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

Falk Martin, Tobias Linden, Dörthe M. Katschinski, Felix Oehme, Ingo Flamme, Chinmay K. Mukhopadhyay, Katrin Eckhardt, Juliane Tröger, Sandra Barth, Gieri Camenisch and Roland H. Wenger

Updated information and services can be found at:
http://bloodjournal.hematologylibrary.org/content/105/12/4613.full.html

Articles on similar topics can be found in the following Blood collections
Gene Expression (1086 articles)
Hematopoiesis and Stem Cells (3094 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://bloodjournal.hematologylibrary.org/site/subscriptions/index.xhtml

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

Falk Martin, Tobias Linden, Dörthe M. Katschinski, Felix Oehme, Ingo Flamme, Chinmay K. Mukhopadhyay, Katrin Eckhardt, Juliane Tröger, Sandra Barth, Gieri Camenisch, and Roland H. Wenger

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 ($CuCl_2$) stabilizes nuclear HIF-1α under normoxic conditions, resulting in hypoxia-response element (HRE)–dependent reporter gene expression. In in vitro hydroxylation assays $CuCl_2$ 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 $CuCl_2$ 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

Introduction

The hypoxia-inducible factor 1 (HIF-1) is an ubiquitously expressed transcriptional master regulator of many genes regulating mammalian oxygen homeostasis.[1] Among others, the corresponding gene products are involved in erythropoiesis, iron metabolism, angiogenesis, control of blood flow, glucose uptake and glycolysis, pH regulation, and cell-cycle control.[2] HIF-1 is a $\alpha_1\beta_1$ heterodimer specifically recognizing the HIF-binding site within *cis*-regulatory hypoxia response elements.[3] Under normoxic conditions, the von Hippel–Lindau tumor suppressor protein (pVHL) targets the HIF-1α subunit for rapid ubiquitination and proteasomal degradation.[4] Binding of the pVHL tumor suppressor protein requires the modification of HIF-1α by prolyl-4-hydroxylation at prolines 402 and 564 of human HIF-1α.[5-8] A family of 3 oxygen- and iron-dependent prolyl-4-hydroxylases called PHD1, PHD2, PHD3, or HPH3, HPH2, HPH1, respectively, has been shown to hydroxylate HIFα.[9,10] A fourth member, called PH-4, regulates HIF-1α in overexpression conditions only.[11] Thus, limited oxygen supply prevents HIFα hydroxylation and degradation.[12] This unusual mechanism of protein regulation provides the basis for the very rapid HIF-1α response to hypoxia.[13] In addition to protein stability, oxygen-dependent C-terminal asparagine hydroxylation of HIF-1α by factor inhibiting HIF (FIH) prevents transcriptional cofactor recruitment, thereby fine-tuning HIF-1 activity following a further decrease in oxygen availability.[14,15]

Among the HIF-1 targets are the genes encoding transferrin, transferrin receptor, heme oxygenase-1, and ceruloplasmin, which coordinately regulate iron metabolism.[16-20] Increased iron uptake, release from the liver, plasma transport, and uptake in the bone marrow are essential to sustain the erythropoietic function of erythropoietin, the prototype HIF-1 target. Ceruloplasmin is a multicopper plasma protein containing ferroxidase activity necessary for Fe^{3+} saturation of transferrin.[21] Hereditary aceruloplasminemia in humans as well as targeted deletion of the ceruloplasmin gene (*Cp*) in mice results in iron metabolism disorders characterized by anemia, hepatic iron overload, and neurodegeneration, demonstrating a tight connection between copper and iron metabolism.[22-26]

Iron deficiency has been known for more than a decade to induce erythropoietin gene expression and HIF-1α protein stabilization.[27] Nowadays, these results are most likely explained by inactivation of the iron-dependent protein hydroxylases PHD1 to 3 and FIH.[12] Iron deficiency also results in mRNA induction of ceruloplasmin by HIF-1–dependent promoter activation and subsequent transcriptional up-regulation of the *Cp* gene.[20,28] These results suggest that PHDs and FIH not only function as oxygen

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.[29] Oxygen partial pressures in the hypoxic workstation (InVivO$_2$-400; Ruskinn Technology, Leeds, United Kingdom) or in the incubator (Model 3319; Forma Scientific, 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 pH3SVL led to HIF-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.[30-32] The luciferase reporter gene pH3SVL is driven by a simian virus 40 (SV40) promoter and contains a total of 6 HIF-1 DNA-binding sites derived from the trans-ferrin gene.[16]

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.[33] 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 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, Schwalbach, Germany) dissolved in dimethyl sulfoxide (DMSO), or DMSO alone, were added in addition to 100 μM CuCl$_2$. Firefly luciferase reporter gene activity was determined as described by the manufacturer (Promega, Madison, WI) using a microplate luminometer (Berthold, Regensdorf, Switzerland). Results were normalized to the solvent-treated normoxic control values, which were arbitrarily defined as 1.

Transient transfections

Hep3B cells were transiently transfected with the *CP* promoter firefly reporter construct pGL-Cp4774[20] by the calcium phosphate coprecipitation method.[34] *CP* enhancer constructs, either containing a wild-type or a mutant HRE, were cloned into pGL3Prom (Promega) as described previously.[20] Semiconfluent cells on a 10-cm dish were 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 detached with 2 mM EDTA (ethylenediaminetetraacetic acid; pH 8.0) in PBS, distributed onto 2 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 ciclopirox olamine (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 reoxygenate. 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.[29] Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as a standard.[35] For immunoblot analysis, cellular protein (100 μg or 50 μg in stability experiments) was electrophoresed through 5% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semidry 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 immunoglobu-lin G$_1$ (IgG$_1$; 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(hy-droxymethyl)aminomethane)–HCl (pH 8.5), 2.65 mM H_2O_2, 0.45 mM luminol, and 0.625 mM coumaric 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.[29] 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 IgG$_1$ (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.[36] 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, thioredoxin-tagged pVHL in complex with elongins B and C was allowed to bind to the hydroxylated peptide, followed by detection of the thioredoxin 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 Sf9 insect cells as described elsewhere.[36,37]

mRNA quantification

Exposure of mice in triplicate to 7.5% oxygen for 0, 24, 48, and 72 hours and excision of their livers was described earlier.[38] Total RNA from frozen mouse liver tissue or cultured Hep3B cells was isolated and analyzed by Northern blotting as described previously.[16] Hybridization probes were obtained by restriction digestion and gel isolation followed by labeling with the random-primed method.[16] The mouse ceruloplasmin cDNA[39] was a kind gift of J. Gitlin (St Louis, MO) and the ribosomal protein L28 cDNA was cloned from a HepG2 cDNA library.[40] Radioactive signals were detected by phosphoimaging 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
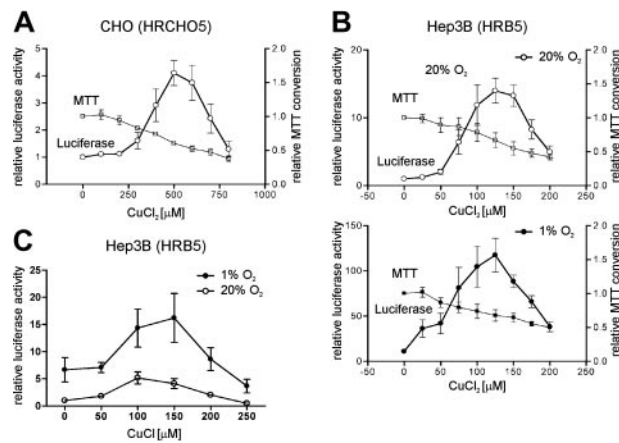
Figure 1. CuCl₂-dependent regulation of a HRE-driven reporter gene. CHO chinese hamster ovary (A) and Hep3B human hepatoma (B-C) cell lines stably transfected with an HRE-containing luciferase reporter gene were treated for 24 hours with the CuCl₂ (A-B) or CuCl (C) concentrations indicated. (A-B) MTT assays were used to assess Cu²⁺ toxicity. Shown are mean values ± SEM of n = 3 (all MTT assays), n = 7 (hypoxic Hep3B), n = 8 (normoxic Hep3B), or n = 10 (CHO) independent experiments. (C) A representative experiment performed in quadruplicate is shown as mean values ± SEM.

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′-gattaattggcccccctgatt-3′ and hCprev 5′-tgcattgtgaggccttgtag-3′; VEGF, hVEGF₁₆₅fwd 5′-gaggagggcagaatcatcac-3′ and hVEGF₁₆₅rev 5′-aggcccacagggattttcttgtc-3′; Glut-1, hGlut1fwd 5′-tcactgtgctcctggttctg-3′ and hGlut1rev 5′-cctgtgctcctgagagatcc-3′; and L28, hL285.1 5′-gcatctgcaatggatggt-3′ and hL283.1 5′-tgttcttgcggatcatgtgt-3′.

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 a HIF-1–dependent 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.[16,20] We thus stably transfected Hep3B cells with the HIF-dependent reporter gene like previously used to generate the HRCHO5 cell line. When the resulting cell line (termed HRB5) was treated with various doses of CuCl₂ for 24 hours, reporter gene expression increased up to 14-fold at 125 μM CuCl₂ (Figure 1B, top graph). Hypoxia alone (24 hours of 1% O₂) induced reporter gene activity 11-fold (Figure 1B, bottom graph) and the combined treatment with 1% O₂ and 125 μM CuCl₂ resulted in a 118-fold reporter gene induction. The CuCl₂ dose-response relationship revealed a relatively narrow window of active CuCl₂ concentrations. Since we suspected CuCl₂ cytotoxicity at higher concentrations, MTT conversion assays were performed to analyze cell

viability (ie, the combined decrease in cell growth and/or the increase in cell death). A dose-dependent reduction of MTT conversion activity was found in both cell lines (Figure 1A-B). At CuCl₂ concentrations that maximally induced reporter gene expression, MTT conversion was still 60% in CHO and 67% in Hep3B cells, respectively, when compared with untreated cells. Hypoxic conditions did not significantly alter MTT conversion activity in Hep3B cells. In addition to Cu²⁺, Cu⁺ also was able to induce reporter gene activity (Figure 1C), which might be due to the disproportionation of cuprous ions to cupric ions and copper in aqueous solutions. Other metal ions tested (Al³⁺, Ca²⁺, Fe²⁺, Fe³⁺, Gd²⁺, Mg²⁺, Mn²⁺, Se²⁺, and Zn²⁺) did not alter reporter gene activity in this type of assay (data not shown).

Nuclear HIF-1α protein induction by CuCl₂

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
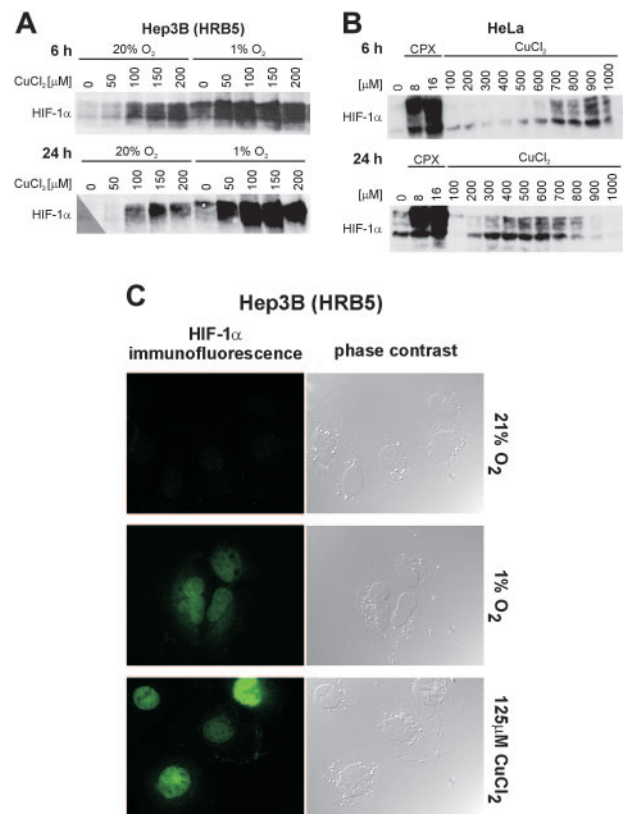

Figure 2. Nuclear HIF-1α protein induction following treatment with CuCl₂. Hep3B hepatoma (subline HRB5) (A) and HeLa cervical carcinoma (B) cell lines were treated with the CuCl₂ concentrations indicated for 6 and 24 hours. HIF-1α protein was detected by immunoblotting using a monoclonal anti–HIF-1α 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.
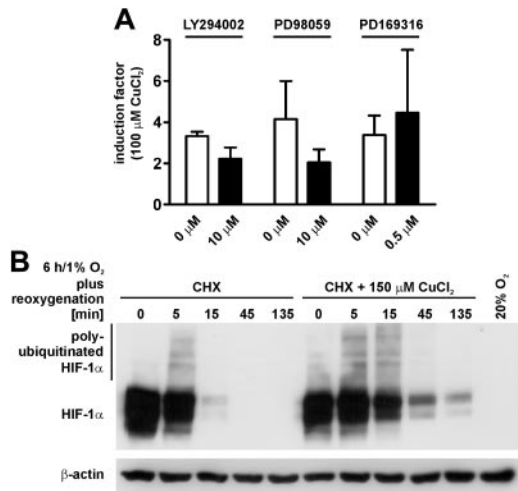
Figure 3. Molecular mechanism of HIF-1α protein stabilization by CuCl₂. (A) Hep3B hepatoma reporter cells (subline HRB5) were treated with or without 100 μM CuCl₂ for 24 hours in the presence or absence of the indicated kinase inhibitors under normoxic conditions. Shown are mean induction factors of luciferase activity ± SD of n = 3 independent experiments. (B) Hep3B cells were kept under hypoxic conditions in the presence or absence of 150 μM CuCl₂ before the addition of 20 μM cycloheximide (CHX) to block translation. Following reoxygenation for the indicated time periods, HIF-1α levels were determined by immunoblotting as in Figure 2. Subsequent detection of β-actin served as control for equal loading.

treated with CuCl₂. As shown in Figure 2B, HIF-1α was also stabilized in HeLa cells, though at a considerably higher CuCl₂ concentration and not as strongly as with the iron chelator CPX that was included as positive control in these experiments. Of note, the window of active CuCl₂ concentrations was shifted to lower values after prolonged incubation, suggesting an overlap with time-dependent cytotoxicity of CuCl₂.

We next determined whether CuCl₂ leads to a nuclear translocation of stabilized HIF-1α protein. Therefore, the Hep3B subline HRB5 was treated with 1% O₂ or 125 μM CuCl₂ for 24 hours. As shown by indirect immunofluorescence analysis, HIF-1α protein accumulated exclusively within the nucleus, excluding the nucleoli, following hypoxia or CuCl₂ treatment but not in normoxic control cells (Figure 2C).

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 3 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-1–dependent manner. Thus, we further analyzed ceruloplasmin regulation, a known HIF-1 target gene inducible by hypoxia and iron depletion in 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.
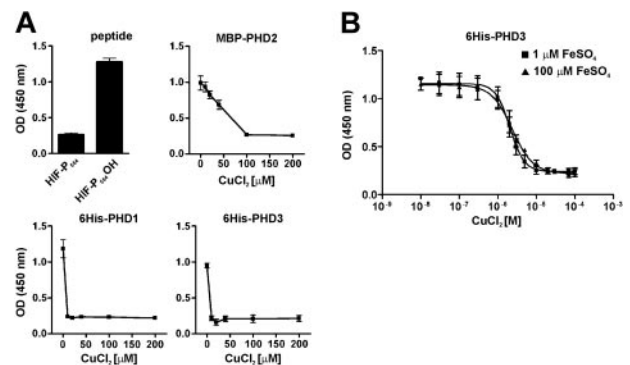

Figure 4. Inhibtion of PHD activity by CuCl₂ in vitro. PHD activity was measured in the presence of oxygen, 2-oxoglutarate, and ascorbate by the binding of a purified pVHL-elonginB-elonginC complex to a HIF-1α–derived peptide bound onto microtiter plates as described in "Materials and methods." (A) PHD enzyme–free assays using a nonhydroxylated peptide or 40 nM of a hydroxyproline-containing peptide served as negative and positive controls (black bars labeled with HIF-P₅₆₄ and HIF-P₅₆₄OH, respectively). PHD1 and PHD3 were obtained from insect cells as 6His fusion proteins and PHD2 was expressed in bacteria as MBP fusion protein. Representative experiments performed in triplicate are shown as mean values ± SD. (B) PHD3-dependent HIF-1α Pro₅₆₄ hydroxylation activity was determined in the presence of either 1 or 100 μM FeSO₄. Shown are mean values ± SD of n = 3 independent experiments. OD indicates optical density.
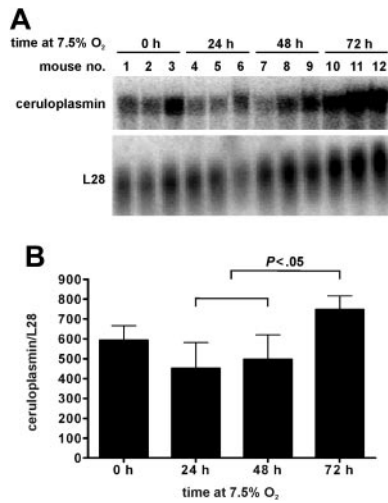
Figure 5. Ceruloplasmin mRNA levels in hypoxic mouse liver. (A) Northern blotting of liver RNA derived from mice exposed to 7.5% O_2 for 0 to 3 days. Hybridization signals obtained with a ribosomal protein L28 probe served as a control for loading and blotting efficiency. (B) Phosphoimager quantification of band intensities shown in panel A. Mean ceruloplasmin to L28 mRNA ratios ± SD of n = 3 mice for each time point are given.

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 $CuCl_2$ 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).
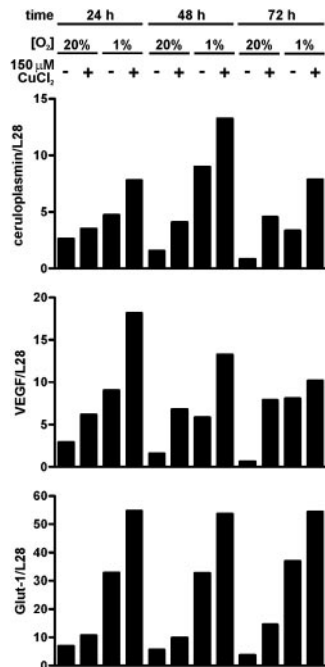
Maximal ceruloplasmin induction by $CuCl_2$ was 5.5-fold after 72 hours of treatment under normoxic conditions.

The ceruloplasmin gene promoter is activated by Cu²⁺

Cu^{2+}-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 $CuCl_2$, reporter gene activity dose-dependently increased up to 4-fold at 200 μM $CuCl_2$ (Figure 7A). Hypoxia alone induced reporter gene activity 3.5-fold and the combination of $CuCl_2$ with hypoxia further increased reporter gene activity. However, $CuCl_2$ 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 $CuCl_2$ or 1% O_2 after 24 hours of treatment. Point mutation of this HRE reduced but did not completely inhibit induction by $CuCl_2$ or hypoxia in these experiments, suggesting that other elements might also be involved in $CuCl_2$-dependent activation of ceruloplasmin gene expression.
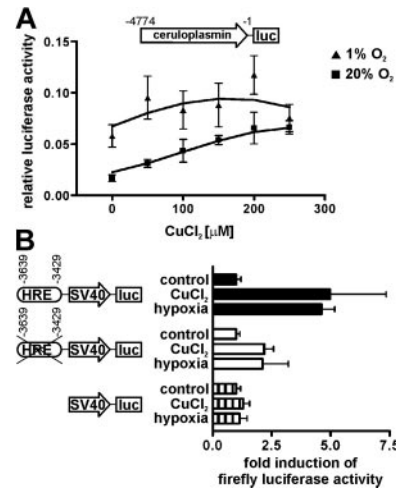

Figure 6. Induction of ceruloplasmin mRNA by hypoxia and $CuCl_2$ in Hep3B hepatoma cells. Real-time PCR quantification of reverse transcribed ceruloplasmin, VEGF, Glut-1, and L28 mRNA levels in Hep3B cells treated with 150 μM $CuCl_2$ under normoxic or hypoxic conditions for the indicated time periods. Shown are mRNA copy numbers relative to the L28 control as measured in duplicate.

Figure 7. Ceruloplasmin promoter regulation by $CuCl_2$. (A) Hep3B hepatoma cells were transiently cotransfected with a ceruloplasmin promoter–firefly luciferase reporter gene construct together with a renilla luciferase control reporter gene driven by the SV40 promoter. Transfected cells were treated with the indicated $CuCl_2$ concentrations under normoxic or hypoxic conditions. After 24 hours, firefly luciferase reporter gene activity was determined and divided by the renilla luciferase values to correct for differences in transfection efficiency. Shown are mean relative luciferase activities ± SEM of 2 transfections performed in quadruplicate. (B) Hep3B cells were transfected with HRE wild-type and HRE mutant ceruloplasmin enhancer SV40 promoter constructs, or the SV40 promoter alone, driving firefly luciferase expression. Following 24 hours of 150 μM $CuCl_2$ or 1% oxygen, luciferase induction factors compared with the untreated controls were determined. A representative experiment performed in triplicate is shown as mean values ± SEM.

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 Co^{2+} or Ni^{2+} and to a lesser extent Mn^{2+} salts.[41] However, Cu^{2+} has not been found to induce erythropoietin gene expression in these early experiments. In our study, we demonstrated that Cu^{2+} 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 Co^{2+}, Ni^{2+}, or Cu^{2+}, several other transition metals have been reported to induce HIF-1α, including vanadate,[42] arsenite,[43] and chromium.[44] A common mechanism by which these transition metals interfere with PHD function is unknown. Salnikow and colleagues reported that intracellular ascorbate required for PHD activity might be depleted by Co^{2+} and Ni^{2+}.[45] Reactive oxygen species, PI3K/Akt, p38, p70[S6K],[1] PKCδ, and adenosine monophosphate (AMP) kinases have been reported to be involved in transition metal-dependent HIF-1α induction.[42-44,46-48] However, several kinase pathway inhibitors did not affect CuCl$_2$-dependent HIF-1 induction in our hands. Moreover, all of these mechanisms are based on an intact cellular environment, but in our experiments we could demonstrate a direct inhibition of PHD activity in a cell-free system in vitro with an IC_{50} value of approximately 2.3 μM and full inhibition at approximately 10 μM CuCl$_2$. These values are somewhat lower than the approximately 150 μM CuCl$_2$ required to obtain maximal induction of HIF-1α in hepatoma cells. However, cell culture conditions, including the Cu^{2+}-binding capacity of serum proteins present in the fetal calf serum (FCS), cannot be directly compared with in vitro assays performed with different kinetics and purified proteins in a cell-free environment. Over all, our data suggest that inhibition of PHD activity is the most likely mechanism by which CuCl$_2$ induced HIF-1α.

Spectroscopic and structural studies on 2-oxoglutarate-dependent dioxygenases showed that Cu^{2+} can substitute active-site Fe^{2+} in this class of enzymes.[12] However, under our experimental conditions Cu^{2+} does probably not compete for the active site of HIF-PHD3 because even the presence of a vast excess of ferrous iron did not reverse the inhibitory function of CuCl$_2$. Although it cannot be excluded that Cu^{2+} ions bind to the active site of PHD with considerably higher affinity compared with Fe^{2+}, it appears to be more likely that other, yet unknown mechanisms are responsible for the inhibition of HIF PHDs by transition metal ions.

Recently, van Heerden and coworkers demonstrated that rainbow trout exposed to CuSO$_4$ induce HIF-1α protein in their gills.[49] These results confirm that Cu^{2+} salts can also induce HIF-1α protein in vivo. van Heerden and coworkers attributed their results to an increased oxygen consumption leading to regional tissue hypoxia. However, our data suggest that a direct inhibition of HIF-1α prolyl-4-hydroxylation by Cu^{2+} salts might also contribute to the accumulation of HIF-1α protein. Whereas the fish were exposed to water containing 1.65 μM CuSO$_4$,[49] optimal CuCl$_2$ concentrations in our experiments were between 100 and 200 μM for hepatoma cells. This concentration range is very similar to the widely used CoCl$_2$ that generally stabilizes HIF-1α in the range between 50-150 μM.[41,50] Of note, under normal conditions the "free" intracellular copper ion concentration is less than 1 atom per cell.[51] Due to its toxicity, intracellular copper is tightly regulated by copper-scavenging metallochaperones.[52] Their copper-binding capacity either needs to be saturated before "free" copper is available to interfere with the PHDs or the copper-bound metallochaperones might be directly involved in PHD regulation. However, the precise molecular mechanisms acting between extracellular 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 Fe^{2+} before it can be bound to transferrin as Fe^{3+}.[21] Previous studies demonstrated that ceruloplasmin is transcriptionally regulated by HIF-1 under hypoxic conditions in vitro.[20] 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,[53-56] 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 Cu^{2+} inhibits PHD activity provides the intriguing possibility that the concentration of free Cu^{2+} regulates the expression of its binding protein in the liver in a HIF-dependent manner. Indeed, when CuSO$_4$ 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 Cu^{2+} in vivo include VEGF which is increased at wound sites topically treated with CuSO$_4$.[58] These data are consistent with our findings and support a physiological role of HIF-1 in Cu^{2+}-dependent gene regulation, extending the sensory functions of the PHD family beyond oxygen sensing.

Acknowledgments

References

1. Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Ann Rev Cell Dev Biol. 1999;15:551-578.

2. Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. FASEB J. 2002;16:1151-1162.

3. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A. 1995;92:5510-5514.

4. Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999;399:271-275.

5. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science. 2001;292:468-472.

6. Ivan M, Kondo K, Yang H, et al. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science. 2001; 292:464-468.

7. Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation. EMBO J. 2001; 20:5197-5206.

8. Yu F, White SB, Zhao Q, Lee FS. HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc Natl Acad Sci U S A. 2001;98: 9630-9635.

9. Epstein AC, Gleadle JM, McNeill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001;107:43-54.

10. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001;294:1337-1340.

11. Oehme F, Ellinghaus P, Kolkhof P, et al. Overexpression of PH-4, a novel putative proline 4-hydroxylase, modulates activity of hypoxia-inducible transcription factors. Biochem Biophys Res Commun. 2002;296:343-349.

12. Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol. 2004;5: 343-354.

13. Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M. Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J. 2001;15:1312-1314.

14. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 2001;15:2675-2686.

15. Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 2002;16:1466-1471.

16. Rolfs A, Kvietikova I, Gassmann M, Wenger RH. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem. 1997;272:20055-20062.

17. Tacchini L, Bianchi L, Bernelli-Zazzera A, Cairo G. Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem. 1999;274:24142-24146.

18. Lok CN, Ponka P. Identification of a hypoxia response element in the transferrin receptor gene. J Biol Chem. 1999;274:24147-24152.

19. Lee PJ, Jiang BH, Chin BY, et al. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J Biol Chem. 1997;272:5375-5381.

20. Mukhopadhyay CK, Mazumder B, Fox PL. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. J Biol Chem. 2000;275:21048-21054.

21. Osaki S, Johnson DA, Frieden E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. J Biol Chem. 1966;241:2746-2751.

22. Miyajima H, Nishimura Y, Mizoguchi K, Sakamoto M, Shimizu T, Honda N. Familial apoceruloplasmin deficiency associated with blepharospasm and retinal degeneration. Neurology. 1987;37: 761-767.

23. Harris ZL, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT, Gitlin JD. Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci U S A. 1995;92: 2539-2543.

24. Harris ZL, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci U S A. 1999;96:10812-10817.

25. Bosio S, De Gobbi M, Roetto A, et al. Anemia and iron overload due to compound heterozygosity for novel ceruloplasmin mutations. Blood. 2002;100: 2246-2248.

26. Garrick MD, Núñez MT, Olivares M, Harris ED. Parallels and contrasts between iron and copper metabolism. Biometals. 2003;16:1-8.

27. Wang GL, Semenza GL. Desferrioxamine in-

duces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood. 1993;82:3610-3615.

28. Mukhopadhyay CK, Attieh ZK, Fox PL. Role of ceruloplasmin in cellular iron uptake. Science. 1998;279:714-717.

29. Katschinski DM, Le L, Heinrich D, et al. Heat induction of the unphosphorylated form of hypoxia-inducible factor-1α is dependent on heat shock protein-90 activity. J Biol Chem. 2002;277:9262-9267.

30. Wanner RM, Spielmann P, Stroka DM, et al. Epolones induce erythropoietin expression via hypoxia-inducible factor-1α activation. Blood. 2000;96: 1558-1565.

31. Stiehl DP, Jelkmann W, Wenger RH, Hellwig-Bürgel T. Normoxic induction of the hypoxia-inducible factor-1α by insulin and interleukin-1β involves the phosphatidylinositol 3-kinase pathway. FEBS Lett. 2002;512:157-162.

32. Linden T, Katschinski DM, Eckhardt K, Scheid A, Pagel H, Wenger RH. The antimycotic ciclopirox olamine induces HIF-1α stability, VEGF expression, and angiogenesis. FASEB J. 2003;17:761-763.

33. Unruh A, Ressel A, Mohamed HG, et al. The hypoxia-inducible factor-1α is a negative factor for tumor therapy. Oncogene. 2003;22:3213-3220.

34. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.

35. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-254.

36. Oehme F, Jonghaus W, Narouz-Ott L, Huetter J, Flamme I. A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity. Anal Biochem. 2004;330:74-80.

37. Hirsilä M, Koivunen P, Günzler V, Kivirikko KI, Myllyharju J. Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J Biol Chem. 2003;278:30772-30780.

38. Wenger RH, Gassmann M. HIF-1 and the molecular response to hypoxia in mammals. In: Storey KB, ed. Environmental Stress and Gene Regulation. Oxford, United Kingdom: BIOS Scientific Publishers;1999:25-45.

39. Klomp LW, Farhangrazi ZS, Dugan LL, Gitlin JD. Ceruloplasmin gene expression in the murine central nervous system. J Clin Invest. 1996;98: 207-215.

40. Wenger RH, Marti HH, Schuerer-Maly CC, et al. Hypoxic induction of gene expression in chronic granulomatous disease-derived B-cell lines: oxygen sensing is independent of the cytochrome b558-containing nicotinamide adenine dinucleotide phosphate oxidase. Blood. 1996;87:756-761.

41. Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science. 1988;242: 1412-1415.

42. Gao N, Ding M, Zheng JZ, et al. Vanadate-induced expression of hypoxia-inducible factor 1 α and vascular endothelial growth factor through phosphatidylinositol 3-kinase/Akt pathway and

reactive oxygen species. J Biol Chem. 2002;277: 31963-31971.

43. Duyndam MC, Hulscher TM, Fontijn D, Pinedo HM, Boven E. Induction of vascular endothelial growth factor expression and hypoxia-inducible factor 1α protein by the oxidative stressor arsenite. J Biol Chem. 2001;276:48066-48076.

44. Gao N, Jiang BH, Leonard SS, et al. p38 Signaling-mediated hypoxia-inducible factor 1α and vascular endothelial growth factor induction by Cr(VI) in DU145 human prostate carcinoma cells. J Biol Chem. 2002;277:45041-45048.

45. Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. J Biol Chem. 2004;279:40337-40344.

46. Gao N, Shen L, Zhang Z, et al. Arsenite induces HIF-1α and VEGF through PI3K, Akt and reactive oxygen species in DU145 human prostate carcinoma cells. Mol Cell Biochem. 2004;255:33-45.

47. Soucy NV, Klei LR, Mayka DD, Barchowsky A. Signaling pathways for arsenic-stimulated vascular endothelial growth factor—a expression in primary vascular smooth muscle cells. Chem Res Toxicol. 2004;17:555-563.

48. Skinner HD, Zhong XS, Gao N, Shi X, Jiang BH. Arsenite induces p70S6K1 activation and HIF-1α expression in prostate cancer cells. Mol Cell Biochem. 2004;255:19-23.

49. van Heerden D, Vosloo A, Nikinmaa M. Effects of short-term copper exposure on gill structure, metallothionein and hypoxia-inducible factor-1α (HIF-1α) levels in rainbow trout (Oncorhynchus mykiss). Aquat Toxicol. 2004;69:271-280.

50. Wenger RH, Marti HH, Bauer C, Gassmann M. Optimal erythropoietin expression in human hepatoma cell lines requires activation of multiple signalling pathways. Int J Mol Med. 1998;2:317-324.

51. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science. 1999;284: 805-808.

52. Prohaska JR, Gybina AA. Intracellular copper transport in mammals. J Nutr. 2004;134:1003-1006.

53. Ragan HA, Nacht S, Lee GR, Bishop CR, Cartwright GE. Effect of ceruloplasmin on plasma iron in copper-deficient swine. Am J Physiol. 1969; 217:1320-1323.

54. Osaki S, Johnson DA, Frieden E. The mobilization of iron from the perfused mammalian liver by a serum copper enzyme, ferroxidase I. J Biol Chem. 1971;246:3018-3023.

55. Young SP, Fahmy M, Golding S. Ceruloplasmin, transferrin and apotransferrin facilitate iron release from human liver cells. FEBS Lett. 1997; 411:93-96.

56. Sarkar J, Seshadri V, Tripoulas NA, Ketterer ME, Fox PL. Role of ceruloplasmin in macrophage iron efflux during hypoxia. J Biol Chem. 2003; 278:44018-44024.

57. Roeser HP, Lee GR, Nacht S, Cartwright GE. The role of ceruloplasmin in iron metabolism. J Clin Invest. 1970;49:2408-2417.

58. Sen CK, Khanna S, Venojarvi M, et al. Copper-induced vascular endothelial growth factor expression and wound healing. Am J Physiol Heart Circ Physiol. 2002;282:H1821–H1827.