the ATG translational start codon.20 A cotransfected constitutive renilla luciferase reporter gene served to normalize for differences in transfection efficiency. Following 24 hours of treatment with CuCl2, reporter gene activity dose-dependently increased up to 4-fold at 200 μM CuCl2 (Figure 7A). Hypoxia alone induced reporter gene activity 3.5-fold and the combination of CuCl2 with hypoxia further increased reporter gene activity. However, CuCl2 concentrations greater than 200 μM were considerably more toxic under hypoxic conditions as could be judged from the decrease in cell density and renilla reporter gene activity (not shown).

The involvement of HIF-1 was further analyzed by using the HRE-containing fragment of the ceruloplasmin −3429 to −3639 upstream region linked to the constitutive SV40 promoter. As shown in Figure 7B, the presence of this fragment was sufficient to induce firefly luciferase activity approximately 5-fold by 150 μM CuCl2 or 1% O2 after 24 hours of treatment. Point mutation of this HRE reduced but did not completely inhibit induction by CuCl2 or hypoxia in these experiments, suggesting that other elements might also be involved in CuCl2-dependent activation of ceruloplasmin gene expression.
Discussion

Molecular mimicry of hypoxia by distinct transition metal ions has been known since 1988, when Bunn and coworkers reported that HepG2 and Hep3B hepatoma cell lines increase erythropoietin gene expression not only under hypoxic conditions but also following treatment with Co2+ or Ni2+ and to a lesser extent Mn2+ salts.43 However, Cu2+ has not been found to induce erythropoietin gene expression in these early experiments. In our study, we demonstrated that Cu2+ can functionally stabilize HIF-1α, leading to target gene induction in hepatoma cells by a mechanism likely to involve the inhibition of prolyl-4-hydroxylation.

Apart from Co2+, Ni2+, or Cu2+, several other transition metals have been reported to induce HIF-1α, including vanadate,42 arsenite,62 and chromium.44 A common mechanism by which these transition metals interfere with PHD function is unknown. Salminen and colleagues reported that intracellular ascorbate required for PHD activity might be depleted by Co2+ or Ni2+, whereas it is increased at wound sites topically treated with CuSO4.58 Other known HIF-1 target genes induced by Cu2+ or Ni2+ has not been found to induce erythropoietin expression.59 Apart from Co2+, Ni2+, or Cu2+, several other transition metal concentrations and intracellular PHD target protein regulation remain unknown.

Liver-derived ceruloplasmin is the main copper-binding plasma transport protein and functions as a ferroxidase essential for the oxidation of Fe2+ before it can be bound to transferrin as Fe3+.-21 Previous studies demonstrated that ceruloplasmin is transcriptionally regulated by HIF-1 under hypoxic conditions in vitro.30 Here, we demonstrated that ceruloplasmin mRNA expression is also induced in the liver of hypoxic mice in vivo. Because both ceruloplasmin and apotransferrin facilitate iron release from liver cells and macrophages,63-66 the coordinate induction of their transcription under hypoxic conditions is likely required to match the increased iron demand of erythropoietic precursor cells in the bone marrow following stimulation by erythropoietin.

The finding that Cu2+ inhibits PHD activity provides the intriguing possibility that the concentration of free Cu2+ regulates the expression of its binding protein in the liver in a HIF-dependent manner. Indeed, when CuSO4 was administered intravenously to copper-deficient pigs, an increase in plasma ceruloplasmin was observed which preceded the increase in plasma iron.57 Other known HIF-1 target genes induced by Cu2+ in vivo include VEGF which is increased at wound sites topically treated with CuSO4.58 These data are consistent with our findings and support a physiological role of HIF-1 in Cu2+-dependent gene regulation, extending the sensory functions of the PHD family beyond oxygen sensing.

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References


27. Wang GL, Semenza GL. Desferrioxamine in-
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