Cisplatin-Induced Nephrotoxicity in Porcine Proximal Tubular Cells: Mitochondrial Dysfunction by Inhibition of Complexes I to IV of the Respiratory Chain

MARIEKE KRUIDERING, BOB VAN DE WATER,2 EMILE DE HEER,3 GERARD J. MULDER and J. FRED NAGELKERKE

Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, 2300 RA Leiden, The Netherlands

Accepted for publication October 21, 1996

ABSTRACT

Cisplatin-induced nephrotoxicity was studied in porcine proximal tubular cells, focusing on the relationship between mitochondrial damage, reactive oxygen species (ROS) and cell death. Cisplatin specifically affected mitochondrial functions: complexes I to IV of the respiratory chain were inhibited 15 to 55% after 20 min of incubation with 50 to 500 μM, respectively. As a result, intracellular ATP was decreased to 70%. The mitochondrial glutathione (reduced form) (GSH) regenerating enzyme GSH-reductase (GSH-Rd) activity was reduced by 20%, which contributed to a 70% reduction of GSH levels and ROS formation. The residual electron flow through the mitochondrial respiratory chain was the source of ROS because additional inhibition of the complexes I to IV reduced ROS formation. Because cisplatin affects both GSH-Rd and complexes I to IV, cells were incubated with N,N′-bis(2-chloroethyl)-N-nitrosourea (inhibitor of GSH-Rd) and inhibitors of the different complexes. Only N,N′-bis(2-chloroethyl)-N-nitrosourea with rotenone (complex I inhibitor) induced ROS formation, which indicates that inhibition of complex I and inhibition of the GSH-Rd is probably the cause of ROS formation. However, the results of ROS is not the cause of cell death because diphenyl-p-phenylene-diamine and deferoxamine, which completely prevented ROS, could not prevent cell death. Similarly, the antioxidants did not completely prevent the decrease in activity of complexes I to IV, ATP or GSH levels. In conclusion, ROS formation does occur during cisplatin-induced toxicity, but it is not the direct cause of cell death.

The antitumor drug cisplatin is used for the treatment of cancer of a wide range of tissues (Borch, 1987). The major side effect, nephrotoxicity, is dose limiting and occurs either acutely or after repeated treatments. In rats, cisplatin exerts its effects mainly in the S3 segment of the proximal tubule, which results in necrotic lesions (Doyban et al., 1980); in man, additional damage is found in the distal part of the tubules (Gonzalez-Vitale et al., 1977). A wealth of histopathological data, mainly in rats and mice, is available about cisplatin-induced nephrotoxicity; in addition, several biochemical effects have been described in vitro as well as in vivo, among which alterations in capacity of several active transport systems (Miura et al., 1987), loss of mitochondrial function (Gordon and Gattone, 1986), Na+,K+-ATPase activity (Brady et al., 1993; Kim et al., 1995) and lipid peroxidation (Zhang et al., 1994; Hannemann and Baumann, 1988). However, the sequence and relative importance of these effects are still unclear.

Mitochondria seem to play an important role in cisplatin-induced nephrotoxicity (Gordon and Gattone, 1986; Brady et al., 1993). Accordingly, we demonstrated that exposure of freshly isolated PPTC in suspension to cisplatin resulted in loss of mitochondrial membrane potential (Δψ) (Kruidering et al., 1994). The decrease in Δψ preceded cell death, implying that damage to the mitochondria is an early event in the cascade of events leading to cell death. However, how cisplatin damages the mitochondria remains unclear.

Oxidative damage has been proposed as a mechanism of cisplatin-induced renal cell death in vitro as well as in vivo (Kameyama and Gemb, 1991a; Zhang and Lindup, 1994;
Sugihara et al., 1987; Gemba et al., 1988). However, these studies did not investigate the relationship between mitochondrial dysfunction and oxidative damage, leaving the question of cause and consequence unanswered. Mitochondria continuously convert 1 to 2% of the consumed oxygen to superoxide (Richter et al., 1995); therefore, they are an important source of ROS. Superoxide anion is produced in the respiratory chain by reaction of oxygen with iron-sulfur centers in complex I and by partially reduced ubiquinone and cytochrome b in complex III. A simplified scheme of the respiratory chain is given in figure 1.

Mitochondria are protected from oxidative damage in several ways. Superoxide and hydrogen peroxide are metabolized by Mn-containing superoxide dismutase and the Se-containing glutathione peroxidase, respectively. Vitamins C and E, glutathione and ubiquinol-10 are important for additional scavenging of ROS. Clearly, mitochondria both form and scavenge ROS; disturbances of the balance, caused by changes in the electron flow or in the defense mechanisms, can lead to an overproduction of ROS. ROS attack lipids, proteins and nucleic acids nonspecifically, which results in (more mitochondrial) dysfunction (Richter et al., 1995).

In this study, we investigated the roles of mitochondrial dysfunction and formation of ROS in cisplatin-induced renal cell death of PPTC in detail. Our results suggest that impaired respiratory activity rather than the formation of ROS is the key event in the pathway leading to renal cell death.

**Materials and Methods**

**Chemicals**

BSA, glycine, collagenase (EC 3.4.24.3) (from Clostridium histolyticum), cis-diamine-dichloroplatinum (II)(Pt), R123, propidium iodide, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid, ubiquinone, cytochrome cIII, N-d-maltoside, Oxa, rot, TTFA, anti, firefly lantern extract (crude luciferin/luciferase), GSH and GSSG, GSH-Rd, TBHP and cumene hydroperoxide were from Sigma Chemical Co. (St. Louis, MO).

DPPD was purchased from Aldrich (Brussels, Belgium). Potassium cyanide (KCN) was from Janssen Chimica (Tilburg, the Netherlands). HEPES and sodium pyruvate were obtained from Boehringer Mannheim (Mannheim, Germany). Nycodenz (Iohexol) was from Nycomed AS (Oslo, Norway). DesferalR (deferoxamine) was from Ciba-Geigy AG (Basle, Switzerland). Diethylmaleate was from Merck (Darmstadt, Germany). (R,S)-3-Hydroxy-4-pentenoic acid was synthesized as described in Shan et al. (1993). Dih123 was from Molecular Probes (Eugene, OR). BCNU was kindly provided by Prof. E.W. Vogel, Leiden University.

**Cell Isolation**

Cell isolation was performed as described before (Kruidering et al., 1993). The kidneys with intact capsule were taken from pigs in the slaughterhouse approximately 15 to 20 min after electrocution; the renal artery and vein were flushed with ice-cold Eurocollins, pH 7.4, consisting of 177 mM glucose, 10 mM NaHCO3, 15 mM KCl, 42 mM K2HPO4 and 15 mM KH2PO4, supplemented with 2 mM glycine. After transport to the laboratory on ice, the capsule was removed and the cortex was dissected on ice. The minced cortex was washed three times with Ca2+-and Mg2+-free Hanks-HEPES buffer, pH 7.4, containing 25 mM HEPES and 2 mM glycine (buffer A). Thirty grams of cortex were incubated at 37°C with 25 ml of 0.07% (w/v) collagenase in buffer A, containing 1 mM deferoxamine and 4 mM CaCl2. After 55 min, 125 ml cold buffer A, including 4 mM CaCl2 and 1.5% BSA (w/v), (buffer C) was used to stop degradation. The cell suspension was filtered through a nylon gauze with a pore size of 80 μm and washed twice by centrifugation (5 min, 80 × g 4°C). The suspension was mixed and proximal tubular cells were purified from this suspension by centrifugation, with use of a discontinuous density gradient consisting of layers of 17%, 11.3% and 8.5% (w/v) Nycodenz. After transport to the laboratory on ice, the cells were resuspended in 20 ml buffer and mixed with 10 ml 34% Nycodenz to obtain 17% and 8.5% (w/v), respectively. The cells were resuspended in 50 ml of a solution containing 6.7 mM KCl, 1.22 mM CaCl2, 10 mM HEPES, pH 7.4. This was mixed 1:1 and 1:3 with buffer C to obtain 17% and 8.5% (w/v), respectively. The cells were suspended in 50 ml of buffer and mixed with 10 ml of 34% Nycodenz to obtain 11.3%. The gradient was layered carefully in two 50-ml glass tubes. First, 5 ml of 17% Nycodenz was put in the tube; on top of this, 10 ml of cells in 11.3% Nycodenz was layered carefully without mixing the layers. Finally, this was topped with 5 ml 8.5% Nycodenz solution. The tubes were centrifuged for 6 min at 2300 × g 4°C. The cells which accumulated in bands at the interface between the upper two layers were removed with a capillary pipette and pooled by centrifugation (90 × g for 5 min at 4°C). Subsequently, cells were washed twice with buffer C (80 × g for 6 min at 4°C). The maximum period between the death of the animals and the end of the isolation was 2.5 h. Viability was determined by trypan blue exclusion. Purity of the resulting cell suspension was determined by counting the percentage γ-glutamyl transpeptidase positive cells (Rutenberg et al., 1969) and by determination of the γ-glutamyl transpeptidase activity.

**Fig. 1.** Scheme of the MRC. Electrons from succinate or NADH enter the chain at complexes II and I, respectively, and are transported via the ubiquinol cycle (Q cycle), complex III, cyt c and complex IV to oxygen. The specific inhibitors of each complex are: rotenone for complex I, TTFA and Oxa for complex II, antimycin for complex III, KCN for complex IV. Q, ubiquinone; QH2, ubiquinol; cyt, cytochrome (Van de Water, 1995).
of the cells before and after purification. The final cell preparation, lacking distal tubular and endothelial cells, had a viability of more than 90% and purity of at least 80% and routinely 90% (i.e., γ-glutamyl transpeptidase positive cells (Kruidering et al., 1993)).

Incubations

Freshly isolated cells were incubated in buffer C, at a density of 0.5-10⁶ cells/ml at 37°C in a humidified atmosphere (95% air/5% CO₂) to allow the cells to recover from isolation stress. After 20 min, cells were centrifuged and resuspended in buffer C without BSA (buffer D). If necessary, cells were preincubated for 20 min as indicated in the legends.

Flow Cytometry

Δψ and viability. Δψ and viability were determined by analyzing the R123 and propidium iodide fluorescence intensity with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser, with the Lysis software program (Becton Dickinson). R123 is a cationic dye that accumulates in the negatively charged inner side of the mitochondria. When the potential drops, less R123 accumulates in the mitochondria, which results in a lower fluorescence signal. The potential was measured as follows: at the indicated time points, samples were taken and analyzed as described below. The samples for flow cytometry were taken directly from the incubation tubes, whereas the samples for determination of enzymatic activities, or ATP and GSH content were washed twice with buffer, snap-frozen in liquid nitrogen and stored at −80°C until use. Incubations for determination of ROS were performed as described below.

Determination of Enzymatic Activity

Determination of Enzymatic Activity of Complexes I to IV of the Respiratory Chain

Enzymatic activities of the complexes I to IV were determined by dual wavelength spectrophotometry with an Amino Dual Wave

Lactate dehydrogenase was determined spectrophotometrically by following the decrease of NADH at 340 nm according to Bergmeyer et al. (1965).

Glucose-6-phosphatase activity was determined by the release of phosphate from 0.03 M glucose 6-phosphate in 0.1 M citrate buffer, pH 6.5, in a volume of 0.5 ml. The reaction was started by addition of the lysed cell homogenate. After precipitation of the protein with 2 ml ice-cold 5% (w/v) TCA, phosphate was determined in the supernatant with the molybdate assay (Harper, 1965).

Acid phosphatase was determined by release of phosphate from 50 mM β-glycerophosphate at 37°C in a 0.1 M citrate buffer, pH 4.6, in a final volume 0.6 ml. The reaction was started by addition of the lysed cell homogenate. After precipitation of the protein with 2 ml ice-cold 5% (w/v) TCA, phosphate was determined in the supernatant with the molybdate assay (Harper, 1965).

Catalase was determined spectrophotometrically by following the decomposition of H₂O₂ in 50 mM potassium phosphate buffer, pH 7.0, at 240 nm as described by Aebi (1974).

GSH-Rd activity was determined spectrophotometrically as described in Bilzer et al. (1984). To 600 μl of 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM KC1 and 1 mM EDTA, 10 μl of 6 mM NADPH and 50-μl sample were added. After stabilization of the signal, 10 μl of 60 mM GSSG (in H₂O) were added and the decrease in absorbency at 340 nm was followed at 25°C.

GSH-Px activity was determined by a modification of the assay described in Lawrence and Burk (1976). To 600 μl of 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM KC1, 10 μl 6 mM NADPH, 60 μl 20 mM GSH, 50 μl GSH-Rd (6 U/ml) and 50 μl sample were added. After stabilization of the signal, 50 μl of 4 mM cumene hydroperoxide was added and the decrease in absorbency at 340 nm followed at 25°C.

Determination of Enzymatic Activity of Complexes I to IV of the Respiratory Chain

Enzymatic activities of the complexes I to IV were determined by dual wavelength spectrophotometry with an Amino Dual Wave

Lactate dehydrogenase was determined spectrophotometrically by following the decrease of NADH at 340 nm according to Bergmeyer et al. (1965).

Glucose-6-phosphatase activity was determined by the release of phosphate from 0.03 M glucose 6-phosphate in 0.1 M citrate buffer, pH 6.5, in a volume of 0.5 ml. The reaction was started by addition of the lysed cell homogenate. After precipitation of the protein with 2 ml ice-cold 5% (w/v) TCA, phosphate was determined in the supernatant with the molybdate assay (Harper, 1965).

Acid phosphatase was determined by release of phosphate from 50 mM β-glycerophosphate at 37°C in a 0.1 M citrate buffer, pH 4.6, in a final volume 0.6 ml. The reaction was started by addition of the lysed cell homogenate. After precipitation of the protein with 2 ml ice-cold 5% (w/v) TCA, phosphate was determined in the supernatant with the molybdate assay (Harper, 1965).

Catalase was determined spectrophotometrically by following the decomposition of H₂O₂ in 50 mM potassium phosphate buffer, pH 7.0, at 240 nm as described by Aebi (1974).

GSH-Rd activity was determined spectrophotometrically as described in Bilzer et al. (1984). To 600 μl of 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM KC1 and 1 mM EDTA, 10 μl of 6 mM NADPH and 50-μl sample were added. After stabilization of the signal, 10 μl of 60 mM GSSG (in H₂O) were added and the decrease in absorbency at 340 nm was followed at 25°C.

GSH-Px activity was determined by a modification of the assay described in Lawrence and Burk (1976). To 600 μl of 100 mM potassium phosphate buffer, pH 7.0, containing 200 mM KC1, 10 μl 6 mM NADPH, 60 μl 20 mM GSH, 50 μl GSH-Rd (6 U/ml) and 50 μl sample were added. After stabilization of the signal, 50 μl of 4 mM cumene hydroperoxide was added and the decrease in absorbency at 340 nm followed at 25°C.
difference in absorbency between 550 and 580 nm according to Birch-Machin et al. (1993a). The assay was performed with 10 to 30 μg protein in a final volume of 1 ml of 25 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM N-D-maltoside. After addition of 10 μl of 1.5 mM cytochrome cII and stabilization of the signal, the reaction was started by the addition of 10 to 30 μg cells. The activity was calculated from the rate of increase in absorbency caused by oxidation of cytochrome cII to cytochrome cIII (ε = 19 mM⁻¹ cm⁻¹).

All activities were expressed per microgram of protein, which was determined according to Lowry et al. (1951).

### Determination of ATP Content

ATP content was determined in 1-ml samples taken from the incubations. The samples were washed once with buffer D, resuspended in 400 μl of 10% (v/v) HClO₄, frozen in liquid nitrogen and stored at −80°C until use. After thawing and addition of 50 μl of 0.5 M potassium phosphate buffer, solutions were further neutralized by addition of 90 μl of 10 N KOH and centrifuged at maximal speed in an eppendorf centrifuge. ATP was assayed by the luciferin/luciferase method optimized according to Kimmich et al. (1975) in buffer consisting of 21 mM glycyglycine, containing 6.6 mM NaN₃HAsO₄ and 4.2 mM MgCl₂, adjusted to pH 8.05 with NaOH. Two milliliters of buffer was mixed with 50 μl of 10 mg/ml firefly extract in H₂O. After addition of 50 μl sample, luminescence was measured. Values were compared with a calibration curve with ATP as standard.

### Determination of GSH

GSH content was determined according to Saville (1958). PPTC were centrifuged at 80 × g for 3 min. The pellet was extracted with 300 μl of 20% w/v TCA. After centrifugation at 14,000 × g for 10 min, GSH was determined in the supernatant.

### Statistical Analysis

All values are expressed as mean ± S.E.M. The statistical evaluation was performed with analysis of variance. Results were considered significant if P < .05.

### Results

#### Effects of cisplatin on mitochondrial activities

**Respiratory activity.** Incubation of freshly isolated PPTC in suspension with cisplatin in concentrations varying from 5 to 100 μM resulted in a concentration-dependent decrease in Δψ and viability. The decrease in Δψ preceded the loss of viability (fig. 2).

The enzymatic activities of complexes I to IV of the MRC were significantly decreased during incubation of the PPTC with cisplatin (table 1). The observed inhibition was dependent on cisplatin concentration and incubation time. At increasing concentrations of cisplatin more inhibition was found, but the concentration dependency was not linear. Within 20 min of incubation with 50 μM cisplatin, the activity of complexes I, II, III and IV were lowered to 85 ± 4%, 67 ± 9%, 70 ± 6% and 63 ± 9% of control, respectively. The viability of the cells at this time point had not changed significantly (table 1), which indicated that the observed loss of activity preceded cell death: at all concentrations, the percentile loss of viability was always less than the decrease in enzymatic activity. When exposed to 500 μM cisplatin for 40 min, the enzymatic activity of complexes I and II of PPTC had decreased to 49 and 40% of control, respectively, which indicated that at that cisplatin concentration, complex II was already maximally inhibited after 20 min (table 1).

**Effects of cisplatin on intracellular ATP content.** The Δψ is the driving force of ATP production. Therefore, the
endoplasmatic reticulum, catalase (peroxisomes) and acid phosphatase (lysosomes) was not altered significantly (n = 4, P ≤ .05).

Control values of the enzymatic activities (mean ± S.E.M.) were: lactate dehydrogenase, 17 ± 2 nmol NADH/min/mg protein; glucose-6-phosphatase, 8 ± 0.9 μmol Pi/min/mg protein; acid phosphatase, 55 ± 8 μmol Pi/min/mg protein; and catalase, 0.7 ± 0.1 mmol/min/mg protein.

Formation of ROS

Time course of decrease in Δψ versus ROS formation. Incubation of the PPTC with cisplatin caused a time- and concentration-dependent generation of ROS, as determined by the fluorescent probe Dih123 with flow cytometry (fig. 4A). The decrease in Δψ occurred before ROS formation (fig. 4B): 10 min after addition of 100 and 500 μM cisplatin, a significant decrease of Δψ to 84 and 65% of control was found; whereas, at this time point, no significant amounts of ROS had been formed (fig. 4B). Only after 20 min, ROS formation became evident at cisplatin concentrations greater than 100 μM; at cisplatin concentrations of 50 μM and lower, ROS formation was not detectable until after 30 to 40 min (fig. 4A).

Total depletion of GSH, both in the cytosol and in the mitochondria of PPTC, by incubation with 1 mM diethyl maleate and 200 μM (R,S)-3-Hydroxy-4-pentenoic acid did not result in ROS formation, but potentiated cisplatin-induced effects on Δψ and viability (not shown).

Effect of cisplatin on GSH-Rd and GSH-Px. One of the mechanisms that may underlie cisplatin-induced oxidative damage is inhibition of enzymes that protect against oxidative stress. Therefore, we determined the effect of cisplatin on GSH-Rd and GSH-Px, responsible for GSH regeneration from GSGS and hydrogen peroxide metabolism, respectively. Incubation of PPTC with cisplatin resulted in inhibition of both enzymes. Within 20 min of exposure to 100 μM cisplatin, GSH-Rd decreased to 80% of control, whereas GSH-Px activity, which was inhibited less, decreased to 90% of control. The inhibition of GSH-Rd was concentration and time dependent (fig. 5).

Effect of cisplatin on GSH content. GSH-Rd regenerates GSH from GSGS; therefore, inhibition of GSH-Rd may lead to decreased GSH levels. Indeed, within 20 min the GSH content of PPTC exposed to 500 μM cisplatin decreased from 4.8 ± 0.8 nmol GSH/mg cell protein to 1.2 ± 0.4 nmol GSH/mg cell protein.
Role of mitochondria in formation of ROS. Mitochondria are often the source of ROS (Dawson et al., 1993). Therefore, we studied the effect of inhibitors of the complexes I to IV of the respiratory chain on cisplatin-induced ROS formation. However, because the probe we used for ROS, Dih123, is cleaved into R123, the resulting fluorescent signal possibly not only depends on ROS formation but also on the Δψ. To assess to what extent the observed effects on ROS production were caused by a lowering of Δψ, we determined the effect of the uncoupler CCCP on the detection of ROS induced by TBHP. Incubation of the PPTC with 500 µM TBHP decreased the Δψ by 15% and caused formation of ROS within 15 min. Incubation of the PPTC with the uncoupler 60 µM CCCP alone caused a decrease in Δψ, but no ROS. However, coinubcation of the PPTC with 500 µM TBHP and 60 µM CCCP showed that CCCP further decreased the Δψ of the PPTC exposed to TBHP, but this decrease did not hamper the detection of ROS by Dih123. The Dih123 fluorescent signal in PPTC exposed to TBHP and CCCP was 172 ± 18% of control, whereas the Dih123 fluorescent signal was 151 ± 13 in PPTC exposed to TBHP alone, which indicated that an effect on Δψ does not interfere with determination of ROS formation measured with Dih123.

After 1 h of exposure to 500 µM cisplatin, the amount of ROS was 371 ± 30% of control; 100% inhibition of complex I with rotenone, complex II with TTFA, complex III with antimycin and complex IV with KCN significantly reduced the ROS formation to 211 ± 18%, 186 ± 15%, 144 ± 22% and 100 ± 2%, respectively (fig. 6A). This indicated that all com-

Fig. 4. Cisplatin-induced ROS formation and decrease of Δψ. (A) The effect of cisplatin on ROS content is shown. (B) The time course of cisplatin-induced effects on Δψ and ROS determined by flow cytometry are depicted in more detail. Each point represents the mean (± S.E.M.) of at least four separate isolations. Statistics: * significantly different from control (P < .05).

Fig. 5. Effect of cisplatin on the activity of GSH-Rd (A) and GSH-Px (B). At the indicated time points, a sample of PPTC was taken, washed twice with ice-cold buffer and stored at −80°C after freezing in liquid nitrogen. Values are given as percentage of control ± S.E.M. (n ≥ 4). Control values of unexposed cells were: GSH-Px, 64 ± 9 nmol/min/mg protein; GSH-Rd, 81 ± 9 nmol/min/mg protein. Statistics: * significantly different from control (P < .05); # significantly different from PPTC treated with 100 µM cisplatin (P < .05).
Complexes of the respiratory chain may contribute to ROS formation.

Apart from participation in the electron transport via the ubiquinol-ubiquinone cycle (the Q cycle, fig. 1), complex II activity also has an antioxidant function by regeneration of α-tocopherol (Van de Water et al., 1995). TTFA inhibits the Q cycle activity of complex II, while Oxa, which competes for succinate, inhibits complex II at another site, which is involved in its antioxidant function. To evaluate at which site cisplatin inhibited complex II, we studied the effect of TTFA and Oxa on cisplatin-induced ROS. Coincubation of the cells with cisplatin and Oxa increased cisplatin-induced ROS formation from 371 ± 30 to 664 ± 40 (fig. 6B). This potentiation of cisplatin-induced ROS was dependent on respiratory activity, since further addition of TTFA, antimycin, rotenone or KCN, reduced the ROS levels from 664% to almost the control level (fig. 6B).

**Contribution of cisplatin-induced effects to ROS content.** The above results showed that cisplatin induced an impaired antioxidant status of the PPTC: the GSH content was reduced and regeneration of GSH was inhibited. In addition, the respiratory activity was decreased. We studied whether decreased GSH-Rd activity alone or in combination with decreased respiratory activity would be sufficient for formation of ROS. PPTC were incubated with BCNU (inhibitor of GSH-Rd) in combination with the inhibitors of complexes I to IV; BCNU alone caused little ROS (fig. 7A).

Coincubation of the PPTC with rotenone and BCNU resulted in formation of ROS: after 90 min ROS levels were 190 ± 13% of control (fig. 7A). BCNU + rot and BCNU + cisplatin had virtually identical effects on Δψ and viability; after 3 h of exposure Δψ decreased to 40 ± 7% of control and viability to 45 ± 9% (fig. 7, B and C).

Coincubation of the PPTC with BCNU and TTFA (II), Oxa (II) or anti (III) did not result in formation of ROS (fig. 7A). Moreover, the effects on Δψ and viability were less pronounced, than those of BCNU and cisplatin. After 3 h of exposure Δψ and viability were 60 ± 7% of control, compared with 45 ± 9% obtained with BCNU/cisplatin (fig. 7, B and C).

KCN is an inhibitor of complex IV activity (Lehninger, 1972). However, it is also known to be a potent displacer of bound cisplatin (Naser et al., 1988). To check whether the observed effects by KCN were caused by inhibition of complex IV or by displacement of cisplatin, we determined cisplatin content of the PPTC in presence and absence of 1 mM KCN.

PPTC exposed to 100 μM cisplatin for 2 h contained 4 nmol Pt/g protein, which was reduced by simultaneous addition of 1 mM KCN to 0.5 nmol Pt/g. Likewise, the Pt content of PPTC exposed to 500 μM was reduced from 9 to 2 nmol Pt/g protein. Values are mean ± S.E.M., n ≥ 3, and were significantly different from cells incubated with cisplatin alone (P < .05). This indicated that prevention of ROS formation by KCN, as shown in fig. 6A, may be merely caused by displacement. Therefore, the exact role of complex IV activity in cisplatin-induced ROS formation can not be studied with KCN.

**Role of ROS formation in cisplatin-induced cell death.** To evaluate the role of ROS formation in cisplatin-induced cell death, we studied the effects the antioxidant DPPD and the iron chelator deferoxamine.

The ROS formation induced by cisplatin could be completely prevented by DPPD and deferoxamine. However, these compounds could not prevent the decrease in Δψ, nor prevent cell death (fig. 8, A–C). Identical results were obtained with rotenone and BCNU; i.e., although formation of ROS was prevented, DPPD and deferoxamine could not prevent the effects on Δψ and viability. As a control, PPTC were incubated with 500 μM TBHP, which is known to cause cell death by peroxidation. Prevention of the TBHP-induced ROS with DPPD and deferoxamine, significantly reduced the loss of Δψ and completely prevented the PPTC from cell death (fig. 9, A–C). This indicated that if ROS formation is the main cause of cell death, its prevention protects PPTC from cell death.

The antioxidants could not prevent the inhibition of complex I to IV activity by cisplatin. At all time points and with
all concentrations cisplatin used, the inhibition of the enzymatic activity of complexes I to IV of the respiratory chain was identical with or without DPPD or deferoxamine (data not shown).

Similarly, the decrease in ATP content, decrease in GSH-Rd and GSH-Px activity or decrease in GSH content, induced by 100 and 500 μM cisplatin could not be prevented by DPPD or deferoxamine.

Discussion

In a previous paper, freshly isolated PPTC were validated as an in vitro model to detect nephrotoxicity by studying the effect of mercuric chloride, cisplatin, para-aminophenol and halogenated alkenes on viability and mitochondrial membrane potential. The cells responded, time- and dose-dependently, to the nephrotic compounds with a decrease in Δψ and loss of viability. The sensitivity of the porcine cells to detect toxic effects corresponded favorably with in vitro systems derived from other animals (Kruidering et al., 1994).

This study provides evidence that cisplatin-induced mitochondrial dysfunction is caused by inhibition of complexes I to IV of the respiratory chain, which results in decreased intracellular levels of ATP. This selectivity for mitochondria is probably caused by accumulation of cisplatin in the negatively charged inner space of the mitochondria because of the positive charge of aquated complexes of cisplatin. Indeed cisplatin has been reported to accumulate in mitochondria of kidney cells in vitro (Gemba and Fukuishi, 1991; Kameyama...
Fig. 8. Effect of antioxidants deferoxamine (1 mM, 20 min preincubation) and DPPD (20 μM, 20 min preincubation) on ROS formation induced by cisplatin (A), decrease in Δψ (B) and loss of viability (C). Each point represents the mean (± S.E.M.) of at least four separate isolations. Statistics: (A) * from 20 min all values with DPPD/des were significantly different from Pt alone (P < .05); (B, C) Values with or without DPPD/des were not significantly different from Pt alone for both Pt concentrations.

Fig. 9. Effect of antioxidants deferoxamine (1 mM, 20 min preincubation) and DPPD (20 μM, 20 min preincubation) on ROS formation induced by 500 μM TBHP (A), decrease in Δψ (B) and loss of viability (C). Each point represents the mean (± S.E.M.) of at least four separate isolations. Statistics: (A) * significantly different from control, (P < .05); (B, C) * significantly different from TBHP alone, (P < .05).
Role of mitochondria in the formation of ROS. Because oxidative damage has been suggested as the main cause of cisplatin-induced renal cell death and several antioxidants and radical scavengers alleviate cisplatin-induced nephrotoxicity in vitro (Zhang et al., 1992; Sadzuka et al., 1992b; Kameyama and Gemba, 1991a) as well as in vivo (Gemba et al., 1988), we studied the role of ROS formation.

We observed formation of ROS after exposure of PPTC to cisplatin. The inhibitory effects of the inhibitors of the respiratory chain complexes I to III on ROS formation suggest that the electron transport chain in the mitochondria is a source of ROS formation in intact PPTC. The contribution of complex IV to ROS formation could not be demonstrated by this study, because inhibition of complex IV with KCN also reduced the cisplatin content of PPTC. Because ROS generation is measured with Dih123 which is cleaved to R123, the signal representing ROS is also dependent on the Δψ. Because the inhibitors of the respiratory chain complexes decrease the Δψ, the lowered ROS production may be the consequence of the effect on Δψ only. Therefore, we determined to what extent the Dih123 signal was dependent on the decrease in Δψ. Exposure of the PPTC to TBHP and the uncoupler CCCP clearly showed that a decrease in Δψ does not directly result in a decreased Dih123 signal and demonstrated that ROS can be detected in spite of a decrease in Δψ.

The detectable amount of ROS is determined by the imbalance between formation and scavenging of ROS. Cisplatin-induced inhibition of complexes I to III will be expected to reduce the amount of ROS by reducing the electron flow. However, exposure of PPTC to cisplatin resulted in an increased ROS content in spite of the inhibition of complexes I to IV, which suggests that the scavenging of ROS is probably even more impaired by cisplatin. The mechanism of decreased oxidant protection in cisplatin-induced ROS formation has been studied extensively. Cisplatin affects many enzymes that protect the cells from oxidative damage, among which Cu/Zn-superoxide dismutase, Mn-superoxide dismutase and catalase (Sadzuka et al., 1992a,b, 1994). In addition, cisplatin decreases intracellular GSH levels (Zhang and Lindup, 1993; Somani et al., 1995), which we also observed.

This study shows that, also in PPTC, cisplatin-induced ROS formation could be partially aggravated by the impaired antioxidant status of the cell, because incubation of the PPTC with cisplatin resulted in a time- and concentration-dependent inhibition of the enzymes GSH-Rd and GSH-Px, in addition to the severe reduction of intracellular GSH levels. GSH-Px is responsible for the detoxification of hydrogen peroxide, resulting in the formation of GSSG. GSH-Rd subsequently regenerates GSH from GSSG.

A decreased GSH content, or inhibition of GSH-Rd alone, is not sufficient to induce ROS formation, as is clear from our observations that maximal depletion of GSH in the cytosol or in the mitochondria of PPTC did not result in ROS formation, nor did incubation of the PPTC with BCNU, which specifically inhibits GSH-Rd (Babson and Reed, 1978). However, coincubation of the PPTC with inhibitors of complexes I to IV clearly showed that only inhibition of complex I in combination with BCNU resulted in ROS production. Because we found that cisplatin inhibits both GSH-Rd and complex I enzymatic activity, our data suggest that this may be the mechanism by which cisplatin-induced ROS formation occurs. This is confirmed by the observations that incubation of the PPTC with the combinations BCNU/cisplatin or BCNU/rotenone had identical toxicity (fig. 6, B–C), whereas BCNU in combination with TTFA, Oxa, anti or KCN had less pronounced effects on Δψ and viability. Inhibition of complex I with rotenone or MPTP (1-methyl-u-phenyl-1,2,3,6-tetrahydropyridine) has been implicated in formation of ROS before (Cortopassi and Wang, 1995). Inhibition of the electron flow through complex I favors electron flow through complex II. Electrons channeled via complex II produce about four times more superoxide than those channeled via complex I (Pryor, 1982); therefore, it is conceivable that inhibition of complex I results in a higher production of superoxide anions, which in combination with depletion of GSH will lead to oxidative damage.

Apart from GSH regeneration, other antioxidant functions exist in PTC. Complex II of the respiratory chain is involved in regeneration of α-tocopherol. Also in PPTC exposed to cisplatin complex II exerts a protective function, because cisplatin-induced ROS formation could be potentiated by the complex II inhibitor Oxa. However, Oxa is also a substrate for the citric acid cycle. Therefore, increased NADH production and subsequent increase in electron flow through the respiratory chain may also cause increased ROS. Our results do not exclude this possibility.

In summary, these results show that only inhibition of complex I in combination with reduced GSH-Rd activity resulted in ROS formation. Cisplatin exerts both effects; therefore, cisplatin-induced ROS formation can be mediated via simultaneous inhibition of complex I and GSH-Rd.

Relevance of ROS for cisplatin-induced cell death. In spite of the observations that several agents such as deferoxamine (Kameyama and Gemba, 1991a), DPPD (Sugihara et al., 1987), α-tocopherol (Hannemann and Baumann, 1988), procaine (Zhang et al., 1992) and dithiothreitol (Zhang et al., 1994) prevented formation of peroxides and cell damage both in vitro and in vivo (Zhang and Lindup, 1994; Sugihara et al., 1987; Gemba et al., 1988) we postulate that peroxide damage is not the cause of cell death.

This study shows that the ROS formation induced by cisplatin could be completely prevented by the antioxidant DPPD and the iron chelator deferoxamine, without any prevention of cell death (fig. 7, A–C). In contrast, TBHP-induced cell death was completely prevented by DPPD and deferoxamine (fig. 8, A–C), which shows that cell death is prevented in PPTC when formation of ROS is the cause of cell death. Therefore, we conclude that the observed ROS formation in PPTC exposed to cisplatin is a consequence rather than the cause of cell death. This would be in agreement with the observations in vivo with long-term studies that protection by antioxidants was far from complete (Reznik et al., 1993) and in vitro in mouse PTC (Liebenthal et al., 1996).

Possible mechanism of cisplatin-induced cell death and clinical relevance. The observation that the decrease in Δψ preceded cell death induced by cisplatin (fig. 2) suggests an important role for mitochondria as primary targets of cisplatin, which is in good agreement with other reports in the mouse and rat in vivo (Singh, 1989; Gordon and Gattone, 1986) as well as in vitro (Zhang and Lindup, 1994;
McGuiness and Ryan, 1994). We showed that cisplatin-induced mitochondrial dysfunction was the result of inhibition of the enzymatic complexes of the respiratory chain by cisplatin, with decreased ATP levels as a consequence. Antioxidants could not fully prevent the inhibition of the mitochondrial enzymes, the decreases in ATP content nor cell death. Therefore, inhibition of respiration probably is an important factor in cisplatin-induced cell death. This hypothesis is supported by the observation that exposure of PPTC to a combination low concentrations of inhibitors of complexes I to IV of the MRC caused reduction of Δψ and cell death similar to cisplatin (fig. 10).

How cisplatin inhibits the enzymatic complexes of the respiratory chain remains to be elucidated. Because of the high affinity of cisplatin for thiol groups (Chu, 1994) present in the active site of many enzymes, cisplatin may exert its effect via inhibition of these enzymes. Studies of the site and type of interaction of cisplatin at the different enzymatic complexes will give more clues about the mechanism of inhibition and the role of this inhibition in the pathway leading to acute cell death. Because cisplatin is known to affect DNA, RNA and protein synthesis, one could argue that the observed inhibition of enzymatic activity is mediated this way. However, toxic effects mediated in this way probably need more time to cause overt toxicity; 10 to 100 μM cisplatin did not have an effect on protein synthesis within 6 h in human kidney cortical slices. Only after 12 h could cisplatin-induced effects on protein synthesis be observed (Fisher et al., 1994), making it less likely, although not excluding, that cisplatin-induced inhibition of respiration, already observed within 20 min, is caused by effects on protein synthesis.

In patients treated with cisplatin, plasma levels reach approximately 10 μM after a single dose. The concentrations we used in this study are higher, but not unrealistic. Cisplatin usually is given to patients in repeated doses with intervals of 1 to 3 weeks (Gandara et al., 1989). However, 21 days after the last dose 13 to 40% of the cisplatin is still present in the body (Fish et al., 1994), which suggests that prolonged treatment may result in accumulation of cisplatin. Indeed accumulation of cisplatin has been shown in kidneys of patients treated with cisplatin (Stewart et al., 1985; Tothill et al., 1992), which implies that studies of the mechanisms underlying cisplatin-induced toxicity should include concentrations higher than 10 μM.

Although this study suggests that ROS formation is not the direct cause of cisplatin-induced toxicity, ROS formation may have significance for cisplatin-induced nephrotoxicity in vivo, because administration of antioxidants can alleviate the nephrotoxicity substantially because of reduction of inflammatory reactions of cell-mediated toxicity and by reduction of the amount of ROS that is released by a damaged cell. Yet, our results demonstrate that the actual cause of cisplatin-induced cellular toxicity is not prevented by antioxidants; therefore, cisplatin will still be nephrotoxic in spite of the use of antioxidants.

Acknowledgments

The enthusiastic support of Jaap van Hellemont (Utrecht University, Utrecht, The Netherlands) with the enzymatic assays of complex I to IV activity is greatly appreciated. We thank Prof. Vogel for the generous gift of BCNU, and Dr. A.M. Fichtinger, TNO, Delft, The Netherlands, for her help with the atomic absorption spectroscopy.

References


