Effect of copper on nuclear translocation of copper chaperone for superoxide dismutase-1

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Abstract

Copper chaperone for superoxide dismutase-1 (CCS-1), facilitating copper insertion into superoxide dismutase 1 (SOD-1), is present in the nucleus. However, it is unknown how CCS-1 is translocated to the nucleus. The present study was undertaken to determine the effect of copper on nuclear translocation of CCS-1. Human umbilical vein endothelial cells (HUVECs) were subjected to hypoxia, causing an increase in both copper and CCS-1 in the nucleus. Treatment with tetraethylenepentamine (TEPA) not only decreased the total cellular concentration and the nuclear translocation of copper, but also completely suppressed the entry of CCS-1 to the nucleus. On the other hand, siRNA targeting CCS-1 neither inhibited the increase in total concentrations nor blocked the nuclear translocation of copper. This study thus demonstrates that under hypoxia condition, both copper and CCS-1 are transported to the nucleus. The nuclear translocation of CCS-1 is copper dependent, but the nuclear translocation of copper could take place alternatively in a CCS-1-independent pathway.

Keywords: Copper, copper chaperone for superoxide dismutase-1, hypoxia-inducible factor-1, human umbilical vein endothelial cells, nucleus, translocation

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Introduction

Previous studies found that copper regulation of hypoxia-inducible factor-1 (HIF-1) transcriptional activity requires the presence of copper chaperone for superoxide dismutase-1 (CCS-1).^{1–3} Both copper chelation and gene silencing of CCS-1 markedly suppress the expression of HIF-1 regulated vascular endothelial growth factor (VEGF), but the former is reversible by copper supplementation and the latter is not.^{1–3} This CCS-1-dependent action of copper on HIF-1 transcriptional activity indicates that CCS-1 must be present in the nucleus simultaneously with copper. The presence of CCS-1 in the nucleus was identified previously,⁴ although the mechanism by which CCS-1 is transported to the nucleus is unknown.

It is possible that CCS-1, as a copper chaperone, not only inserts copper ions to SOD-1,^{5,6} but also transfers copper into the nucleus. In this context, the nuclear translocation of CCS-1 would be associated with the need for copper presence in the nucleus, indicating copper would affect the process of CCS-1 nuclear translocation. The present study was undertaken to determine the effect of copper on CCS-1 transportation to the nucleus. We subjected human

umbilical vein endothelial cells (HUVECs) to hypoxia that requires the presence of copper in the nucleus for HIF-1 transcriptional activity. Under this condition, the nuclear transportation of copper and CCS-1 can be manipulated genetically and biochemically. The data obtained indicates that CCS-1 transportation to the nucleus is copper-dependent.

Materials and methods

Cell culture and treatment

HUVECs (American Type Culture Collection, VA) were cultured in H-Dulbecco's modified Eagle's medium (H-DMEM, Invitrogen, CA) and supplemented with 10% fetal bovine serum (FBS, Invitrogen, Auckland, NZ), 100 µg/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. Stock cultures were maintained at 80–90% confluence and passaged by 0.25% Trypsin (GIBCO, USA) containing 1% EDTA in Ca²⁺ and Mg²⁺ free phosphate buffered saline (PBS). Experimental cells were cultured in 25 cm² or 75 cm² flasks. Cells were placed into a hypoxia incubator chamber

(Stem Cell Technologies, WA) full of mixture gas (1% O₂, 5% CO₂, 94% N₂) for hypoxia exposure. To reduce cellular concentrations of copper, tetraethylenepentamine (TEPA) at a final concentration of 50 μ mol/L in the cultures was used for 24 h exposure.

Nuclear extraction

The nucleus was extracted by sucrose density gradient centrifugation. Cells were scraped and washed three times with cold PBS. The cell pellet was re-suspended with 0.25 mol/L sucrose in TKM buffer solution (50 mmol/L Tris-HCl, pH 7.4, 25 mmol/L KCl, 5 mmol/L MgCl₂) and homogenized with 20 strokes in a glass douncer. After centrifugation at 800 g for 10 min, the pellet was re-suspended with 1 mL 0.25 mol/L sucrose. The supernatant was carefully laid onto 5 mL 2.1 mol/L sucrose, centrifuged at 70,000 g for 1 h at 4°C. The pellet was washed with 0.25 mol/L sucrose for three times. The final pellet contained the purified nuclei. The pellet was lysed using 1% SDS buffer to extract the nuclear protein.

Measurement of copper concentration

Copper concentrations in the isolated nucleus and in the cell lysate were determined using a graphite furnace atomic absorption spectrophotometer (AAS). Briefly, the freeze-dried nucleus or cell samples were dissolved in 100 μ L concentrated HNO₃ for three days. Before the analysis, de-ionized water of 500 μ L was added and copper concentrations were directly determined.

Western blotting analysis of proteins

Protein extracts were obtained after lysing cells in the RIPA lysis buffer (Beyotime, Jiangsu, CN) containing 1% complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, DE) for 30 min on ice. Equal loading of protein was assured by prior quantitation using Bio-Rad assay. An aliquot of proteins was resolved in a SDS-polyacrylamide electrophoresis gel and transferred onto a polyvinylidene difluoride membrane (Bio-RAD, USA). Membranes were blocked for 1 h in Tris-buffered saline/Tween 20 (TBST) (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk, and incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: anti-CCS-1 (Santa Cruz, TX); anti- β -actin (ZSGB-BIO, Beijing, CN); anti-HIF-1 α (Abcam, Cambridge, UK) anti-GAPDH (ZSGB-BIO, Beijing, CN), and anti-COX17 (Santa Cruz, TX). After incubation, the membranes were washed with TBS-T six times for 10 min each. Then the blots were incubated for 2 h at room temperature with appropriate secondary antibody. After washing six times, target proteins were visualized using a chemiluminescence HRP substrate (Millipore, Billerica, MA) and analyzed by densitometry using a Quantity One Software.

Gene silencing of CCS-1

The siRNA targeting human CCS-1 and mismatched control were designed as previously reported,¹

and synthesized by RiboBio (Guangzhou, CN). The siRNA targeting CCS-1 sequences for CCS-1 were: sense, GGACCAGAUGGUCUUGGUAtt, and antisense, UACC AAGACCAUCUGGUCCtt. Both siRNA targeting CCS-1 and mismatched controls were transfected into cells by using lipofectamine 2000 (Invitrogen, Auckland, NZ) according to the manufacturer's instruction. Briefly, HUVECs were transfected with 50 nmol/L of the selected siRNAs targeting hCCS-1 or negative-mismatched siRNA in antibiotic-free media when the confluence of the 75 cm² culture flask reached 30–50%. After 48 h transfection, cells were collected for further analysis as described in the experimental procedure.

Statistical analysis

Data were initially evaluated using one-way ANOVA, followed by Dunnett *t* test. *P* < 0.05 was considered significant.

Results

Quality control of nuclear isolation

The purity of the isolated nuclei was tested using GAPDH and COX17 as cytoplasmic and mitochondrial markers, respectively. These tests verified that the isolated nuclei were essentially free of cytoplasmic and mitochondrial contaminations (Figure 1). In addition, HIF-1 α was probed in both total cellular and nuclear fractions under either normoxic or hypoxic condition. HIF-1 α was slightly detectable under normoxic condition in either total cellular or nuclear fraction, but was markedly elevated after 24 h hypoxia; being highly concentrated in the nuclear fraction (Figure 1). Furthermore, β -actin was more in the total cellular fraction than in the nuclear fraction. However, the concentration of this protein was not affected by hypoxia (Figure 1), allowing β -actin to be used as a reference in the following experiments.

Elevation of copper and CCS-1 in the nucleus under hypoxic condition

After the HUVECs were subjected to hypoxia for 24 h, both total cellular and nuclear concentrations of copper were significantly elevated. However, the intensity of this

Figure 1 Determination of the purity of isolated nuclear fraction by Western blot analysis of the cytoplasmic and mitochondrial markers. GAPDH: cytoplasmic marker; COX17: mitochondrial marker; and β -actin: internal standard marker

elevation was much higher in the nucleus than in the cytosol (Figure 2a). Under the same hypoxic condition, the protein content of CCS-1 was found to be significantly increased in the nucleus, although its total concentration was not significantly elevated (Figure 2b). Under this condition, the expression of VEGF is highly up-regulated in these cells.^{2,7}

Correlation between copper reduction and suppression of CCS-1 nuclear translocation

Treatment of HUVECs with TEPA for 24 h significantly reduced copper concentrations in both total cellular and nuclear fractions (Figure 3a). Under the TEPA treatment condition, hypoxia did not cause an increase in copper concentrations in the nucleus. Under this condition, the expression of VEGF in these cells is down regulated.^{2,7} Interestingly, the total cellular concentrations of CCS-1 were significantly elevated in response to copper depression (Figure 3b), but hypoxia-induced nuclear translocation of CCS-1 was completely blocked (Figure 3c).

Effect of CCS-1 deficiency on copper transportation to the nucleus

Transfection with siRNA targeting CCS-1 for 48 h significantly reduced the protein level of CCS-1 in either total cellular or nuclear fraction, as shown in Figure 4(a). Hypoxia affected neither the concentration nor the localization of CCS-1 in these cells. Under this CCS-1 deficiency condition, total cellular copper concentrations remained elevated under hypoxic condition (Figure 4b), however, it was interesting to observe that copper concentrations in the nucleus were even further elevated (Figure 4c).

Discussion

The presence of both copper and CCS-1 in the nucleus was observed in previous studies,^{4,8} but the mechanism by which CCS-1 is transported to the nucleus has not been explored. In addition, the significance of this nuclear translocation is also elusive. Copper is required for HIF-1 transcriptional activity,^{1,2} indicating an important role for copper in the nucleus as the same action site as HIF-1.⁹ The action of copper in the regulation of HIF-1 transcriptional activity is CCS-1 dependent,¹ suggesting a possible co-translocation of copper and CCS-1 into the nucleus. The present study specifically addressed this possibility. The data presented demonstrate a correlation between copper and CCS-1 in their translocation into the nucleus.

The data here clearly showed that under hypoxic condition, in which the level of HIF-1 α was markedly increased, both copper and CCS-1 were significantly increased in the nucleus. This hypoxia-driven nuclear presence of copper and CCS-1 suggests a concomitant mobilization and translocation of the two in response to the same trigger signal. Therefore, we made first effort to determine under the condition of copper depression, whether or not CCS-1 remained increased and translocated into the nucleus under hypoxic condition.

TEPA has been shown to enter cells and be able to reduce both copper levels and SOD-1 activity in a time-dependent manner without affecting cell viability or cell differentiation.¹⁰ In previous studies, we have used 50 μ mol/L TEPA to treat the same cells used in the present study and found that it does not cause any significant changes in cellular metabolism and function.⁷ The result obtained here shows that in the presence of 50 μ mol/L TEPA, copper

Figure 2 Quantitation of the total cellular and nuclear copper concentrations and CCS-1 protein levels. (a) Copper concentrations in the total cellular fraction (including nuclear component) or in the isolated nuclear fraction from HUVECs subjected to hypoxia for 24 h in relation to normoxia as control. (b) Western blot analysis of CCS-1 protein levels in the total cellular and the isolated nuclear fractions and semiquantitative analysis of the intensity changes on the blot. The bar graphs present the data obtained from three independent experiments. Normoxia: cells were cultured under normal condition for 24 h; hypoxia: cells were cultured under hypoxia condition for 24 h. All of the data are expressed as means $\pm$ SEM. *Significantly different from normoxia condition ($P < 0.05$); #significantly different from total cellular fraction under hypoxia condition ($P < 0.05$)

Figure 3 Effects of TEPA on copper and CCS-1 concentrations and their translocation to the nucleus. (a) The effect of the TEPA on copper concentrations in the total cellular and the isolated nuclear fractions. (b) The effect of the TEPA on the protein levels of CCS-1 in the total cellular fraction. (c) The effect of the TEPA on the protein levels of CCS-1 in the isolated nuclear fraction. The bar graphs present the data obtained from three independent experiments. Con: cells were cultured for 24 h; TEPA: cells were exposed to 50 $\mu\text{mol/L}$ TEPA for 24 h. Data are expressed as means $\pm$ SEM. *Significantly different from control groups ($P < 0.05$); #significantly different from normoxia control group ($P < 0.05$)

concentrations in the cell were significantly decreased, accompanied by an elevation of CCS-1 concentrations. This agrees with a previous report that CCS-1 elevation serves as an index for copper depression.^{11–13} Under this copper depression condition, hypoxia did not cause an increase in copper concentrations in the nucleus. It is interesting to observe that the hypoxia-driven elevation of CCS-1 in the nucleus was completely suppressed under the copper depression condition, indicating CCS-1 translocation to the nucleus is copper-dependent.

Since CCS-1 is a copper chaperone,^{5,6} it was speculated that CCS-1 would carry copper into the nucleus. As discussed above, copper depression blocked CCS-1 translocation into the nucleus. Therefore, we made another effort to determine whether copper translocation to the nucleus could be affected by CCS-1 depression. We used siRNA targeting CCS-1 to depress this protein and surprisingly found that under the CCS-1 deficient condition, copper

remained elevated in the nucleus. This strongly suggests that copper translocation to the nucleus might take place in both CCS-1-dependent and -independent pathways. Although CCS-1 may play a primary role under normal conditions, a compensatory mechanism may be activated in the absence of CCS-1 under hypoxia condition to help copper translocation to the nucleus.

It thus indicates that the nuclear transport of CCS-1 under hypoxic condition is dependent on copper translocation to the nucleus. But in contrary, the nuclear transport of copper is not solely dependent of CCS-1, although the nuclear action of copper is CCS-1 dependent as demonstrated in our previous studies.^{1,2} This suggests that when copper is required by the nucleus, its translocation would trigger the accompanied transport of CCS-1 into the same location. Without the entry of copper to the nucleus, the translocation of CCS-1 to the nucleus is inhibited.

Figure 4 Effects of CCS-1 depression on copper concentrations in the total cellular and isolated nuclear fractions. (a) The effects of siRNA targeting CCS-1 on the protein levels of CCS-1 in the HUVECs. The bar graphs present the data obtained from three independent experiments. MM-siRNA: cells were exposed to mismatch siRNA for 48 h; CCS-siRNA: cells were exposed to CCS siRNA for 48 h. (b) Copper concentrations in the total cellular fraction after transfection with siRNAs for 48 h followed by hypoxia for 24 h. (c) Copper concentrations in the nucleus fraction under the same treatment protocol above. Both bar graphs present the data obtained from four independent experiments. Data are expressed as means $\pm$ SEM. *Significantly different from mm siRNA under normoxic condition ($P < 0.05$); #significantly different from mm siRNA under hypoxic condition ($P < 0.05$)

Therefore, there would be at least two different mechanisms for copper translocation to the nucleus: one is CCS-1 independent, which not only helps copper transport to nucleus but also serves as a trigger for subsequent copper nuclear translocation, and the other is CCS-1 dependent; by its own need, CCS-1 carries copper to the nucleus during the process of its translocation to the nucleus. Although this study addressed the copper-dependent nuclear translocation of CCS-1, it remains unknown how copper is transported into the nucleus by the CCS-1 independent mechanism. This is a limitation of the present study. To understand this mechanism, copper chaperones besides CCS-1 need to be identified, an important and time-consuming undertaking in our future studies.

In summary, the present study shows that under hypoxia condition, both copper and CCS-1 are transported to the nucleus. In this process, the nuclear translocation of CCS-1 is copper dependent, but copper can be transported to the nucleus through a CCS-1 independent mechanism. Future studies will focus on identification of copper chaperones, besides CCS-1, which can transfer copper to the nucleus.

Authors' contributions: The first two authors made equal contributions to this study. All authors participated in the design, interpretation of the results, analysis of the data, and review of the manuscript; LW and YG carried out the experiments; YJK and LW wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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