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Elevated Glutathione Levels Confer Cellular Sensitization to Cisplatin Toxicity by Up-Regulation of Copper Transporter hCtr1

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ABSTRACT

Previous studies have demonstrated that treating cultured cells with cisplatin (CDDP) up-regulated the expression of glutathione (GSH) and its de novo rate-limiting enzyme glutamate-cysteine ligase (GCL), which consists of a catalytic (GCLC) and a modifier (GCLM) subunit. It has also been shown that many CDDP-resistant cell lines exhibit high levels of GCLC/GCLM and GSH. Because the GSH system is the major intracellular regulator of redox conditions that serve as an important detoxification cytoprotector, these results have been taken into consideration that elevated levels of GCL/GSH are responsible for the CDDP resistance. In contrast to this context, we demonstrated here that overexpression of GSH by transfection with an expression plasmid containing the *GCLC* cDNA conferred sensitization to CDDP through up-regulation of human copper transporter (hCtr) 1, which is also a transporter for CDDP. Depleting GSH levels in these transfected cells reversed CDDP sensitivity with concomitant reduction of hCtr1 expression. Although rates of copper transport were also up-regulated in the transfected cells, these cells exhibited biochemical signature of copper deficiency, suggesting that GSH functions as an intracellular copper-chelator and that overexpression of GSH can alter copper metabolism. More importantly, our results reveal a new role of GSH in the regulation of CDDP sensitivity. Overproduction of GSH depletes the bioavailable copper pool, leading to up-regulation of hCtr1 and sensitization of CDDP transport and cell killing. These findings also have important implications in that modulation of the intracellular copper pool may be a novel strategy for improving chemotherapeutic efficacy of platinum-based antitumor agents.

Cisplatin (CDDP) has been recognized as an important antitumor agent because of its activity against many human malignancies, including testicular, ovarian, cervical, bladder, head and neck, and small-cell lung cancers (SCLC) (Prestayko et al., 1979; Kollmannsberger et al., 2006). However, many patients eventually relapse and develop resistance to the treatment. It is well known that CDDP acts on multiple cellular targets representing diverse signal transduction pathways. It is therefore conceivable that multiple mechanisms have been proposed for CDDP resistance, including reduction of drug transport and increased DNA adduct tolerance and repair (for reviews, see Giaccone, 2000; Kartalou and Essigmann, 2001; Siddik, 2003; Wang and Lippard, 2005; Kelland, 2007).

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ABBREVIATIONS: CDDP, cisplatin; SCLC, small-cell lung cancer; GSH, glutathione; GCL, glutamate-cysteine ligase; GCLC, catalytic glutamate-cysteine ligase; BSO, buthionine sulfoximine; hCtr, human copper transporter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SOD, superoxide dismutase; siRNA, small interfering RNA; CCO, cytochrome *c* oxidase; apo, apoenzyme; Cp, ceruloplasmin, tet, tetracycline; rTA, tet-regulatable transactivator.

Another CDDP resistance mechanism that has been widely described in the literature is the detoxification through glutathione (GSH) system. GSH is the most abundant thiol-containing antioxidant ($1 \sim 10$ mM). De novo biosynthesis of GSH is controlled by the rate-limiting enzyme, glutamate-cysteine ligase (GCL), which consists of a catalytic (GCLC) and a modifier subunit. Previous studies demonstrated that exposure of cultured cells to CDDP led to the development of CDDP resistance that was closely correlated with increased cellular GSH levels. Moreover, GSH depletion by buthionine sulfoximine (BSO) is associated with increased sensitivity to CDDP. In many cases when GCL mRNA contents were measured, elevated levels of GCL mRNA were also correlated with CDDP resistance. These studies have been widely taken to suggest that intracellular GSH levels play an important role in regulating CDDP resistance (for reviews, see Perez, 1998; Kartalou and Essigmann, 2001; Siddik, 2003; Kelland, 2007; Rabik and Dolan, 2007; Stewart, 2007, and references therein in). However, these studies frequently used CDDP-treated cells, and the observations were mostly correlational in nature. To investigate the cause-effect relationships between elevated GSH and CDDP sensitivity, we used GCLC-overexpressing cell lines established by stably transfecting with the *GCLC* cDNA plasmid. We observed that the transfected cells, which displayed elevated levels of GSH, in fact exhibited elevated sensitivity to CDDP because of up-regulation of its transporter, human copper transporter (hCtr) 1 (Ishida et al., 2002; Song et al., 2004). Thus, our results provide a previously undiscovered new role of GSH in the regulation of CDDP sensitivity.

Materials and Methods

Cell Cultures and Transduction with Glutamate-Cysteine Ligase Modifier-Inducible Recombinant Adenovirus. SR3A and the development of its *GCLC* stably transfected cell lines SR3A-13, SR3A-14, and SR3A-15 have been described previously (Yamane et al., 1998). The cell lines have been kept in liquid N_2. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C in 5% CO_2 atmosphere, except as otherwise described and with 400 μg/ml Geneticin (G418; Invitrogen, Carlsbad, CA) for the transfected cell lines. Construction and preparation of recombinant adenovirus AdE1.tTA.*GCLC* [previously referred to as AdE1.tTA.γ-GCSh, with γ-GCSh being the catalytic (heavy) subunit of GCL] were described previously (Savaraj et al., 2005).

Determination of Drug Sensitivity. Cells grown in 96-well plates (10^4 cells/ml medium) were continuously exposed to various concentrations of drugs for 72 h, 200 μl of 0.5 mg/ml MTT (Sigma-Aldrich, St. Louis, MO) was added to each well, and the plate was incubated for 4 h. The medium was removed, and the formazan products were solubilized with 120 μl of dimethyl sulfoxide. The cell contents were measured by absorbance at 570 nm. The IC_{50} value (micromolar) was calculated by the Hill plot method with linear regression.

Measurements of Copper and CDDP Uptake. Measurements of ^{64}Cu and CDDP uptake followed the procedures described previously (Song et al., 2004). In brief, 10^6 cells were plated in a 12-well plate. After 24 h, cells were treated with 20 μM CDDP or 2 μM ^{64}CuSO$_4$ in the culture medium for various time intervals every hour from 0 to 4 h. Cells were harvested for CDDP and ^{64}CuSO$_4$ measurements as follows. Plates were placed on ice and rinsed three times with 3 ml of ice-cold phosphate-buffered saline. Cell lysis buffer (0.1% Triton X-100 and 1% SDS in phosphate-buffered saline) in a volume of 800 μl was added to the well, and the radioactivity of cell lysates was determined by scintillation counter. For measurement of

CDDP contents, cell lysates were acidified with 200 μl of 0.3 N HCl and determined in an atomic absorption spectrometer (SpectrAA300; Varian, Inc., Palo Alto, CA). Rates of uptake were calculated from the slopes of plot using amounts of ^{64}Cu of CDDP contents versus time (in hours).

RNase Protection Assay and Immunoblots. The procedures for preparation of RNA and RNase protection assay have been described previously (Yamane et al., 1998; Song et al., 2004). Western blottings were carried out according to the procedures described previously (Song et al., 2004) using the following antibodies: rabbit polyclonal antibodies against hCtr1 (Klomp et al., 2002), ATP7A (1:500; Orbigen, San Diego, CA), ATP7B (1:500; Orbigen), copper/zinc superoxide dismutase (SOD) (1:5000; Calbiochem, Darmstadt, Germany) and mouse monoclonal β-actin (Pierce Chemical, Rockford, IL) antibody.

Small Interfering RNA Transfection. Cells ($5-10 \times 10^6$) were transfected with hCtr1-specific siRNA, a control (scrambled) sequence at a 100 nM concentration or without any siRNA by using Lipofectamine 2000 (Invitrogen). Transfected cells were maintained in the regular culture medium for 2 days, and transfection was repeated once more as described above. After 24 h of transfection, cells were divided into 96-well plates. Different concentrations of CDDP were added to each well the following day. Cell killing was measured by MTT assay 3 days thereafter.

Measurements of Cytochrome *c* Oxidase and Superoxide Dismutase 1 Activities and Ceruloplasmin Enzyme Profile. CCO activity was determined using an assay kit obtained from Sigma-Aldrich according to the procedure provided by the vendor. SOD1 activity was measured by the procedure described by Chen et al. (2001). Measurements of Cp holoenzyme and apoenzyme by non-reducing gel electrophoresis followed the procedures described previously (Hellman et al., 2002; Nose et al., 2006).

Results

Elevated Expression of GSH in *GCLC*-Transfected Cells Sensitizes Cells to CDDP but Not to Copper. We previously performed transfection of *GCLC* recombinant cDNA into a human SCLC cell line, SR3A. Several GCLC-overproducing cell lines were obtained. Among them, SR3A-13, SR3A-14, and SR3A-15 exhibited 5.1-, 9.6-, and 2.2-fold, respectively, increased GCLC mRNA levels, corresponding to 2.3-, 3.0-, and 2.1-fold increases of GSH levels (Yamane et al., 1998) compared with those in SR3A cell line and the SR3A cells transfected with empty vector. Consistent with a previous study (Mulcahy et al., 1995), all the transfected cells exhibited resistance to the alkylating agents melphalan and chlorambucil (data not shown). It is noteworthy that these cells exhibited elevated sensitivity to the toxicity of CDDP (Fig. 1A), but not to copper, except for SR3A-15 (Fig. 1B). Similar results were observed in *GCLC*-transfected rat hepatoma cell lines H9 and H17 (Yamane et al., 1998; data not shown). It may be important to note that although SA3A-13 and SR3A-15 exhibited similar levels of elevated GSH, the IC_{50} values were not comparable. This may reflect clonal variation in the context of multiple mechanisms of CDDP resistance as alluded to above (Giaccone, 2000; Kartalou and Essigmann, 2001; Siddik, 2003; Wang and Lippard, 2005; Kelland, 2007). These results demonstrated that elevated expression of GSH by transfection enhanced cellular sensitivity to CDDP treatment.

***GCLC*-Transfected Cells Showed Increased Transport Activities of CDDP and Copper.** To investigate whether enhanced CDDP sensitivity was due to increased drug accumulation, we determined the rates of drug uptake.

SR3A and its three transfected lines were treated with 20 μM CDDP or 2 μM ^{64}Cu. At different time intervals, the cellular CDDP and copper contents were measured by atomic absorption spectroscopy and scintillation counting, respectively. The rates of CDDP (Fig. 1C) and ^{64}Cu (Fig. 1D) transport were both significantly increased in the transfected cells compared with those in the untransfected SR3A cells. The rates of CDDP efflux were not significantly different among these cells (data not shown).

Recent studies have demonstrated that many copper transporters, including import transporter (hCtr1) and export transporters (ATP7A and ATP7B), are also involved in CDDP transports (for review, see Kuo et al., 2007). To investigate the roles of these CDDP transporters in CDDP accumulation in the transfected cells, we first performed RNase protection assay to determine the steady-state mRNA levels encoding these transporters. Figure 2A shows that levels of hCtr1 mRNA were elevated in the transfected cells, whereas the levels of ATP7A and ATP7B mRNA show no significant difference compared with those in SR3A cells. Elevated expression of hCtr1 (Fig. 2B), but not ATP7A and ATP7B, was also evidenced by immunoblottings (Fig. 2C). Although it has been demonstrated that hCtr1 exits in oligomeric conformation and previous reports showed multiple species of hCtr1 signal in the SDS-polyacrylamide gel electrophoresis gels (Puig and Thiele, 2002; Kuo et al., 2007), in our hands, we only detected a single band in the SDS gels (Fig. 2B). The elevated expression of hCtr1 but not ATP7A and ATP7B could be detected by RNase protection and by Western blotting. The increases of hCtr1 expression in the three transfected cells did not correlate precisely with increased rate of CDDP and copper uptake; these increases may be due to clonal variations among the clones.

To validate these results, we used tetracycline (tet)-inducible GCLC expression system. Recombinant adenoviral vector AdE1.tTA.*GCLC* (Savaraj et al., 2005) contains two expression cassettes: one constitutively expresses tet-regulatable transactivator (rTA), and the other contains a *GCL*-expression cassette whose expression is under the control of rTA. In the absence of tet, the constitutively expressed rTA binds to the promoter of GCLC and activates the transcription of GCLC, whereas in its presence, expression of GCLC was inhibited. SR3A cells were transduced with AdE1.tTA. *GCLC* in the presence ($+$) or absence ($-$) of tet for 24 or 48 h. Levels of GCLC expression were increased approximately 11- and 30-fold in cells grown in the absence of tet for 24 and 48 h, respectively, compared with those treated with tet. Comparable levels of elevated GSH were found in the GCLC-overexpressing samples (Savaraj et al., 2005). Levels of hCtr1 expression were elevated in the GCLC-overexpressing cells (Fig. 2D). These results confirmed that elevated GCLC levels induce the expression of hCtr1. Low levels of hCtr1 up-regulation were also observed in cells treated with tet, which suppress the induced expression of GCLC. The precise mechanism of this induction is not known, but it is perhaps due to adenoviral transduction-associated oxidative stress, which induces the expression of GCLC because similar low levels of GCLC expression were also increased when the cells were transduced with recombinant adenovirus encoding β-globin gene (Savaraj et al., 2005). Alternatively, incomplete repression of GCLC by tet could not be ruled out.

Down-Regulation of the Overexpressed hCtr1 mRNA in the *GCLC*-Transfected Cells by siRNA Resulted in Enhanced Resistance to CDDP. We have shown previously that overexpression of hCtr1 in SCLC cells and their CDDP-resistant variants (SR2 line) enhanced the transport
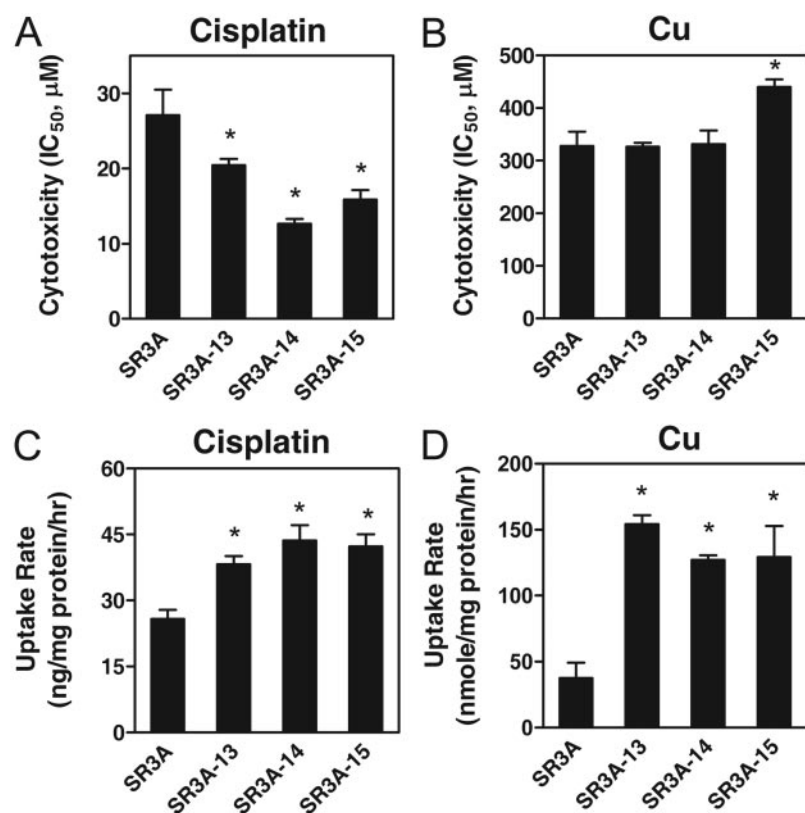

Fig. 1. Measurements of sensitivities to CDDP (A) and copper (B) and uptake of CDDP (C) and copper (D) in SR3A and the *GCLC*-transfected (SR3A-13, SR3A-14, and SR3A-15) cell lines. Measurements of sensitivities to CDDP and CuSO4 and rates of uptakes of CDDP and ^{64}Cu are described under *Materials and Methods*. Each bar represents mean $\pm$ S.D. $*$, $P < 0.01$, significantly different from SR3A cells when tested by Student's t test.

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of CDDP and enhanced cell killing by CDDP to these cells (Song et al., 2004). To further demonstrate that the causal link between increased expression of hCtr1 and enhanced sensitivity to CDDP in the *GCLC*-transfected cells, we used siRNA approach to down-regulate the elevated expression of hCtr1. Figure 3 shows that down-regulation of hCtr1 in SR3A-13, SR-3A-14, and SR3A-15 by hCtr1 siRNA increased levels of resistance to CDDP, compared with untreated, mock-treated, and control siRNA-treated cells (Fig. 3B). Similar results were obtained in SR-3A cells. These results demonstrated that the hCtr1 is responsible for the elevated sensitivities to CDDP.

Elevated GSH Levels Reduces Bioavailable Pool of Copper That Up-Regulates hCtr1 Expression. Glutathione can form a GSH-Cu(I) complex by directly interacting with its internal cysteine-thiole residue. The formation of this complex is almost a spontaneous reaction and requires no enzymatic involvement (Freedman et al., 1989; Harris, 2000), resulting in reduced intracellular bioavailability of copper. It has been well established that Ctr1 expression is regulated by intracellular available copper levels (Kuo et al., 2007). Expression of hCtr1 is up-regulated in copper-depleted cultured cells but down-regulated under copper-replete conditions (Song et al., 2008). To investigate whether up-regulation of hCtr1 in the *GCLC*-transfected cells is due to the reduction of available copper, we analyzed three biochemical

markers associated with copper deficiency. 1) We examined the enzymatic activity of the well-characterized copper-dependent enzyme SOD1, which is reduced in copper-deficient animals (Prohaska et al., 2003; Reeves et al., 2004). As shown in Fig. 4A (open bars), the activity of SOD1 was significantly reduced in the SR3A-13, SR3A-14, and SR3A-15 cells compared with that in SR3A cells. 2) The mitochondrial CCO requires copper for its activity. It has been demonstrated that reduced CCO activity is associated with copper depletion (Cobine et al., 2006). We found that CCO activity was significantly reduced in the *GCLC*-transfected cells compared with that in the nontransfected cells (Fig. 4B). 3) Ceruloplasmin is a copper-containing ferroxidase that plays an important role in mammalian iron homeostasis. This protein uses the bound copper ions to couple iron oxidation with four-electron reduction of dioxygen. Cultured cells grown in copper-depleted medium severely comprise the incorporation of seven copper atoms into the Cp as it transverses the secretary pathway (Hellman et al., 2002), resulting in increased apo-Cp at the expense of holo-Cp. This phenomenon is also observed in the serum from animals of intestinal epithelial cell-specific *ctr1*
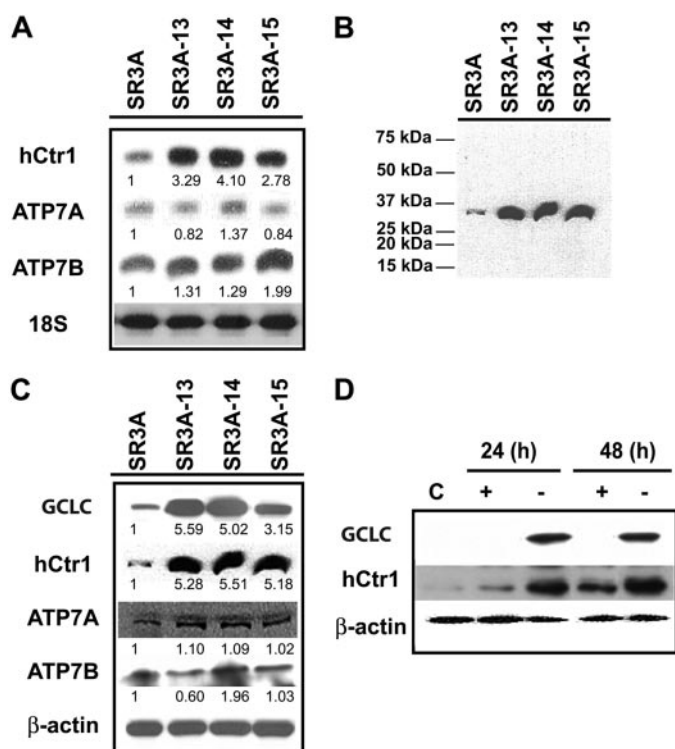
Fig. 2. Analyses of the expression of various copper transporters in SR3A and *GCLC*-transfected variants. Messenger RNA (A) and protein (B–D) levels of hCtr1, ATP7A, and ATP7B were determined by the RNase protection assay and by immunoblots using 18S RNA and β-actin as loading controls, respectively. Numbers underneath are -fold increased estimated by filmless autoradiographic analyses. D, analysis of hCtr1 expression in AdE1.tTA. *GCLC*-transduced SR3A cells. The cells were treated with recombinant adenovirus at 50 multiplicity of infection for 24 and 48 h as indicated either in the presence (+) or absence (−) of 1 μg/ml tet. Total cell lysates were prepared and probed by using anti-GCLC, anti-hCtr1, and anti-β-actin antibodies.
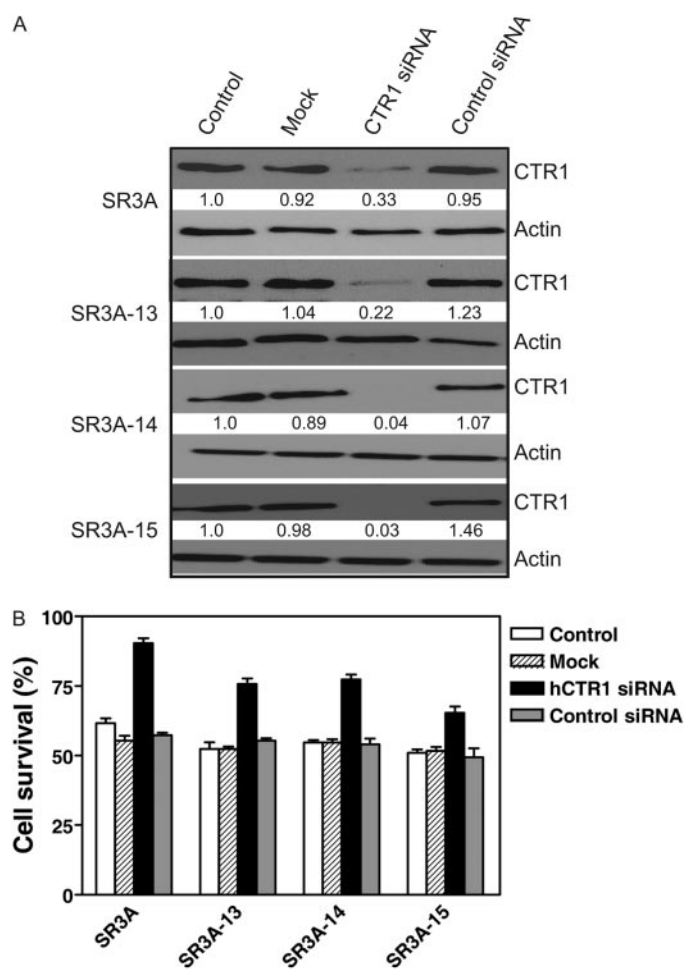
Fig. 3. hCtr1 knockdown decreases sensitivity against CDDP. A, cell extracts prepared from cells treated with 100 mM hCTR1-specific siRNA or mock, scrambled siRNA were subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and immunoblotted by anti-hCtr1-specific antibody. Anti-β-actin antibody was used as a loading control. Bands were visualized with SuperSignal West Femto Western blot detection kit. B, reduction of sensitivity of *GCLC*-transfected cells to CDDP by knockdown of hCtr1. All results are expressed as the means ± S.D. of the results of at least three experiments.

knockout mice (Nose et al., 2006). We performed immunoblot analysis of Cp from cultured media of *GCLC*-transfected cells after SDS-polyacrylamide gel electrophoresis under nonreducing conditions that resolve the apo- and holoenzymes into molecular masses corresponding to 135 and 85 kDa, respectively (Hellman et al., 2002; Nose et al., 2006). As shown in Fig. 4C (control), the majority of Cp secreted into medium from cultured SR3A cells was holoenzyme (apoCP:holoCp, 1:2.5). However, in the *GCLC*-transfected cells, almost all the secreted Cp was in apo-Cp form. Thus, the analyses of three copper-containing enzymes strongly suggested that elevated GSH levels reduced intracellular available copper and led to the induction of hCtr1 expression, resulting in increased uptake of CDDP and the sensitization of cells to CDDP toxicity.

Reduction of GSH Levels in the *GCLC*-Transfected Cells Reverses CDDP Sensitivity and hCtr1 Expression. To validate the above-mentioned results, we used BSO to deplete intracellular GSH levels and analyzed the sensitivities of the treated cells to copper and CDDP. Figure 4, A and B, shows that BSO treatment restored the reduced SOD and CCO activities in the *GCLC*-transfected cells compared with their respective untransfected cells. Moreover, almost all the secreted Cp in the BSO-treated, *GCLC*-transfected cells was in the holo-form, whereas the nontransfected SR3A cells contain approximately 50% holoenzyme (Fig. 4C). These results demonstrated BSO treatments enhanced the bioavailable copper pools in the *GCLC*-transfected cells. Taken together, these results showed, for the first time, that intracellular copper availability is inversely regulated by GSH levels.

Figure 5 shows that treatment of SR3A cells with BSO greatly sensitized SR3A cells to CDDP (90% decrease in IC_{50} value compared with those in the untreated cells). Increased CDDP sensitivity is correlated with increased expression of hCtr1 (Fig. 5E). The precise mechanisms underlying the induction of hCtr1 expression by BSO treatment are not known, but it could be due to a consequence of oxidative stress, as suggested from the results shown in Fig. 2D. Paradoxically, increased hCtr1 expression in the BSO-treated SR3A cells did not show increased uptakes of CDDP and ^{64}Cu (Fig. 5, C and D). In contrast, the IC_{50} values of CDDP in the BSO-treated SR3A-13, SR3A-14, and SR3A-15 cells increased to the level comparable with that of untreated SR3A (Fig. 5A). Strikingly, expression levels of hCtr1 were reduced in the BSO-treated transfected cells as determined by Western blotting (Fig. 5E). Reduction of hCtr1 expression in the *GCLC*-transfected by BSO treatment was correlated with the reduced uptake rates of CDDP and ^{64}Cu (Fig. 5, C and D). Levels of ATP7A and ATP7B were not changed in all the cell lines investigated, regardless whether BSO were used or not (Fig. 5E). These results demonstrated that treating the *GCLC*-transfected cells with GSH-depleting agent reversed the acquired CDDP and copper sensitivity (Fig. 5, A and B), further supporting the role of GSH in the regulation of hCtr1 expression and CDDP sensitivity.

The elevated expression of hCtr1 in the *GCLC*-transfected cells and down-regulation of hCtr1 in the BSO treatment were also demonstrated by immunofluorescent microscopy (Fig. 6). Although the majority of overexpressed hCtr1 was associated with membrane, as consistent with previous observations (Klomp et al., 2002), cytoplasmic location of hCtr1
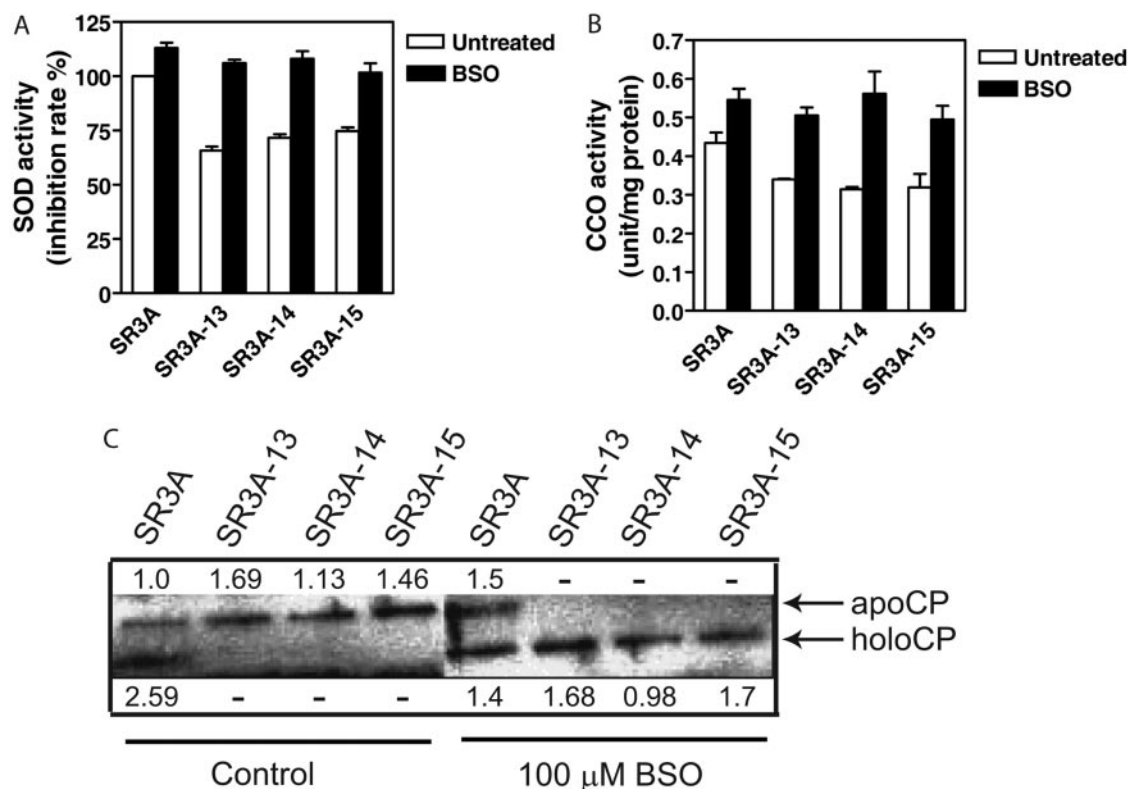
Fig. 4. Measurements of biochemical signature for copper availability in the *GCL*-transfected cells either in the presence of absence of BSO. SOD1 activities (A), CCO activities (B), and Western blotting analysis (C) of ceruloplasmin. Numbers in C denote the relative band intensity determined by filmless autoradiographic analysis. ∗, $P < 0.05$, significantly different from SR3A cells when tested by Student's t test.
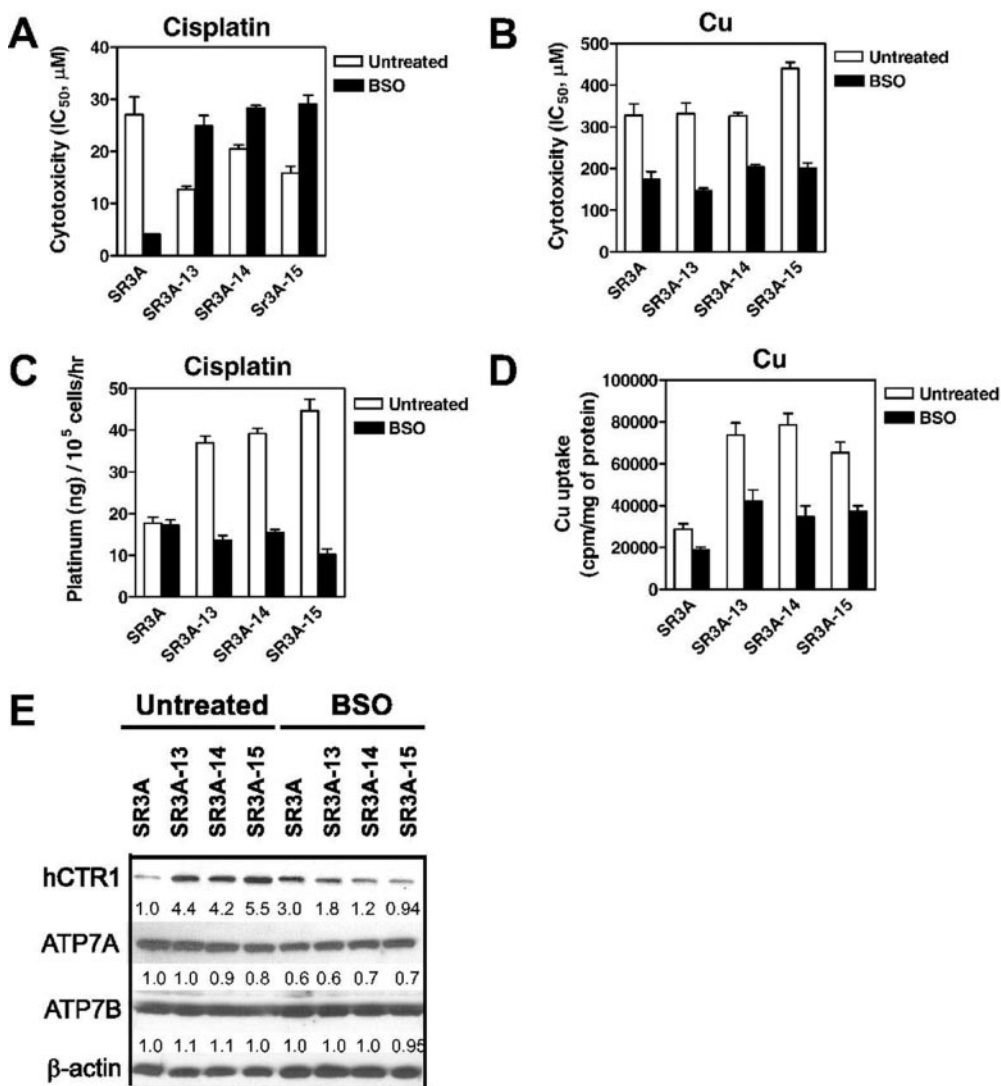
Fig. 5. Determinations of the effects of BSO treatments on the cytotoxicity of CDDP (A) and copper (B), and rates of uptake of CDDP (C) and copper (D), expression of hCtr1, ATP7A, and ATP7B by Western blots (E) and hCtr1. For cytotoxicity assays, cells grown in 96-well plates (104 cells/well) were continuously exposed to various concentrations of copper and CDDP in the presence or absence of 100 μM BSO. After 72-h incubation, cytotoxicity was measured by MTT assay. The IC_{50} value (micromolar) was calculated by the Hill plot method with linear regression. ∗, $P < 0.01$, significantly different from SR3A cells. #, $P < 0.01$, significantly different from SR3A cell in the presence of BSO.
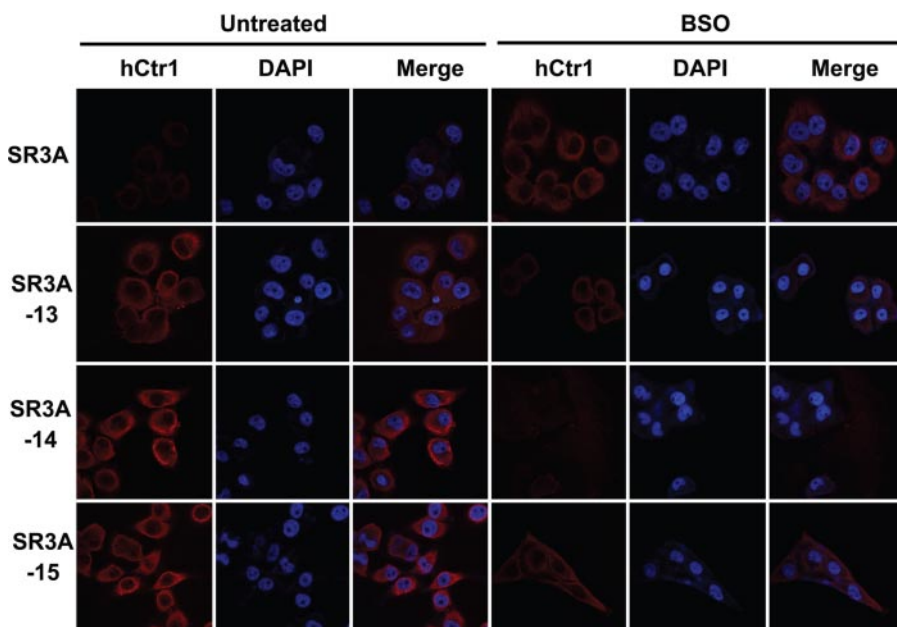

Fig. 6. Immunofluorescence staining in SR3A and *GCLC*-transfected cells. SR3A, SR3A-13, SR3A-14, and SR3A-15 treated with or without BSO were stained with anti-hCtr1 anti-hCtr1 antibody and counterstained with 4,6-diamidino-2-phenylindole (DAPI) for nucleus. Fluorescence signals were viewed by an Nikon Eclipse TE2000 confocal microscope.

was also evidenced (Fig. 6). Taken together, these results show that depleting GSH levels by BSO in the GCLC-transfected cells is associated with reduced hCtr1 expression and thus reverses the CDDP sensitivity.

Discussion

The discovery that hCtr1 can transport platinum-based antitumor agents (Ishida et al., 2002) underscores the importance of this metal transporter in cancer chemotherapy. In this communication, we report that overexpression of GSH sensitizes cancer cells to the treatment of CDDP by up-regulating hCtr1 expression. Although the rates of copper transport were also elevated in these hCtr1-overproducing cells, no enhanced sensitization to copper toxicity was found in these cells. These results suggest that the imported copper was nontoxic, most likely because of detoxification either by direct metallation of GSH or by GSH-mediated sequestration of undefined compartments. Consistent with this notion, the bioavailable copper was reduced in these cells.

Our results showed, for the first time, that GSH can interfere with intracellular copper physiology by depleting the pool of bioavailable copper (Fig. 7), leading to the increased import of CDDP, which enhances the cell-killing effects of CDDP. This observation is in contrast to those described previously in the CDDP-treated cells, which showed elevated expression of GCL/GSH. Strikingly speaking, there is no direct proof that increased GCL/GSH is responsible for CDDP resistance, yet this association is commonly made and has almost become a running theme (for reviews, see Perez, 1998; Kartalou and Essigmann, 2001; Siddik, 2003; Kelland, 2007; Rabik and Dolan, 2007; Stewart, 2007). Several mechanisms have been proposed in previous studies to account for GSH-mediated CDDP resistance: 1) The GSH system is a major cellular detoxification machinery by means of redox chemistry; increased CDDP resistance by elevated GSH levels is due to reduced redox conditions (Giaccone, 2000; Siddik, 2003; Wang and Lippard, 2005). However, the mechanisms by which redox regulates CDDP sensitivity have not

been vigorously proven. 2) CDDP may be detoxified by glutathionation, and the platinum-GSH complex is then eliminated by the ATP-dependent efflux pump MRP/GS-X (Ishikawa and Ali-Osman, 1993; Minamino et al., 1999). However, unlike redox-active metals, such as copper and cadmium ions, reaction of platinum-GSH formation is a very slow process (Ishikawa and Ali-Osman, 1993), and overexpression of MRP1 in cultured cells did not always confer resistance to CDDP (Cole et al., 1994). 3) GSH may protect cells by maintaining proper nucleotide pool for DNA repairing system for CDDP-induced DNA damage (Lai et al., 1989).

It is important to note that all those previous studies were done before the discovery of hCtr1 as a CDDP transporter. The expression levels of hCtr1 in these CDDP-resistant variants were not measured. We recently analyzed the expression levels of hCtr1, ATP7A, and ATP7B in five pairs of CDDP-resistant human cell lines by the RNase protection assay. We found that four of these cell lines exhibited reduced levels of hCtr1 mRNA expression to various extents compared with those in their respective parental cell lines, whereas levels of ATP7A and ATP7B expression were not reduced (Song et al., 2004). These results demonstrated that reduced expression of hCtr1 mRNA is frequently observed in CDDP-resistant variants.

Normal cells contain millimolar concentrations of endogenous GSH. From stoichiometric considerations, one could argue that these abundant amounts of thiol compound may already be sufficient to neutralize the cytotoxic effects of CDDP, which are in micromolar ranges. The severalfold increases of GSH content at most found in the CDDP-treated cells may not be necessary to have a major impact in the detoxification mechanism of CDDP. We reason that elevated expression of GCL, and thus GSH, in CDDP-treated cells is an oxidative stress-induced phenomenon, because GCLC is a sensor/regulator of reactive oxygen species imbalance; and its expression can be induced by a wide array of cytotoxic insults, including antitumor agents, carcinogens, metal ions, antioxidants, pro-oxidants, and others (Song et al., 2005, and references therein). In many cases, the elevated expression of GCLC, GSH, or both did not confer CDDP resistance in the treated cells.

Our observations that the depleted pool of bioavailable copper by GSH results in up-regulation of hCtr1 expression is consistent with the well documented phenomenon that intracellular copper content plays an important role in regulating hCtr1 expression. Available information regarding mechanisms that regulate *hCtr1* expression in response to copper concentrations suggests that the regulation is mainly controlled at the post-translational levels, but the results are controversial (Petris et al., 2003; Eisses et al., 2005). Our results showing that the steady-state levels of hCtr1 mRNA were elevated in the *GCLC*-transfected cells (Fig. 2A) and down-regulated in these cells treated with GSH clearly demonstrate that hCtr1 mRNA levels can be modulated in accordance to intracellular concentration fluctuation. These results are consistent with our recent finding showing up-regulation of hCtr1 mRNA in SCLC cells treated with copper depletor (100 μM bathocuproine disulfonic acid for 16 h) and down-regulation of hCtr1 mRNA in SCLC cells treated with copper (Song et al., 2008). It is important to note that previous studies have also demonstrated that expression of *Saccharomyces cerevisiae yCtr1* and *yCtr3* (for review, see Rutherford and Bird, 2004) and *Drosophila melanogaster*
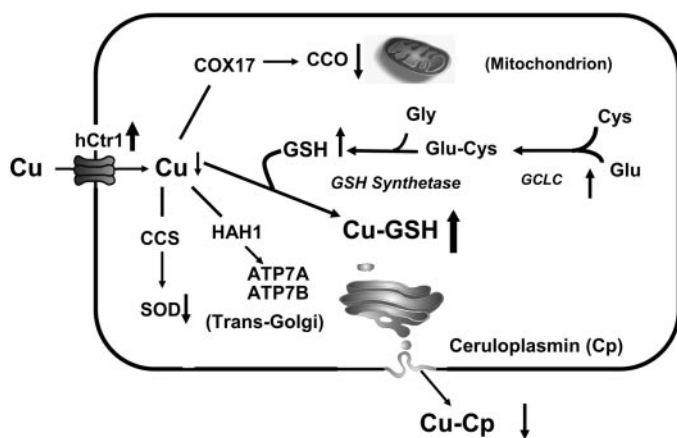

Fig. 7. Schematic illustration showing the effects of *GCLC* overexpression on cellular copper metabolism. Overexpression of GCLC, which catalyzes the ligation of cysteine (Cys) and glutamate (Glu), results in increased GSH levels. Excess GSH functions as a copper depletor, as evidenced by the reduction of CCO and SOD activity, and holo-ceruloplasmin (Cu-Cp) contents. Intracellular copper deficiency up-regulates hCtr1 expression resulting in elevated sensitivity to CDDP treatment. CCS, HAH1, and COX17 are copper chaperones that shuffle copper to their respective targets as indicated by the arrows.

dCtrB (Selvaraj et al., 2005) was also regulated by intracellular copper homeostasis. These findings, collectively, suggest that an evolutionarily conserved intracellular copper pool-sensing mechanism is involved in the regulation of Ctr1 expression from yeast to mammals, although it has been reported that almost all the intracellular copper is occupied and free copper pool is limited to less than one molecule per cell (Rae et al., 1999). In light of current finding, it is important to investigate how intracellular copper pool is mobilized in response to copper stress challenges, leading to regulation of Ctr1 expression accordingly requires future investigations.

In summary, we have revealed a novel mechanism on regulation of CDDP sensitivity by GSH. Moreover, our results show that it is feasible to modulate cellular sensitivities to CDDP treatment by using the copper chelator GSH. Development of small molecules targeting the pool of intracellular available copper may be a novel approach toward improving the efficacy of platinum-based antitumor agents. Such approaches have been used in the first-line treatment of copper toxicosis in Wilson's disease (Das and Ray, 2006) and may be an effective strategy in cancer chemotherapy.

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