The copper transporter Ctr1 contributes to cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity

Navjotsingh Pabla, Robert F. Murphy, Kebin Liu, and Zheng Dong

Departments of 1Cellular Biology and Anatomy and 2Biochemistry and Molecular Biology, Medical College of Georgia and Charlie Norwood Veterans Affairs Medical Center, Augusta, Georgia; and 2National Cancer Institute, National Institutes of Health, Bethesda, Maryland

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Pabla N, Murphy RF, Liu K, Dong Z. The copper transporter Ctr1 contributes to cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity. Am J Physiol Renal Physiol 296: F505–F511, 2009. First published January 14, 2009; doi:10.1152/ajprenal.90545.2008.—The usefulness and efficacy of cisplatin, a chemotherapeutic drug, are limited by its toxicity to normal tissues and organs, including the kidneys. The uptake of cisplatin in renal tubular cells is high, leading to cisplatin accumulation and tubular cell injury and death, culminating in acute renal failure. While extensive investigations have been focused on the signaling pathways of cisplatin nephrotoxicity, much less is known about the mechanism of cisplatin uptake by renal cells and tissues. In this regard, evidence has been shown for the involvement of organic cation transporters (OCT), specifically OCT2. The copper transporter Ctr1 is highly expressed in the renal tubular cells; however, its role in cisplatin nephrotoxicity is not known. In this study, we demonstrate that Ctr1 is mainly expressed in both proximal and distal tubular cells in mouse kidneys. We further show that Ctr1 is mainly localized on the basolateral side of these cells, a proposed site for cisplatin uptake. Importantly, downregulation of Ctr1 by small interfering RNA or copper pretreatment results in decreased cisplatin uptake. Consistently, downregulation of Ctr1 suppresses cisplatin toxicity, including cell death by both apoptosis and necrosis. Cimetidine, a pharmacological inhibitor of OCT2, can also partially attenuate cisplatin uptake. Notably, cimetidine can further reduce cisplatin uptake and cisplatin toxicity in Ctr1-downregulated cells. The results have demonstrated the first evidence for a role of Ctr1 in cisplatin uptake and nephrotoxicity.

The copper transporter Ctr1 contributes to cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity. (see Refs. 2 and 27 for a recent review). However, much less is known about the mechanism underlying cisplatin uptake by tubular cells. Earlier studies have shown that cisplatin is transported, preferentially from the basolateral side in renal tubular cells (24). More recently, it has been suggested that the organic cation transport system present on the basolateral side might be responsible for cisplatin uptake (5, 7, 16, 23). Specifically, evidence has been suggested that the organic cation transporter OCT2 is involved in cisplatin transport in tubular cells (5).

On the other hand, the high-affinity copper transporter Ctr1 has been recently implicated in cisplatin transport in nonrenal cells (12, 17, 22). Ctr1 is a 23-kDa protein that has three transmembrane segments and oligomerizes to form a functional trimer, which has a unique pore for the transport of copper (1, 20, 25). Interestingly, it has been reported that in various cell lines overexpressing Ctr1 accumulate significantly higher levels of cisplatin (17). Notably, while Ctr1 is expressed in a variety of tissues, its expression is particularly high in the kidneys (18, 19). However, the role of Ctr1 in cisplatin uptake by renal tubular cells during cisplatin nephrotoxicity is not known.

This study sought to 1) examine the expression and localization of Ctr1 in renal tubular cells and 2) determine the role of Ctr1 in cisplatin uptake and subsequent tubular cell death. Our results indicate that Ctr1 is expressed on the basolateral side of the renal tubular cells. Importantly, knockdown of Ctr1 by small interfering (si)RNA reduced cisplatin uptake in renal tubular cells and blocked cisplatin-induced renal cell death. Importantly, the OCT2 inhibitor cimetidine and Ctr1 knockdown had additive inhibitory effects on cisplatin uptake and cell death. These results have therefore suggested a dual pathway of cisplatin uptake in renal tubular cells via Ctr1 and OCT2.

MATERIALS AND METHODS

Reagents. Antibodies were from the following sources: polyclonal Ctr1 antibody was from Dr. Jane Gitschier at University of California San Francisco (18); polyclonal p53 antibody was from Cell Signaling Technology (Beverly, MA), and the monoclonal β-actin antibody was from Sigma (St. Louis, MO). Cy3-labeled secondary antibody was from Chemicon (Temecula, CA), and FITC-labeled Phaseolus vulgaris agglutinin (PHA) or peanut agglutinin (PNA) was purchased from Vector (Burlingame, CA). The primary antibody to Ctr1 was from Dr. Jane Gitschier at University of California San Francisco (18). The copper chelator dipyridamole was from Sigma (St. Louis, MO) and cimetidine was from Research Biochemicals (Natick, MA).

METHODS

THE PLATINUM-BASED DRUG CISPLATIN and its analogs are one of the most widely used and successful drugs for the treatment of solid tumors (29, 30). They are used alone or in combination with other drugs for the treatment of testicular, head and neck, ovarian, cervical, and many other types of malignancies (29, 30). However, acquired resistance by cancer cells and side effects to normal tissues limit the use of cisplatin (29). One of the most important dose-limiting factor in the use of cisplatin is its toxicity to the kidneys (2, 27). During cisplatin treatment, over a quarter of patients develop impairment in renal function, finally resulting in acute renal failure (2, 27).

Tubular damage is recognized as a major pathogenic factor in cisplatin nephrotoxicity. Under the condition, cisplatin is accumulated in renal tubular cells at high concentrations, leading to tubular injury and cell death. Research during recent years has revealed multiple signaling pathways that are responsible for tubular cell injury and death during cisplatin nephrotoxicity (see Refs. 2 and 27 for a recent review). However, much less is known about the mechanism underlying cisplatin uptake by tubular cells. Earlier studies have shown that cisplatin is transported, preferentially from the basolateral side in renal tubular cells (24). More recently, it has been suggested that the organic cation transport system present on the basolateral side might be responsible for cisplatin uptake (5, 7, 16, 23). Specifically, evidence has been suggested that the organic cation transporter OCT2 is involved in cisplatin transport in tubular cells (5).

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from Vector Laboratories (Burlingame, CA). Other reagents including cisplatin and cimetidine were purchased from Sigma.

**Animals and cells.** C57BL/6 mice were housed in the animal facility of the Charlie Norwood Veterans Affairs (VA) Medical Center (Augusta, GA). Animal experiments were conducted under an approved animal user protocol in accordance with the guidelines established by the VA Medical Center. Primary proximal tubular cells were isolated from male C57BL/6 mice and cultured as described recently (31, 32). The immortalized rat kidney proximal tubular cell (RPTC) line was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH). Human embryonic kidney (HEK293) cells were purchased from American Type Culture Collection (Manassas, VA). RPTC and HEK293 cells were cultured as shown previously (3, 28) and plated to reach ~90% confluence for the experiment.

**Experimental models of cisplatin nephrotoxicity.** C57BL/6 mice were injected with a single dose of 30 mg/kg cisplatin to induce nephrotoxicity or injected with saline as a control as described recently (31, 32). In vitro, RPTC cells and HEK293 cells were incubated with 20 and 40 μM cisplatin, respectively, for the indicated times to induce apoptosis. At these concentrations, little or no necrosis was observed. To induce necrosis, RPTC and HEK293 cells were treated for 8 h with 100 and 150 μM cisplatin, respectively.

**Transfection.** RPTC and HEK293 cells were transiently transfected with siRNA plasmids from SA Biosciences (Frederick, MD) using Lipofectamine 2000 reagent from Invitrogen. Twenty-four hours later, the transfected cells were identified by green fluorescence since the siRNA plasmids also had a GFP-expressing sequence. The transfection efficiency of RPTC cells was ~30%, and for HEK293 cells it was >80%.

**Immunofluorescence staining.** C57BL/6 mice were killed to collect kidneys, which were immediately frozen in liquid nitrogen. The freshly frozen tissues were cryosectioned and fixed with 4% paraformaldehyde, followed by permeabilization with 1% Triton X-100. Subsequently, the tissue sections were sequentially incubated with a blocking buffer, the Ctrl antibody, and Cy3-labeled secondary antibody. For costaining, the tissue sections were first stained for Ctrl1 immunofluorescence as described above and then incubated with FITC-labeled PHA or PNA as described recently (31, 32). The staining was then examined by confocal microscopy.

**Immunoblot analysis.** Equal amounts of proteins were loaded for SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were then incubated with blocking buffer, primary antibodies, and with horseradish peroxidase-conjugated secondary antibody. Chemiluminescent substrates (Pierce) were then used to detect the levels of various proteins.

**Measurement of apoptosis.** Apoptosis in RPTC and HEK cells was analyzed by standard methods, including morphological examination and caspase activity. Briefly, after cisplatin treatment the cells were stained with Hoechst 33342 and the number of cells showing morphological features of apoptosis including cellular and nuclear condensation and fragmentation were counted to determine percent apoptosis. For biochemical measurement of apoptosis, the caspase activity was measured enzymatically as described previously (13, 14). Briefly, untreated and cisplatin-treated cells were lysed by 1% Triton X-100 containing buffer and then 25 μg protein were added to an enzymatic reaction containing a fluorogenic peptide substrate of caspases. The fluorescence at excitation 360 nm/immision 535 nm was detected using a GENios plate-reader (Tecan US, Research Triangle Park, NC).

**Measurement of necrosis.** Control and cisplatin-treated cells were stained with 1 μg/ml propidium iodide (PI) for 10 min, and the percentage of cells showing positive PI staining were counted as necrotic cells by fluorescence microscopy. For biochemical analysis of necrosis, the release of lactate dehydrogenase (LDH) from the cells was determined. LDH was measured enzymatically using a kit from Sigma. Briefly, one group of cells was lysed with Triton X-100 to obtain the reading of total LDH activity, and the media from the other groups were collected to determine the enzymatic activity of LDH. The released LDH signals were divided by the total LDH activity to determine the percent LDH released under various experimental conditions.

**Statistics.** Data are expressed as means ± SD; n = ≥3. Statistical differences between two different groups was determined by Student’s t-test using Microsoft Excel 2000. P < 0.05 was considered statistically significant.
RESULTS

**Ctrl1 is highly expressed in both proximal and distal tubular cells.** Ctrl1 is a ubiquitously expressed protein, but its expression is reported to be particularly high in the kidneys (18, 19). To determine the expression and localization of Ctrl1 in the kidneys, we first analyzed the whole-cell lysates of mice kidney cortex and also mouse primary cultured tubular cells using a well-characterized Ctrl1 antibody (18). Monomeric, dimeric, and trimeric forms of Ctrl1 were detected in both the kidney cortex and the cultured tubular cells, although the signal of the dimeric 50-kDa band was much weaker (Fig. 1A). These results confirmed that the Ctrl1 is highly expressed in the renal cortex. Next, we determined the localization of Ctrl1 in the renal tubular cells. As shown in Fig. 1B, Ctrl1 was mostly localized on the basolateral side of renal tubules. Some staining was also seen in the cytoplasm, especially in the perinuclear region of the tubular cells. However, Ctrl1 staining in the apical region was minimal. To confirm this observation and also determine the cell types that express Ctrl1, we costained the tissues with Ctrl1 (red) and FITC-labeled lectin markers of proximal (PHA) and distal (PNA) tubular cells. As shown in Fig. 1C, the proximal tubule-specific lectin PHA stained the apical brush-border membrane, whereas Ctrl1 was expressed on the basolateral side. In contrast, PNA-stained distal tubules showed Ctrl1 expression all over the cells except the nuclei (Fig. 1D).

**Ctrl1 expression during cisplatin treatment in renal cells and tissues.** Cisplatin treatment is known to alter the expression of Ctrl1 in HEK293 and human ovarian carcinoma cells (9, 10). To determine the effects of cisplatin on Ctrl1 expression in renal cells, we first analyzed RPTC and HEK293 cells, which have been used to study cisplatin-induced apoptosis (28). Cisplatin treatment in these cell lines induced ~40–50% apoptosis after 16-h treatment (28). Whole-cell lysates collected at different time points did not show obvious differences in the expression of Ctrl1 following cisplatin treatment (Fig. 2A). To determine the levels of Ctrl1 expression in vivo during cisplatin treatment, we used a mouse model of cisplatin nephrotoxicity described previously (31, 32). As shown in Fig. 2B, Ctrl1 expression in renal tissues did not show significant changes 1–3 days after cisplatin treatment, a time period of acute nephrotoxicity. **Cisplatin treatment induced Ctrl1 downregulation.** Higher extracellular copper has been reported to downregulate Ctrl1 protein levels (8). Consistently, we showed that incubation of HEK cells with 100 μM copper led to significant decreases in both monomeric and trimeric Ctrl1 (Fig. 3A). Importantly, the copper-pretreated cells showed signific-
cantly lower cisplatin uptake than control cells (Fig. 3B). To further determine the role of Ctrl in cisplatin uptake, we transfected HEK293 cells with a Ctrl-specific siRNA to knock down Ctrl (Fig. 3A). The Ctrl knockdown cells accumulated ~50% less cisplatin compared with nonspecific siRNA-transfected cells (Fig. 3B). Cisplatin is known to cause DNA damage, and p53 has been established as an early marker of DNA damage during cisplatin nephrotoxicity (14, 15, 32). As expected, we detected p53 stabilization and accumulation in both untransfected and control siRNA-transfected cells, whereas p53 accumulation was significantly attenuated in Ctrl knockdown cells (Fig. 3C). These results suggest that Ctrl contributes to cisplatin uptake and subsequent nephrotoxic signaling.

Ctrl knockdown ameliorates cisplatin-induced tubular cell apoptosis. Renal tubular cell apoptosis is a main event leading to acute kidney injury during cisplatin nephrotoxicity (27). We reasoned that Ctrl knockdown and the subsequent decrease in cisplatin uptake would ameliorate cisplatin-induced renal cell apoptosis. To test this possibility, we examined the effects of Ctrl knockdown. By morphology, HEK293 cells transfected with control siRNA showed ~60% apoptosis after 20 h of cisplatin treatment, which was reduced to 30% in Ctrl siRNA-transfected cells (Fig. 4A). To biochemically verify the results, we analyzed caspase activity. Consistent with the morphological examination, Ctrl knockdown reduced the caspase activation to about half of that in control siRNA-transfected cells (Fig. 4B). To confirm the results in RPTC cells, we transfected the cells with control or Ctrl-specific siRNA and then treated the cells with 20 μM cisplatin for 16 h. After incubation, the cells were fixed and the transfected (GFP-positive) cells were examined for the level of apoptosis. As shown in Fig. 4C, Ctrl siRNA-transfected RPTC cells had significantly lower apoptosis compared with control transfected cells. Representative images are shown in Fig. 4D.

Ctrl knockdown abrogates cisplatin-induced necrosis. Cisplatin induces both apoptosis and necrosis during cisplatin nephrotoxicity (27). Generally, necrosis is induced by higher doses of cisplatin (21). To test whether Ctrl also contributes to cisplatin uptake and toxicity at high doses, we treated HEK293 cells with 150 μM cisplatin for 8 h. About 70% of the cells showed PI staining under this experimental condition. Importantly, Ctrl knockdown significantly reduced high dose-induced necrosis as measured by PI staining (Fig. 5A). Similar results were seen in RPTC cells treated with 100 μM cisplatin (data not shown). The LDH release assay, which is a biochemical test of necrosis, also showed that Ctrl knockdown cells had significantly less necrosis compared with control siRNA-transfected cells (Fig. 5B).

Ctrl knockdown and cimetidine have additive effects on cisplatin uptake and cell death in HEK293 cells. The organic cation transport system has been implicated in cisplatin uptake in renal tubular cells (5, 26, 33). Specifically, cimetidine, a pharmacological inhibitor of OCT2, has been shown to reduce cisplatin uptake and subsequent apoptosis (5). To understand the roles of Ctrl and OCT2 in cisplatin transport, we treated control and Ctrl knockdown HEK293 cells with cimetidine and measured cisplatin uptake. Ctrl siRNA or 1 mM cimetidine alone reduced the uptake of cisplatin by ~50 and 40%, respectively (Fig. 6A). Importantly, when added in Ctrl knockdown cells, cimetidine further reduced the cisplatin uptake by ~75% (Fig. 6A). In addition, we showed that cimetidine could further reduce apoptosis and caspase activation in Ctrl knockdown cells (Fig. 6B and C). Similar additive effects were shown on necrosis induced by higher concentrations of cisplatin, as indicated by LDH release (Fig. 6D). Together with previous findings (5, 26, 33), our results suggest that both Ctrl and OCT2 may be involved in cisplatin uptake in renal tubular cells and contribute to subsequent nephrotoxicity.

DISCUSSION

Nephrotoxicity is a major limiting factor in the clinical use of cisplatin (2, 27). Renal tubular cell death is considered as a
major pathophysiological basis of cisplatin toxicity (27). High sensitivity of tubular cells to cisplatin is partly attributed to the high uptake of cisplatin by these cells. This study has demonstrated the first evidence that the copper transporter Ctr1 plays an important role in tubular uptake of cisplatin during nephrotoxicity. Ctr1 knockdown by siRNA significantly reduced cisplatin uptake and renal cell death.

Ctr1 is a highly conserved protein essential for copper import in mammalian cells (20, 25). It forms a homotrimeric complex that is responsible for high-affinity copper transport (1). Although Ctr1 transports copper with very high specificity, recent studies showed that it is also involved in the transport of cisplatin (12). Indeed, mouse embryonic fibroblasts from Ctr1 knockout mice have markedly reduced cisplatin uptake compared with wild-type cells (11, 12). Interestingly, Ctr1 is highly expressed in the kidney (18, 19), but its role in cisplatin transport in the kidneys is not known. Our study corroborates the earlier studies that Ctr1 is highly expressed in the mouse kidney cortex and in primary tubular cells (Fig. 1). Moreover, our results indicate that Ctr1 is expressed on the basolateral side of the renal tubular cells. Weaker Ctr1 staining was also seen in the cytoplasm of tubular cells, but not on the apical membrane. This observation was in contrast to a previous study that suggested an apical membrane localization of Ctr1 in renal proximal tubular cells. The exact cause of the discrepancy between these two studies is unclear, although different mouse strains were used in the two studies. In addition, we used lectin markers to further confirm the basolateral localization of Ctr1, whereas the previous study examined various tissues without focusing on subcellular levels. Consistent with our results, Zimnicka et al. (34) recently also demonstrated basolateral localization of Ctr1 in several cell types including Madin-Darby canine kidney cells and opossum kidney cells. Together, the results indicate that Ctr1 is expressed in both the proximal and distal tubules, and in proximal tubules it is mainly localized on the basolateral membrane. This localization is consistent with a role for Ctr1 in transporting cisplatin from the basolateral side of renal tubular cells (7, 23).

Fig. 5. Suppression of cisplatin-induced necrosis in Ctr1 knockdown cells. HEK293 cells transfected with control or Ctr1 siRNA were treated with 150 μM cisplatin for 8 h, and necrosis was measured by propidium iodide (PI) staining (A) and LDH assay (B). Values are means ± SD; n = 3. *Statistically significant difference from the untreated group (P < 0.05). #Statistically significant difference from the control siRNA treated group (P < 0.05).

Fig. 6. Additive effects of Ctr1 knockdown and cimetidine on cisplatin uptake and cell death in HEK293 cells. A: cisplatin uptake. Control and Ctr1 siRNA-transfected cells were treated with either 40 μM cisplatin alone or 1 mM cimetidine + 40 μM cisplatin for 12 h to measure cisplatin uptake. B: apoptosis. Control and Ctr1 siRNA-transfected cells were treated with either 40 μM cisplatin alone or 40 μM cisplatin with different concentrations of cimetidine for 20 h. Apoptosis was determined by a morphological method. C: caspase activity. Control and Ctr1 siRNA-transfected cells were treated with either 40 μM cisplatin alone or 1 mM cimetidine + 40 μM cisplatin for 20 h to measure caspase activity. D: necrosis. Control and Ctr1 siRNA-transfected cells were treated with either 150 μM cisplatin alone or 1 mM cimetidine + 150 μM cisplatin for 8 h to determine LDH release to indicate plasma membrane breakdown or necrosis. Values are means ± SD; n = 3. *Statistically significant difference from the untreated group (P < 0.05). #Statistically significant difference from the cisplatin-treated group (P < 0.05). @Statistically significant difference compared with control siRNA-treated group (P < 0.05).
Although it has been reported that cisplatin induces the degradation or stabilization of Ctrl (9, 10), we did not detect significant changes in the expression of Ctrl in RPTC, HEK293 cells, or mouse tissues during cisplatin nephrotoxicity (Fig. 2). We did not detect significant changes in Ctrl oligomerization, either. The discrepancy could be caused by differences in the experimental models. For example, the earlier study analyzed overexpressed Ctrl (9), whereas we examined the endogenous protein. On the other hand, we showed that treatment with 100 μM copper resulted in a significant decrease in Ctrl protein levels (Fig. 3A). This observation is consistent with a previous study, suggesting that high extracellular copper results in Ctrl degradation (8). Functionally, we showed that the cells pretreated with copper had significantly reduced cisplatin uptake (Fig. 3B).

To further study the role of Ctrl in cisplatin uptake in renal cells, we used a siRNA approach to knock down Ctrl. HEK293 cells transfected with Ctrl-specific siRNA, but not the control siRNA, showed an obvious decrease in Ctrl expression (Fig. 3B). Importantly, Ctrl knockdown significantly reduced cisplatin uptake in HEK293 cells, to half of the control level (Fig. 3B). Our and other laboratories have shown that p53 is activated, stabilized, and accumulated during cisplatin treatment of renal tubular cells (6, 15, 32). In the present study, we showed that p53 was induced by cisplatin in control siRNA-transfected cells, but this induction was blocked in Ctrl-siRNA-transfected cells. These results suggest that indeed Ctrl knockdown reduces cisplatin uptake in renal cells and subsequent nephrotoxic signaling. Depending on the dosage, cisplatin is known to induce apoptosis, necrosis, or both in renal tubular cells (21). Our results show that Ctrl knockdown significantly reduced apoptosis during 20- to 40-μM cisplatin treatment in both HEK293 and RPTC cells (Fig. 4). Moreover, Ctrl knockdown also ameliorated 100- to 150-μM cisplatin-induced necrosis (Fig. 5).

The organic cation transporter OCT2 has been reported to contribute to cisplatin uptake in renal tubular cells (5). Specifically, cimetidine, a pharmacological inhibitor of OCT2, was shown to reduce cisplatin uptake in cultured renal tubular cells (5, 23). In line with these observations, we demonstrated partially inhibitory effects of cimetidine on cisplatin uptake in HEK293 cells. Importantly, we showed that cimetidine could further reduce cisplatin uptake in Ctrl knockdown HEK293 cells. The additive effects of cimetidine and Ctrl knockdown were also shown for the toxicity of cisplatin (Fig. 6 and data not shown). In this regard, both apoptosis and necrosis induced by cisplatin were further reduced by cimetidine in Ctrl knockdown HEK293 cells (Fig. 6). These results raise the intriguing possibility that both Ctrl and OCT2 contribute to cisplatin transport in renal tubular cells, resulting in nephrotoxicity. However, the results should be interpreted with caution, because, as a pharmacological agent, cimetidine may have off-target effects. OCT2 expression in HEK293 cells is generally low (4), and whether cimetidine specifically inhibits OCT2 in these cells can be questioned. Therefore, further studies should determine the involvement of OCT2 in cisplatin nephrotoxicity in more detail by using molecular- or genetic-inhibitory approaches.

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REFERENCES


