Cisplatin-induced renal injury in LLC-PK_{1} cells

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Abstract

Nephrotoxicity is one of the important adverse effects induced by cisplatin (CDDP), a potent anticancer drug. CDDP causes a disorder of renal proximal tubules. So, we evaluated mechanisms of renal tubular injury induced by CDDP using established epithelial cell line, LLC-PK\(_{1}\), which is characteristic of renal proximal tubular epithelium. LLC-PK\(_{1}\) cells were incubated in culture media except CDDP subsequently after exposure to CDDP for five hours. The production of reactive oxygen species (ROS) increased in LLC-PK\(_{1}\) cells which was an earlier event than the increase in the release of LDH occurring at 48 hr after the exposure. CDDP induced also the activation of MAP kinase, ERK1/2. U0126, an ERK inhibitor, inhibited LDH leakage from the cells 48hr after the CDDP exposure. An antioxidant, tempol, significantly prevented CDDP-induced activation of ERK1/2 and cell injury. BAPTA-AM, an intracellular calcium chelator, prevented the increases in production of ROS and cell injury induced by CDDP. The results suggest that CDDP –induced increase in intracellular calcium causes oxidative stress to induce renal tubular injury via ERK1/2 activation leaded by ROS. Furthermore, these results indicate that established renal cell line, LLC-PK\(_{1}\), is very effective method for elucidation of nephrotoxic mechanisms.

Keywords: nephrotoxicity, cisplatin, renal cell culture, reactive oxygen species, intracellular calcium

Introduction

Cisplatin (CDDP) is one of the effective antitumor agents in the treatment of various solid tumors (Rosenberg, 1977, Boulikas, 2004). It is effective for testis, bladder, head and neck, lung and ovarian cancer. However, CDDP causes sever adverse effects, such as acute renal failure, gastrointestinal toxicity and ototoxicity (Hill, 1982). In particular, its clinical use is limited due to the acute renal failure induced by CDDP. Therefore, we think that an elucidation of mechanisms for nephrotoxicity induced by CDDP is necessary for its effective clinical use. CDDP nephrotoxicity has been shown to be dose-related in both animals and humans (Dobyan, 1980, Chopra, 1982). CDDP is mainly excreted into urine and kidney tissue content of CDDP is higher than the concentration in other organs (Jacobs, 1980). The major site of morphological damage is the S3 segment of the proximal tubule. CDDP stimulates production of reactive oxygen species in epithelial cells (Kawai, 2006, Tsutsumishita, 1998). We also think that oxidative stress plays an important role in the pathogenesis of CDDP-induced nephrotoxicity. Furthermore, it has been reported that CDDP increases the intracellular calcium level in the renal cortical slices and causes an abnormality of intracellular signal transduction pathway leading to renal cell injury (Kim, 1997). Reactive oxygen species (ROS) have recently been demonstrated to act as second messengers in signal transduction pathways, including MAP kinase (Ramachandrian, 2002). In particular, oxidative stress has been reported to induce activation of extracellular signal-regulated kinase (ERK), one of the MAP kinase. And activated ERK has been shown to result in cell injury (Matsunaga, 2005). Therefore we investigated the role of activated ERK for nephrotoxicity induced by CDDP.

We showed \textit{in vitro} methods in renal toxicology (Table 1). Because CDDP causes renal damage in proximal tubule, we used the established renal epithelial cells, LLC-PK\(_{1}\), which is characteristic of proximal epithelial cells. It is easy to grow, maintain, and handle renal epithelial cell lines which retain some of the basic renal epithelial functions. So, we used established cell lines, LLC-PK\(_{1}\) and investigated the mechanisms for tubular epithelial cell injury caused by CDDP.
Materials and methods

Animal experiments
We used male Sprague-Dawley (SD) rats weighing 180 to 230 g. The experiment was conducted in accordance with the Osaka University of Pharmaceutical Sciences Animal Experiment Guidelines. CDDP (7.5 mg/kg) was administered intravenously to rats. Urine was collected in metabolic cages for 18 hr after administration of CDDP. After urine volume was measured, urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) in urine was measured as an index of nephrotoxicity.

Cell culture experiments
LLC-PK₁ cells were cultured for 4 days for reaching confluence and then the culture medium was exchanged for serum-free medium with CDDP. Final concentration of CDDP was 20 μM in culture medium. When the medium was exchanged, an antioxidant, tempol, and an ERK inhibitor, U0126, were added to the medium. LLC-PK₁ cells were cultured in medium containing CDDP for 5 hr. After exposure to CDDP for 5 hr, medium was exchanged for medium without CDDP. LLC-PK₁ cells were incubated consecutively in culture medium except CDDP. After exposure to CDDP, we measured a rate of lactate dehydrogenase (LDH) release from cells to the medium as an index of cytotoxicity. The quantity of intracellular ROS was detected using DCFH-DA and ERK activity was detected using western blotting analysis.

Next, we examined an increase in intracellular calcium in the CDDP-exposed renal cell. Confluent LLC-PK₁ cells were exposed to 500 μM CDDP for 1 and 5 hr.

Results
CDDP increased LDH release from LLC-PK₁ cells 48 hr after exposure to CDDP (Fig. 1A). Increases in LDH release induced by CDDP were further augmented at 72 hr. Fig. 1B shows effect of CDDP on urinary excretion of NAG in rats. Urinary excretion of NAG of nephrotoxic index was increased 48 hr after treatment of CDDP with rats, being comparable with the time-course of CDDP injury in LLC-PK₁ cells.

We examined whether ERK involved in renal cell
An ERK inhibitor, U0126, significantly inhibited renal cell injury by CDDP (Fig. 2). In order to prove a detailed role of ERK in renal cell injury by CDDP, we investigated an effect of an antioxidant, tempol, on ERK activity in LLC-PK1 cells exposed to CDDP. ERK activity was stimulated transiently about 3-fold compared to control 8 hr after CDDP addition. Tempol inhibited ERK activation induced by CDDP.

Next, we examined participation of an increase in intracellular calcium in production of ROS in the CDDP-induced renal cell injury. Confluent LLC-PK1 cells were exposed to 500 μM CDDP for 5 hr and then we measured rate of LDH release as cytotoxic index. We examined the change of intracellular ROS level exposed to CDDP. In order to measure intracellular ROS, we used a fluorescence dye, CM-H2DCFDA. CM-H2DCFDA is changed intracellularly to CM-H2DCF and it is oxidized by ROS to be displayed by fluorescence. Therefore, an extent of fluorescence intensity shows a level of intracellular ROS. BAPTA-AM, an intracellular calcium chelator, ameliorated cell injury induced by CDDP (Table 2). CDDP increased ROS generation from 1 hr to 5 hr after its exposure to the cells (data not shown). Such increase in ROS level by CDDP was prevented completely by BAPTA-AM 1 hr after the exposure (Fig. 4). CDDP increased the generation of ROS prior to cell injury in cultured renal epithelial cells. BAPTA-AM inhibited the ROS production in LLC-PK1 cells exposed to CDDP for 1 hr.

Table 2. Effect of BAPTA-AM on cell injury induced by CDDP

<table>
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<tr>
<th>LDH release</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>500 μM CDDP</td>
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<tr>
<td>CDDP + BAPTA-AM</td>
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<td>100 μM BAPTA-AM</td>
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Values represent the mean ± SEM. * P < 0.001 compared to control.

Discussion

We investigated the roles of a level of intracellular calcium and an activity of MAP kinase, ERK, in oxidative injury induced CDDP. CDDP induced increases in ROS production and activation of ERK. An antioxidant, tempol, inhibited cell injury and activation of ERK induced by CDDP. Furthermore, an ERK inhibitor, U0126, ameliorated cell injury induced by CDDP.

We have reported that cell injury is caused 3 hr after exposure to CDDP and intracellular calcium level and production of ROS increased 1 hr after the exposure, which is earlier than the time of the increase in release of LDH (Kawai, 2006). However, an antioxidant, DPPD, did not inhibit CDDP-induced increase in the intracellular calcium level. In this study, an intracellular calcium chelator, BAPTA-AM, prevented the cell injury induced by CDDP. Therefore, the increase in intracellular calcium concentration in the early phase induced by CDDP has been suggested to play an important role in the generation of ROS in LLC-PK1 injury exposed to CDDP.

Recently, it has been demonstrated that ROS acts as second messengers in signal transduction pathways, including MAP kinase (Ramachandrian, 2002). In particular, oxidative stress induces activation of extracellular signal-related kinase (ERK), one of the MAP kinase. Therefore, we examined the role of activated ERK in renal cell injury induced by CDDP. An ERK was activated in LLC-PK1 cells 8 hr after exposure to CDDP. Antioxidant tempol completely inhibited ERK activation by CDDP. An ERK inhibitor, U0126, ameliorated cell injury induced by CDDP. These results suggest that CDDP-...
induced increase in ROS level causes activation of ERK in LLC-PK₁ cells.

The kidney is a highly complex organ, so the assessment of impaired organ function cannot easily be explored in in vivo studies. Evaluation of renal injury using such in vitro models would neglect the hemodynamic, extrarenal variables and reduce the number of animals used in research (Prieto, 2002, Pfaller, 1998). In particular, established renal epithelial cell lines are very useful as models for the study on tubular damage caused by drugs and chemicals. The time-course of in vitro cell injury induced by CDDP in LLC-PK₁ cells agreed with the nephrotoxic results obtained in vivo with rats treated with CDDP.

In conclusion, CDDP-induced increase in intracellular calcium level causes ROS production to induce renal tubular injury via ERK activation. This study indicates that using established renal cell line, LLC-PK₁, is very effective method for elucidation of nephrotoxic mechanism caused by CDDP.

Acknowledgements

This research was supported by a Grant-in-Aid for High Technology Research from the Ministry of Education, Sciences, Sports, and Culture, Japan.

References
