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ORIGINAL ARTICLE

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Role of Na^+, K^+-ATPase α1 subunit in the intracellular accumulation of cisplatin

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Abstract The present study was undertaken to identify what regulates intracellular cisplatin (CDDP) accumulation and what changes in membrane fraction of CDDDP-resistant cell line. The CDDP-resistant rat hepatoma cell line, H4-II-E/CDDP, shows a significant decrease in intracellular platinum accumulation compared with parental H4-II-E cells, although there was no difference in the efflux of CDDP between these two cell lines. In this study, we examined the contribution of functional change in active transport to the CDDP resistance of H4-II-E/CDDP cells. Compared with the resistant cells, platinum accumulation in the parental cells was clearly decreased by low temperature or ATP depletion. In addition, the Na^+, K^+-ATPase inhibitor ouabain and the K^+ channel inhibitor tetraethylammonium decreased platinum accumulation in parental cells but did not change the accumulation in resistant cells. Amphotericin B, an antifungal agent, increased the intracellular platinum accumulation in resistant cells to the same level as in parent cells. Western blot analysis demonstrated that the Na^+, K^+-ATPase α_1 subunit was reduced in resistant cells compared with the parental cells, although there was no difference in the expression of the β1 subunit between the two cell lines. Furthermore, the Na^+, K^+-ATPase α1 subunit of H4-II-E was decreased following a 24-h exposure to CDDP. These results suggest that Na^+, K^+-ATPase-dependent active transport of CDDP does not occur in resistant cells, and, furthermore, our findings provide the first evidence that the Na^+, K^+-ATPase α1 subunit plays an important role in the transport of CDDP.

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Introduction

Cisplatin (CDDP) is one of the most effective antitumor agents for the treatment of human malignancies. However, many tumors show a natural or acquired resistance to CDDP that is independent of multidrug resistance and is responsible for most therapy failures. Different mechanisms of resistance have been described, including reduced intracellular accumulation of CDDP; elevated intracellular levels of glutathione and metalothioneins; increased DNA-repair activity; and increased tolerance to DNA damage [1, 2]. Attempts have been made to overcome CDDP resistance using novel platinum compounds and various agents that affect these resistance mechanisms [3, 4]. Understanding the mechanism of intrinsic and acquired resistance to CDDP is thus critical for developing more effective treatments for cancer.

Several authors have suggested that reduced CDDP uptake is an important factor that can result in CDDP resistance [5]. Although some findings have led to the suggestion that passive diffusion is a major mechanism for CDDP uptake, other studies have suggested a role for active transport. In addition, specific changes in the plasma membrane have been reported in association with reduced CDDP accumulation in resistant cells [6]. It has been demonstrated that ouabain, a Na^+, K^+-ATPase inhibitor, inhibits CDDP uptake and that high potassium media causes an increase in CDDP accumulation [7, 8]. These findings suggest some component of CDDP accumulation is dependent on the membrane potential, which is maintained by Na^+, K^+-ATPase and other channels. Furthermore, there is increasing evidence that CDDP may influx and efflux from cells by utilizing transporters that have evolved for the management of copper (Cu^{2+}) homeostasis [9, 10]. Indeed, CDDP uptake is impaired in yeast and mammalian cells

in which the gene encoding the major copper importer CTR1 has been deleted.

We reported that our established rat hepatoma CDDP-resistant subline has a significantly reduced accumulation of intracellular platinum [11]. This CDDP-resistant cell line did not show an increased efflux of CDDP due to improved MRP2 expression. We speculated that there were some functional or structural changes in active transport of CDDP into cells. In this study, we validate this hypothesis by demonstrating some differences in the responses of the parent and the CDDP-resistant cell lines to temperature, ATP depletion, and transporter inhibitors. We also show that there is no difference in copper transport, although there is sharp contrast in the level of Na^+, K^+-ATPase α1 subunit expression. These studies strongly suggest that the level of membrane potential-regulating transporters is important for determining the sensitivity to platinum compounds.

Materials and methods

Drugs

CDDP was purchased from Kyowa Hakko Kogyo (Tokyo, Japan). Carboplatin (CBDCA) was purchased from Bristol Pharmaceuticals (Tokyo, Japan). Nedaplatin (CDGP) was purchased from Shionogi (Osaka, Japan).

Cell culture

Rat hepatoma H4-II-E cells were purchased from Dainippon Pharmaceutical (Osaka, Japan). H4-II-E cells were maintained in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2 in air. CDDP-resistant H4-II-E/CDDP cells were established by a stepwise exposure of H4-II-E cells to increasing concentrations of CDDP over 12 months; the cells were initially treated with 0.3 to 0.5 μg/ml of CDDP for 3 months, and the CDDP concentration was gradually increased up to a final concentration of 1.0 μg/ml. Established H4-II-E/CDDP cells were maintained MEM containing 10% FBS with 1.0 μg/ml of CDDP.

Intracellular accumulation of CDDP and copper

Cells (4×10^7) were seeded into 75-cm^2 tissue culture flasks and incubated overnight. Next, the cells were incubated with CDDP, CBDCA, CDGP, or copper sulfate for 2 h. A single culture was used to determine the accumulation of each drug in each experiment. Immediately thereafter, the cells were harvested and washed three times with cold PBS(−). The resulting cell pellets were lysed in digestion buffer (10 mM Tris–HCl, 10 mM EDTA, 0.15 M NaCl, and 0.5% sodium lauryl sulfate) in the presence of 0.1 mg/ml proteinase K for 1 h at 55°C and then overnight at 37°C. The protein content was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The amounts of platinum and copper in the samples were determined by a Hitachi Z-9000 flameless atomic absorption spectrometry (FASS, Tokyo, Japan). The results were expressed as ng platinum or copper per mg protein.

The effects of coincubation with antimycin A, ouabain, tetraethylammonium chloride (TEA), and amphotericin B on the intracellular accumulation of platinum were investigated. These agents were added at 60 min prior to a 2-h exposure to CDDP. Also, the influence of temperature reduction on the accumulation of platinum was studied by incubation of cells with CDDP at 4°C. Experiments at 4°C were performed simultaneously with incubations at 37°C and using the same batch of cells. After exposure, cells were collected and washed. Subsequently, platinum accumulation was determined by FASS as described above.

Western blotting

Membrane fractions of cells were extracted using a plasma membrane protein extraction kit (MBL, Tokyo, Japan). Membrane proteins (10 μg) were subjected to SDS-PAGE and transferred to polyvinyllidene difluoride membranes. The membrane was blocked with Blocking Reagent (Roche Diagnostics, Indianapoilis, IN, USA) in maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, pH 7.5) for 1 h at room temperature and then incubated overnight at 4°C with 1:2000 monoclonal antibody against Na^+, K^+-ATPase α1 or β1 subunit (Novus Biologicals, Littleton, CO, USA). The membrane was rinsed with PBS(-) containing 0.1% Tween 20 and then incubated for 1 h with 1:50,000 horseradish peroxidase-conjugated anti-mouse IgG (Amersham Biosciences, Piscataway, NJ, USA). The membrane was then washed and visualized with the ECL Plus Western blotting detection system and an ECL mini-camera (Amersham Biosciences).

Results

Relationship between concentration and accumulation of CDDP

The total amount of intracellular platinum after a 2-h exposure to CDDP (10, 20, 40, or 60 μg/ml) for H4-II-E and CDDP-resistant H4-II-E/CDDP cells is shown in Fig. 1. Within this concentration range, intracellular platinum levels increased linearly with increasing dose. Intracellular platinum levels differed by approximately tenfold between the two cell lines. These levels were significantly lower ($P < 0.05$) in H4-II-E/CDDP compared with the parental cells at each tested concentration.

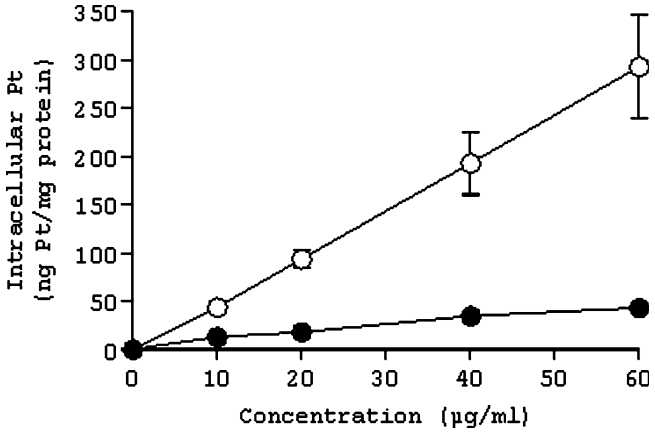

Fig. 1 Intracellular platinum accumulation following CDDP exposure. Cells were incubated with CDDP for 2 h. The amount of accumulated platinum was determined by FAAS. The symbols used are as follows: *open circle*, H4-II-E; *filled circle*, H4-II-E/CDDP. Data represent the mean ± SD for three individual experiments

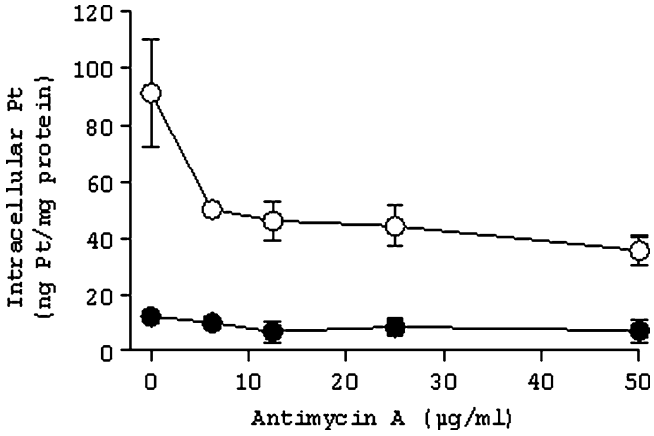

Fig. 3 Influence of antimycin A on platinum accumulation. Antimycin A was added 60 min prior to a 2-h exposure of cells to CDDP (10 µg/ml). The amount of accumulated platinum was determined by FAAS. The symbols used are as follows: *open circle*, H4-II-E; *filled circle*, H4-II-E/CDDP. Data represent the mean ± SD for three individual experiments

Energy dependency of platinum accumulation

Lowering the temperature to 4°C resulted in a significantly reduced intracellular accumulation of CDDP in both H4-II-E and resistant H4-II-E/CDDP cells (81 and 50% of the accumulation at 37°C, respectively; Fig. 2). The difference between H4-II-E and H4-II-E/CDDP cells was less substantial at 4°C than at 37°C.

We next examined the effect of ATP depletion on CDDP accumulation using antimycin A, an inhibitor of respiratory complex III of the electron transport system. Treatment with antimycin A reduced ATP levels in H4-II-E and H4-II-E/CDDP cells by approximately 98%. Under the ATP-deprived conditions, the intracellular accumulation of CDDP in H4-II-E was reduced to 39% of the level obtained under standard culture conditions (Fig. 3). However, in the H4-II-E/CDDP cell line, depletion of ATP did not result in a significantly decreased accumulation of CDDP. We also assessed the effect of antimycin A on the accumulation of CDDP derivatives (CBDCA and CDGP). The intracellular accumulation of CBDCA and CDGP in H4-II-E was reduced to 46% and 48%, respectively, of the standard level.

Copper transport

The ability of H4-II-E and H4-II-E/CDDP cells to accumulate copper was assessed by exposing the cells to $CuSO_4$ for 2 h (Fig. 4). Every copper contents were enough over the limit for detection. There was no significant difference in the basal copper level between the two cell lines. When treated with 8, 16, 32, or 64 µg/ml $CuSO_4$, the intracellular copper levels increased linearly with increasing dose. At the highest concentration, the level of intracellular copper was significantly higher ($P < 0.05$) in the H4-II-E/CDDP cells than in the parental cells.

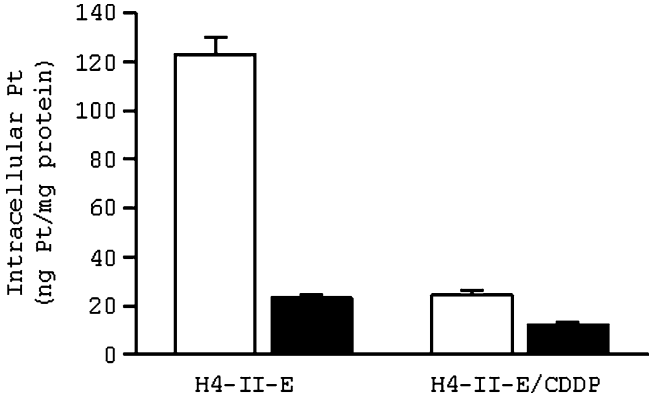

Fig. 2 Influence of temperature reduction on platinum accumulation. Cells were incubated with CDDP (10 µg/ml) at individual temperatures for 2 h. The amount of accumulated platinum was determined by FAAS. The symbols used are as follows: *open square*, 37°C; *filled square*, 4°C. Data represent the mean ± SD for three individual experiments

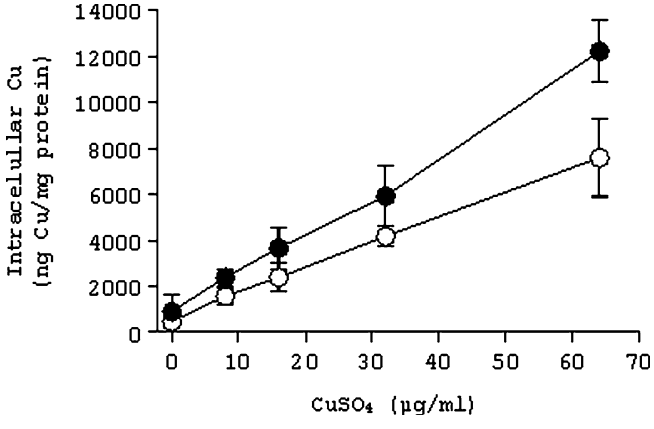

Fig. 4 Intracellular copper accumulation following $CuSO_4$ exposure. Cells were incubated with $CuSO_4$ for 2 h. The amount of accumulated copper was determined by FAAS. The symbols used are as follows: *open circle*, H4-II-E; *filled circle*, H4-II-E/CDDP. Data represent the mean ± SD for three individual experiments

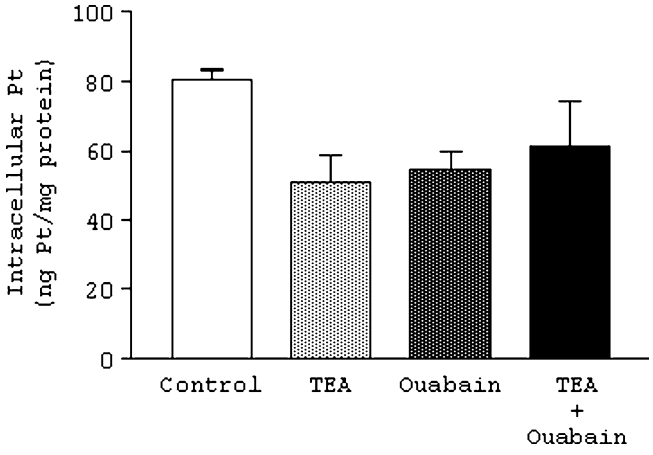

Fig. 5 Influence of TEA and/or ouabain on platinum accumulation. These agents (TEA: 20 mg/ml, ouabain: 0.73 mg/ml) were added 60 min prior to a 2-h exposure of cells to CDDP (10 μg/ml). The amount of accumulated platinum amounts was determined by FAAS. The symbols used are as follows: *open rectangle*, Control; *filled rectangle*, TEA; *filled rectangle*, ouabain; *filled rectangle*, TEA + ouabain. Data represent the mean ± SD for three individual experiments. The statistical significance of differences between control and test groups was determined by Dunnet's multiple comparison tests (*$P < 0.05$)

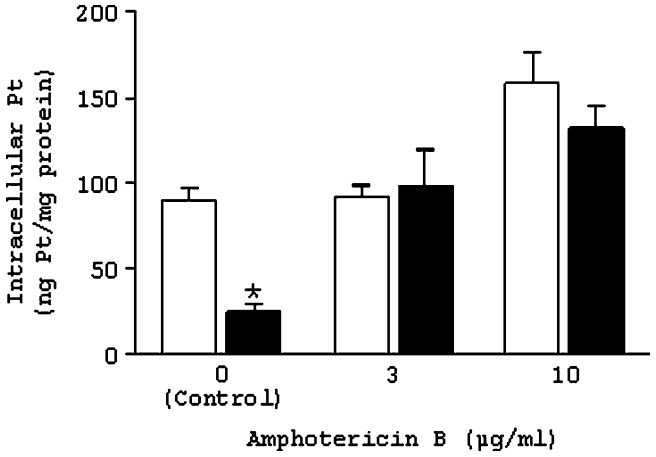

Fig. 6 Influence of amphotericin B on platinum accumulation. Amphotericin B was added 60 min prior to a 2-h exposure of cells to CDDP (10 μg/ml). The amount of accumulated platinum was determined by FAAS. The symbols used are as follows: *open square*, H4-II-E; *filled square*, H4-II-E/CDDP. Data represent the mean ± SD for three individual experiments. Statistical significance of the differences between H4-II-E and H4-II-E/CDDP was determined by Student's *t* test (*$P < 0.05$)

Effect of transporter inhibitors

Coincubation of CDDP with the Na^+, K^+-ATPase inhibitor, ouabain, or the K^+ channel inhibitor, TEA, resulted in a significant decrease in platinum accumulation in the H4-II-E cells (Fig. 5). In the presence of both ouabain and TEA, the platinum concentration in H4-II-E was not significantly different than with either ouabain or TEA alone. However, in H4-II-E/CDDP cells, ouabain and TEA did not result in a significantly decreased accumulation of platinum (data not shown).

Effect of amphotericin B

The effect of amphotericin B, a Na^+ ionophore, on the accumulation of CDDP was assessed in H4-II-E and H4-II-E/CDDP cells (Fig. 6). Amphotericin B dose-dependently enhanced the platinum accumulation. At 3 μg/ml of amphotericin B, the platinum accumulation in H4-II-E/CDDP was increased approximately fourfold over the control group, although accumulation in H4-II-E cells was unaffected. Amphotericin B at 10 μg/ml elicited approximately two- and fivefold increases in the platinum accumulation in the H4-II-E and H4-II-E/CDDP cells, respectively.

Expression of Na^+, K^+-ATPase

Expression of Na^+, K^+-ATPase α1 and β1 subunits were assessed in the membrane fraction of H4-II-E and H4-II-E/CDDP cells (Fig. 7a). Immunoblot analysis revealed greatly reduced levels of Na^+, K^+-ATPase α1 subunit in H4-II-E/CDDP cells compared with the parental cells. However, there was no difference in Na^+, K^+-ATPase β1 subunit expression between the two cell lines. Also, amphotericin B caused no change in the levels of Na^+, K^+-ATPase α1 or β1 subunit (data not shown). Furthermore, the effects of CDDP exposure on Na^+, K^+-ATPase subunits expression were assessed in H4-II-E (Fig. 7b). Following a 24-h exposure to CDDP (10 μg/ml), the level of Na^+, K^+-ATPase α1 subunit was markedly decreased, but that of β1 subunit had no change.

Discussion

We previously established the novel CDDP-resistant cell line H4-II-E/CDDP by continuous exposure to CDDP [11]. Compared with the parental cell line, H4-II-E/CDDP cells exhibited an approximately ninefold increase in resistance to CDDP. The dominant factor in CDDP resistance in these cells is a reduced uptake of CDDP, resulting in a reduction of platinum-DNA adduct formation.

There have been many studies on the mechanisms of resistance to CDDP, and decreased intracellular accumulation of CDDP has been reported in a large number of them. There is increasing evidence that some component of CDDP influx must be mediated by active transport. Dornish and Pettersen [12] reported that aldehyde derivatives reduced the cytotoxicity of CDDP by interacting with a factor on the extracellular surface of the cell. In addition, Mann et al. [6] reported that the membrane fluidity was not significantly different in the sensitive and resistant cells, although there were slight differences in the lipid compositions of the cells.

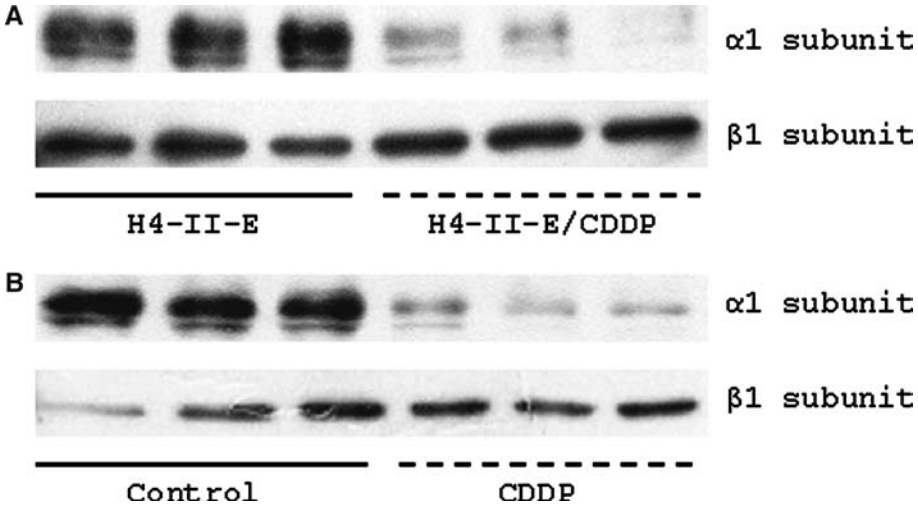

Fig. 7 Expression of the Na^+, K^+-ATPase α1 and β1 subunit proteins: **A** H4-II-E and H4-II-E/CDDP; **B** H4-II-E control cells and cells following a 24-h exposure to CDDP (10 μg/ml). The membrane fraction was isolated from each cell line. Membrane proteins (10 μg) were subjected to SDS-PAGE and analyzed by Western blotting with antibodies specific for each of the Na^+, K^+-ATPase subunits

Andrews et al. [7] pointed out the significance of a CDDP transport system being dependent on the function of Na^+, K^+-ATPase. Furthermore, many studies have confirmed the importance of a membrane potential maintained by Na^+, K^+-ATPase in CDDP accumulation [13–15]. However, why there down-regulation of Na^+, K^+-ATPase occurs and what differences there are in the membranes of the H4-II-E/CDDP cells is not currently clear.

This study showed that, following a 2-h exposure to CDDP, the intracellular accumulation of platinum was significantly lower in the resistant cell line than in the parental cell line. We have already reported that there was no difference in CDDP efflux between two cell lines [11]. The difference in CDDP accumulation may be due to the ability of the cells to take up CDDP via active transport. Uptake by the parental cell line was significantly affected by lowering the temperature or depleting the ATP with antimycin A. In contrast, ATP depletion did not affect platinum accumulation in the H4-II-E/CDDP cell line. Thus, it is obvious that the resistant cells have lost the system for actively transporting CDDP and other platinum compounds.

It has been pointed out that copper transporter CTR1 function influences the uptake of all of the clinically used platinum-containing drugs [16–19]. Beretta et al. [18] reported that impairment of intracellular CDDP accumulation is not mediated by CTR1 and that it plays a marginal role in the cellular pharmacology of CDDP. In this report, copper accumulation was compared between H4-II-E and H4-II-E/CDDP cells following exposure to $CuSO_4$. Although H4-II-E/CDDP showed markedly lower platinum accumulation than the parent cells, the copper contents of H4-II-E/CDDP in basal or following exposure to $CuSO_4$ were rather a little higher than those of H4-II-E. These results suggest that CTR1 is not down-regulated in the H4-II-E/CDDP cell line. We do not know whether other copper transporting factors (ATP7A and ATP7B) associated with CDDP resistance [20] have been affected in the H4-II-E/CDDP cells. However, there did not appear to be a relationship between platinum and copper influx in our resistant cell line.

Na^+, K^+-ATPase plays an important role in active transport through the cell membrane and in supporting physical ion balances [21, 22]. In addition, its enzyme activity is reported to be essential for the intracellular accumulation of CDDP except for the passive transport. Na^+, K^+-ATPase consists of α and β subunits present in a 1:1 molar ratio. Although the α subunit is the catalytic subunit of Na^+, K^+-ATPase, its assembly with a β subunit in the endoplasmic reticulum is required for stable expression, full function, and transport to the cell membrane [23, 24]. Binding of ouabain to the extracellular domain of the α subunit inhibits the activity of the Na^+, K^+-ATPase [25]. Ouabain has been used for the identification of active transport of CDDP in a number of cell lines [26–28]. TEA is a K^+ channel inhibitor that can inhibit the function of Na^+, K^+-ATPase indirectly. Kolb et al. [29] demonstrated that TEA reduced the accumulation of CDDP in the renal proximal tubule. We found that these inhibitors significantly decreased the platinum accumulation exclusively in H4-II-E cells. The effect of these drugs was not additive. These results suggest that the important factor in CDDP influx may be not only the function of Na^+, K^+-ATPase but also the electrochemical gradient across the membrane.

Amphotericin B, an antifungal agent, has previously been shown to reverse resistance to CDDP [30, 31]. Both the rate of ouabain-sensitive potassium uptake and Na^+, K^+-ATPase activity are markedly elevated by amphotericin B [32]. We found that platinum accumulation in the H4-II-E/CDDP cells was recovered by amphotericin B to the same extent as in the parental cells. These results suggest that H4-II-E has an active transport system for CDDP that is dependent on the membrane potential generated by Na^+, K^+-ATPase. Also, The effects of amphotericin B on the membrane structure and other transporters should be paid much attention to.

Gately and Howell [5] have postulated a gated channel dependent on the function of the Na^+, K^+-ATPase as the route for CDDP influx. Also, although there was no difference in ATP content between two cell lines (data not shown), based on the results from coexposure with antimycin A or ouabain there may be an abnormality in the function of the Na^+, K^+-ATPase in H4-II-E/CDDP cell lines. Oubain is a representative agent that inhibit Na^+, K^+-ATPase. Although we have not assessed the effect of ouabain on the activity of Na^+, K^+-ATPase, it has been confirmed that ouabain shows the inhibitive effect on CDDP accumulation in a dose-dependent manner and that its effect has reached the maximum level at 1 mM. The relationship between the activity of Na^+, K^+-ATPase and CDDP accumulation may give the important information about the route of CDDP influx. Moreover, coexposure with amphotericin B showed that there may be some residual Na^+, K^+-ATPase in the H4-II-E/CDDP cells. We speculated that down-regulation of Na^+, K^+-ATPase might be caused by a decreased expression of either the α or β subunits of Na^+, K^+-ATPase. We found no difference in the expression of the β1 subunit, but there was a significant decrease in the level of the α1 subunit in the H4-II-E/CDDP cells. Also, CDDP exposure decreased the level of the α1 subunit in the H4-II-E cells. Thus, it was suggested that the continuous exposure to CDDP transformed H4-II-E cells into cells with a permanent decreased α1 subunit level. Given that the α subunit is the catalytic subunit, the decreased level of the α1 subunit may result in resistance to CDDP in H4-II-E/CDDP cells by reducing the membrane potential and, thus, CDDP influx via a voltage-sensitive channel.

In the present study, we assessed the route of CDDP influx and the mechanism of CDDP resistance using the rat hepatoma cell lines H4-II-E and H4-II-E/CDDP. The H4-II-E cell line showed active transport of CDDP that was dependent on the function of Na^+, K^+-ATPase. We found a decrease in the expression of the Na^+, K^+-ATPase α1 subunit in the CDDP-resistant H4-II-E/CDDP cells. Therefore, our results suggest that the Na^+, K^+-ATPase α1 subunit is a key factor in CDDP transport.

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