Cisplatin-induced injury of the renal distal convoluted tubule is associated with hypomagnesaemia in mice

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ABSTRACT

Background. Cisplatin is an effective anti-neoplastic drug, but its clinical use is limited due to dose-dependent nephrotoxicity. The majority of cisplatin-treated patients develop hypomagnesaemia, often associated with a reduced glomerular filtration rate (GFR), polyuria and other electrolyte disturbances. The aim of this study is to unravel the molecular mechanism responsible for these particular electrolyte disturbances.

Methods. Two groups of 10 mice were injected intraperitoneally three times, once every 4 days, with cisplatin (5 mg/kg body weight) or vehicle. Serum and urine electrolyte concentrations were determined. Next, renal mRNA levels of distal convoluted tubule (DCT) genes epithelial Mg2+ channel TRPM6, the Na+-Cl− cotransporter (NCC), and parvalbumin (PV), as well as marker genes for other tubular segments were measured by real-time qPCR. Subsequently, renal protein levels of NCC, PV, aquaporin 1 and aquaporin 2 were determined using immunoblotting and immunohistochemistry (IHC).

Results. The cisplatin-treated mice developed significant polyuria (2.5 ± 0.3 and 0.9 ± 0.1 mL/24 h, cisplatin versus control, P < 0.05), reduced creatinine clearance rate (Ccr) (0.18 ± 0.02 and 0.26 ± 0.02 mL/min, cisplatin versus control, P < 0.05) and a substantially reduced serum level of Mg2+ (1.23 ± 0.03 and 1.58 ± 0.03 mmol/L, cisplatin versus control, P < 0.05), whereas serum Ca2+, Na+ and K+ values were not altered. Measurements of 24 h urinary excretion demonstrated markedly increased Mg2+, Ca2+, Na+ and K+ levels in the cisplatin-treated group, whereas P levels were not changed. The mRNA levels of TRPM6, NCC and PV were significantly reduced in the cisplatin group. The expression levels of the marker genes for other tubular segments were unaltered, except for claudin-16, which was significantly up-regulated by the cisplatin treatment. The observed DCT-specific down-regulation was confirmed at the protein level.

Conclusions. The present study identified the DCT as an important cisplatin-affected renal segment, explaining the high prevalence of hypomagnesaemia following treatment.

INTRODUCTION

cis-Diaminedichloroplatinum(II) (cisplatin) is a widely used cytotoxic agent with a broad range of actions in the treatment of solid tumours, including ovarian, endometrial, cervical, ur- othelial, testicular, head/neck and lung cancer [1]. Cisplatin causes cytotoxic lesions in rapidly dividing cells, such as tumour cells, due to the formation of cross-links with RNA, DNA and protein [2]. Despite its effect as an anti-cancer agent, clinical use is limited as ~20% of the patients who receive high-dose cisplatin develop severe renal dysfunction, often leading to acute renal failure [3–5]. DNA-damaging agents usually have a less toxic effect on non-proliferating cells, yet, selective tubular epithelial cell damage has been demonstrated. Treatment with cisplatin or related compounds cause tissue damage by inflammation, oxidative stress injury, necrosis and/or apoptosis [4] of particularly the renal proximal tubule (PT) [6, 7] and/or the distal convoluted tubule (DCT) [8–13] in humans and a variety of animal models.
ORIGINAL ARTICLE

The renal phenotype associated with treatment of cisplatin is multifold. The majority of cisplatin-treated patients suffer from hypomagnesaemia [14, 15], often associated with a reduced glomerular filtration rate (GFR), polyuria and electrolyte disturbances such as sodium (Na⁺), calcium (Ca²⁺) and magnesium (Mg²⁺) wasting and/or hypokalaemic alkalosis [16–19]. Previously, it was suggested that the DCT could play a prominent role in causing cisplatin-induced hypomagnesaemia [20–22].

The observed nephrotoxicity likely results from cisplatin accumulation in the kidneys, which is five times higher in comparison with other tissues. Although platinum compounds are bound to proteins in the plasma, most cisplatin is unbound [5] and is freely filtered by the glomerulus. Next to filtration, renal cells secrete cisplatin from the blood to the tubular lumen [23]. The extent to which either pathway is responsible for the induction of cisplatin-induced renal cell death is unclear. Cisplatin is rapidly removed from the body as ~65% of the cisplatin is excreted via the urine within the first 4 h following treatment [24]. Cisplatin enters the tubular cell by passive diffusion and active uptake via specific transport mechanisms [25].

The present study aims to elucidate the molecular mechanism responsible for the cisplatin-induced hypomagnesaemia. In recent years, the study of patients with hypomagnesaemia has led to the identification of genes involved in Mg²⁺ handling. A large fraction of these genes localize to the early part of the DCT (DCT1), indicating its prominent role in Mg²⁺ reabsorption [26]. Examples are the transient receptor potential channel melastatin subtype 6 (TRPM6), the epidermal growth factor, the voltage-gated K⁺ channel Kv1.1 and the Na⁺/K⁺-ATPase γ-subunit (γ-Na⁺/K⁺-ATPase). We hypothesize that cisplatin-induced hypomagnesaemia is caused by specific damage to the DCT. The effect of chronic cisplatin treatment on serum Na⁺, potassium (K⁺), Ca²⁺ and Mg²⁺ and urine Na⁺, K⁺, Ca²⁺, Mg²⁺ and phosphate (P) levels was evaluated in mice. The mRNA and protein levels of renal Mg²⁺ and Na⁺ transporting proteins were studied to indicate which segments of the tubule were affected.

**METHODS**

**Animal studies**

Female C57BL/6J mice (10 weeks of age) were purchased from Charles River (L’Arbresle Cedex, France) and housed in a temperature- and light-controlled room with ad libitum access to standard pellet chow (SSNIF Spezialdiäten GmbH, Soest, Germany) and drinking water. Mice were randomly assigned to a control and a cisplatin treatment group (n = 10 per group). Cisplatin was administered using an intraperitoneal injection (5-mg/kg body weight per injection), while control animals received an intraperitoneal injection of vehicle only (0.9% (w/v) NaCl solution). The injections were administrated on days 0, 4 and 8. Before the start of the experiment, and on day 12, the mice were individually housed in metabolic cages enabling 24-h urine collections (under mineral oil to prevent evaporation) and to measure water and food intake. On day 13, blood samples were taken under isoflurane anaesthesia, after which the mice were sacrificed. Subsequently, kidneys were frozen immediately in liquid nitrogen or incubated in periodate-lysine-paraformaldehyde (PLP) solution for the isolation of mRNA and protein or immunohistochemistry (IHC) analysis, respectively. Blood was led to clot at room temperature, incubated overnight at 4°C and spun down for 5’ at 13 250 × g. The serum was collected and used for analytical procedures. The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

**Analytical procedures**

Serum and urinary Mg²⁺ concentrations were determined using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, the Netherlands). Serum and urine Ca²⁺ concentrations were measured as described previously [27]. A flame spectrophotometer (FCM 6343; Eppendorf) was used to measure serum and urine Na⁺ and K⁺ concentrations. Urine P concentrations were measured by the phosphomolybdate method with an Aerosem analyser (Abbott Diagnostics, Abbott Park, IL).

**Total kidney RNA isolation and cDNA synthesis**

Total RNA was extracted from the kidneys using TriZol. Total RNA isolation reagent according to standard procedures (Gibco BRL, Breda, the Netherlands). The obtained RNA was subjected to DNase treatment (Promega, Madison, WI) to prevent genomic DNA contamination. All samples were resolved on a 1% w/v formaldehyde agarose gel to evaluate the RNA quality, while RNA concentration was determined by measuring the ratio of the UV absorbance at 260 and 280 nm using the NANODROP 2000c (Thermo scientific, Wilmington, DE). Thereafter, 1.5 µg of RNA was reverse transcribed by Molony murine leukemia virus reverse transcriptase (Invitrogen, Breda, the Netherlands) into cDNA according to the manufactures recommendations.

**SYBR Green real-time quantitative (q) PCR**

Total kidney cDNA was used to determine the mRNA expression levels of genes of interest, as well as the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design real-time qPCR primers according to the general criteria for RT-primers. All primer sequences used in this study are listed in Table 1 and Figure 1 shows an overview of all markers used. Prior to real-time qPCR reactions the efficiency (95–105%) and dynamic range (R² > 0.98) were evaluated for each primer set. Real-time qPCR reactions were performed on a Bio-Rad CFX96™ Real-Time qPCR and Bio-Rad C1000™ Thermal Cycler system, using Bio-Rad iQ™ SYBR® Green Supermix. All amplicons showed the correct sizes after gel electrophoresis, and the dissociation curves showed one distinct melting peak, ensuring the absence of a non-specific by-product or primer dimers. Moreover, no reverse transcription and no template controls were taken along.
Immunoblotting

Kidneys of control and cisplatin-treated mice were homogenized in homogenization buffer A (HbA; 20 mM Tris/HCl (pH = 7.4), 5 mM MgCl₂, 5 mM NaH₂PO₄, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, 1 µg/mL leupeptin and 10 µg/mL pepstatin). Protein concentration of the homogenates was determined using the Bio-Rad Protein Assay, (Bio-Rad, Munich, Germany). The proteins were solubilized by 30 incubation at 37°C in Laemmli buffer. Each protein sample (60 µg) was separated on a SDS–PAGE gel and blotted to a

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TAAACATCAATGGGGTGAGG-3'</td>
<td>5'-GGTTTACCCCAATCACAAC-3'</td>
</tr>
<tr>
<td>Kim-1</td>
<td>5'-TGTCGAGTGAGATTCCTTGGGTG-3'</td>
<td>5'-GGTTTACCCCAATCACAAC-3'</td>
</tr>
<tr>
<td>TRPM6</td>
<td>5'-AAAAGCATTGGAGATCGGATC-3'</td>
<td>5'-TTTACAAGGAAACCTTGCCC-3'</td>
</tr>
<tr>
<td>PV</td>
<td>5'-CGCTGAGGACATCAAGAAGG-3'</td>
<td>5'-CCGGGTTCTTTTCTTCAGG-3'</td>
</tr>
<tr>
<td>NCC</td>
<td>5'-CTTCGGCCACTGGCATTCTG-3'</td>
<td>5'-GATGGCAAGGGTAGGATGGG-3'</td>
</tr>
<tr>
<td>NKCC2</td>
<td>5'-GGCTTGTATCTTTCCTTGGTCG-3'</td>
<td>5'-CCATCTTGAATTCCTCC-3'</td>
</tr>
<tr>
<td>NHE3</td>
<td>5'-TGCTTGTGGTGATCTCTG-3'</td>
<td>5'-CCGCTCTCTTCAACCTCAG-3'</td>
</tr>
<tr>
<td>CLDN16</td>
<td>5'-GGCTCATTGATCTTCCTCC-3'</td>
<td>5'-CGGCCACTTAAACAACAGG-3'</td>
</tr>
<tr>
<td>CLDN19</td>
<td>5'-GGTTACCTTCTCTGTCGAC-3'</td>
<td>5'-TGATACGGCCAGCCTGAT3'</td>
</tr>
<tr>
<td>AQP1</td>
<td>5'-CCGAGCTTAGGGTGGTCAG-3'</td>
<td>5'-TCAGGCCAGGATGAAACAC-3'</td>
</tr>
<tr>
<td>SGLT1</td>
<td>5'-CTCTTGCAGGCACTGACAG-3'</td>
<td>5'-GGATTCTGCTCCTGGTG-3'</td>
</tr>
<tr>
<td>TRPV5</td>
<td>5'-CATGCTCTGAGTCAACATG-3'</td>
<td>5'-CCATAAGAGCAGCCTCACCC-3'</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>5'-CTTCACACTGCGCACAGC-3'</td>
<td>5'-GAGCGACCCGTCGATGAC-3'</td>
</tr>
<tr>
<td>AQP2</td>
<td>5'-GACCAAGTCAGGAACAGAAGG-3'</td>
<td>5'-GCCATGTCAGGATGAC-3'</td>
</tr>
<tr>
<td>OCT2</td>
<td>5'-CCCTACACTGCGCACAG-3'</td>
<td>5'-TCTCGGGGTATCTGAGTCC-3'</td>
</tr>
<tr>
<td>CTR1</td>
<td>5'-ACACCTATGCGGCTTACACT-3'</td>
<td>5'-GAGCGACCCGTCGATGAC-3'</td>
</tr>
</tbody>
</table>

Mouse primers used to perform SYBR Green real-time qPCR. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Kim-1, kidney injury molecule-1; TRPM6, transient receptor potential melastatin member 6; PV, parvalbumin; NCC, Na⁺-Cl⁻ cotransporter; NKCC2, Na⁺-K⁺-Cl⁻ cotransporter; NHE3, Na⁺-H⁺ exchanger 3; CLDN16, claudin-16; CLDN19, claudin-19; AQP1, aquaporin 1; SGLT1, sodium-glucose cotransporter 1; TRPV5, transient receptor potential vanilloid member; α-ENaC, epithelial Na⁺ channel alfa subunit; AQP2, aquaporin 2; OCT2, organic cation transporter; CTR1, copper transporter 1.

**FIGURE 1:** Schematic overview of a nephron depicting the expression of gene products used as marker to determine in which segments cisplatin exerts its effect. Nephron segments: PCT, proximal convoluted tubule; PST, proximal straight tubule; TAL, thick ascending limb of Henle; DCT, distal convoluted tubule; CNT, connecting tubule; CD, collecting duct. Gene products used as marker: NHE3, Na⁺-H⁺ exchanger 3; AQP1, aquaporin 1; SGLT1, sodium-glucose cotransporter 1; TRPM6, transient receptor potential melastatin member 6; PV, parvalbumin; NCC, Na⁺-Cl⁻ cotransporter; TRPV5, transient receptor potential vanilloid member 5; α-ENaC, epithelial Na⁺ channel alfa subunit; OCT2, organic cation transporter; CTR1, copper transporter 1.
PVDF-nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA). Blots were incubated for 16 h with either a rabbit NCC antibody (1:500 dilution; Millipore, Billerica, MA), rabbit parvalbumin antibody (PV-28, 1:500 dilution; Swant, Bellinzona, Switzerland), mouse monoclonal aquaporin 1 (AQP1) antibody (1:100 dilution) [28], rabbit aquaporin 2 (AQP2) antibody (1:500 dilution) or mouse tubulin antibody (1:20 000 dilution); (these antibodies were provided by Prof. Deen). Thereafter, blots were incubated with peroxidase-conjugated secondary antibodies after which proteins were visualized by chemiluminescence (Pierce, Rockford, IL). Immunopositive bands were scanned using ChemiDoc XRS (Bio-Rad) and signals were analysed with the Quantity One software (Bio-Rad). The amount of NCC, PV, AQP1 and AQP2 protein was normalized for the corresponding total amount of protein, using tubulin protein levels or Coomassie staining. Data are based on two independent experiments in which four samples per group were analysed.

Immunohistochemistry

IHC staining was performed on 7-µm cryosections PLP-fixed kidney samples. Sections were stained with rabbit anti-NCC [29] and rabbit PV antibody (1:200; PV-28, Swant, Bellinzona, Switzerland), following standard procedures. Images were made using a Zeiss fluorescence microscope (Sliedrecht, the Netherlands) equipped with an AxioCam digital photo camera.

Statistical analysis

Data are expressed as mean ± SEM. Overall statistical analyses were performed by Student’s t-test. P < 0.05 was considered statistically significant. All calculations were accomplished using the InStat 3 for Macintosh software.

RESULTS

Serum and urine electrolyte levels of cisplatin-treated mice

Ten-week-old C57Bl/6J mice were intra-peritoneally injected with either 5-mg/kg body weight cisplatin on days 0, 4 and 8 or vehicle. After 12 days, the animals were placed into metabolic cages for the collection of 24 h urine. Subsequently, the mice were sacrificed to collect blood and to harvest the kidneys. Serum electrolyte and creatinine levels and body weight are displayed in Table 2, while urinary electrolyte levels are presented in Table 3. The body weight was significantly decreased in the cisplatin group compared with the control group (Table 2). Besides the weight loss, no signals of severe illness were perceived. Compared with control mice, serum Mg²⁺ levels were substantially reduced in the cisplatin-treated mice (n = 10), while no differences were observed in serum Ca²⁺, Na⁺ and K⁺ levels (Table 2). The mice chronically treated with cisplatin displayed polyuria (Table 3). Moreover, these mice showed markedly increased serum creatinine levels (Table 2) in combination with reduced urinary creatinine excretion (Table 3), indicating a significantly reduced creatinine clearance (C₀ CR) (Table 3). The excretion of Mg²⁺, Ca²⁺, Na⁺, K⁺ and P₄ was corrected by urinary concentration of creatinine to compensate for changes in GFR. In comparison with the control group, the renal excretion of Mg²⁺, Ca²⁺, Na⁺, K⁺ and P₄ was corrected by urinary concentration of creatinine to compensate for changes in GFR. In comparison with the control group, the renal excretion of

### Table 2. Serum analysis and body weight of control and cisplatin-treated mice

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Mg²⁺] (mmol/L)</td>
<td>1.58 ± 0.03</td>
<td>1.23 ± 0.03*</td>
</tr>
<tr>
<td>[Ca²⁺] (mmol/L)</td>
<td>2.12 ± 0.03</td>
<td>2.19 ± 0.03</td>
</tr>
<tr>
<td>[Na⁺] (mmol/L)</td>
<td>137.1 ± 0.5</td>
<td>138.7 ± 0.8</td>
</tr>
<tr>
<td>[K⁺] (mmol/L)</td>
<td>6.7 ± 0.1</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>[Creatinine] (µmol/L)</td>
<td>6.4 ± 0.3</td>
<td>10.4 ± 0.9*</td>
</tr>
<tr>
<td>Weight loss (g)</td>
<td>0.1 ± 0.1</td>
<td>2.3 ± 0.8*</td>
</tr>
</tbody>
</table>

Control, mice receiving vehicle injections only; cisplatin, mice receiving 5 mg/kg cisplatin injections on days 0, 4 and 8. Values are presented as average ± SEM. *P < 0.05 versus control.

### Table 3. Urine analysis of control and cisplatin-treated mice

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Control</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume (mL/24 h)</td>
<td>0.9 ± 0.1</td>
<td>2.5 ± 0.3*</td>
</tr>
<tr>
<td>[Creatinine] (mmol/L)</td>
<td>2.4 ± 0.2</td>
<td>0.9 ± 0.1*</td>
</tr>
<tr>
<td>Mg²⁺ excretion (µmol/24 h)</td>
<td>24 ± 2</td>
<td>42 ± 2*</td>
</tr>
<tr>
<td>Mg²⁺/creatinine</td>
<td>10.6 ± 0.3</td>
<td>16.0 ± 0.5*</td>
</tr>
<tr>
<td>Ca²⁺ excretion (µmol/24 h)</td>
<td>4.7 ± 0.4</td>
<td>7.2 ± 0.3*</td>
</tr>
<tr>
<td>Ca²⁺/creatinine</td>
<td>2.1 ± 0.1</td>
<td>3.2 ± 0.4*</td>
</tr>
<tr>
<td>Na⁺ excretion (µmol/24 h)</td>
<td>110 ± 13</td>
<td>163 ± 18*</td>
</tr>
<tr>
<td>Na⁺/creatinine</td>
<td>48 ± 3</td>
<td>69 ± 3*</td>
</tr>
<tr>
<td>K⁺ excretion (µmol/24 h)</td>
<td>344 ± 38</td>
<td>464 ± 21</td>
</tr>
<tr>
<td>K⁺/creatinine</td>
<td>150 ± 6</td>
<td>186 ± 4*</td>
</tr>
<tr>
<td>P₄ excretion (µmol/24 h)</td>
<td>43 ± 8</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>P₄/creatinine</td>
<td>17 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>6.7 ± 0.2</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>Urinary osmolality (Osmol/kg)</td>
<td>2.8 ± 0.1</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>C₀ CR (mL/min)</td>
<td>0.26 ± 0.02</td>
<td>0.18 ± 0.02*</td>
</tr>
</tbody>
</table>

Control, mice receiving vehicle injections only; cisplatin, mice receiving 5 mg/kg cisplatin injections on days 0, 4 and 8; C₀ CR, creatinine clearance. Values are presented as average ± SEM. *P < 0.05 versus control.
Mg^{2+}, Ca^{2+}, Na^+ and K^+ was increased on treatment with cisplatin, whereas the excretion of Pi was not affected (Table 3). The urinary osmolality was substantially decreased when the mice were treated with cisplatin (Table 3).

### The effect of cisplatin on proximal tubular injury

To evaluate whether cisplatin treatment caused tubular injury, the expression level of kidney injury molecule-1 (Kim-1) was measured. Kim-1 is a type I membrane protein, which is expressed at very low levels in normal kidney. As a result of renal injury, Kim-1 is strongly up-regulated in predominantly PT cells [30]. Compared with the control group, our study showed markedly increased Kim-1 mRNA levels upon treatment with cisplatin (3378 ± 1101 and 100 ± 30%, cisplatin versus control, P < 0.05) (Figure 2).

### The effect of cisplatin on mRNA expression of renal electrolyte transporters

The mRNA expression levels of TRPM6, NCC and parvalbumin (PV) and different Ca^{2+} and Na^+ transporters as well as AQP2, were determined by real-time qPCR (Figure 3). The cisplatin group showed significantly lower expression levels of TRPM6 (60 ± 7 and 100 ± 5%, cisplatin versus control, P < 0.05) and NCC (63 ± 7 and 100 ± 5%, cisplatin versus control, P < 0.05). Additionally, the DCT specific protein PV was markedly down-regulated in cisplatin-treated mice (38 ± 12 and 100 ± 6%, cisplatin versus control, P < 0.05). Subsequently, the mRNA expression levels of the Na^{+}-K^{+}-2Cl^{-} cotransporter (NKCC2) and the tight junction proteins claudin-16 (CLDN16) and claudin-19 (CLDN19) [31], all located in the thick ascending limb of Henle (TAL), were determined. The NKCC2 and CLDN19 expression levels were not significantly changed (103 ± 5 versus 100 ± 5% for NKCC2 and 120 ± 9 versus 100 ± 5% for CLDN19, cisplatin versus control, P > 0.2), whereas CLDN16 was substantially up-regulated in the cisplatin-treated group (163 ± 16 and 100 ± 3%, cisplatin versus control, P < 0.05). The mRNA level of the Na^{+}-H^{+} exchanger 3 (NHE3), which is expressed in the proximal convoluted tubule (PCT) and the TAL [32, 33] was not affected by cisplatin (100 ± 4 and 100 ± 3%, cisplatin versus control, P > 0.2). Moreover, the mRNA expression levels of AQP1, expressed in the PCT and the proximal straight tubule (PST) and of the sodium-glucose cotransporter 1 (SGLT1), exclusively expressed in the PST, were also unaffected by cisplatin treatment (96 ± 6 versus 100 ± 5% for AQP1 and 98 ± 3 versus 100 ± 5% for SGLT1,
cisplatin versus control, P > 0.2). The mRNA level of the late DCT (DCT2) and CNT marker TRPV5 was not changed either upon cisplatin treatment (122 ± 11 and 100 ± 5%, cisplatin versus control, P > 0.2). Furthermore, the mRNA level of the epithelial Na⁺ channel (ENaC), which localizes to the connecting tubule (CNT) and collecting duct (CD), was not significantly changed by cisplatin (89 ± 5 and 100 ± 5%, cisplatin versus control, P > 0.2). Finally, the mRNA level of AQP2, as a measure of CD integrity, was unchanged (125 ± 13 and 100 ± 7%, cisplatin versus control, P > 0.2).

**The effect of cisplatin on protein expression of renal electrolyte transporters**

The effect of cisplatin treatment on NCC, PV, AQPI and AQP2 protein expression was investigated by immunoblotting. We were able to confirm the DCT-specific down-regulation on protein level. NCC and PV were significantly reduced in the cisplatin-treated group (38 ± 9 versus 100 ± 19% for NCC and 62 ± 5 versus 100 ± 6% for PV, cisplatin versus control, P < 0.05) (Figures 4A and B), whereas the AQPI and AQP2 protein expression in the cisplatin-treated group did not significantly differ from the control group (116 ± 23 and 100 ± 10% for AQPI and 120 ± 18 and 100 ± 16% for AQP2, cisplatin versus control, P > 0.2) (Figure 4C and D).

Next, to establish whether the reduction of mRNA encoding for DCT transporters is dependent on protein down-regulation or on cellular damage immunohistochemical staining was performed for NCC and PV. Figure 5 shows that NCC and PV are localized in the same cell compartment when treated with cisplatin, compared with control mice. It is clear that the tubules of the cisplatin-treated mice have a larger lumen and that the epithelial cells are more flat. In addition, there were indications of minor tubular damage with individual necrotic tubular epithelial cells in the cisplatin kidneys (data not shown).

**The effect of cisplatin on mRNA expression of known cisplatin transporters**

Previous studies suggested that the uptake of cisplatin is mediated by a specific transport mechanism [34, 35]. Exclusive expression of cisplatin transporters to the DCT may explain the damage to particularly this tubule segment. The organic cation transporter 2 (OCT2) and the copper transporter 1 (CTR1) are identified to contribute to the uptake of cisplatin [36, 37]. The mRNA expression level of OCT2 was not changed in the cisplatin group, compared with the control group (93 ± 10 and 100 ± 7%, cisplatin versus control, P > 0.2) (Figure 6A). In contrast, the mRNA level of CTR1 was significantly down-regulated by treatment with cisplatin (82 ± 4 and 100 ± 6%, cisplatin versus control, P < 0.05) (Figure 6B).

**DISCUSSION**

Our study provides further evidence that the DCT segment is affected by cisplatin treatment. We demonstrated that cisplatin treatment in mice induces hypomagnesaemia, whereas serum Ca²⁺, Na⁺ and K⁺ levels were unaffected. The mice developed polyuria, reduced GFR and the renal excretion of Mg²⁺, Ca²⁺, Na⁺ and K⁺ was increased, whereas P₁ excretion was not changed, after the chronic treatment with cisplatin. Investigation of the mRNA expression levels of the DCT marker genes NCC, TRPM6 and PV showed a significant decline in the cisplatin-treated mouse group. In line with these data, we observed markedly reduced NCC and PV protein levels by immunoblotting experiments. IHC staining for NCC and PV suggested that specific protein down-regulation rather than major cellular damage explains the underlying mechanism.

The DCT plays a key role in determining the final plasma Mg²⁺ concentration, as the more distal parts of the tubule are largely impermeable to Mg²⁺. In DCT, Mg²⁺ reabsorption occurs in an active trans-cellular manner initiated by TRPM6 [38]. TRPM6 localizes to the luminal membrane where it facilitates transport of Mg²⁺ from the pro-urine into the cell [39]. The importance of Mg²⁺ in cisplatin-induced renal injury has been underlined by several studies. A recent study demonstrated that cisplatin-induced nephrotoxicity is enhanced due to Mg²⁺ depletion [21, 40]. In addition, nephro-protection can be attained in patients who are supplemented with Mg²⁺ during and between courses of cisplatin treatment [41]. In addition, NCC is responsible for the reabsorption of 10–15% of the filtered NaCl [42]. Its importance is illustrated by the fact that NCC is the target of the thiazide diuretics, which are commonly prescribed drugs in the treatment of high blood pressure [43].

Furthermore, the effect of cisplatin treatment on mRNA and protein expression in other tubule segments, including PT, TAL, CNT and CD, was investigated. The PT can be severely affected by cisplatin treatment, especially morphological changes in the PT have been observed [6, 7]. The cisplatin-induced increase of the Kim-1 expression level suggests that the PST segment is also affected in our study [30, 44]. The NHE3 expression levels were similar in the kidneys of control and cisplatin-treated groups. IHC for NHE3 previously showed strong staining on the apical membrane of the CPT, while staining was absent in the PST [32]. Therefore, we also determined the mRNA expression level of SGLT1, which is exclusively expressed in the PST segment [45], and of AQPI, which is present throughout the PT [46]. However, also for these genes we did not observe a significant effect by cisplatin treatment. Moreover, the urinary excretion level of P₁ was not affected in cisplatin-treated mice. P₁ is mainly reabsorbed in the PT in a trans-cellular, Na⁺-dependent manner [47]. These results suggest that cisplatin injury to the PST might be less severe or different from injury to the DCT.

We did not observe expression differences for the CNT marker TRPV5 or for the CNT and CD marker ENaC, suggesting that these tubular segments are not significantly affected by cisplatin treatment in our study. Additionally, the mRNA expression level of NKCC2 was unchanged following cisplatin treatment. Our observations are in line with a study by Ecelbarger et al. which showed normal protein expression of NKCC2 in the medullary TAL segment [48]. The same
study also demonstrated that the protein abundance of NHE3, the renal outer medullary K⁺ channel (ROMK), localized in TAL and CNT and the α subunit of the Na⁺/K⁺ ATPase (α-Na⁺/K⁺ ATPase), expressed throughout the nephron, were not affected. On the other hand, the expression of IMCD proteins AQP2 and AQP3 and urea transporters (UT-A) was reduced. This is in contrast with our results, which showed no effect of cisplatin treatment on AQP2 mRNA and protein level. Remarkably, a study by Lajer et al. displayed lower protein abundance for all investigated proteins, including the α-Na⁺/K⁺ ATPase, NHE3, NKCC2, AQP1 and AQP2 [40]. It is important to note that these discrepancies may result from a species-specific effect because they all used rats reporting a more severe polyuria. Proper interaction of CLDN16 and CLDN19 in TAL is essential for the cation selectivity of paracellular route and consequently reabsorption of divalent cations in this tubule segment [31]. Upregulation of CLDN16 levels in our cisplatin-treated mouse group suggested that TAL might compensate for decreased reabsorption of Mg²⁺ in DCT. Altogether, our results suggest a specific down-regulation of proteins that reside in the DCT. To our knowledge, this is the first study that specifically investigates the expression levels of transport proteins in the DCT following cisplatin treatment.

![Figure 4: Effect of cisplatin on protein expression levels of renal marker proteins.](https://example.com/figure4.png)

**FIGURE 4:** Effect of cisplatin on protein expression levels of renal marker proteins. The effect of cisplatin (Cisp) (5 mg/kg/injection on days 0, 4 and 8) on protein expression levels of the Na⁺-Cl⁻ cotransporter (NCC) (A), parvalbumin (PV) (B), aquaporin 1 (AQP1) (C) and aquaporin 2 (AQP2) (D). The upper part of each figure shows the immunoblot, with on the left side the molecular mass (in kDa) and the lower parts depict the expression levels as percentage of control (Cont). Values are presented as average ± SEM (n = 4), while experiments are performed in duplo. *P < 0.05 compared with control.
There are several mechanisms that can explain the polyuria, which is caused by cisplatin administration. In rats it has been shown that polyuria is associated with decreased expression of AQP1, 2 and 3 [49]. However, we did not observe a change in mRNA or protein level of AQP1 and AQP2. Additionally, IHC staining displayed similar AQP2 localization and quantity in kidneys of cisplatin-treated or control animals (data not shown). We also determined whether the polyuria might be secondary to osmotic diuresis. As depicted in Table 3, the urinary osmolality is markedly reduced after cisplatin treatment that renders this assumption unlikely. Reduced urinary osmolality in combination with a decline of Ccr indicate decreased GFR due to acute kidney failure in the cisplatin-treated animals. Another explanation might, therefore, be that a decreased GFR, secondary to cisplatin-induced nephrotoxicity, is associated with medullary urea cycling defect. This results in reduced tonicity of the medulla and as a consequence increased excretion of water [4]. Moreover, we showed that cisplatin treatment causes hypomagnesaemia and increased urinary excretion of K+ and Na+ in combination with polyuria. Interestingly, in patients suffering from Gitelman Syndrome (GS), besides tetany and muscle weakness, similar findings were observed [16, 18, 50]. GS results from homozygous loss-of-function mutations in NCC [51]. Absence of NCC causes renal NaCl wasting and activation of the renin–angiotensin–aldosterone system [52, 53]. The high Na+ load in the CNT/CD region will cause increased reabsorption by ENaC, in exchange for K+ [54]. The present study demonstrated a lower NCC mRNA and protein expression levels, following treatment with cisplatin. Another characteristic of GS is hypocalciuria. Nijenhuis et al. demonstrated that hypocalciuria in NCC-deficient mice is caused by enhanced Ca2+ reabsorption in the PT, secondary to the hypovolaemia [55]. The fact that we did observe hypercalciuria in the cisplatin-treated mice, instead of hypocalciuria as shown in GS, suggests tubular damage to the PT, as also confirmed by the upregulation of Kim-1. Hence, it might be that the polyuria is primarily caused by impaired NaCl reabsorption via NCC, in combination with insufficient compensation via ENaC, AQP2 and NHE-3.

Taken together, our study identified for the first time a specific effect of cisplatin on the expression level of DCT markers. It is unclear whether this is caused by specific down-regulation of Na+ and Mg2+ transport proteins in the DCT or a cisplatin-induced DCT-specific cell death. The procedure for cisplatin-mediated cell death is still unknown. Apoptosis can be induced by the formation of chromosomal DNA/cisplatin adducts and/or mitochondria-induced...
pathways [56, 57]. Mitochondria-induced apoptosis is of interest as the large size and high density of mitochondria characterizes DCT cells [58]. For this reason, the DCT may be more susceptible to mitochondria-induced apoptosis, as shown for cell lines with a relatively high density of mitochondria [59]. Alternatively, as only minor cellular damage was detected, specific down-regulation of proteins in DCT might indeed be the mechanism causing loss of function of this part of the nephron.

Research focuses on the identification of cisplatin transport mechanism contributing to its segment-specific nephrotoxicity and ways to block these in a competitive manner. Recent studies identified OCT2 [36] and the copper transporter 1 (CTR1) [37] as cisplatin-transporting proteins. Interestingly, both OCT2 and CTR1 are expressed in the kidney as well as in malignant tissues [60–62] and are, for this reason, candidates to facilitate the entry of cisplatin into renal cells. Our results showed that the mRNA level of OCT2 was not affected, whereas the CTR1 expression level was slightly but significantly decreased by cisplatin treatment. Recently, Pabla et al. identified PKCζ as a critical regulator of cisplatin nephrotoxicity [63]. Future studies are needed to confirm the role of CTR1 in the entry of cisplatin in DCT cells and if targeting PKCζ could be an effective way to reduce cisplatin-induced side effects.

In summary, cisplatin treatment results in polyuria, hypomagnesaemia and renal Mg²⁺, Ca²⁺, Na⁺ and K⁺ wasting. These defects likely arise from impaired functionality of the renal PCT and DCT segments. As long as the exact mechanism that causes hypomagnesaemia is not resolved, it is important that patients treated with cisplatin are frequently checked for their serum Mg²⁺ level and supplemented with Mg²⁺ accordingly.

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CONFLICT OF INTEREST STATEMENT

None declared.

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Biological responses to PDGF-AA versus PDGF-CC in renal fibroblasts

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ABSTRACT

Background. Platelet-derived growth factors (PDGF)-AA and -CC mediate renal fibroblast proliferation and/or renal fibrosis. Whereas PDGF-CC binds to both the PDGF receptors (PDGFRs)-αα- and -αβ, PDGF-AA binds more selectively to the αα-receptor, suggesting potential differences in the biological activities.

Methods. We compared signal transduction, gene expression as well as changes in the proteome induced by PDGF-AA and -CC in rat renal fibroblasts, which express both PDGFR subunits. The growth factor concentrations used were chosen based on their equipotency in inducing rat renal fibroblast proliferation.

Results. Both PDGF-AA and PDGF-CC induced phosphorylation and activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2. Renal fibroblast proliferation induced by either PDGF-AA or -CC could be blocked by signal