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# CISPLATIN NEPHROTOXICITY: DECREASES IN MITOCHONDRIAL PROTEIN SULPHYDRYL CONCENTRATION AND CALCIUM UPTAKE BY MITOCHONDRIA FROM RAT RENAL CORTICAL SLICES

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**Abstract**—The effects of cisplatin on several aspects of the function of mitochondria isolated from the rat renal cortex have been investigated *in vitro*. Incubation of renal cortical slices with cisplatin (2 mM) caused a rapid loss of mitochondrial protein-SH followed by a substantial decrease in $Ca^{2+}$ uptake by the mitochondria and a decline in the mitochondrial membrane potential, which was assessed by rhodamine 123 uptake by the slices. Dithiothreitol, a glutathione (GSH)-reducing agent, significantly reversed the alterations in protein-SH, $Ca^{2+}$ accumulation and rhodamine 123 uptake. There was also a marked amelioration of cisplatin-induced cytotoxicity, as shown by the decreased leakage of several enzymes from the slices. Diethylmaleate, a GSH depletor, enhanced both the cisplatin-induced increase in toxicity, as assessed by enzyme leakage, and also the decreases in protein-SH, $Ca^{2+}$ accumulation and rhodamine 123 uptake. The antioxidant *N,N'*-diphenylphenylenediamine substantially alleviated cisplatin toxicity but did not protect against cisplatin-induced alterations to protein-SH and $Ca^{2+}$ uptake. In addition, the cytotoxicity caused by cisplatin was not affected by cyclosporin A, an inhibitor of $Ca^{2+}$ release from mitochondria and ruthenium red, an inhibitor of the reuptake of $Ca^{2+}$. It was concluded that loss of mitochondrial protein-SH and a decrease of $Ca^{2+}$ uptake are implicated in the toxicity of cisplatin and that mitochondrial GSH is an important factor in relation to oxidative stress to mitochondria and cytotoxicity.

*Key words:* cisplatin; nephrotoxicity; mitochondria; protein sulphydryl; rat kidney; cortical slice

Cisplatin is an anticancer drug which causes severe kidney damage in both humans and experimental animals [1–3]. Recent investigations have suggested that cisplatin-induced nephrotoxicity is related to oxidative stress and that lipid peroxidation is one of the mechanisms [4–7]. The role of lipid peroxidation and its position in the chain of events leading to nephrotoxicity, in particular, whether it is a cause or consequence of toxicity, remains controversial. Vermeulen and Baldew [8] reported that cisplatin did not induce lipid peroxidation in rat kidney microsomes nor did it inhibit the activity of a microsomal GSH†-dependent protective factor against lipid peroxidation induced by $Fe^{2+}$-ascorbate. In addition, the decrease of pyruvate-stimulated gluconeogenesis was independent of lipid peroxidation in renal slices *in vitro* [5, 7]. These results suggest that there may be another mechanism of cisplatin-induced nephrotoxicity in addition to lipid peroxidation.

Cisplatin-induced mitochondrial damage has been demonstrated in previous work [9–11] and our recent study [12, 13] showed that cisplatin significantly depleted the GSH and increased lipid peroxidation of mitochondria isolated from rat renal cortical slices. GSH depletion was an early event and appeared to be a determinant step in this oxidative stress to mitochondria, because cisplatin-induced cytotoxicity occurred subsequent to both the depletion of GSH and the lipid peroxidation.

GSH is the most important cellular thiol which can play a critical role in the defence against oxidative stress by a number of chemicals. Mitochondrial GSH may be pivotal in the regulation of inner membrane permeability by keeping intramitochondrial-SH groups in the reduced state [14]. The expression of chemically induced toxicity correlates strongly with mitochondrial GSH depletion, whereas the loss of protein-SH appears to occur after the loss of GSH [15, 16]. So far, the effect of cisplatin on the protein-SH of mitochondria has not been studied *in vitro* although Levi *et al.* [17] reported that cisplatin administration *in vivo* decreased the concentration of protein-SH in the kidney.

Furthermore, perturbation of intracellular thiols is also involved in the disturbance of $Ca^{2+}$ homeostasis [18, 19]. The homeostasis of $Ca^{2+}$ and of thiols in the mitochondria are believed to be closely linked either directly or through the pyridine nucleotides, and so an imbalance in one could affect the status of the other and diminish cell viability [20]. Gemba's

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† Abbreviations: GSH, glutathione; Tris, tris-(hydroxymethyl) aminomethane; DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); DTT, dithiothreitol; DEM, diethylmaleate; DPPD, *N,N'*-diphenylphenylenediamine; RR, ruthenium red; CSA, cyclosporin A; SH, sulphydryl; NAG, *N*-acetyl-β-glucosaminidase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.

group [21] found that co-incubation of cisplatin and DEM with slices of rat kidney cortex decreased the ability of the mitochondria to accumulate $Ca^{2+}$, but cisplatin alone had no significant effect on the uptake of $Ca^{2+}$ and there seems to be no relationship between both time- and concentration-related effects, especially, the role of $Ca^{2+}$ uptake in the nephrotoxicity of cisplatin.

This article therefore describes the effects of cisplatin on the concentration of protein-SH and $Ca^{2+}$ uptake of mitochondria and their roles in toxicity and preliminary results have been reported [22]. We show below that cisplatin added *in vitro* markedly depleted the protein-SH groups of mitochondria and inhibited $Ca^{2+}$ uptake by the mitochondria and that it also collapsed the mitochondrial potential. Cisplatin-induced cytotoxicity was closely associated with these biochemical events.

## MATERIALS AND METHODS

*Chemicals.* The following reagents were obtained from the Sigma Chemical Co. (Poole, U.K.): DTT, DEM, DPPD, RR, rhodamine 123, DTNB (Ellman's reagent), ATP disodium salt, Tris, *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and L-cysteine. Cisplatin was a generous gift from the Johnson Matthey Research Centre (Reading, U.K.). CSA was obtained from Sandoz Products Ltd (Middlesex, U.K.). L-Glutamic acid, sodium salt and glycine were from Merck Ltd (Poole, U.K.). $^{45}CaCl_2$ (sp. act.: 13.12 mCi/mg; stated radiochemical purity >99%) was from Du Pont Ltd (Herts, U.K.). All other reagents were commercial products of the highest available grade of purity.

*Preparation of renal cortical slices and isolation of mitochondria.* The preparation of renal cortical slices from female Wistar rats, 240–270 g body wt, and isolation of mitochondria from the slices were carried out as described previously [13].

*Incubation of renal cortical slices.* After preparation, the slices (three to five slices; total weight about 100–200 mg) were added to glass conical flasks (25 mL Erlenmeyer flasks, Duran, Astell Scientific, Kent, U.K.) which contained 5 mL of the first incubation medium (97 mM NaCl, 40 mM KCl, 0.74 mM $CaCl_2$, 1 mM glycine, 1 mM glutamate, 0.2 mM L-cysteine and 7.5 mM sodium phosphate buffer, pH 7.4, gassed with pure oxygen). Cisplatin (up to 2 mM), DTT and RR were dissolved in the first incubation medium, DPPD and CSA were dissolved in methanol and DEM in dimethyl sulphoxide prior to use. The flasks were oxygenated with 100% oxygen for 1 min after addition of the slices and then stoppered immediately with rubber bungs. The slices were incubated in a shaking waterbath at 100 cycles/min at 37° for various times. After incubation, mitochondria were isolated from the slices and mitochondrial protein was determined by the method of Lowry *et al.* [23] with bovine serum albumin as standard and the concentration of mitochondrial protein-SH groups and the accumulation of $Ca^{2+}$ by the mitochondria were examined as described below.

For the assay of enzyme leakage, the slices were gently removed from first incubation medium, blotted on wetted filter paper and transferred to another flask containing 4 mL of the second incubation medium (this was the first incubation medium without glycine, glutamate and L-cysteine). The flask was gassed with pure oxygen for 1 min and incubated at 25° for 90 min. After incubation, enzyme activity in the second incubation medium was determined.

*Mitochondrial calcium uptake.* The mitochondrial pellets were suspended in ice-cold medium (1.5 mL) which was composed of 100 mM KCl, 5 mM sodium succinate, 4 mM ATP, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 20 mM Tris–HCl (pH 7.0) until the assay (0.5 mg/mL of mitochondrial protein) was initiated by the addition of $^{45}CaCl_2$ (0.05 mL; 0.1 μCi/mL of final mixture; 0.2 μCi/μmol) to the mixture. The tubes were incubated at 37° for 15 min in a shaking waterbath. The reaction was terminated by removal of aliquots (0.5 mL) which were vacuum filtered through Millipore (0.45 μm) cellulose nitrate filters. The filters were washed four times with 10 mL of cold 100 mM KCl–20 mM Tris–HCl (pH 7.5) (10 mL used each time), dried in an oven at 50° for 10 min and placed in scintillation vials with 4 mL liquid scintillation fluid (Fisons PLC, Loughbrough, U.K.). Radioactivity was measured with a Packard 1500 TRI-CARB liquid scintillation analyser and $Ca^{2+}$ uptake was expressed as nmol of $Ca^{2+}$/mg per mitochondrial protein per 15 min. All uptake values were corrected for nonspecific binding of radioactivity to the filter when the same medium without mitochondria was used.

*Mitochondrial protein-SH.* The protein-SH was determined by the method of Monte *et al.* [16] with little modification. Mitochondrial suspension (0.5 mL; 0.5–1 mg/mL of mitochondrial protein) was added to 0.5 mL of 13% (w/v) trichloroacetic acid and the protein sedimented by centrifugation (MSE Micro Centaur). The supernatant was discarded and the pellet washed twice with 6.5% (w/v) trichloroacetic acid. The final pellets were suspended in 1 mL of 0.5 M Tris–HCl (pH 7.5). An aliquot (0.1 mL) of 10 mM DTNB in methanol was added and then mixed well. The absorbance was then measured at 412 nm within 5 min and the protein-SH concentration was calculated with L-cysteine as the standard.

*The uptake of rhodamine* 123. The uptake of this cationic fluorescent dye by the slices was used to estimate mitochondrial membrane potential by the method of Carini *et al.* [24]. After the first incubation, the slices were transferred to another flask and incubated in 4 mL of a second incubation medium containing 1.5 μM rhodamine 123, gassed with oxygen, at 25° for 90 min. After incubation, the medium was centrifuged at 3000 *g* for 10 min to remove particulate matter. The relative fluorescence intensity remaining in the medium was measured with a Perkin-Elmer 203 fluorescence spectrophotometer with a Perkin-Elmer 150 xenon power supply set at 490 nm excitation and 525 nm emission wavelengths. The capacity of mitochondria to take up rhodamine 123 was expressed as the difference (ΔF) in the fluorescence intensity of the medium, before and after incubation, per milligram of protein of the slices.

*Assay of enzyme activity.* The cytotoxicity of

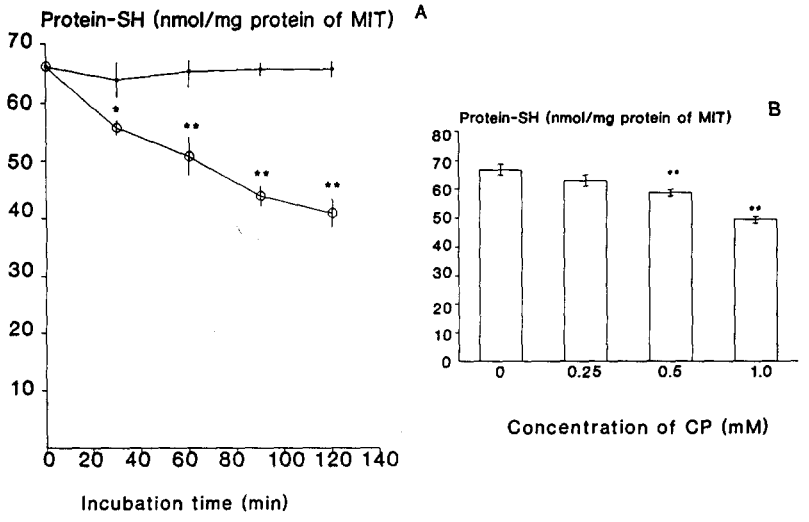


Fig. 1. (A) Time- and (B) concentration-courses of the loss of protein-SH from mitochondria (MIT) caused by cisplatin (CP). The mitochondria were isolated from renal cortical slices which were incubated in the medium (for 2 hr in Fig. 1B) with (-⊖-) or without 2 mM CP (-●-) at 37°. Each value represents the mean ± SE of five determinations. Significant differences from control are indicated by * $P < 0.05$, ** $P < 0.01$.

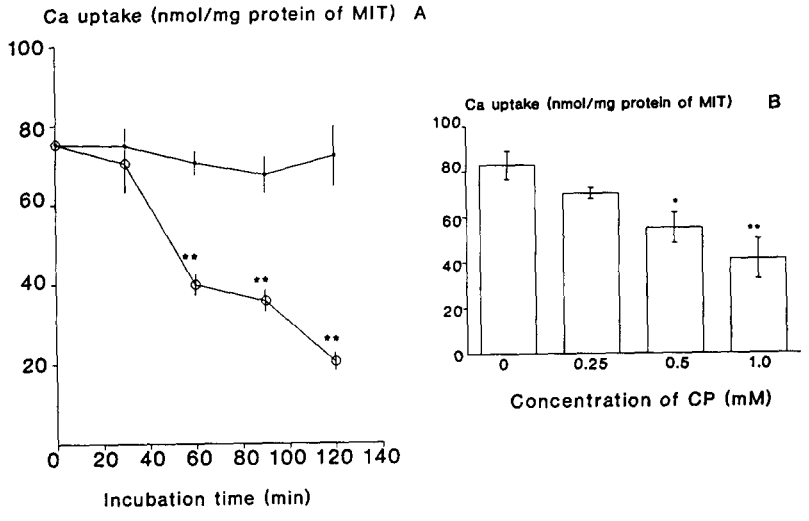


Fig. 2. (A) Time- and (B) concentration-courses of the decrease of $Ca^{2+}$ uptake by mitochondria. The other conditions were as in Fig. 1.

cisplatin was estimated by measurement of three enzyme activities released into the second incubation medium. The activity of AST (EC 2.6.1.1) was assayed by a colorimetric method based on the reaction of oxaloacetate with 2,4-dinitrophenylhydrazine (Sigma Kit catalogue No. 505) and likewise LDH (EC 1.1.1.27) was assayed by a kinetic ultraviolet method based on the formation of NAD (Sigma catalogue No. DG 1340-K). NAG (EC 3.21.30) activity was colorimetrically determined as described before [13].

*Statistics.* The renal cortical slices for each experiment were prepared from at least three rats and the results are expressed as means ± SE from four to five samples. The data were analysed with either an unpaired two-tailed Student's *t*-test or linear correlation or Pearson's correlation.

## RESULTS

### *Loss of protein-SH in the mitochondria*

Figure 1A shows the time-course of loss of

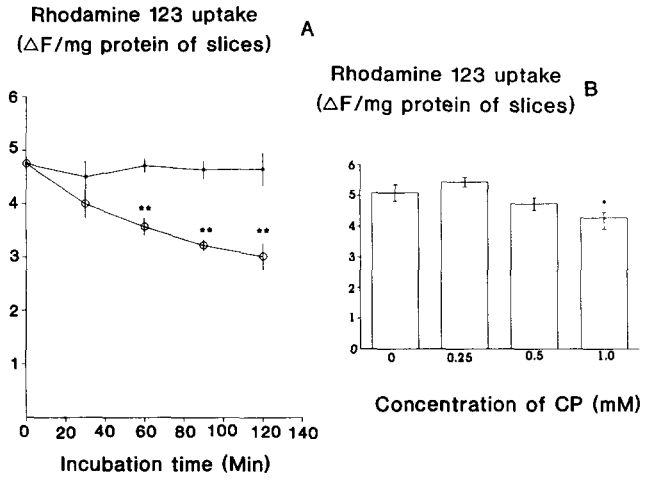


Fig. 3. (A) Time- and (B) concentration-courses of the decline of rhodamine 123 uptake by renal cortical slices. The other conditions were as in Fig. 1.

mitochondrial protein-SH caused by cisplatin. The concentration of protein-SH in the control group was about 65 nmol/mg of mitochondrial protein and this concentration remained constant during the various periods of incubation. Mitochondria from rat renal cortical slices incubated with 2 mM cisplatin suffered a dramatic depletion of protein-SH. A significant decrease ($P < 0.05$) was observed after 30 min of incubation and the protein-SH was decreased to 41 nmol/mg protein of mitochondria after 120 min of incubation. The depletion of mitochondrial protein-SH was concentration dependent and became statistically significant ($P < 0.01$) above a cisplatin concentration of 0.5 mM (Fig. 1B) and a further loss (to 70% of control) occurred with 1 mM after 120 min of incubation (Fig. 1B).

The concentration of cisplatin of up to 2 mM (600 μg/mL) that was used is about 10–20-fold higher than that of about 10 μg platinum/g wet tissue which is found in the rat *in vivo* 4–5 days after a moderately severe nephrotoxic dose (5 mg/kg) [6, 25]. Tumour concentrations of platinum can exceed 6 μg platinum/g wet tissue in patients after conventional cisplatin treatment [26] and after administration of a cisplatin–albumin complex the tumour concentration can exceed 50 μg platinum/g wet tissue [27]. Other investigators have used similar concentrations *in vitro* [28, 29].

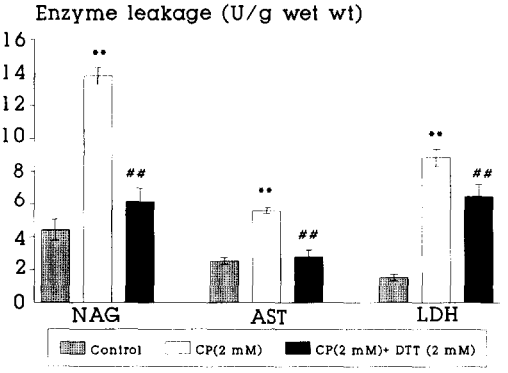


Fig. 4. Effect of DTT on cisplatin (CP)-induced increases of NAG, AST and LDH activities in the incubation medium. The renal cortical slices were incubated in the first incubation medium which contained either 2 mM CP or 2 mM CP + 2 mM DTT at 37° for 120 min. After this first incubation, the slices were incubated in second incubation medium at 25° for 90 min and leakages of enzymes into this second incubation medium were used to assess the extent of CP-induced cellular damage. Significant differences from control are indicated by ** $P < 0.01$. Significant differences from CP alone are indicated by **$P < 0.01$. The other conditions were as in Fig. 1.

*Inhibition of* $Ca^{2+}$ *uptake by mitochondria*

The ability of the mitochondria from control slices to accumulate $Ca^{2+}$ was not affected by the duration of incubation over a period between 15 and 120 min and the control amount of $Ca^{2+}$ uptake was about 70 nmol/mg protein of mitochondria (Fig. 2A). Cisplatin inhibited the uptake of $Ca^{2+}$ in both a time- and concentration-dependent way. The depression of $Ca^{2+}$ uptake was statistically significant ($P < 0.01$) by 60 min with 2 mM cisplatin and timewise this depression was subsequent to depletion of protein SH (see Fig. 1A). The depression of uptake was 70% (i.e. 30% of control) after 120 min of exposure to 2 mM cisplatin (Fig. 2A). Incubation of slices with lower concentrations (0.5 and 1.0 mM) of cisplatin for 120 min also decreased $Ca^{2+}$ uptake by mitochondria ($P < 0.05$ and $P < 0.01$, respectively) although cisplatin (0.25 mM) had no effect on the uptake of $Ca^{2+}$ (Fig. 2B).

*Collapse of mitochondrial potential*

The mitochondrial membrane potential was

Table 1. Effect of DTT, DEM and DPPD on cisplatin (CP)-induced changes of protein-SH, $Ca^{2+}$ accumulation and rhodamine 123 uptake by mitochondria (MIT) isolated from rat renal cortical slices

| | Protein-SH (nmol/mg protein of MIT) | $Ca^{2+}$ uptake (nmol/mg protein of MIT) | Rhodamine uptake ($\Delta F$/mg protein of slice) |
|---|---|---|---|
| Experiment 1 | | | |
| Control | 63.3 ± 1.8 | 69.4 ± 2.4 | 4.83 ± 0.25 |
| CP (2 mM) | 33.9 ± 0.5* | 17.6 ± 3.0* | 3.25 ± 0.06* |
| CP (2 mM) + DTT (2 mM) | 44.2 ± 2.9‡ | 36.9 ± 1.4‡ | 4.21 ± 0.16‡ |
| Experiment 2 | | | |
| Control | 67.7 ± 1.4 | 68.1 ± 7.4 | 4.83 ± 0.25 |
| CP (2 mM) | 41.0 ± 1.5* | 22.5 ± 7.2* | 3.25 ± 0.06* |
| CP (2 mM) + DEM (2 mM) | 31.6 ± 2.2‡ | 10.8 ± 2.1‡ | 2.92 ± 0.05† |
| Experiment 3 | | | |
| Control | 65.8 ± 2.5 | 70.5 ± 3.5 | 4.83 ± 0.25 |
| CP (2 mM) | 38.8 ± 1.3* | 20.0 ± 1.9* | 3.25 ± 0.06* |
| CP (2 mM) + DPPD (5 $\mu$M) | 39.5 ± 0.9 | 20.4 ± 1.0 | 3.60 ± 0.11† |

The slices were incubated in the medium with 2 mM of CP or CP+ one of the agents (DTT, DEM or DPPD) at 37° for 120 min. Each value represents the mean ± SE of five determinations.
Significant differences from control are indicated by *$P < 0.01$.
Significant differences from CP are indicated by †$P < 0.05$, ‡$P < 0.01$.

estimated by the measurement of the uptake of rhodamine 123 by the slices. The addition of 2 mM cisplatin to the incubation medium significantly decreased the fluorescence intensity of rhodamine 123 in the slices after 60 min of incubation, which indicated that the mitochondrial membrane potential had collapsed. After 120 min the uptake of rhodamine was only about 65% of control (Fig. 3A). Cisplatin at 1 mM decreased the uptake of rhodamine 123 (82% of control) but lower concentrations (0.25 and 0.5 mM) did not (Fig. 3B).

Since cisplatin-induced alterations in the protein-SH concentration, $Ca^{2+}$ accumulation and rhodamine 123 uptake of mitochondria were similar in both the time- and concentration-dependent experiments the correlations among them were analysed. A significant correlation was observed between protein-SH and $Ca^{2+}$ uptake at all times examined ($r$ =0.884, $P < 0.05$). Significant correlations were also found between $Ca^{2+}$ uptake and rhodamine 123 uptake ($r = 0.892$, $P < 0.02$) and also between protein-SH and rhodamine 123 uptake ($r = 0.997$, $P < 0.001$).

*The relationship between the biochemical events and cytotoxicity*

To obtain more precise information about the roles of protein-SH and $Ca^{2+}$ uptake in cisplatin-induced cytotoxicity, the effects of DTT, DEM and DPPD on the leakage of marker enzymes were studied. Control experiments where these three compounds were incubated with kidney slices revealed that in no case did they cause any increase in the leakage of the enzymes AST and LDH. Co-incubation of DTT (2 mM), a SH-reducing agent, and cisplatin (2 mM) with the slices markedly protected against leakages of NAG, AST and LDH from the slices into the incubation medium (Fig. 4).

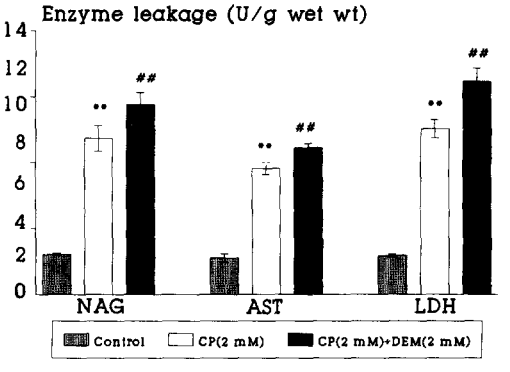


Fig. 5. Effect of DEM on cisplatin-induced increases of NAG, AST and LDH activities in the incubation medium. The other conditions were as in Figs 1 and 4.

Furthermore, such treatment also ameliorated the decreases of protein-SH concentration, and also the uptake of both $Ca^{2+}$ and rhodamine 123, as compared to cisplatin alone (Table 1). In addition, treatment of slices with DEM (2 mM), a GSH depletor, significantly increased cisplatin-induced toxicity (Fig. 5) and the loss of protein-SH and inhibition of the uptake of both $Ca^{2+}$ and rhodamine 123 by cisplatin (2 mM) was potentiated in DEM-treated slices (Table 1). Further experiments were done with the antioxidant DPPD. A preliminary study showed that DPPD at only 5 $\mu$M completely protected against cisplatin-induced lipid peroxidation (data not shown) and addition of DPPD (5 $\mu$M) protected against

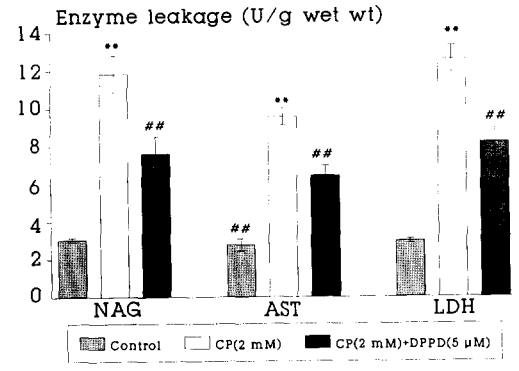


Fig. 6. Effect of DPPD on cisplatin-induced increases of NAG, AST and LDH activities in the incubation medium. The other conditions were as in Figs 1 and 4.

cisplatin-induced cytotoxicity (Fig. 6). We also investigated the effects of DPPD on the cisplatin-induced alterations of protein-SH, $Ca^{2+}$ accumulation and rhodamine 123 uptake by mitochondria. As shown in Table 1, DPPD had little effect on the loss of protein-SH and inhibition of $Ca^{2+}$ uptake caused by cisplatin, but significantly reduced the cisplatin-induced decrease of rhodamine 123 uptake.

As far as the evaluation of $Ca^{2+}$ homeostasis is concerned, the release of $Ca^{2+}$ from mitochondria is considered an important factor which can lead to disturbances of $Ca^{2+}$ homeostasis [30]. Unfortunately, the present *in vitro* slice model did not allow measurement of $Ca^{2+}$ fluxes in mitochondria. However, information about whether or not cisplatin increases $Ca^{2+}$ release from the mitochondria during the process of pathogenesis was obtained by evaluation of the effects of CSA and RR on cisplatin-induced toxicity. As shown in Table 2, CSA (500 nM), an inhibitor of the release of $Ca^{2+}$, did not prevent cisplatin-induced toxicity, and similarly it had no effect on the alterations of protein-SH, $Ca^{2+}$ uptake and rhodamine 123 uptake which were induced by cisplatin. Furthermore, RR (50 μM), an inhibitor of the reuptake of $Ca^{2+}$ uptake did not ameliorate the cisplatin-induced leakage of NAG [15.3 ± 2.6 (SE) (U/g wet wt) in cisplatin alone; 15.0 ± 1.5 (SE) (U/g wet wt) in cisplatin + RR; c.f. Table 2].

Table 2. Effect of CSA on cisplatin (CP)-induced changes of protein-SH, calcium accumulation and rhodamine 123 uptake of mitochondria (MIT) and enzyme leakage from renal cortical slices

| | Protein-SH (nmol/mg protein of MIT) | $Ca^{2+}$ uptake (nmol/mg protein of MIT) | Rhodamine 123 uptake (ΔF/mg protein of slices) | NAG (U/g wet wt of slice) | AST (U/g wet wt of slice) | LDH (U/g wet wt of slice) |
|---|---|---|---|---|---|---|
| Control | 69.2 ± 2.7 | 70.8 ± 4.2 | 4.52 ± 0.16 | 3.1 ± 0.5 | 3.2 ± 0.2 | 3.0 ± 0.5 |
| CP (2 mM) | 42.7 ± 1.0* | 22.0 ± 2.5* | 3.35 ± 0.18* | 14.6 ± 0.7* | 9.0 ± 0.4* | 14.2 ± 0.7* |
| CP (2 mM) + CSA (500 nM) | 39.2 ± 0.9 | 20.6 ± 2.2 | 3.45 ± 0.14 | 16.1 ± 2.0 | 7.5 ± 0.7 | 14.4 ± 0.9 |

The slices were incubated in the medium with 2 mM of CP or CP + 500 nM of CSA at 37° for 120 min. Each value represents the mean ± SE of four or five determinations. Incubation of slices with CSA alone in control experiments had no effect on enzyme leakage.
Significant differences from control are indicated by *$P < 0.01$.

## DISCUSSION

The aim of our recent work [12, 13, 22] was to define the mechanisms of cisplatin-induced renal cytotoxicity *in vitro*, based on our hypothesis that the mitochondrion is a primary target for cisplatin-induced oxidative stress. The results of the present work have shown that cisplatin significantly decreased the concentration of mitochondrial protein-SH, inhibited $Ca^{2+}$ uptake by mitochondria and collapsed the mitochondrial potential, and also that correlations appear to exist among them. Furthermore, cisplatin-induced kidney damage, characterized by the

increases of NAG, LDH and AST was associated with these biochemical indicators.

The importance of mitochondrial GSH in relation to cisplatin-induced peroxidation and toxicity has been demonstrated in the renal cortical slices under the same experimental conditions [12, 13]. Cisplatin depleted mitochondrial GSH within 15 min of incubation and loss of protein-SH was significant at 30 min of incubation (Fig. 1A), which indicated that loss of protein-SH took place only after the depletion of the mitochondrial GSH. In addition, DTT, which has been reported to restore the depletion of GSH in kidney slices caused by cisplatin [31], also protected against the loss of protein-SH, probably by scavenging cisplatin and its transformation products. On the other hand DEM, a GSH depletor, potentiated the loss of protein-SH (Table 1). These results are similar to the observations that menadione-induced intracellular GSH depletion in isolated hepatocytes preceded the loss of protein thiol groups [16]. This suggests that the thiol group of GSH is more readily available than the thiol groups of proteins for reaction with cisplatin, such a mechanism would protect against the depletion of protein-SH.

SH groups in both proteins and nonproteins (mainly GSH) are involved in the maintenance of various cellular functions, including many enzyme activities. The depletion of protein-SH usually occurs by its oxidation, the formation of protein disulphides and mixed disulphides. The fact that DTT, a known SH-reducing agent can protect against cisplatin-induced depletion of GSH [31] and protein-SH does not mean that an oxidation of -SH is implicated in such a depletion. Cisplatin can readily react with DTT to form an opaque, slightly pink complex, either in the incubation medium alone or in the medium which contains slices, after 30 min of incubation. These results are in agreement with the observation of Corden [32] who showed that cisplatin and DTT form a precipitate. Some uptake of this colloidal complex may occur by a phagocytotic mechanism which would explain why DTT enhances the uptake of cisplatin [31].

Another piece of evidence is that the powerful antioxidant DPPD has little effect on cisplatin-induced loss of protein-SH. Similarly, procaine, which is also an antioxidant failed to prevent GSH depletion by cisplatin [6], which suggests that the depletion of GSH is unrelated to its oxidation, whereas depletion of GSH and loss of protein-SH in hepatocytes by the $Ca^{2+}$ ionophore, A23187 were preventable by DPPD [15]. Thus, the mechanisms by which cisplatin (and its transformation products) depletes mitochondrial protein-SH, and possibly GSH, may be by its direct binding with -SH rather than by oxidation.

Perturbation of cellular $Ca^{2+}$ homeostasis is an important mechanism involved in many chemical-induced changes in cellular metabolism and in some cases, cell toxicity. Mitochondria, driven by the proton translocation-generated membrane potential, can take up large amounts of $Ca^{2+}$. This has led to the general belief that the mitochondrion can be a major regulator of cytosolic $Ca^{2+}$ concentration [15]. Thus both a decrease of mitochondrial $Ca^{2+}$ uptake and an increase of $Ca^{2+}$ release from the mitochondria can impair the ability of mitochondria to sequester $Ca^{2+}$.

In the present study, we found that cisplatin dramatically inhibited $Ca^{2+}$ uptake by mitochondria in both a time- and concentration-dependent way (Fig. 2). On the other hand, a variety of chemically diverse peroxidants cause $Ca^{2+}$ release from mitochondria by stimulating the hydrolysis of oxidized pyridine nucleotides, and pyridine nucleotide hydrolysis is a prerequisite but not a consequence of $Ca^{2+}$ release. Neither changes of the GSH redox state nor of the membrane potential are the primary cause of $Ca^{2+}$ release [32]. Beatrice *et al.* [14] have suggested, however, that the mitochondrial GSH/GSSG ratio is important in the control of $Ca^{2+}$ flux. In this study, the major question to be resolved is whether the depletion of mitochondrial GSH and protein-SH may increase the release of $Ca^{2+}$. This possibility was investigated with two different types of reagents. One was CSA, a cyclic unadecapeptide, which has been shown to prevent prooxidant-mediated $Ca^{2+}$ release from mitochondria by an inhibitory effect on pyridine nucleotide hydrolysis and the other was RR, an inhibitor of the $Ca^{2+}$ reuptake process in mitochondria. It has been demonstrated that CSA completely prevented the quinone imine-stimulated release of sequestered $Ca^{2+}$ [33]. However, neither the cisplatin-induced cytotoxicity nor the attendant biochemical events were prevented by CSA (Table 2), which suggests that cisplatin did not cause $Ca^{2+}$ release from mitochondria under these experimental conditions. Alternatively, it may be that the release of $Ca^{2+}$ by cisplatin is less important. This assumption was supported by the study with RR, because RR also did not ameliorate the cisplatin-induced leakage of enzymes from the slices.

Cisplatin also decreased mitochondrial potential (Fig. 3) as monitored by the uptake of the fluorescent substance rhodamine 123 by the slices. Our previous work showed that cisplatin induced changes in the charge density of mitochondrial membranes [13] and Singh [11] reported that incubation of cisplatin *in vitro* with cultured human fibroblast cells caused a decline in the uptake of rhodamine 123 with time, which indicated mitochondrial damage. Several mechanisms could be responsible for the decline of rhodamine 123 uptake by mitochondria. $Ca^{2+}$ overload can cause the collapse of mitochondrial membrane potential. Although we do not know the concentration of $Ca^{2+}$ in the mitochondria of the slices damaged by cisplatin, Gordon and Gattone [9] reported that administration of cisplatin to rats increased mitochondrial $Ca^{2+}$ accumulation. Moreover, collapse of mitochondrial potential may be associated with lipid peroxidation, since DPPD protected against cisplatin-induced inhibition of rhodamine 123 uptake.

There were good correlations among the results of measurements of protein-SH, $Ca^{2+}$ uptake and rhodamine 123 uptake. Mitochondrial sulphydryl groups have been implicated in the control of $Ca^{2+}$ homeostasis. The release of $Ca^{2+}$ that is induced by *p*-benzoquinone is related to inhibition of mitochondrial NAD(P)H dehydrogenases by arylation of critical SH groups which will decrease the

$NAD(P)^+$-reducing capacity, and possibly lower the $NAD(P)H/NAD(P)^+$ redox status in favour of $Ca^{2+}$ release [18, 33]. Moreover, thiol oxidizing agents can impair the active $Ca^{2+}$ sequestration by microsomal vesicles because the activity of $Ca^{2+}$, $Mg^{2+}$-ATPase in either the cell membrane and/or the endoplasmic reticulum, which is responsible for the transport of $Ca^{2+}$, is modulated by redox transitions of its protein-SH group [34]. Chavez *et al.* [35] suggested that dithiol groups in mitochondria are involved in the transport of $Ca^{2+}$ and that cadmium competitively inhibits $Ca^{2+}$ uptake into mitochondria. Therefore, the cisplatin-induced decrease of $Ca^{2+}$ uptake may be due to such a mechanism caused by loss of mitochondrial SH. The increase in cisplatin uptake by the slices in the presence of DTT [31] supports our proposition. On the other hand, the fact that the cisplatin-induced decrease of $Ca^{2+}$ uptake seems to be independent of lipid peroxidation is based on the following evidence. First, the cisplatin-induced decrease of $Ca^{2+}$ uptake preceded lipid peroxidation which was significantly increased after 60 min of incubation [13]. Secondly, the antioxidant DPPD failed to protect against cisplatin-induced loss of protein-SH and inhibition of $Ca^{2+}$ uptake.

To examine the role played by protein-SH and $Ca^{2+}$ homeostasis in the cellular toxicity induced by cisplatin, we investigated the effect of several agents on its toxicity to kidney slices. Cisplatin markedly increased the leakage of NAG, AST and LDH from the slices and this is evidence of cellular damage. This cisplatin-induced cell injury was eliminated by DTT while DEM potentiated the toxicity caused by cisplatin (Figs 4 and 5). These results suggest that cisplatin-induced loss of mitochondrial SH and disturbance of $Ca^{2+}$ homeostasis are important in the nephrotoxicity of cisplatin.

In conclusion, cisplatin caused significant loss of mitochondrial protein-SH, inhibited the uptake of $Ca^{2+}$ by mitochondria and decreased the mitochondrial membrane potential. These events may be responsible for cisplatin-induced kidney damage in addition to lipid peroxidation and if so, the depletion of mitochondrial GSH is an early and critical event in toxicity which results from oxidative stress to mitochondria.

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