0026-895X/09/7501-174–182$20.00
MOLECULAR PHARMACOLOGY
Vol. 75, No. 1
51516/3418360
Mol Pharmacol 75:174–182, 2009
Printed in U.S.A.

Copper Regulation of Hypoxia-Inducible Factor-1 Activity

Wenke Feng, Fei Ye, Wanli Xue, Zhanxiang Zhou, and Y. James Kang

Departments of Medicine (W.F., F.Y., W.X., Z.Z., Y.J.K.) and Pharmacology and Toxicology (Y.J.K.), University of Louisville School of Medicine, Louisville, Kentucky

Received August 22, 2008; accepted October 8, 2008

ABSTRACT

Previous studies have demonstrated that copper up-regulates hypoxia-inducible factor 1 (HIF-1). The present study was undertaken to test the hypothesis that copper is required for HIF-1 activation. Treatment of HepG2 cells with a copper chelator tetraethylenepentamine (TEPA) or short interfering RNA targeting copper chaperone for superoxide dismutase 1 (CCS) suppressed hypoxia-induced activation of HIF-1. Addition of excess copper relieved the suppression by TEPA, but not that by CCS gene silencing, indicating the requirement of copper for activation of HIF-1, which is CCS-dependent. Copper deprivation did not affect production or stability of HIF-1α but reduced HIF-1α binding to the hypoxia-responsive element (HRE) of target genes and to p300, a component of HIF-1 transcriptional complex. Copper probably inhibits the factor inhibiting HIF-1 to ensure the formation of HIF-1 transcriptional complex. This study thus defines that copper is required for HIF-1 activation through the regulation of HIF-1α binding to the HRE and the formation of the HIF-1 transcriptional complex.

Previous studies have shown that pressure overload causes copper decrease in the heart in association with an inhibition of myocardial angiogenesis and the transition from cardiac hypertrophy to heart failure in a mouse model (Jiang et al., 2007). Dietary supplementation with physiologically relevant levels of copper replenishes cardiac copper and reverses hypertrophic cardiomyopathy in the presence of pressure overload induced by ascending aortic constriction in the mouse model (Jiang et al., 2007). Further studies have shown that dietary copper supplementation increases myocardial vascular endothelial growth factor (VEGF) levels along with an enhanced angiogenesis. It is noteworthy that systemic administration of anti-VEGF antibody blunts copper regression of hypertrophic cardiomyopathy (Jiang et al., 2007). It has also been shown that dietary copper restriction causes suppression of VEGF expression in the heart, and copper replenishment recovers myocardial VEGF expression (Elsherif et al., 2004). In addition, copper stimulates VEGF expression in cultured human keratinocytes and enhances angiogenesis and promotes wound healing (Sen et al., 2002).

In an attempt to understand the molecular mechanisms by which copper stimulates VEGF expression, we studied the role of transcription factor, hypoxia-inducible factor 1 (HIF-1), in regulation of copper-induced VEGF expression. Copper chelation suppresses the transcription activity of HIF-1, and gene silencing of α subunit of HIF-1 (HIF-1α) blocks copper stimulation of VEGF expression (Jiang et al., 2007). Other studies have shown that excess copper stabilizes HIF-1α and stimulates VEGF expression under both normoxic and hypoxic conditions (Martin et al., 2005). However, it is important to know how copper regulates HIF-1 transcription activity.

HIF-1 is composed of HIF-1α and HIF-1β (Wang et al., 1995). The synthesis and accumulation of HIF-1α is a rate-limiting step for activation of HIF-1 (Wang and Semenza, 1993b; Huang et al., 1998) . The expression level of HIF-1α is undetectable in most cell types under normoxic conditions because of its degradation by the ubiquitin-proteasome pathway, which is mediated by proline hydroxylation of HIF-1α catalyzed by three HIF prolyl hydroxylases (PHDs) (Huang et al., 1998; Ivan et al., 2001; Jaakkola et al., 2001). The hydroxylated HIF-1α is recognized by a von Hippel-Lindau protein, which is a constitute of an ubiquitin ligase complex, targeting HIF-1α subunit for degradation by proteasome in cytosol (Maxwell et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000; Masson et al., 2001). Under hypoxic conditions, HIF-1α escapes from the degradation pathway, accumulates, and translocates into the nucleus, where it dimerizes with

Support for this study was provided in part by National Institutes of Health grants HL59225 and HL63760 (to Y.J.K.), Kentucky Science and Engineering Foundation grant KSEF-888-RDE-008 (to W.F. and Y.J.K.), and American Diabetes Association grant 7-07-JF-23 (to W.F.).

Y.J.K. is a Distinguished University Scholar at the University of Louisville.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.051516.

ABBREVIATIONS: VEGF, vascular endothelial growth factor; TEPA, tetraethylenepentamine; HIF, hypoxia-inducible factor; HRE, hypoxia-responsive element; DFO, deferoxamine; FIH-1, factor inhibiting hypoxia-inducible factor 1; CCS, copper chaperone for superoxide dismutase 1; IGF, insulin-like growth factor; PHD, hypoxia-inducible factor prolyl hydroxylase; EMSA, electrophoretic mobility shift assay; siRNA, short interfering RNA; PBS, phosphate-buffered saline; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

HIF-1β and interacts with cofactors to assemble the HIF-1 transcriptional complex. Some transition metals such as cobalt and nickel enhance HIF-1 transcription activity by stabilizing HIF-1α protein (Yuan et al., 2003; Maxwell and Salnikow, 2004; Hirsila et al., 2005; Ke et al., 2005). In the nucleus, HIF-1α is hydroxylated on its C-terminal asparagine by another dioxygenase, factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). FIH-1 prevents the interaction between HIF-1α and its cofactors, such as p300, and inhibits the transcription activity of HIF-1 (Dames et al., 2002; Freedman et al., 2002; Lando et al., 2002a,b; McNeill et al., 2002). FIH-1 is an iron-dependent enzyme. Therefore, iron chelation enhances HIF-1 transcription activity (Wang and Semenza, 1993a).

Copper is capable of stabilizing HIF-1α by a mechanism involving the inhibition of prolyl hydroxylases (van Heerden et al., 2004; Martin et al., 2005). However, this action of copper would be the same as other transition metals such as cobalt and nickel, which are not essential for HIF-1 activation but enhance HIF-1 activity when the cells are exposed to excess amounts of these transition metals (Yuan et al., 2003; Maxwell and Salnikow, 2004; Hirsila et al., 2005; Ke et al., 2005). The critical question addressed here is whether copper is essential for HIF-1 transcription activity.

Our previous studies have suggested that copper is required for HIF-1 transcription activity. Copper chelation in cultured cells blocks insulin-like growth factor-1 (IGF-1)-induced HIF-1 binding to hypoxia responsive element (HRE) and VEGF expression. This inhibitory effect can be relieved by the addition of excess copper in cultures (Jiang et al., 2007). In addition, we have found that this copper action depends on a copper chaperone for superoxide dismutase 1 (CCS), and CCS gene silencing blocks IGF-1-induced HIF-1 binding to the HRE and VEGF expression, mimicking the effect of copper chelation. Furthermore, CCS directly interacts with HIF-1α, as revealed by an immunoprecipitation assay (Jiang et al., 2007).

There are multiple sites that potentially require copper for activation of HIF-1, including HIF-1α synthesis, stabilization, translocation from cytosol to nucleus, binding to the HRE sequence of target genes, and HIF-1 transcriptional complex formation. In the present study, we report that copper is required for HIF-1 binding to the HRE sequence of target genes and for the HIF-1 transcriptional complex formation in a HepG2 human hepatoma cell line.

Materials and Methods

Cell Culture and Treatments. Human hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were routinely cultured in 95% air/5% CO_2 at 37°C. Hypoxic conditions were introduced by incubating cells in a tightly sealed chamber maintained at 1% O_2, 5% CO_2, and balanced with N_2 at 37°C. For treatment with reagents, cells were seeded in culture dishes and grown overnight. Thereafter, tetraethylenepentamine (TEPA) or $CuSO_4$ was added to the cultures, followed by further incubation for the time periods indicated in each experiment. In some experiments, deferoxamine (DFO) was added at 16 h before cell harvesting to induce HIF-1α accumulation.

RNA Interference. siRNAs targeting human FIH-1 and CCS and a negative mismatched control were designed and synthesized by Ambion, Inc. (Austin, TX). The siRNA sequences for FIH-1 were as follows: sense, GCUUAUUGAGAAUGAGGAGtt; antisense, CUCCUCAUUCUCAAUAAGCtc. The siRNA sequences for CCS were as follows: sense, GGACCAGAUGGUCUUGGUAtt; antisense, UACCAAGACCAUCUGGUCCtt. After monolayer cultures reached 50% confluence, the cells were transfected with 100 nM FIH-1, CCS, or negative mismatched siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Antibiotics were added to the medium 24 h after transfection, and cells were used for experimental procedures 48 h after transfection.

Nuclear Extract Preparation. Nuclear extracts were prepared as described previously (Hellwig-Burgel et al., 1999) with minor modifications. In brief, cells were washed once on dish with ice-cold phosphate-buffered saline (PBS). Ice-cold buffer (10 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$, and 10 mM KCl) containing freshly added 0.4 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol (DTT), and 1% protease inhibitor cocktail (Sigma, St. Louis, MO) was overlaid on cells in the dish and incubated for 10 min. The cells were then harvested by scraping with a rubber cell policeman and lysed by Dounce homogenization. Nuclei were pelleted by centrifugation and then resuspended in ice-cold buffer (20 mM Tris-HCl, pH 7.8, 420 mM KCl, 1.5 mM $MgCl_2$, and 20% glycerol) containing freshly added 0.4 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 1% protease inhibitor cocktail, and 1 mM Na_3VO_4 and incubated for 30 min on ice with occasional tapping. The extracts were clarified by centrifugation at 12,000*g* for 15 min at 4°C, placed in aliquots, and stored at −80°C.

Quantitative Analysis of VEGF. VEGF levels were determined using a Quantikine Human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Western Blotting. Whole-cell lysates were prepared as described previously (Jaakkola et al., 2001). Appropriate amount of protein in total cell lysates was resolved in a SDS-polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene difluoride membrane (Whatman, Sanford, ME). Membranes were blocked for 1 h in Tris-buffered saline/Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: HIF-1α (BD Biosciences, San Jose, CA); p300, FIH-1, and CCS (Santa Cruz Biotechnology, Santa Cruz, CA); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; StressGen, Victoria, Canada). After washing with Tris-buffered saline/Tween 20, the membranes were incubated with a horseradish peroxidase-linked anti-mouse or anti-rabbit IgG antibody (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for 1 h at room temperature. Proteins were visualized using an enhanced chemiluminescence system (GE Healthcare).

Immunoprecipitation. Immunoprecipitation was performed using anti-HIF-1α or anti-p300 (BD Biosciences) antibodies as described previously (Feng et al., 2005). The immunoprecipitates were subjected to Western blotting analysis as described above.

Transient Transfection and Luciferase Reporter Assay. HepG2 cells were transfected with a luciferase reporter gene construct pH3SVL plasmid (a kind gift from Professor R. H. Wenger) by Lipofectamine 2000 according to the instruction provided by the manufacturer (Invitrogen). After recovering, cells were treated with different reagents as described in each experiment. Thereafter, cells were incubated for 16 h under hypoxia. In some experiments, cells were treated with DFO for induction of HIF-1α. After washing with PBS, cells were lysed with a passive lysis buffer provided by Promega (Madison, WI). Luciferase activities were determined using a luciferase assay system (Promega) and normalized to total cellular protein. In some experiments, pH3SVL luciferase reporter plasmid was cotransfected with siRNAs, as described above.

Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides containing the HIF-1 binding sequence from erythropoietin HRE (5′-TCT GTA CGT CAC ACT CAC CTC-3′) (Santa Cruz Biotechnology) were end-labeled with [γ-^{32}P]ATP (GE Healthcare) using T4-polynucleotide kinase (Promega). Unincorporated nucleo-

tide was removed by gel-filtration using a Nick column (GE Healthcare). The γ-^{32}P-labeled HRE probe was incubated with 5 to 10 μg of nuclear extract and 1 μg of poly(dI-dC)·(dI-dC) (GE Healthcare) in a buffer containing 10 mM Tris-HCl, pH 7.8, 20 mM KCl, 1 mM EDTA, 5 mM DTT, 1 mM $MgCl_2$, and 5% glycerol overnight at 4°C in a total volume of 20 μl. For competition assay, a 200-fold molar excess of unlabeled HRE was added to the reaction before the addition of labeled probe. For supershift assay, 2 μl of mouse monoclonal antibody against HIF-1α (BD Biosciences) was added to the reaction 2 h before electrophoresis. Electrophoresis was performed using 5% nondenaturing polyacrylamide gel at 200 V in 0.5× Tris-buffered EDTA for 2 h at 4°C. The gels were dried and subjected to autoradiography for visualization.

Immunocytochemistry. HepG2 cells grown on a chamber slide were incubated under normoxia or hypoxia with or without additional treatments. After incubation, media were removed, and cells were fixed with 4% paraformaldehyde. The fixed cells were washed three times with PBS plus 3% bovine serum albumin and incubated at 4°C overnight with rabbit polyclonal anti-HIF-1 antibody (BD Biosciences). Then the cells were washed three times with PBS plus 3% bovine serum albumin and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 h. Reaction omitting the primary antibody served as a negative control.

Statistical Analysis. Data were evaluated using Student's *t* test. $p < 0.05$ was considered significant.

Results

Copper Deprivation Suppresses HIF-1 Transcription Activity. To define the essential role of copper in activation of HIF-1 in HepG2 cells in response to hypoxia, we treated cultured HepG2 cells with a copper chelator, TEPA, to decrease intracellular levels of labile copper. The efficacy of TEPA in reducing cellular copper was determined by the effect of TEPA on accumulation of a copper-sensitive CCS in the cells. CCS levels increase when copper levels decrease, which has been used as an indicator of copper depletion (Prohaska et al., 2003). The result presented in Fig. 1A shows that the accumulation of CCS in HepG2 cells increased after the cells were treated with 50 μM TEPA for 24 h and further increased after treatment for 72 h, indicating that TEPA treatment effectively decreased cellular copper levels. Under this experimental condition, TEPA up to a final concentration of 100 μM in the cultures did not cause cytotoxicity, as evaluated by a lactate dehydrogenase release assay (data not shown).

The effect of copper deprivation on HIF-1 transcription activity was evaluated by two different assays. In the first, hypoxia-induced VEGF production in cultured HepG2 cells was determined, and in the second, a HIF-1-specific reporter assay was performed. In the first experiment, the presence of 50 μM $CuSO_4$ in cultures enhanced hypoxia-induced VEGF production, but the presence of 50 μM TEPA suppressed the production. To confirm that the TEPA-suppressed VEGF production results from copper deprivation, varying concentrations of $CuSO_4$ were added to the TEPA-treated cultures, and a copper concentration-dependent recovery of TEPA suppression of VEGF production was observed (Fig. 1B), indicating a selective effect of copper chelation by TEPA.

We next determined the effect of copper deprivation by TEPA on HIF-1 transcription activity using a luciferase reporter assay. The HepG2 cells were transfected with a reporter plasmid containing 6× HIF-1 binding site fused with a luciferase gene (Wanner et al., 2000). As expected, the luciferase reporter gene was activated by hypoxia, and TEPA treatment significantly reduced the luciferase activity. This effect of TEPA on luciferase activity was also relieved by the addition of excess copper in a copper concentration-dependent manner (Fig. 1C), further demonstrating the selective effect of copper deprivation on HIF-1 transcription activity.

To further verify the effect of copper on HIF-1 transcription

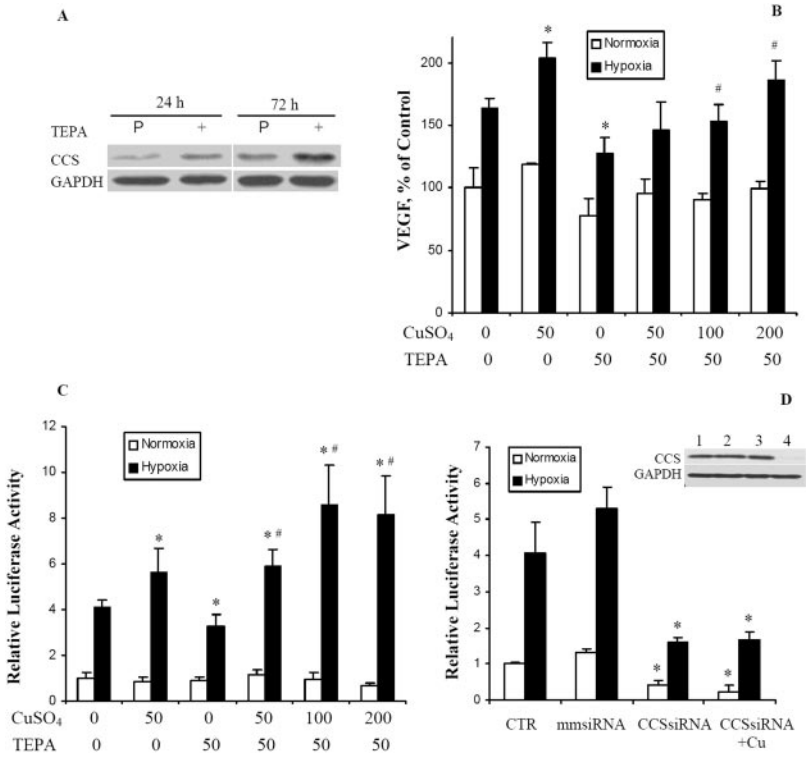

Fig. 1. Effects of TEPA and siRNA targeting CCS on HIF-1 activation. A, effects of TEPA treatment on CCS levels. Cells were incubated in the presence or absence of 50 μM TEPA for 24 or 72 h, and CCS levels were determined by immunoblotting in whole-cell extracts. GAPDH was probed as a loading control. B, effects of excess copper on TEPA-suppressed VEGF expression. HepG2 cells were cultured with TEPA (in micromoles) or $CuSO_4$ (in micromoles) alone or in combination at the concentrations indicated for 24 h before incubation under hypoxia for 16 h. VEGF levels in the culture supernatants were determined as described under *Materials and Methods*. C, effects of excess copper on TEPA-suppressed HIF-1 activation. Cells were transfected with the pH3SVL reporter plasmid containing the luciferase gene under the control of six HIF-1 binding sites from transferrin 3′ enhancer and then treated as described under *Materials and Methods*. Luciferase activities were measured and normalized to sample protein concentration. D, effects of siRNA targeting CCS on HIF-1 activation. Cells were cotransfected with CCS siRNA or mismatched siRNA (mmsiRNA) and the luciferase plasmid before the addition of $CuSO_4$ (100 μM) for 24 h. Cells were then subjected to hypoxia for 16 h, and luciferase activities were determined. D, inset, efficiency of CCS siRNA on CCS protein levels were determined by immunoblotting. 1, control; 2, transfection reagent; 3, mmsiRNA; 4, CCS siRNA. Data are the mean ± S.D. of three separate culture dishes from one representative experiment. Each experiment was performed three times with consistent results. *, $p < 0.05$ versus controls; #, $p < 0.05$ versus $CuSO_4$ 0 μM and TEPA 50 μM.

activity, we determined the effect of CCS gene silencing on HIF-1-specific HRE binding activity. We have shown previously that CCS is required for copper activation of HIF-1 (Jiang et al., 2007). Therefore, CCS gene silencing should block hypoxia-induced activation of HIF-1, but the addition of excess copper should not overcome this effect because the action of copper is mediated by CCS. The data presented in Fig. 1D show that a siRNA targeting CCS effectively blocked CCS production in these cells and suppressed hypoxia-induced activation of HIF-1. Addition of excess copper did not relieve the inhibitory effect of CCS-silencing on HIF-1 activation.

Copper Deprivation Affects neither Production nor Stability of HIF-1α. To define the action site at which copper is required for activation of HIF-1 transcription activity, we examined whether copper chelation by TEPA affects HIF-1α protein expression or stability. Under normoxic conditions, HIF-1α protein undergoes a degradation process mediated by hydroxylation of the proline residues 402 and 564 of HIF-1α by PHDs. HepG2 cells were treated with or without 100 μM TEPA under normoxic conditions for 24 h and subsequently cultured for 5 h under hypoxic conditions (1% O_2). Hypoxia-induced HIF-1α accumulation was detectable at 5 min and reached a peak level 5 h after hypoxia (data not shown). After hypoxic treatment for 5 h, the cells were exposed to normoxic conditions for varying periods of time. There were no differences between the control and TEPA-treated cultures in hypoxia-induced HIF-1α production. Upon reoxygenation, the HIF-1α protein was rapidly decreased in a time-dependent manner and became undetectable 20 min after reoxygenation. TEPA treatment did not affect HIF-1α protein degradation (Fig. 2A). We noted that when the cells were reoxygenated for 2 min, higher molecular mass bands appeared, most likely representing the ubiquitinated HIF-1α. The data presented here thus demonstrate that copper deprivation by TEPA affects neither expression nor stability of HIF-1α.

Cytosolic accumulation of HIF-1α results in its nuclear translocation. To determine whether TEPA treatment affects HIF-1α nuclear localization, immunohistochemistry was performed on HepG2 cells using an anti-HIF-1α antibody (Fig. 2B). Under normoxic conditions, HIF-1α protein was undetectable. Hypoxia induced a remarkable accumulation of HIF-1α in the nucleus. TEPA treatment changed neither HIF-1 protein levels nor localization. The addition of $CuSO_4$ to TEPA-treated cells did not affect either HIF-1 protein levels or localization (data not shown).

Copper Deprivation Decreases HIF-1 Binding to the HRE. Because copper deprivation did not change the expression or stability of HIF-1α, we continued our effort to further define a possible action site for copper in the activation of HIF-1. We examined the DNA binding activity of HIF-1 using nuclear extracts from TEPA-treated HepG2 cells by an electrophoretic mobility shift assay (EMSA). The result presented in Fig. 3A shows that hypoxia increased the binding of HIF-1 to HRE (lane 2). The treatment with TEPA decreased the binding intensity (Fig. 3A, lane 6). To confirm the specificity of HIF-1 binding to the DNA sequence, a competition assay using 200-fold unlabeled HRE and a supershift assay using a monoclonal anti-HIF-1α antibody were performed. Unlabeled HRE competed off the binding of the labeled HRE to HIF-1 (Fig. 3A, lane 3), and the antibody shifted the DNA-protein complex to a higher molecular mass band (Fig. 3A, lanes 4 and 5), indicating the specific binding of HIF-1 to the HRE (Fig. 3A). The addition of copper to TEPA-treated cells relieved the inhibition of HRE binding by TEPA, as shown in Fig. 3C.

Copper Deprivation Reduces HIF-1 Transcriptional Complex Formation. An important mechanism for the regulation of HIF-1 transcription activity is the HIF-1 transcriptional complex formation. Among the cofactors involved in the formation of HIF-1 transcriptional complex, p300 is essential for HIF-1 transcription activity. We examined whether copper deprivation by TEPA affects the interaction between HIF-1 and p300. We used an anti-HIF-1α antibody to perform an immunoprecipitation assay. Under hypoxic condition, p300 was coimmunoprecipitated with anti-HIF-1α antibody (Fig. 4A). However, the amount of p300 that was precipitated by the anti-HIF-1α antibody was dramatically decreased under the treatment with TEPA (Fig. 4A). The addition of $CuSO_4$ to the TEPA-treated cells elevated the amount of p300 protein precipitated by anti-HIF-1α antibody (Fig. 4A). A retroimmunoprecipitation was also performed using anti-p300 antibody to confirm the HIF-1α/p300 interaction. As shown in Fig. 4B, TEPA treatment decreased the amount of HIF-1α protein precipitated by anti-p300 antibody, and the addition of $CuSO_4$ relieved this inhibition.

Under normoxic conditions, FIH-1 hydroxylates Asn803 residue in HIF-1α and therefore inhibits the binding of HIF-1α to

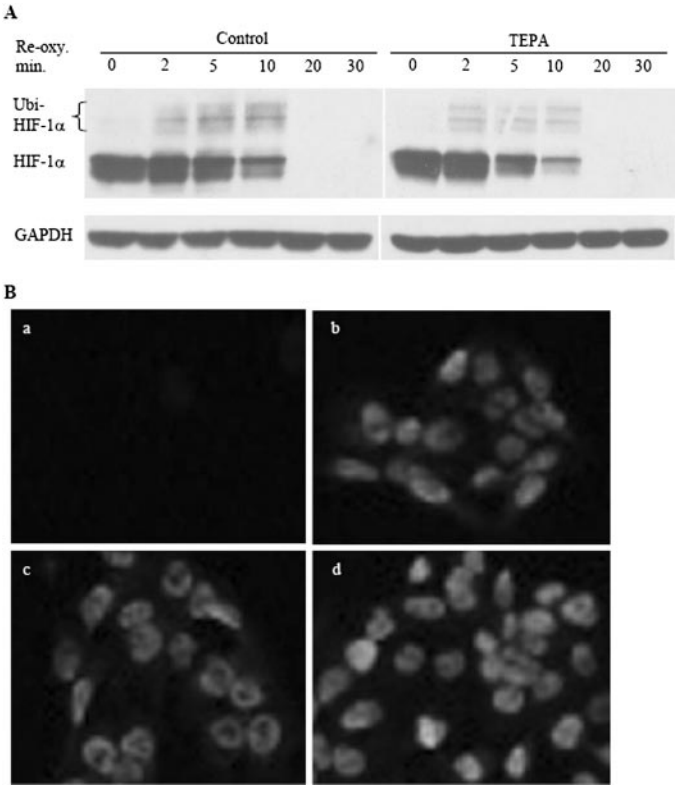

Fig. 2. Effects of TEPA on HIF-1α expression and stability. A, Western blot analysis for HIF-1α. HepG2 cells were cultured with TEPA (100 μM) for 24 h and then subjected to hypoxia for 5 h. Reoxygenation was performed by exposing the cells to the air for varying times as indicated. HIF-1α protein levels were analyzed by immunoblotting. GAPDH was probed as a loading control. The immunoradiograph is a representative from two separate experiments with consistent results. B, immunofluorescent staining of HIF-1α. HepG2 cells were cultured with TEPA (100 μM) or TEPA in combination with $CuSO_4$ (100 μM) for 24 h and then subjected to hypoxia for 5 h. a, normoxia; b, hypoxia; c, TEPA treatment followed by hypoxia; d, copper addition to TEPA-treated cells followed by hypoxia.

p300. However, we found that copper deprivation did not affect the level of FIH-1 (Fig. 5A). We then determined whether copper inhibits the activity of FIH-1. If copper inhibits FIH-1 activity, TEPA should relieve this inhibition, leading to the recovery of FIH-1 activity, and enhance the inhibition of HIF-1α binding to p300 along with a suppression of HIF-1 transcription activity. In this context, if FIH-1 activity is inhibited by a different mechanism, copper deprivation should not suppress the HIF-1 transcription activity. To test this hypothesis, we used an siRNA targeting FIH-1 to delete FIH-1 in HepG2 cells (Fig. 5A) and then examined the effect of TEPA on HIF-1α transcription activity under FIH-1 deletion condition. The results presented in Fig. 5, B and C, show that deletion of FIH-1 made TEPA treatment ineffective in suppression of HIF-1 transcription activity, suggesting that FIH-1 is a possible target of copper action.

To further define the role of copper in FIH-1 activity, we determined the effect of copper deprivation on iron chelation-induced activation of HIF-1. It has been shown that iron is required for the activity of FIH-1, and iron chelation results in an inhibition of FIH-1, leading to the activation of HIF-1. If the action of copper is mediated by the inhibition of FIH-1, the activation of HIF-1 by iron chelation should not be affected by TEPA treatment, because iron chelation-induced FIH-1 inhibition cannot be recovered by copper deprivation. We used an iron chelator, DFO, which has been shown to induce HIF-1 activation through the inhibition of FIH-1 (Hirsila et al., 2005). The treatment with DFO increased HIF-1 transcription activity in HepG2 cells, as determined by VEGF expression (Fig. 6, top) and luciferase reporter assay (Fig. 6, bottom). Pretreatment with TEPA did not affect DFO-induced VEGF expression or luciferase reporter activation (Fig. 6).

Discussion

In the present study, we report that copper deprivation suppresses hypoxia-induced activation of HIF-1 and thus inhibits the expression of genes such as VEGF controlled by this transcription factor. We have presented several pieces of evidence that demonstrate copper is required for HIF-1 activation. In the presence of the copper chelator TEPA, the expression of VEGF and the luciferase reporter activity were suppressed under hypoxic conditions. This suppression was relieved by the addition of excess copper to cell cultures, indicating the selective effect of copper chelation by TEPA and the important role of copper in HIF-1α activation. The action of copper is mediated by CCS, as indicated by the facts that CCS gene-silencing suppressed HIF-1 activity and that the addition of excess copper did not relieve this suppression. These results thus demonstrate that copper is required for HIF-1 activation. The important question is how copper is involved in HIF-1 activation.

The essential role of copper in HIF-1 activation is different from that of excess copper-enhanced HIF-1 transcription activity. The activation of HIF-1 under hypoxic conditions is proceeded by two distinct processes. The level of the α subunit of HIF-1 is an important determining factor for HIF-1 activation, and it is controlled by von Hippel-Lindau protein-associated protein degradation mediated by PHDs. Under hypoxic conditions, this pathway is inhibited so that HIF-1α accumulates in the cell. In the nucleus, the transactivity of HIF-1 is regulated by HIF-1 binding to HRE and HIF-1 transcriptional complex formation, which is regulated by FIH-1. Exposure to excess copper results in an accumulation of HIF-1α in cells (van Heerden et al., 2004; Martin et al., 2005). Other transition metals such as nickel and cobalt have also been shown to activate HIF-1 by increasing the accumulation of HIF-1α in cells. This activation of HIF-1 by transition metals, including copper, is mediated by increasing the stability of HIF-1α by inhibiting the degradation process (Yuan et al., 2003; Maxwell and Salnikow, 2004; Salnikow et al., 2004; Hirsila et al., 2005; Ke et al., 2005; Martin et al., 2005).

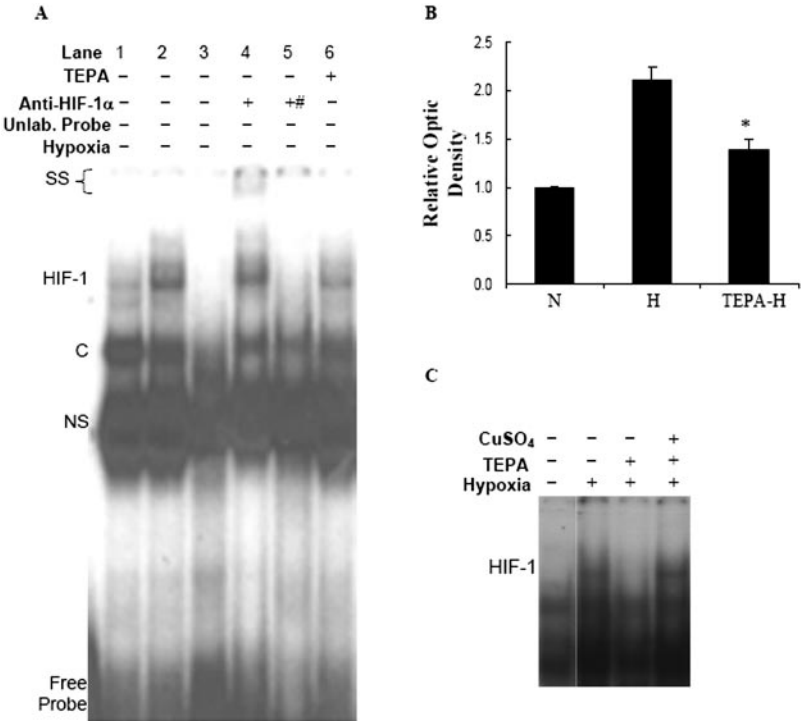

Fig. 3. Effect of TEPA on HIF-1 HRE-binding activity. A, HepG2 cells were cultured with TEPA (100 μM) for 24 h before exposure to hypoxia for 5 h. Nuclear extracts were prepared and analyzed by EMSA for HRE binding to a ^{32}P-labeled oligonucleotide containing the HIF-1 binding site of the erythropoietin 3′ enhancer. The presence of HIF-1 was verified in a competition assay by adding 200-fold molar excess of unlabeled oligonucleotides to the binding reaction before the addition of labeled probe, and a supershift assay (SS), in which a HIF-1α-specific antibody was added to the binding reaction and incubated for 2 h before resolved, #, overnight incubation with the antibody. Lane 1, normoxia control; lane 2, hypoxia control; lane 3, competition with unlabeled probe; lanes 4 and 5, supershift; lane 6, TEPA-hypoxia. C, constitutive binding; NS, nonspecific binding. B, quantitative analysis of the effect of TEPA on HIF-1 HRE binding was performed from the data obtained from all three independent experiments and expressed as the mean ± S.E. *, $p < 0.05$ versus hypoxia alone. C, the effect of readdition of $CuSO_4$ on HIF-1 HRE binding under hypoxic conditions was evaluated by adding 100 μM CuSO4 after the cells were treated with 100 μM TEPA, as described above.

Hypoxia is a major regulatory mechanism of HIF-1 activity. Hypoxia stabilizes HIF-1α protein but has no effect on HIF-1α transcription, which has been shown by unchanged HIF-1α mRNA levels under hypoxic conditions (Wenger et al., 1997). HIF-1α is also regulated by a mammalian target of rapamycin-dependent pathway (Hudson et al., 2002), which enhances HIF-1α protein expression (Laughner et al., 2001; Treins et al., 2002; Phillips et al., 2005). However, copper deprivation affected neither synthesis nor stability of HIF-1α, indicating that copper is required at different steps of HIF-1 activation.

The present study provides important insights into the mechanism of action of copper in the process of HIF-1 activation. First, copper may be required for HIF-1 binding to HRE in the target genes. In previous studies, we have observed that copper deprivation decreases the binding of HIF-1 to HRE in cultured cells in response to IGF-1 stimulation, as determined by an enzyme-linked immunosorbent assay assay, but the total level of HIF-1α in cells was not reduced by copper deprivation (Jiang et al., 2007). In the present study, we observed that under hypoxic conditions, copper deprivation also decreased the binding of HIF-1 to HRE, but we used a different assay, EMSA, to determine the binding. Therefore, these complementary results

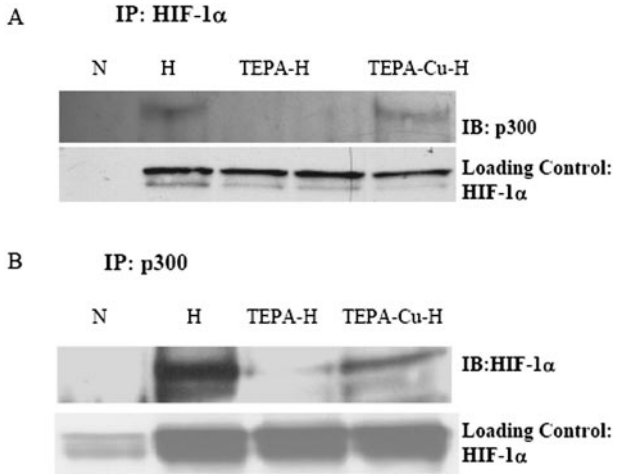

Fig. 4. Effects of TEPA on p300-binding in HIF-1 transcriptional complex. A, HepG2 cells were cultured in the presence or absence of TEPA (100 μM) for 24 h, and then $CuSO_4$ (100 μM) was added to the TEPA-treated cells, and cells were then subjected to hypoxia (H) or normoxia (N) for 16 h. Coimmunoprecipitation was performed using a HIF-1α-specific antibody in the whole-cell lysates. The immunoprecipitants were resolved by SDS-polyacrylamide gel electrophoresis and p300 was immunoblotted. The lysates were also subjected to Western blot, HIF-1α was probed for loading control. Immunoblot is a representative from three separate experiments with consistent results. B, coimmunoprecipitation of HIF-1α by anti-p300. N, normoxia control; H, hypoxia control; TEPA-H, hypoxia after TEPA treatment; TEPA-Cu-H, hypoxia after addition of $CuSO_4$ (100 μM) to the TEPA-treated cells.

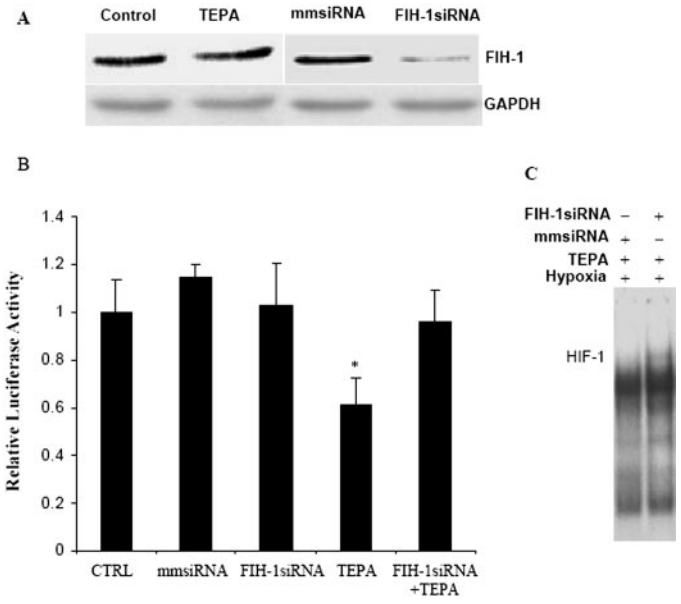

Fig. 5. Effects of siRNA targeting FIH-1 on HIF-1 transcriptional activity. HepG2 cells were cotransfected with the luciferase reporter plasmid and FIH-1 siRNA. Cells were then incubated with TEPA (100 μM) for 24 h and then subjected to hypoxia for 16 h. A, Western blotting of FIH-1 protein levels. Left, the effect of TEPA on FIH-1 protein level; right, efficiency of FIH-1 gene silencing by siRNA. B, luciferase activity. Data are the mean ± S.D. of three separate culture dishes from one representative experiment, which was performed two times with consistent results. *, $p < 0.05$ versus control. C, effect of FIH-1 siRNA on HRE binding. Procedures were the same as described in Fig. 3.

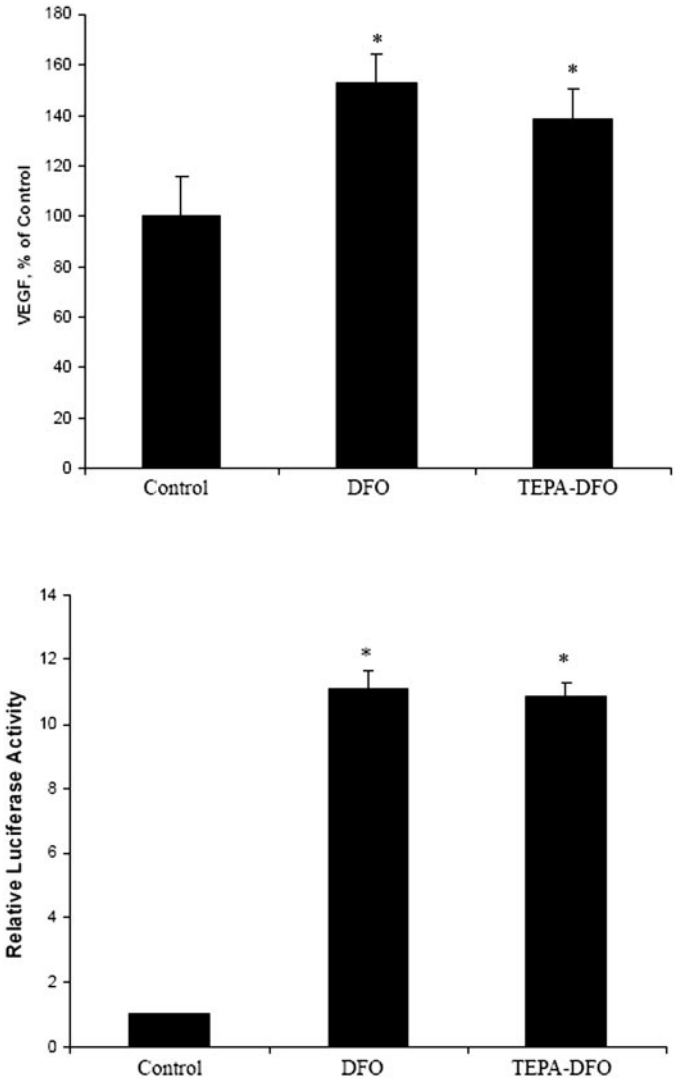

Fig. 6. Effects of TEPA on DFO-induced VEGF expression and HIF-1 activation. HepG2 cells were cultured with TEPA (100 μM) for 24 h before the addition of DFO (100 μM) for 16 h. VEGF levels in the culture supernatants (top) and luciferase activities (bottom) were determined. Data are mean ± S.D. of three separate culture dishes from one representative experiment that was performed two times with consistent results. *, $p < 0.05$ versus controls.

obtained from different cells under different experimental conditions confirm the inhibitory effect of copper deprivation on the binding of HIF-1 to HRE. It seems that this process is copper chaperone CCS-dependent. It has been shown that CCS is present in the nucleus (Casareno et al., 1998), and we have also observed the appearance of CCS in the nucleus by an immunocytochemical procedure (data not shown). Although CCS may be important for copper transport into the nucleus, it is apparently required for copper interaction with HIF-1α, because CCS is coimmunoprecipitated with HIF-1α (Jiang et al., 2007), and CCS gene-silencing suppresses HIF-1 activity.

Second, copper apparently plays an important role in the formation of HIF-1 transcriptional complex. The formation of HIF-1 transcriptional complex is a critical step for HIF-1 activation of target gene expression. HIF-1α enters into the nucleus and associates with HIF-1β to assemble HIF-1. Although the binding of HIF-1 to HRE is important, the initiation of the transcription of target genes requires a transcriptional complex (Kallio et al., 1998; Roth et al., 2004). This complex includes several proteins, and among these proteins is a p300 (Arany et al., 1996; Forsythe et al., 1996; Kallio et al., 1998; Carrero et al., 2000; Gray et al., 2005). In this process, a critical regulatory factor is FIH-1, which causes hydroxylation of the Asn803 of HIF-1α to inhibit the recruitment of cofactors, including p300 to HIF-1 transcriptional complex. It seems that this is a process involving the action of copper, as indicated by the result that copper deprivation reduced the binding of HIF-1 to p300. This result suggests that either copper is required for the binding of HIF-1 to p300 or copper inhibits FIH-1 activity, relieving the binding capacity of HIF-1 to its cofactors.

The results here suggest that copper is probably involved in the cofactor-recruiting step of activation of HIF-1 through the inhibition of FIH-1 activity. We have observed that FIH-1 gene-silencing using RNA interference technique blocked the inhibitory effect of copper deprivation by TEPA on hypoxia-induced luciferase reporter activity. To further confirm the possibility of copper regulation of HIF-1 transcription activity through its action on FIH-1, we examined the effect of copper deprivation on iron chelation-induced activation of HIF-1. It has been shown that FIH-1 activity is iron-dependent. Previous studies have suggested that PHDs are iron-dependent, so that iron chelation can inhibit the activity of PHDs, leading to an accumulation of HIF-1α (Wang and Semenza, 1993a). However, recent studies have shown that iron chelation by DFO mainly inhibited FIH-1 activity (Hirsila et al., 2005). If copper acts through its inhibition of FIH-1 activity, copper deprivation should not affect iron-chelation-induced HIF-1 activation. This indeed was observed in the present study, and the result suggests that copper inhibition of FIH-1 activity is probably involved in the regulation of HIF-1 transcription activity.

A recent study showed that a copper-zinc chelator, clioquinol, increased functional HIF-1α protein, leading to the target gene expression (Choi et al., 2006). It is interesting that clioquinol inhibited ubiquitination of HIF-1α in a Cu(II)- and Zn(II)-dependent manner, but it prevented FIH-1 from hydroxylation of the asparagine residue (803) in a Cu(II)- and Zn(II)-independent fashion (Choi et al., 2006). This implies that clioquinol inhibition of FIH-1 activity was not by the chelation of metals but by an unknown mechanism. The logK_i values of the copper chelator TEPA are 23.1 for Cu(II) and 15.3 for Zn(II) (Smith and Martell, 1976). The logK_i values of clioquinol are 15.8 for Cu(II) and 12.5 for Zn(II) (Cherny et al., 1999). Apparently, the K_i value of TEPA- copper is approximately 7 orders of magnitude higher than that of clioquinol-copper ($K_{i\ \text{TEPA-copper}}/K_{i\ \text{clioquinol-copper}}$). TEPA also has a remarkably higher selectivity for Cu(II) over Zn(II) ($K_{i\ \text{TEPA-copper}}/K_{i\ \text{TEPA-zinc}}$: approximately 8 orders of magnitude) than clioquinol ($K_{i\ \text{clioquinol-copper}}/K_{i\ \text{clioquinol-zinc}}$: approximately 3 orders of magnitude). In addition, zinc has been shown to be a negative regulator of HIF-1 activation (Chun et al., 2000). Taken together, the discrepancy between TEPA and clioquinol in the regulation of FIH-1 may be, at least in part, due to a remarkable difference in copper selectivity and affinity.

It is important to note that TEPA is also able to bind other transition metals, such as iron, cobalt, and nickel, but with much lower affinity compared with copper (Smith and Martell, 1976). Iron chelation inactivates PHD and causes HIF-1α accumulation and activation (Fig. 6). Although previous studies showed that exogenous cobalt and nickel treatment caused HIF-1 accumulation and activation (Maxwell and Salnikow, 2004), there is yet no evidence that shows the effect of cobalt or nickel chelation on HIF-1 activation. The physiological concentrations of cobalt and nickel are very low, and TEPA chelation of cobalt or nickel is unlikely to affect HIF-1 activation.

In summary, we show here that copper chelation suppresses hypoxia-induced HIF-1 transactivity and VEGF pro-

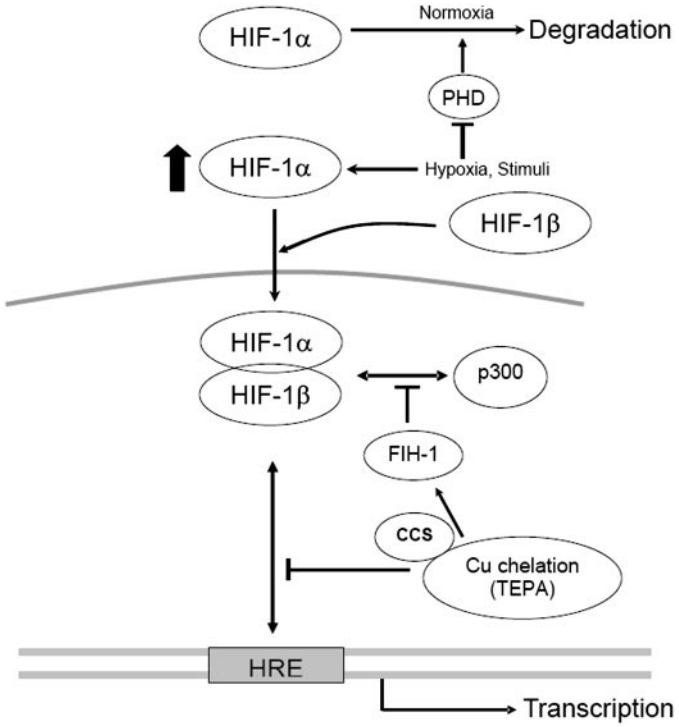

Fig. 7. Graphical representation of the proposed mechanisms of effects of copper chelation by TEPA on HIF-1 transactivity. Upon stabilized by hypoxia or other stimuli, HIF-1α binds HIF-1β and translocates into nucleus. The heterodimer then binds to HRE sequence and cofactor p300 and starts the transcription of target genes. Copper chelation by TEPA would activate FIH-1, resulting in a hydroxylation of HIF-1α and inhibition of its binding to p300. It would also attenuate the binding of HIF-1α to its target gene sequences, leading to suppression of its transcriptional activity. CCS is a copper chaperone, and its deletion mimics the same effect as copper chelation by TEPA.

duction in HepG2 cells. The requirement of copper for HIF-1 activation and for the target gene expression provides a novel insight into the mechanism by which copper manipulation affects various clinical practices, in particular, when angiogenesis is concerned. In this study, we have identified two action sites of copper in HIF-1α activation (Fig. 7) but cannot exclude other possibilities. First, copper is probably required for the binding of HIF-1 to the HRE sequence of target genes, a process that requires the copper chaperone CCS. Second, copper apparently is involved in the regulation of HIF-1 transcriptional complex formation. In this regard, copper may inhibit the activity of FIH-1 to retain the capacity of HIF-1α binding to its cofactors. However, copper deprivation does not affect the expression or stability of HIF-1α, indicating that these processes are not copper-dependent, although excess copper can stabilize HIF-1α through a mechanism shared by other transition metals that are not essential for HIF-1 activation. Although more detailed mechanistic insights are required to provide a comprehensive understanding of copper requirement for HIF-1 activation, the present study sheds the light for copper manipulation of cancer treatment and cardiovascular diseases in clinical practice.

Acknowledgments

We thank Prof. Roland H. Wenger for providing the luciferase reporter plasmid.

References

Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, and Livingston DM (1996) An essential role for P300/CBP in the cellular response to hypoxia. *Proc Natl Acad Sci U S A* **93:**12969–12973.

Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H, and Poellinger L (2000) Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Mol Cell Biol* **20:**402–415.

Casareno RL, Waggoner D, and Gitlin JD (1998) The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. *J Biol Chem* **273:**23625–23628.

Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, Beyreuther K, Tanzi RE, Masters CL, and Bush AI (1999) Aqueous dissolution of Alzheimer's disease Aβ amyloid deposits by biometal depletion. *J Biol Chem* **274:**23223–23228.

Choi SM, Choi KO, Park YK, Cho H, Yang EG, and Park H (2006) Clioquinol, a Cu(II)/Zn(II) chelator, inhibits both ubiquitination and asparagine hydroxylation of hypoxia-inducible factor-1α, leading to expression of vascular endothelial growth factor and erythropoietin in normoxic cells. *J Biol Chem* **281:**34056–34063.

Chun YS, Choi E, Kim GT, Lee MJ, Lee MJ, Lee SE, Kim MS, and Park JW (2000) Zinc induces the accumulation of hypoxia-inducible factor (HIF)-1alpha, but inhibits the nuclear translocation of HIF-1beta, causing HIF-1 inactivation. *Biochem Biophys Res Commun* **268:**652–656.

Dames SA, Martinez-Yamout M, De Guzman RN, Dyson HJ, and Wright PE (2002) Structural basis for Hif-1α/CBP recognition in the cellular hypoxic response. *Proc Natl Acad Sci U S A* **99:**5271–5276.

Elsherif L, Jiang Y, Saari JT, and Kang YJ (2004) Dietary copper restriction-induced changes in myocardial gene expression and the effect of copper repletion. *Exp Biol Med (Maywood)* **229:**616–622.

Feng W, Cai J, Pierce WM, Franklin RB, Maret W, Benz FW, and Kang YJ (2005) Metallothionein transfers zinc to mitochondrial aconitase through a direct interaction in mouse hearts. *Biochem Biophys Res Commun* **332:**853–858.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, and Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* **16:**4604–4613.

Freedman SJ, Sun ZY, Poy F, Kung AL, Livingston DM, Wagner G, and Eck MJ (2002) Structural basis for recruitment of CBP/P300 by hypoxia-inducible factor-1α. *Proc Natl Acad Sci U S A* **99:**5367–5372.

Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, and Gallick GE (2005) HIF-1alpha, STAT3, CBP/P300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. *Oncogene* **24:**3110–3120.

Hellwig-Bürgel T, Rutkowski K, Metzen E, Fandrey J, and Jelkmann W (1999) Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. *Blood* **94:**1561–1567.

Hirsilä M, Koivunen P, Xu L, Seeley T, Kivirikko KI, and Myllyharju J (2005) Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway. *FASEB J* **19:**1308–1310.

Huang LE, Gu J, Schau M, and Bunn HF (1998) Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* **95:**7987–7992.

Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ, and Abraham RT (2002) Regulation of hypoxia-inducible factor 1{alpha} expression and function by the mammalian target of rapamycin. *Mol Cell Biol* **22:**7004–7014.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG Jr (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* **292:**464–468.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim Av, Hebestreit HF, Mukherji M, Schofield CJ, et al. (2001) Targeting of HIF-alpha to the Von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292:**468–472.

Jiang Y, Reynolds C, Xiao C, Feng W, Zhou Z, Rodriguez W, Tyagi SC, Eaton JW, Saari JT, and Kang YJ (2007) Dietary copper supplementation reverses hypertrophic cardiomyopathy induced by chronic pressure overload in mice. *J Exp Med* **204:**657–666.

Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, and Poellinger L (1998) Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/P300 coactivator by the hypoxia-inducible factor-1alpha. *EMBO J* **17:**6573–6586.

Ke Q, Kluz T, and Costa M (2005) Down-regulation of the expression of the FIH-1 and ARD-1 genes at the transcriptional level by nickel and cobalt in the human lung adenocarcinoma A549 cell line. *Int J Environ Res Public Health* **2:**10–13.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, and Bruick RK (2002a) FIH-1 Is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* **16:**1466–1471.

Lando D, Peet DJ, Whelan DA, Gorman JJ, and Whitelaw ML (2002b) Asparagine hydroxylation of the HIF transactivation domain: a hypoxic switch. *Science* **295:**858–861.

Laughner E, Taghavi P, Chiles K, Mahon PC, and Semenza GL (2001) HER2 (Neu) signaling increases the rate of hypoxia-inducible factor 1{alpha} (HIF-1{alpha}) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol* **21:**3995–4004.

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

Martin F, Linden T, Katschinski DM, Oehme F, Flamme I, Mukhopadhyay CK, Eckhardt K, Tröger J, Barth S, Camenisch G, et al. (2005) Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation. *Blood* **105:**4613–4619.

Masson N, Willam C, Maxwell PH, Pugh CW, and Ratcliffe PJ (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J* **20:**5197–5206.

Maxwell P and Salnikow K (2004) HIF-1: an oxygen and metal responsive transcription factor. *Cancer Biol Ther* **3:**29–35.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399:**271–275.

McNeill LA, Hewitson KS, Claridge TD, Seibel JF, Horsfall LE, and Schofield CJ (2002) Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. *Biochem J* **367:**571–575.

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, and Kaelin WG (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the Von Hippel-Lindau protein. *Nat Cell Biol* **2:**423–427.

Phillips RJ, Mestas J, Gharaee-Kermani M, Burdick MD, Sica A, Belperio JA, Keane MP, and Strieter RM (2005) Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1α. *J Biol Chem* **280:**22473–22481.

Prohaska JR, Broderius M, and Brokate B (2003) Metallochaperone for Cu,Zn-superoxide dismutase (CCS) protein but not MRNA is higher in organs from copper-deficient mice and rats. *Arch Biochem Biophys* **417:**227–234.

Roth U, Curth K, Unterman TG, and Kietzmann T (2004) The transcription factors HIF-1 and HNF-4 and the coactivator P300 are involved in insulin-regulated glucokinase gene expression via the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* **279:**2623–2631.

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

Sen CK, Khanna S, Venojarvi M, Trikha P, Ellison EC, Hunt TK, and Roy S (2002) Copper-induced vascular endothelial growth factor expression and wound healing. *Am J Physiol Heart Circ Physiol* **282:**H1821–H1827.

Smith RM and Martell AE (1976) *Critical Stability Constants, Volume 4: Inorganic Complexes.* Plenum Press, New York.

Tanimoto K, Makino Y, Pereira T, and Poellinger L (2000) Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the Von Hippel-Lindau tumor suppressor protein. *EMBO J* **19:**4298–4309.

Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, and Van Obberghen E (2002) Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem* **277:**27975–27981.

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

Wang GL, Jiang BH, Rue EA, and Semenza GL (1995) 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* **92:**5510–5514.

Wang GL and Semenza GL (1993a) Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* **82:**3610–3615.

Wang GL and Semenza GL (1993b) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A* **90:**4304–4308.
Wanner RM, Spielmann P, Stroka DM, Camenisch G, Camenisch I, Scheid A, Houck DR, Bauer C, Gassmann M, and Wenger RH (2000) Epolones induce erythropoietin expression via hypoxia-inducible factor-1 alpha activation. *Blood* **96:**1558–1565.
Wenger RH, Kvietikova I, Rolfs A, Gassmann M, and Marti HH (1997) Hypoxia-inducible factor-1 alpha is regulated at the post-MRNA level. *Kidney Int* **51:**560–563.
Yuan Y, Hilliard G, Ferguson T, and Millhorn DE (2003) Cobalt inhibits the interaction between hypoxia-inducible factor-α and Von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-α. *J Biol Chem* **278:**15911–15916.

Address correspondence to: Dr. Y. James Kang, 511 South Floyd Street, MDR530, University of Louisville School of Medicine, Louisville, KY 40202. E-mail: yjkang01@louisville.edu