Stress response inhibits the nephrotoxicity of cisplatin

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Hanigan, Marie H., Mei Deng, Lei Zhang, Peyton T. Taylor Jr., and Maia G. Lapus. Stress response inhibits the nephrotoxicity of cisplatin. Am J Physiol Renal Physiol 288: F125–F132, 2005. First published September 7, 2004; doi:10.1152/ajprenal.00041.2003.—Salt loading and saline hydration are used to protect patients from cisplatin-induced nephrotoxicity. The mechanism by which salt exerts its protective effect is unknown. As part of an ongoing study of cisplatin nephrotoxicity, an in vitro assay system was developed that models the in vivo exposure and response of proximal tubule cells to cisplatin. In this study, it was discovered that the toxicity of cisplatin toward LLC-PK1 cells varied dramatically according to the tissue culture media used for 3-h cisplatin exposure. Further experiments revealed that minor variations in the sodium concentration among standard tissue culture media modulated cisplatin nephrotoxicity. NaCl has been shown to protect against cisplatin-induced nephrotoxicity in vivo but has never before been demonstrated in vitro. NaCl did not alter the cellular accumulation of cisplatin. NaCl altered the osmolarity of the external media, and its effect was replicated by substituting equiosmolar concentrations of impermeant anions or cations. The change in osmolarity triggered a stress response within the cell that modulated sensitivity to cisplatin. These data resolve several long-standing controversies regarding the mechanism by which salt loading protects the kidney from cisplatin-induced nephrotoxicity.

Despite the widespread use of salt, the mechanism by which salt protects the kidney has never been identified. Several theories have been proposed to explain the protective effect of hydration against cisplatin nephrotoxicity. Some investigators have suggested that hypertonic saline and saline hydration protect the kidney by increasing the rate of cisplatin excretion (9). Others have proposed that the salt provides a high concentration of chloride ions, which prevents the dissociation of the chloride ions from the platinum molecule, thereby reducing the formation of the reactive, aquated species of cisplatin (5, 10). There has been debate as to whether the sodium or the chloride ion is responsible for the protective effect. Litterst’s (21) mouse data indicated that the sodium ion is the important molecule, whereas Earhart and co-workers (10) reported data from rat studies suggesting that the chloride is the protective ion.

As part of ongoing studies on the mechanism of cisplatin nephrotoxicity, we developed an in vitro model system of cisplatin-induced toxicity toward proximal tubule cells (33). Confluent monolayers of cells are treated for 3 h with cisplatin. The drug is removed and cell viability is assessed at 3 days. This treatment mimics the in vivo exposure. In this system, cisplatin toxicity is dose and time dependent. During the course of our studies, we found that treating the cells with cisplatin in RPMI-1640 medium was more toxic than treating the cells with cisplatin in Dulbecco’s Modified Eagle Medium (DMEM). Both RPMI-1640 medium and DMEM are commonly used to maintain mammalian cells in culture. Their formulations are very similar. In this study, we analyzed the effect of four commonly used tissue culture media on the toxicity of cisplatin. We tested individual components and found that the NaCl concentration in the media modulated cisplatin toxicity. The robust effect of NaCl on cisplatin toxicity in this in vitro system is similar to that observed in vivo. This model system enabled us to test directly several of the hypotheses regarding the mechanism by which NaCl protects the kidney from cisplatin toxicity.

MATERIALS AND METHODS

Cell culture. LLC-PK1 [American Type Culture Collection (ATCC) CRL 1392], a pig kidney cell line, was purchased from ATCC (Manassas, VA). LLC-PK1 cells were grown in DMEM supplemented with 4 mM l-glutamine (DMEM, GIBCO/BRL, Grand Island, NY), 5% FBS (Hyclone Laboratories, Logan, UT), penicillin (50 U/ml), and streptomycin (50 μg/ml, GIBCO/BRL). Cells were maintained in 5% CO2-95% air at 37°C. Subconfluent cultures were passaged every 3 to 4 days. For toxicity experiments, LLC-PK1 cells

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were seeded in 96-well plates at 5 × 10^3 cells/well. The cells formed confluent monolayers on day 3 after plating. The medium was replaced with fresh medium on day 4, and the cells were used for experiments on day 7 after plating.

**Cisplatin toxicity.** Cisplatin (Sigma, St. Louis, MO) was prepared as a 3.3 mM stock solution in 0.9% saline daily. RPMI-1640 Medium (RPMI, Cat. No. 31800), DMEM (Cat. No. 12800), Leibovitz’s L-15 Medium (L-15, Cat. no. 41300), Nutrient Mixture Ham’s F-12 (F-12, Cat. no. 21700), and HBSS (Cat. no. 11201) were prepared from powder (GIBCO/BRL). L-15 was supplemented with 18 mM HEPES (Cat. no. 21700), and HBSS (Cat. no. 11201) were prepared from RPMI, HBSS, and DMEM were prepared according to their formulaations in the GIBCO/BRL catalogue, but without sodium bicarbonate and the pH adjusted to pH 7.4. For toxicity experiments, the media on the cells was changed to the test solution and cisplatin was added to the wells. Cells treated with only the test solution served as controls. The cells were incubated at 37°C in 5% CO_2, except cells in L-15 medium that were incubated at 37°C without CO_2. After 3 h, the cisplatin-containing solution was removed from the cells and replaced with DMEM containing 5% FBS and antibiotics. The cells were incubated for an additional 69 h at 37°C in 5% CO_2. The number of viable cells was then determined with the MTT assay (23). A standard curve showed a direct correlation between the number of viable LLC-PK1 cells and the OD_270 in the MTT assay.

**Effect of NaCl concentration and osmolality on cisplatin toxicity.** Solutions containing the inorganic salt components of the media were tested showed that the NaCl concentration in the cisplatin-containing solution was the same as its corresponding control. Solutions containing NaCl and mannitol or NaCl and N-methyl-d-glucamine were prepared with 5 mM HEPES, pH 7.4. Equismolar solutions were prepared by decreasing the concentration of mannitol or N-methyl-d-glucamine as the concentration of NaCl increased to maintain the osmolality of the solution at 288 mosmol/kgH_2O. The osmolality of the tissue culture media and salt solutions was determined with a Wescor 5520 VAPOR Vapor Pressure Osmometer (Logan, UT).

**Cisplatin uptake.** LLC-PK_1 cells were seeded in 24-well plates at 5 × 10^4 cells/well. On day 3, 100 μM cisplatin in 125 to 150 mM NaCl in DMEM with 5% FBS and antibiotics. The cells were treated with cisplatin for 3 h and then returned to complete DMEM. Toxicity was assessed at 72 h. The media on the cells in the four experimental groups varied only during the 3-h cisplatin exposure. Prior to and following cisplatin treatment, all cells were in complete DMEM. The data in Fig. 1A show that cisplatin in RPMI or F-12 media was significantly more toxic to LLC-PK_1 cells than equimolar cisplatin in DMEM or L-15 media. A dose-dependent toxicity of cisplatin was observed with RPMI and F-12 media containing cisplatin. At 100 and 150 μM, cisplatin was significantly less toxic in DMEM or L-15 media (P < 0.001).

To determine which components of the media modulated cisplatin toxicity, components of the media were tested with the same protocol used for the media. The cells were exposed to cisplatin in the test solutions for 3 h, and toxicity was measured at 72 h. Data from experiments in which the inorganic salt components of the media were tested showed that the salt solutions modulated cisplatin toxicity (Fig. 1B). The composition of the solutions tested is shown in Table 1. There was a direct correlation between the concentration of NaCl in the solution and the reduction in cisplatin toxicity: saline > L-15 > HBSS > F-12 > DMEM > RPMI. The higher the concentration of NaCl, the less toxic cisplatin was to the cells. The

Pretreatment with salt. DMEM was prepared according to the formulation in the GIBCO/BRL catalogue with the exception of NaCl, which was omitted from the stock solution so that the concentration could be varied within the experiment. Three hours before cisplatin treatment, the medium was changed to fresh DMEM containing 5% FBS, antibiotics, and various concentrations of NaCl. After 3 h, the media were removed and the cells treated with 100 μM cisplatin in DMEM. At the end of the 3-h cisplatin exposure, the cisplatin was removed and cells returned to DMEM containing 5% FBS and antibiotics for 69 h. The number of viable cells was determined with the MTT assay.

**DNA platination.** LLC-PK_1 cells were seeded in P-60 plates at 5.7 × 10^4 cells/plate. On day 5, the confluent cells were exposed to 100 μM cisplatin in 125 to 150 mM NaCl with 5 mM HEPES, pH 7.4, at 37°C without CO_2. The cells were washed with PBS and trypsinized. DNA was purified by the method of McKeage and co-workers (22). Briefly, cells were lysed (10 mM Tris, 10 mM EDTA, 0.15 M NaCl, and 0.5% SDS, pH 8.0) in the presence of 1 mg/ml protease K. The lysis mixture was incubated at 60°C for 10 min and then 37°C overnight. DNA was purified by phenol extraction and ethanol precipitation. The DNA pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0, with addition of 100 μg/ml RNase and incubation at 37°C for 1 h. DNA was then precipitated and hydrolyzed in 0.2% HNO_3. DNA concentration was estimated by measuring the absorbance at 260 nm, and the platinum content was measured by FAAS as described above.

**Data analysis.** Each experiment was done a minimum of three times. Each data point within the experiment represents the mean value of three to six replicate wells. To detect statistically significant effects of medium or inorganic salt solutions on cisplatin nephrotoxicity, the toxicity of each concentration of cisplatin in each of the solutions was compared with the toxicity of cisplatin in L-15 by a Student’s t-test. Significant correlations between the salt concentration in the solution and cisplatin toxicity, platinum accumulation or platinum-DNA binding were detected by one-way ANOVA and posttest for a linear trend. Statistically significant difference between curves were detected by an F-test. All statistical analyses were done using GraphPad Prism version 3 (GraphPad Software, San Diego, CA).

**RESULTS**

**Effect of medium on cisplatin toxicity.** Tissue culture media modulated the toxicity of cisplatin toward proximal tubule cells. LLC-PK_1 cells were grown to confluence and maintained in DMEM with 5% FBS and antibiotics. The cells were treated with cisplatin for 3 h and then returned to complete DMEM. Toxicity was assessed at 72 h. The media on the cells in the four experimental groups varied only during the 3-h cisplatin exposure. Prior to and following cisplatin treatment, all cells were in complete DMEM. The data in Fig. 1A show that cisplatin in RPMI or F-12 media was significantly more toxic to LLC-PK_1 cells than equimolar cisplatin in DMEM or L-15 media. A dose-dependent toxicity of cisplatin was observed with RPMI and F-12 media containing cisplatin. At 100 and 150 μM, cisplatin was significantly less toxic in DMEM or L-15 media (P < 0.001).

To determine which components of the media modulated cisplatin toxicity, components of the media were tested with the same protocol used for the media. The cells were exposed to cisplatin in the test solutions for 3 h, and toxicity was measured at 72 h. Data from experiments in which the inorganic salt components of the media were tested showed that the salt solutions modulated cisplatin toxicity (Fig. 1B). The composition of the solutions tested is shown in Table 1. There was a direct correlation between the concentration of NaCl in the solution and the reduction in cisplatin toxicity: saline > L-15 > HBSS > F-12 > DMEM > RPMI. The higher the concentration of NaCl, the less toxic cisplatin was to the cells. The
concentrations of the other components did not correlate with cisplatin toxicity. It should be noted that DMEM reduced the toxicity of cisplatin (Fig. 1A). However, when the inorganic salt solution of DMEM was tested, it potentiated cisplatin toxicity (Fig. 1B). DMEM contains 44 mM NaHCO₃, which is higher than the concentration in F-12 or RPMI (14 and 24 mM, respectively). NaHCO₃ increased the Na⁺ ion concentration in DMEM media by 39 mM compared with the Na⁺ ion concentration in the DMEM inorganic salt solution tested. The inorganic salt fraction of L-15 is also somewhat less protective than L-15 medium. There may be additional components in L-15 medium that modulate cisplatin toxicity.

To directly analyze the effect of NaCl on the toxicity of cisplatin, cells were incubated for 3 h in a 100 μM solution of cisplatin containing 130 to 155 mM NaCl and 5 mM HEPES, pH 7.4. As shown in Fig. 2, increasing the concentration of NaCl from 130 to 155 mM increased cell survival in 100 μM cisplatin from 22 ± 3 to 98 ± 3% (P < 0.001). In the absence of cisplatin, the 3-h incubation in the NaCl solutions had no effect on cell viability (P = 0.6).

**Effect of NaCl concentration on cisplatin accumulation.**
NaCl did not alter the accumulation of cisplatin in LLC-PK₁ cells. Platinum levels were measured in confluent monolayers treated with cisplatin solutions containing NaCl. Increasing the NaCl concentration from 125 to 155 mM had no significant effect on platinum accumulation (Fig. 3), despite the dramatic effect on toxicity (Fig. 2).

**Preconditioning cells with various concentrations of NaCl.**
A series of experiments was undertaken to determine whether the effect of NaCl on cisplatin toxicity was due to interaction of the NaCl with the cisplatin or to an effect of the salt on the cells. The cells were incubated in media with various concentrations of NaCl for 3 h before the cisplatin treatment. The preincubation media consisted of DMEM formulated with various concentrations of NaCl plus 5% FBS. At the end of the preincubation period, the media were removed and all the cells were treated with 100 μM cisplatin in DMEM containing the standard amount of NaCl. After the cisplatin treatment, the cisplatin-containing solution was removed and all cells were incubated for an additional 69 h in DMEM with 5% FBS and antibiotics. The data in Fig. 4 show that the concentration of NaCl reduced the toxicity of cisplatin in LLC-PK₁ cells when added 3 h before cisplatin or in the HEPES/NaCl solutions without cisplatin.

### Table 1. Composition of salt solutions tested in Fig. 1B

<table>
<thead>
<tr>
<th>Component</th>
<th>Saline</th>
<th>L-15</th>
<th>HBSS</th>
<th>F-12</th>
<th>DMEM</th>
<th>RPMI</th>
</tr>
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<tbody>
<tr>
<td>CaCl₂</td>
<td>—</td>
<td>1.3mM*</td>
<td>1.3</td>
<td>0.3</td>
<td>1.8</td>
<td>—</td>
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<tr>
<td>KCl</td>
<td>—</td>
<td>5.4</td>
<td>5.4</td>
<td>3.0</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
<td>0.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>—</td>
<td>0.8</td>
<td>—</td>
<td>0.8</td>
<td>0.4</td>
<td>—</td>
</tr>
<tr>
<td>NaCl</td>
<td>145.4</td>
<td>136.9</td>
<td>136.9</td>
<td>130.0</td>
<td>109.5</td>
<td>102.7</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>1.5</td>
<td>1.3</td>
<td>—</td>
<td>1.0</td>
<td>3.9</td>
<td>5.6</td>
</tr>
<tr>
<td>Na₃PO₄</td>
<td>0.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>HCl to adjust pH</td>
<td>—</td>
<td>0.2</td>
<td>—</td>
<td>0.1</td>
<td>—</td>
<td>1.0</td>
</tr>
<tr>
<td>NaOH to adjust pH</td>
<td>0.3</td>
<td>—</td>
<td>0.3</td>
<td>—</td>
<td>0.6</td>
<td>—</td>
</tr>
<tr>
<td>Na⁺</td>
<td>149.2</td>
<td>139.5</td>
<td>137.8</td>
<td>132.0</td>
<td>119.1</td>
<td>113.9</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>145.4</td>
<td>147.1</td>
<td>144.9</td>
<td>134.9</td>
<td>118.5</td>
<td>109.1</td>
</tr>
</tbody>
</table>

*All concentrations are mM.*
NaCl in the preincubation medium had a dramatic effect on the toxicity of cisplatin. Pretreatment of the cells with higher concentrations of Na\(^+\) ions in DMEM was correlated with greater cisplatin toxicity. Cell survival decreased from 80.4 ± 7.6 to 2.4 ± 2.9%. The linear trend was highly significant (P < 0.0001). All of the cells were treated with the same cisplatin solution. These data show that the NaCl concentration is modulating cisplatin toxicity through its effect on the cell rather than through a direct interaction with the cisplatin. Controls showed that the pretreatment with the media alone (no cisplatin) had no significant effect on cell viability (P = 0.18).

Higher concentrations of NaCl during the pretreatment period resulted in increased sensitivity to cisplatin toxicity (Fig. 4), whereas higher NaCl concentrations during the cisplatin exposure were protective (Fig. 2). These data suggest a correlation between osmotic induction of changes in cell volume and sensitivity to cisplatin. In the experiment shown in Fig. 2, all of the cells were incubated in DMEM, which contains 156 mM sodium, before the cisplatin exposure. The medium was then changed to solutions with 130 to 155 mM NaCl during the cisplatin exposure. The decrease in extracellular salt concentration may have caused cell swelling. The data show that there was a dose-response relationship between the increase in cisplatin susceptibility and the decrease in extracellular salt. Conversely, in the experiment shown in Fig. 4, the extracellular NaCl was varied for 3 h before the cisplatin exposure. All of the cells were then treated with 100 μM cisplatin in a solution containing 156 mM sodium. Cells that were pretreated with media containing less than 156 mM sodium experienced an increase in extracellular salt when switched to the cisplatin solution. These cells may have responded by cell shrinkage which protected them from cisplatin toxicity. The cells that had been pretreated with media containing more than 156 mM sodium experienced a decrease in extracellular salt when switched to the cisplatin solutions. These cells may have responded by swelling which made them more susceptible to cisplatin toxicity. The data in Fig. 3 demonstrate that the changes in extracellular salt concentration alters the sensitivity of the cell to cisplatin without altering the accumulation of platinum in the cell. The changes in cell volume may have modulated stress responses within the cell that altered sensitivity to cisplatin.

**Effect of osmolality on cisplatin toxicity.** An analysis of the osmolality of the four tissue culture media tested in Fig. 1A revealed a correlation between the osmolality of the media and the toxicity of cisplatin (Table 2). These data suggested that the NaCl concentration in the extracellular solution may be modulating cisplatin toxicity through an osmotic effect. To compare the effect of NaCl vs. osmolality on cisplatin toxicity, one set of cells was treated for 3 h in a 100 μM solution of cisplatin containing 115 to 155 mM NaCl and 5 mM HEPES, pH 7.4. Two additional sets of cells were incubated in the same solutions balanced with mannitol or N-methyl-D-glucamine such that the osmolality of each solution was 288 mosmol/kg H\(_2\)O. As shown in Fig. 5, maintaining the osmolality of the solution with either mannitol or N-methyl-D-glucamine protected the cells from cisplatin toxicity, indicating that the osmotic effect of NaCl modulated cisplatin toxicity. In the absence of cisplatin, the 3-h incubation in the salt solutions had no effect on cell viability (data not shown).

Four compounds were tested for their ability to modulate cisplatin toxicity by modulating the osmolality. Cells were incubated for 3 h in solutions containing 100 μM cisplatin, 115

### Table 2. Osmolality of tissue culture media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Osmolality, mosmol/kg H(_2)O</th>
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</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>286</td>
</tr>
<tr>
<td>F-12</td>
<td>298</td>
</tr>
<tr>
<td>L-15</td>
<td>330</td>
</tr>
<tr>
<td>DMEM</td>
<td>350</td>
</tr>
</tbody>
</table>
mM NaCl, 5 mM HEPES, pH 7.4 and increasing concentrations of mannitol (a sugar), sucrose (a sugar), N-methyl-D-glucamine (an impermeant cation), or gluconate (an impermeant anion). At equivalent osmolality, all four compounds reduced cisplatin toxicity to a similar level (Fig. 6). In the absence of cisplatin, the 3-h incubation in the solutions had no effect on cell viability (data not shown). The osmolality range tested in these experiments (214 to 288 mosmol/kgH₂O) is equivalent to the osmolality range of the NaCl solutions tested in Fig. 5 (∩, 115 to 155 mM). These data show that an osmotic stress response modulates the sensitivity of renal cells to cisplatin toxicity.

Effect of osmolality on cisplatin accumulation. Platinum levels were measured in confluent monolayers of cisplatin-treated cells incubated in 115 mM NaCl with mannitol. Increasing the mannitol concentration from 19 to 75 mM increased the osmolality of the solution from 232 to 288 mosmol/kgH₂O. Over this concentration range, there appears to be a decrease in platinum accumulation per cell (Fig. 7A). However, when the data are graphed with a volume correction to account for the osmolarity-induced change in cell volume, the data show that the platinum accumulation does not change over this osmolality range (Fig. 7B).

Effect of NaCl on platinum bound to DNA. Increasing the NaCl concentration resulted in a decrease in the amount of platinum bound to DNA. Increasing the NaCl concentration from 125 to 150 mM decreased the level of DNA platination from 153 ± 11 to 98 ± 15 ng/mg DNA, a 36% decrease (Fig. 8, P < 0.0001). The decrease in DNA platination is less than the 65% decrease in cisplatin toxicity over this same range of extracellular NaCl. However, altered DNA platination may be part of the mechanism by which changes in extracellular NaCl exert its protective effect.

DISCUSSION

Small variations in the osmolality among tissue culture media account for their modulation of cisplatin nephrotoxicity. NaCl and mannitol have been shown to protect against cisplatin-induced nephrotoxicity in vivo, but not in vitro (5). Our data have shown that the NaCl does not alter the accumulation of cisplatin. The salt did not exert its effect by interacting directly with cisplatin, but rather by altering the osmolality of the extracellular solution, thereby modulating a stress response within the cell.

It has been proposed that volume repletion of patients with isotonic saline protects against cisplatin-induced nephrotoxicity by flushing the cisplatin out of the body, limiting uptake into the proximal tubule cells (9). Patients treated with cisplatin are generally given up to 6 l saline/day iv (28, 34). The diuretic mannitol is also commonly used to decrease the nephrotoxicity of cisplatin. In our assay system, NaCl and mannitol protected the cells from cisplatin toxicity. The amount of cisplatin in the medium was constant during the 3-h treatment; therefore, the protective effect of these compounds was not due to decreased exposure of the cells to the drug.

The in vitro studies presented here demonstrated a large effect of NaCl on cisplatin toxicity over a narrow concentration range (130 to 155 mM). The normal reference range for sodium in serum is 135–145 mM. Sodium concentrations below 120 or above 160 mM are considered pathological (13).
Physiological saline (0.85% NaCl) is 145 mM NaCl. The sodium concentration in the tissue culture media we tested ranged from 138 to 156 mM. In calculating the sodium and the chloride concentration in each medium, the total amount of each ion included the ions contributed by the sodium salts of amino acids and the sodium hydroxide or hydrochloric acid solutions used to adjust the pH. Studies analyzing the protective effect of a variety of compounds on cisplatin toxicity often do not take into account the amount of sodium added when the sodium salt of the compound is used. Sodium selenite protected rats against cisplatin-induced nephrotoxicity without changing the pharmacokinetics of the cisplatin (35). The investigators demonstrated that selenite does not react with the cisplatin and speculated that the selenite requires bioactivation to exert its protective effect. It is possible that the sodium rather than the selenite is responsible for the protection. This scenario has been demonstrated with aminoglycosides, which are also toxic to the proximal tubules. Ticarcillin has been shown to protect against gentamicin nephrotoxicity. Sabra and Branch (31) demonstrated that the salt load associated with ticarcillin administration was the primary mechanism by which ticarcillin protected against aminoglycoside nephrotoxicity.

Clinical protocols have administered cisplatin in solutions containing as much as 3% NaCl (2, 28). Some investigators have proposed that the salt provides a high concentration of chloride ions, which prevents the dissociation of the chloride ions from the platinum molecule, thereby reducing the formation of the reactive, aquated species of cisplatin (5). In vivo NaCl must be injected shortly before or in the same solution as the cisplatin to exert its nephroprotective effect (5). Our studies in which the NaCl concentration was varied before cisplatin treatment showed that the extracellular NaCl is affecting the cell and not interacting directly with the cisplatin.

Both NaCl and mannitol protected against cisplatin-induced toxicity. At equiosmolar concentrations, they were equally protective. Studies of platinum accumulation showed that increasing the extracellular concentration of NaCl did not change the total amount of platinum in cells at 3 h. In contrast, increasing the extracellular concentration of mannitol decreased cellular platinum accumulation. These data indicate that total platinum accumulation is not the mechanism by which osmolality alters cisplatin toxicity, as these two compounds differed in their effect on accumulation while having the same effect on toxicity. There have been conflicting data regarding the mechanism by which cisplatin is taken up into cells (1). Recently, two groups have shown that cisplatin can be transported into the cell by the copper transporter Ctr1, an energy and sodium-independent transporter (17, 19, 20). Permeant ions such as sodium and chloride and impermeant ions such as mannitol have different effects on membrane permeability and transport systems (12).

We propose that osmotic stress responses are induced in proximal tubule cells by increased or decreased osmolality of the extracellular solution. These responses may be triggered by cell swelling or cell shrinkage as the osmolality of the extracellular solution changes. The volume changes are transient. Epithelial cells can readjust their volume by regulatory volume

Fig. 7. Effect of osmolality on the accumulation of platinum. Confluent monolayers of LLC-PK1 cells were incubated for 3 h in solutions containing 100 μM cisplatin, with 115 mM NaCl, 5 mM HEPES, pH 7.4. Mannitol was used to vary the osmolality of the solutions. At the end of 3 h, the cisplatin was rinsed off the cells and the amount of platinum that had accumulated in the cells was determined by FAAS. Each point is the average of 3 to 6 values ± SD. Data are expressed as the platinum accumulation per cell (A) and platinum accumulation per cell with a volume correction (B).

Fig. 8. Effect of NaCl on platinum bound to DNA. Confluent monolayers of LLC-PK1 cells were incubated for 3 h in solutions containing 100 μM cisplatin, with 5 mM HEPES, pH 7.4 and varying amounts of NaCl. At the end of 3 h, the cisplatin was rinsed off the cells, DNA was isolated, and the amount of platinum bound to the DNA was determined by FAAS. Each point is the average of 3 values ± SD.
decrease or regulatory volume increase, active processes involving ion channels and other cotransporter-mediated mechanisms (3, 25, 37). These transient changes in cell volume may affect cisplatin nephrotoxicity by any of several mechanisms. Changes in cell volume have been shown to modulate cell survival and apoptotic pathways (32). Cisplatin kills LLC-PK1 cells by a caspase-3-dependent apoptosis (18, 26, 38). Resistance to cisplatin has been associated with induction of proteins associated with other stress responses. Wachsberger and co-workers (36) reported that mammalian cells grown at pH 6.7 had elevated levels of HSP27 and were resistant to cisplatin. Changes in the osmolality of the extracellular medium have also been shown to alter chromatin structure and free radical-induced DNA-protein crosslinks (27, 30). Alteration of chromatin structure may alter the accessibility of platinum to DNA. The data in this study showed increased platinum-DNA binding at low NaCl concentrations, the conditions under which the cells were the most sensitive to cisplatin toxicity. Alternatively, an osmotic response may be modulating components of the pathway through which cisplatin is metabolized to a nephrotoxin. We have shown that the nephrotoxicity of cisplatin is due to the conjugation of cisplatin to glutathione and the subsequent metabolism of that conjugate to a cysteinyl-glycine conjugate, then to a cysteine conjugate, and finally to a reactive thiol, which is toxic to the cell (14, 15, 33). This pathway is analogous to the metabolic activation of nephrotoxic alkenes (7). In this pathway, the glutathione conjugates are formed intracellularly then excreted. The metabolism of the glutathione conjugate and the cysteinyl-glycine conjugate occurs extracellularly, catalyzed by the cell-surface enzymes GGT and aminopeptidase. The cysteine conjugates are metabolized to reactive thiols intracellularly by the cysteine-S conjugate β-lyase. The osmotic stress response could be affecting any of several steps in this pathway.

In the clinic, hydration by intravenous infusion of saline is the method most commonly used to prevent cisplatin-induced nephrotoxicity (29). Our ongoing studies into the molecular mechanism of this stress response will elucidate this pathway and provide novel strategies to block the nephrotoxic effects of cisplatin.

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