Copper Is Required for Cobalt-Induced Transcriptional Activity of Hypoxia-Inducible Factor-1

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ABSTRACT

Cobalt inhibits prolyl hydroxylases, leading to the accumulation of hypoxia-inducible factor-1α (HIF-1α) and a concomitant increase in the transcriptional activity of HIF-1. Therefore, cobalt has been under development as a drug for activating HIF-1 under some disease conditions. However, it has been shown that ischemic conditions resulted in the loss of copper, and the activation of HIF-1 would not occur unless copper was supplemented. The present study was undertaken to test the hypothesis that copper is also required for the cobalt activation of HIF-1 transcriptional activity. Human umbilical vein endothelial cells subjected to treatment with cobalt chloride (CoCl2) at concentrations above 25 μM for 2 h resulted in an accumulation of HIF-1α, which was determined by Western blot analysis, and an increase in the expression of vascular endothelial growth factor (VEGF), which was determined by real-time reverse transcription-polymerase chain reaction analysis for mRNA levels and enzyme-linked immunosorbent assay analysis for protein levels. The copper chelator tetraethylenepentamine at 25 μM did not significantly affect the accumulation of HIF-1α but blocked increases in VEGF mRNA and protein levels, an effect that could be reversed by the addition of 25 μM copper sulfate (CuSO4). In addition, gene silencing of the copper chaperone for Cu,Zn-superoxide dismutase blocked VEGF expression with little effect on cobalt-induced HIF-1α accumulation. The present study thus demonstrates that copper was required for cobalt-activated transcriptional activity of HIF-1, although copper did not affect cobalt-induced accumulation of HIF-1α in the cells.

Introduction

Hypoxia-inducible factor-1 (HIF-1) plays a critical role in the regulation of cellular metabolism, homeostasis, and responses to stresses (Semenza, 2000; Hirsila et al., 2005). Therefore, the regulation of HIF-1 transcriptional activity has been a major focus in understanding the fundamental process of HIF-1 activation and its therapeutic significance. HIF-1 is composed of HIF-1α and HIF-1β. The cellular stability of HIF-1α determines the ultimate activation of HIF-1. The level of HIF-1α is undetectable in most cell types under normoxic conditions because of its degradation by the ubiquitin-proteasome pathway, which is catalyzed by three HIF prolyl hydroxylases (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 constituent of an ubiquitin ligase complex, targeting the 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 in the cytosol, and translocates into the nucleus, where it dimerizes with HIF-1β and interacts with cofactors to assemble the HIF-1 transcriptional complex, leading to transcriptional activation.

Cobalt is capable of stabilizing HIF-1α, thereby stimulating the transcriptional activity of HIF-1. The mechanism of action of cobalt involves the inhibition of prolyl hydroxylases (Yuan et al., 2003; Maxwell and Salnikow, 2004; Hirsila et al., 2005; Ke et al., 2005). Because of the pivotal role of HIF-1α in multiple cellular function and homeostasis, cobalt has been extensively studied for its potential as a drug to stimulate cellular function regulated by HIF-1 (Endoh et al., 2000; Ohtomo et al., 2008).

Several transition metals share the same inhibitory action

ABBREVIATIONS: HIF-1, hypoxia-inducible factor-1; VEGF, vascular endothelial growth factor; TEPA, tetraethylenepentamine; SOD, superoxide dismutase; CCS, copper chaperone for Cu,Zn-SOD; HUVEC, human umbilical vein endothelial cell; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; RT, reverse transcription; siRNA, short interfering RNA; mmsiRNA, mismatched siRNA; TBST, Tris-buffered saline/Tween 20; COX, cyclooxygenase; HRE, hypoxia-responsive element.
on prolyl hydroxylases with cobalt (Yuan et al., 2003; Maxwell and Salnikow, 2004; Hirsila et al., 2005; Ke et al., 2005). Among these metals is copper (van Heerden et al., 2004; Martin et al., 2005). However, other studies have demonstrated that cobalt is required for HIF-1 transcriptional activity under hypoxic conditions in addition to its inhibitory action on prolyl hydroxylases (Feng et al., 2009). This observation raises an interesting question: is copper required for HIF-1 transcriptional activity under the condition of exposure to transition metals including cobalt?

The mechanism of action of copper on HIF-1 transcriptional activity involves its role in the interaction between HIF-1 and the hypoxia-responsive element (HRE) sequence of target genes and the formation of HIF-1 transcriptional complex (Feng et al., 2009). These processes take place in the nucleus, thus copper has to be transported into the nucleus to ensure the transcriptional activity of HIF-1. The copper chaperone for Cu,Zn-SOD (CCS) most likely functions as a copper nuclear chaperone. It has been shown that gene silencing of CCS inhibited HIF-1 transcriptional activity under hypoxic conditions (Feng et al., 2009). The inhibitory effect of CCS deletion on HIF-1 transcriptional activity cannot be relieved by excess copper, although the inhibitory effect of copper chelator on the transcriptional activity can be reversed by excess copper (Feng et al., 2009), indicating that CCS is essential in this action.

Cobalt activates HIF-1 by increasing the cytosolic accumulation of HIF-1α, but it has not been shown to be required for the activity of HIF-1 in the nucleus. Therefore, although cobalt and copper share the same inhibitory action on prolyl hydroxylases, the essential role of copper in HIF-1 transcriptional activation would be unique. In the present study, we used human umbilical vein endothelial cells (HUVECs) to test the hypothesis that copper is required for HIF-1 transcriptional activation induced by cobalt. The results obtained demonstrate that copper indeed is required for cobalt-induced HIF-1 transcriptional activation, although copper did not affect cobalt-induced HIF-1α accumulation in the human umbilical vein endothelial cells.

Materials and Methods

Cell Culture and Treatment. HUVECs (American Type Culture Collection, Manassas, VA) were chosen for this study because endothelial cells critically contribute to angiogenesis, which is a major target for the development of cobalt as a drug in ischemic diseases. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal serum (Invitrogen, Auckland, NZ), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were routinely maintained in a standard culture incubator with humidified air containing 5% CO2 at 37°C. For treatment with reagents, cells were seeded in six-well plates and grown overnight. Thereafter, CoCl2 (Kelong, Chengdu, China), tetraethylpentamine (TEPA; Sigma, St. Louis, MO), or CuSO4 (Kelong) was added to the cultures, followed by further incubation for the periods indicated. Cell numbers were counted by a hemocytometer.

Western Blotting Analysis of Proteins. Protein extracts were obtained after lysing cells in the radioimmunoprecipitation assay lysis buffer (Beyotime, Jiangsu, China) containing 1% complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM phenylmethylsulfonyl fluoride for 30 min on ice. Equal loading of protein was assured by prior quantitation using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). An appropriate amount of protein in total cell lysates was resolved in a SDS-polyacrylamide electrophoresis gel and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked for 1 h in Tris-buffered saline/Tween 20 (TBST) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: mouse anti-human HIF-1α (BD Biosciences, San Jose, CA), rabbit anti-CCS polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and mouse anti-β-actin monoclonal antibody (ZSGB-BIO, Beijing, China). After washing with TBST, the membranes were incubated with a horseradish peroxidase-linked anti-mouse or anti-rabbit IgG antibody (ZSGB-BIO) diluted in TBST for 1 h at room temperature. Proteins were visualized by using a chemiluminescence horseradish peroxidase substrate (Millipore Corporation, Billerica, MA).

Enzyme-Linked Immunosorbent Assay. VEGF in culture media was measured by using the Quantikine Human VEGF Immunoassay (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Each sample was measured in triplicate, and the VEGF concentrations were normalized by total protein content in each culture sample. Protein concentration was measured by using a Bio-Rad Laboratories protein assay kit.

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNAs were extracted from HUVECs harvested after various treatments by using the RNAiso Plus method (TaKaRa Biotechnology, Dalian, China). Two-step real-time PCR was performed in this study. The reverse transcription (RT) reaction was carried out by using 900 ng of total RNA following the protocol for the M-MLV RTase cDNA Synthesis Kit (TaKaRa Biotechnology). A primer optimization step was tested for each set of primers to determine the optimal primer concentrations. Once the optimal primer concentration was determined, 1.0 μL of primers, 10 μL of SYBR Green Master Mix (TaKaRa Biotechnology), and 20 to 100 ng of cDNA sample were applied to a total volume of 20 μL of PCR amplification. Reactions were run on a Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories), under the following cycling conditions: denaturation at 95°C (30 s), 45 cycles at 95°C (5 s) and 60°C (20 s). Cycle threshold (Ct) values were obtained from Bio-Rad Laboratories CFX Manager software. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide (YWHAZ) was also determined for each RNA sample as control. Fold change of relative mRNA expression was determined by using the 2^-ΔΔCT method. Primers were designed by TaKaRa Biotechnology, as follows: VEGF sequences, forward, 5'-TTGCTTTGCTGCTGC- TACCTCCA-3', reverse, 5'-GATGGCACTAGTGCCTGATA-3'; and YWHAZ sequences, forward, 5'-ACCTTATCCTGCTGAGGTTG- TCC-3', reverse, 5'-CTGACTGCTGAAGATCTTG-3'.

Gene Silencing of CCS. The siRNA targeting human CCS and mismatched control were designed and synthesized from Ribobio (Guangzhou, China). The siRNA sequences for CCS were as follows: sense, GGACCAGAUGGGCUUUGGUAtt; antisense, UACCAAGAC- CAUCUGGUCtt. The optimal transfection efficiency was determined from our preliminary study testing the range from 10 to 100 nM, and we selected the condition in which the siRNA caused an optimal silencing effect with minimal cytotoxicity. After HUVECs were transfected with 25 nM annealed siRNA targeting human CCS or negative mismatched control siRNA in serum- or antibiotics-free media, a Lipofectamine 2000 reagent (Invitrogen) was used as the transfection reagent according to the manufacturer's instructions. After 48-h transfection, cells were trypsinized and collected for further analysis as described above.

Statistical Analysis. Data were evaluated by using Dunnnett t test. p < 0.05 was considered significant.

Results

CoCl2 Increased the Level of HIF-1α and Promoted VEGF Expression in Cultured HUVECs. The treatment of HUVECs with varying concentrations of CoCl2 for 2 h
showed that at concentrations above 25 μM in cultures CoCl₂ caused significant increases in the level of HIF-1α in a dose-dependent fashion (Fig. 1A). Further analysis showed that cellular accumulation of HIF-1α reached the highest level after 2-h exposure to 50 μM CoCl₂ and remained at the same high level thereafter (Fig. 1B). Corresponding to the cellular accumulation of HIF-1α, the treatment with 50 μM CoCl₂ increased the levels of VEGF mRNA in the cells 4 h after the treatment (Fig. 1C) and VEGF protein in the culture media 24 h after the treatment (Fig. 1D).

**Copper Chelation by TEPA Suppressed Cobalt-Induced VEGF Expression.** To define the requirement of copper for cobalt-induced HIF-1 transcriptional activation, a copper chelator, TEPA, was used. The HUVECs were first exposed to varying concentrations of CuSO₄ for 2 h to examine copper’s effects on HIF-1α accumulation or for 24 h to examine its effects on VEGF expression. The concentrations of CuSO₄ used here ranged from nontoxic to toxic in the HUVECs as determined in our recent studies (Li et al., 2012). It has been specifically shown that CuSO₄ concentrations lower than 100 μM in cultures did not cause toxicity, but at 200 μM CuSO₄ was toxic in the HUVECs. The results showed that copper did not cause HIF-1α accumulation at any concentration applied in this experiment, but it caused an increased expression of VEGF at the concentration of 5 μM only in cultures (Fig. 2). The HUVECs in cultures were then treated with 50 μM CoCl₂ for 2 h, then some cobalt-treated cultures were exposed to 25 μM TEPA or 25 μM TEPA plus 25 μM CuSO₄ for an additional 2 h. As negative or positive controls, some HUVECs in cultures were, respectively, treated with the same volume of saline for 4 h or 50 μM CoCl₂ for 4 h. The results showed that the addition of 25 μM TEPA in cultures after cobalt treatment for 2 h had little effect on cobalt-induced accumulation of HIF-1α (Fig. 3, A and B). However, the treatment with 25 μM TEPA completely inhibited cobalt-induced VEGF expression. But in the presence of equal molar concentrations of CuSO₄ (25 μM) the TEPA effect was suppressed (Fig. 3, C and D).

**CCS Gene Silencing Also Inhibited Cobalt-induced VEGF Expression.** To further verify the effect of copper on HIF-1 transcription activity, the effect of CCS gene silencing on HIF-1α accumulation and VEGF gene expression was determined, because it has been shown that the

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*Fig. 1.* Cobalt-induced HIF-1α accumulation and VEGF expression. A, effects of treatment with CoCl₂ for 2 h at varying concentrations on HIF-1α accumulation. B, effects of 50 μM CoCl₂ on HIF-1α accumulation as a function of treatment time. Data in A and B were determined by Western blotting, and the intensity of HIF-1α was quantified by a densitometry and normalized to control. C, effects of treatment with 50 μM CoCl₂ for 4 h on the level of mRNA for VEGF detected by real-time RT-PCR analysis. D, effects of treatment with 50 μM CoCl₂ for 24 h on VEGF concentrations in culture media determined by ELISA. All of the data were collected from three independent experiments and are expressed as mean ± S.E.M. *p* significantly different from untreated controls (*p* < 0.05).
Fig. 2. Effects of CuSO₄ on HIF-1α accumulation and VEGF expression. A, effects of treatment with varying concentrations of CuSO₄ for 2 h on HIF-1α accumulation. B, effects of treatment with different concentrations of CuSO₄ for 24 h on VEGF concentrations in culture media, determined by ELISA. All of the data were obtained from three independent experiments. *, significantly different from untreated controls (P < 0.05).

Fig. 3. Effects of TEPA on CoCl₂-induced HIF-1α accumulation and VEGF expression. A, Western blot analysis of HUVECs treated for 4 h with 50 μM CoCl₂ only, for 2 h with 25 μM TEPA only, and with 50 μM CoCl₂ for 2 h first, then for an additional 2 h with 25 μM TEPA without or with an additional 25 μM CuSO₄. B, semiquantitative analysis of HIF-1α protein detected by Western blotting. C, effects of TEPA on the level of mRNA for VEGF detected by a real-time RT-PCR analysis. The treatment protocol was the same as described for A. D, effects of TEPA on VEGF concentration in culture media determined by ELISA. The cells were treated for 24 h with 50 μM CoCl₂ only, for 22 h with 25 μM TEPA only before collection, or with 50 μM CoCl₂ for 2 h first, then for additional 22 h with 25 μM TEPA without or with an additional 25 μM CuSO₄. All of the data were obtained from three independent experiments. *, significantly different from untreated controls (P < 0.05). Co, cobalt; Cu, copper.
copper chaperone CCS would transfer copper into the nucleus. A siRNA targeting human CCS, along with a mismatched siRNA as a negative control, was used to silence the expression of CCS in cultured HUVECs. After transfection with siRNAs for 48 h, the cells were exposed to 50 μM CoCl₂, 25 μM CuSO₄, or their combination as described in Fig. 4. The data presented in Fig. 4A show that the siRNA targeting CCS effectively blocked CCS production in these cells, as determined by Western blot. The gene silencing of CCS did not affect cobalt-induced accumulation of HIF-1α in the cells (Fig. 4, A and B) but completely suppressed cobalt-induced VEGF gene expression (Fig. 4, C and D). However, it was interesting to note that 25 μM CuSO₄ alone did not cause any change in HIF-1α accumulation or VEGF expression, but recovered cobalt-induced VEGF expression under the condition of CCS deletion.

**Discussion**

Cobalt is of significant concern not only in environmental toxicology but also in clinical application. Cobalt contamination in the environment has been a focus for a long time in the toxicological field. One of the mechanisms of action of cobalt is its inhibition of prolyl hydroxylases, leading to the accumulation of HIF-1α and gene expression regulated by HIF-1 (Yuan et al., 2003; Maxwell and Salnikow, 2004; Hirsila et al., 2005; Ke et al., 2005). Because of this action, cobalt becomes a candidate for the development of drugs to stimulate HIF-1 transcriptional activity to promote cellular metabolism and function. The data obtained from the present study showed that copper is a critical factor in the regulation of cobalt-induced HIF-1 transcriptional activation. Copper had little effect on the cobalt-induced accumulation of HIF-1α, but it was required for the transcriptional activity of HIF-1.

**Fig. 4.** Effects of CCS gene silencing on CoCl₂-induced HIF-1α accumulation and VEGF expression. A, effects of CCS gene silencing on HIF-1α accumulation detected by Western blotting. HUVECs were transfected with CCS siRNA or mismatched siRNA (mmRNA) for 48 h before the treatment with 50 μM CoCl₂ only, with 25 μM CuSO₄ only, or in combination for 4 h. B, semiquantitative analysis of HIF-1α accumulation detected by Western blotting as described for A. C, effects of CCS gene silencing on the level of mRNA for VEGF detected by a real-time RT-PCR analysis. The treatment protocol was as the same as described for A. D, effects of CCS gene silencing on VEGF expression in culture media determined by ELISA. The treatment protocol described for A was used with the exception that the cells were treated with 50 μM CoCl₂ only, 25 μM CuSO₄ only, or in combination for 24 h. All of the data were obtained from three independent experiments. * significantly different from untreated controls (P < 0.05). #, significantly different from CCS siRNA (P < 0.05). Co, cobalt; Cu, copper.
Limiting the availability of copper by TEPA and interfering with copper trafficking by CCS gene silencing resulted in the inhibition of HIF-1-controlled VEGF gene expression, confirming the requirement of copper for HIF-1 transcriptional activity.

Under physiological conditions, copper deficiency would not be a major concern among general populations. However, it has been shown in human biopsy and autopsy tissue samples that under certain disease conditions copper depletion occurred in those tissues. For instance, under ischemic or hypertrophic conditions, copper levels in myocardium are significantly reduced (Wester, 1965; Zama and Towns, 1986). The same observation has been found in animal models of ischemic heart disease and pressure overload-induced heart hypertrophy (Chipperfield and Chipperfield, 1978; Chevion et al., 1993; Jiang et al., 2007). It has been shown that under ischemic conditions HIF-1α levels increase in the heart (Kim et al., 2002; Su et al., 2002). However, a puzzling problem is that in the ischemic tissue blood vessels do not increase but are decreased. The high levels of HIF-1α should predispose angiogenesis, because HIF-1 is a master transcription factor that regulates a myriad of genes involved in angiogenesis and vasculogenesis. This discrepancy between high levels of HIF-1α and the suppression of angiogenesis would at least partially be attributed to the loss of copper in the ischemic tissue. In our previous studies, we observed that supplementation with copper in the pressure overload-induced heart hypertrophic mouse model can replenish the lost copper, promote VEGF expression, and stimulate myocardial angiogenesis (Jiang et al., 2007).

TEPA has been shown to enter cells and be able to reduce both copper levels and Cu,Zn-superoxide dismutase mutismutase activity in a time-dependent manner (Percival and Layden-Patrice, 1992). However, TEPA-induced copper deficiency in HL-60 cells did not result in changes in the cell viability or alterations in the stage of cell differentiation (Percival and Layden-Patrice, 1992). Therefore, it has been recommended that TEPA is an ideal chelator for effectively removing copper from a cell without affecting its viability or causing any fundamental changes in the cell phenotype (Ding et al., 2011). In the present study, TEPA at the concentration used (25 μM) would not cause any significant changes in cellular metabolism and function in the cultured HUVECs. In the presence of TEPA, cobalt-induced VEGF expression was completely suppressed. It is possible that TEPA also chelates cobalt. Therefore, the experimental design in the present study took this possible confounding effect under consideration. First, the cells were treated with CoCl2 for 2 h before being exposed to TEPA, at which time HIF-1α had accumulated to the highest level in the cells so that the change in gene expression would not result from an alteration in HIF-1α accumulation. Second, the addition of equal molar concentration of copper did not affect cobalt-induced HIF-1α accumulation or VEGF gene expression, but reversed the TEPA inhibition of VEGF gene expression. Thus, the data obtained from TEPA demonstrated the significance of copper in the regulation of HIF-1 transcriptional activity.

It has been known that there is virtually no free copper in mammalian cells. Copper trafficking in the cells is tightly regulated, and copper chaperones mediate copper intracellular transport. There are three extensively characterized copper chaperones: Atox1 (ATX1 antioxidant protein 1 homolog), transfers copper to ATPase proteins such as ATP7A and ATP7B in trans-Golgi network, Cox17 (COX17 cytochrome c oxidase assembly homolog or cytochrome c oxidase copper chaperone) transfers copper to Sco1 (SCO cytochrome oxidase-deficient homolog 1) and Sco2 (SCO cytochrome oxidase-deficient homolog 2), and Cox11 (COX11 cytochrome c oxidase assembly homolog) in mitochondria. CCS transfers copper to Cu,Zn-SOD in cytosol. There are no defined copper chaperones that transfer copper into the nucleus. However, it has been shown that CCS can be found in the nucleus (Casarenco et al., 1998). In our previous studies, we found that CCS interacts with HIF-1α, and deletion of CCS inhibits HIF-1α interaction with the HRE sequences of the target genes (Jiang et al., 2007; Feng et al., 2009), indicating that CCS would transfer copper into the nucleus. In the present study, gene silencing of CCS resulted in no significant changes in cobalt-induced accumulation of HIF-1α but completely suppressed cobalt-activated VEGF gene expression. This further demonstrated the role of CCS in copper transport into the nucleus, and importantly it confirmed the requirement of copper for cobalt-induced HIF-1 transcriptional activation.

The most important finding of this study is that copper is required for cobalt-induced HIF-1 transcriptional activation. This is of significant clinical relevance. Because cobalt has been used as a drug to promote HIF-1 transcriptional activity (Endoh et al., 2000; Ohtomo et al., 2008), factors affecting its efficacy and long-term safety have to be considered. It is reasonable to consider the use of cobalt to promote angiogenesis in ischemic tissues. However, as discussed above, copper depletion is often found in these tissues. It is also important to emphasize that the blood concentrations of copper have no way to predict a particular tissue level of copper. For instance, under ischemic conditions, it is often found that the blood concentrations of copper are increased, but the myocardial tissue levels of copper decrease. Why copper found in the blood cannot be available to ischemic myocardium or other ischemic tissues is an important question. Our understanding of copper intracellular trafficking and interorgan transport has not been fully achieved. However, the results obtained in the present study suggest that when one proposes the use of cobalt as a drug to promote HIF-1 transcriptional activity tissue copper status needs to be considered. It is an important issue related to how to replenish copper in a particular tissue.

This study raises two critical issues in considering the role of copper in the regulation of HIF-1 transcriptional activity. The first is the sufficiency of copper in a particular organ, and the second is the availability of copper for a particular subcellular organelle or a copper-acquiring molecule. The first was demonstrated by the use of the copper chelator TEPA; copper deficiency caused suppression of HIF-1 transcriptional activity, and copper supplementation reversed the suppression. The second was demonstrated by the deletion of CCS; in the absence of a specific copper chaperone, suppression of HIF-1 transcriptional activity was not reversed by copper supplementation. Therefore, copper concentration in a particular organ is only one aspect to consider, and its availability has to be taken into account overall.

It was interesting to note that copper alone did not cause any change in HIF-1α accumulation, but led to an increase in the VEGF protein level only at 5 μM in cultures. The expression of VEGF is regulated by HRE and the Sp1 responsive
element (Grzenkowicz-Wydra et al., 2004). It seems that the regulation of VEGF expression by either HRE or the Sp1 responsive element is copper-dependent, so that without HIF-1α accumulation, which acts through HRE, copper stimulates VEGF expression by affecting Sp1 regulation. However, it will be interesting to determine in future studies why copper shows a stronger induction of HIF-1α-independent VEGF expression only at 5 µM in cultures. Another interesting observation in the present study was that CCS deletion suppressed cobalt-induced VEGF expression, but copper addition recovered the expression. Previous studies have shown that CCS knockout suppressed Cu,Zn-SOD activity (Wong et al., 2000). However, the mice could adapt to the CCS-null condition by using glutathione as a substitute chaperone for transferring copper to the enzyme (Carroll et al., 2004). The exogenously added copper might activate the glutathione-dependent chaperone mechanism to overcome the effect of CCS deletion, and that will be tested in our future studies.

In summary, the present study described a thorough analysis of the requirement of copper for cobalt-induced HIF-1α transcriptional activation. From the approach using the copper chelator TEPA to limit the availability of copper to that of gene silencing of CCS to restrict the nuclear transport of copper, the role of copper in the regulation of HIF-1α transcriptional activity has been demonstrated. Copper did not affect the accumulation of HIF-1α induced by cobalt, but it was required for cobalt-activated VEGF gene expression. It would be misleading if the accumulation of HIF-1α is used as the index for the efficacy of cobalt as a drug to promote HIF-1α transcriptional activity under tissue copper depletion conditions. Therefore, in the development of cobalt as a proangiogenic drug, it is important to consider the status of copper in a particular tissue.

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Authorship Contributions
Participated in research design: Qiu, Ding, and Kang.
Conducted experiments: Qiu, Ding, Zhang, and Kang.
Performed data analysis: Qiu and Kang.
Wrote or contributed to the writing of the manuscript: Qiu, Ding, and Kang.

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