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THE ROLE OF METALLOTHIONEIN IN THE REDUCTION OF CISPLATIN-INDUCED NEPHROTOXICITY BY Bi^{3+}-PRETREATMENT IN THE RAT *IN VIVO* AND *IN VITRO*

ARE ANTIOXIDANT PROPERTIES OF METALLOTHIONEIN MORE RELEVANT THAN PLATINUM BINDING?

PIETER J. BOOGAARD,*† ANJA SLIKKERVEER,‡ J. FRED NAGELKERKE* and GERARD J. MULDER*

*Division of Toxicology, Center for Bio-Pharmaceutical Sciences and ‡Toxicology Laboratory, University Hospital, Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands

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Abstract—Nephrotoxicity induced by cisplatin (CDDP) was reported to be reduced by Bi^{3+}-pretreatment, which selectively induces renal metallothionein (MT). In the present study renal MT had increased to 250% of control in rats that received bismuth subnitrate (50 μmol/kg/day, orally) for 8 days. *In vitro* experiments demonstrated that the reduction of CDDP-induced toxicity is a renal effect: in proximal tubular cells (PTC) isolated from Bi^{3+}-treated rats the toxicity of CDDP, and also of $HgCl_2$, $CdCl_2$ and *p*-aminophenol, was reduced as compared to PTC from untreated rats. In contrast to the reduction in CDDP, Hg^{2+} and Cd^{2+} toxicity, the reduction in *p*-aminophenol toxicity cannot be explained by the metal-binding properties of MT. MT was reported to act as a free radical scavenger, which may explain our observation since *p*-aminophenol toxicity is thought to be a consequence of the generation of oxygen radicals. *In vivo* experiments showed that the overall renal Pt-content as well as the Pt bound to renal MT is lower in Bi^{3+}-pretreated rats than in untreated rats, 24 hr after administration of CDDP (12 mg/kg), suggesting that the reduction in nephrotoxicity is not due to increased binding of Pt^{2+} to renal MT. Renal superoxide dismutase (SOD) activity was increased in rats that had only received CDDP. Such a rise in SOD may result from peroxidative damage caused by exposure to CDDP. The fact that SOD was not elevated in rats that received Bi^{3+} prior to CDDP suggests that (i) peroxidation contributes to CDDP-induced nephrotoxicity and (ii) the anti-oxidant properties of MT are responsible for the reduction of this toxicity.

cis-Diamminedichloroplatinum(II) (CDDP§) is a potent cytostatic drug with therapeutic activity against a wide variety of tumours, including testicular, ovarian, bladder, head and neck, cervix and osteogenic carcinomas [1]. The anti-tumour effect is most likely due to an inhibition of DNA synthesis by formation of bifunctional interstrand cross-links [2]. The use of CDDP is restricted by its severe side-effects [3, 4]: the serious nephrotoxicity is dose-limiting. The mechanism underlying this nephrotoxicity still remains unclear [4–6]. Despite aggressive hydration and hypersalination, which are routinely applied in the clinical situation to control nephrotoxicity [7, 8], renal failure, especially after repeated administration of CDDP, still occurs.

Several attempts have been made to reduce nephrotoxicity by coadministration of other compounds, which, however, reduce the oncolytic effect of CDDP as well [9, 10]. So far, only the dithiocarbamates, selenium and 4-methylthiobenzoic acid seem successful *in vivo* [11–13]. However, dithiocarbamates and selenium have toxic side-effects themselves at doses needed to prevent nephrotoxicity.

Recently it was demonstrated that in mice oral administration of Bi^{3+}-salts gave a substantial reduction in CDDP-induced nephrotoxicity without compromising its anti-tumour activity [14, 15]. Bi^{3+}-salts, which have been used in the treatment of peptic ulcers and gastro-intestinal complaints for many years, are relatively non-toxic in man [16]. Treatment with bismuth subsalicylate before chemotherapy with CDDP gave a small but significant reduction in nephrotoxicity in women with advanced ovarian cancer [17]; bismuth subnitrate treatment before CDDP administration prevented renal toxicity in patients with lung cancer [18]. In both trials the anti-tumoral response was unaffected. A mechanism

† To whom correspondence should be addressed. Present address: Shell Int. Petroleum Co., HSE/241, P.O. Box 162, 2501 AN The Hague, The Netherlands.

§ Abbreviations: AAS, atomic absorption spectroscopy; BSA, bovine serum albumin; CDDP, *cis*-diammine-dichloroplatinum (II); DCDFE-Cys, *S*-(1,1-difluoro-2,2-dichloroethyl)-L-cysteine; GSH, glutathione; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; HH-BSA-buffer, Hanks' buffer supplemented with 25 mM HEPES and 2.5% (w/v) BSA (pH 7.4); α-MG, α-methyl-glucose; MT, metallothionein; PTC, proximal tubular cell(s); SOD, superoxide dismutase; TCA-SB, 50 mM L-serine.borate in 0.4 M trichloroacetic acid; Tris–ME buffer: 10 mM Tris–HCl buffer supplemented with 1 mM mercaptoethanol (pH 7.4).

for this protective effect, however, has not been established as yet.

One possible mechanism is provided by the fact that bismuth very selectively induces metallothionein (MT) in the kidney, as it does (albeit to a much lesser extent) in the heart, but not in the liver [15, 19–21]. MT is a small (≈7 kDa), heat-stable protein, that contains about 30% cysteine residues and can bind a wide variety of heavy metals. Its role in the metal homeostasis, especially of Cu^{2+} and Zn^{2+}, and in the detoxification of heavy metals has been established, but many other functions have been suggested e.g. scavenging of free radicals and control of the cellular redox potential and sulphur metabolism [22, 23]. In many cell culture systems MT protects against CDDP-induced cytotoxicity [24–26]. As expected Pt^{2+}-ions have a high affinity for the cysteine thiolates: at pH 7.2 K_2PtCl_4 could replace half of the Cd and Zn in native MT [27]. CDDP binds *in vivo* as well as *in vitro* to MT [28–31]. Thus, the amelioration of CDDP-induced toxicity may be explained by its metal-binding characteristics, if it is assumed that MT-bound Pt is not toxic [14, 32]. Litterst *et al.* [33] suggested that both GSH and MT play a protective role in CDDP nephrotoxicity through formation of Pt—S bonds. CDDP reacts readily with cysteine and GSH *in vitro*. However, reports on the effect of CDDP on renal GSH concentration *in vivo* are conflicting [6, 33, 34]. Depletion of GSH resulted in a large increase of CDDP toxicity *in vitro* [35] as well as *in vivo* [33]; nevertheless, the induction of MT by Cd^{2+}-pretreatment, which protects against CDDP toxicity, lowers GSH concentrations. Unfortunately, these results are difficult to interpret since Cd itself is nephrotoxic.

Recent studies indicated that pretreatment with Bi^{3+}-salts protects against Adriamycin®-induced cardiotoxicity as well. This protection is correlated with the extent of MT induction [15, 36]. Adriamycin® exerts its cardiotoxic side effects via redox-cycling of the semi-quinone that is formed upon reduction [37, 38]. Therefore, the reduction of cardiotoxicity by MT may be due to the fact that MT acts as a free radical scavenger of $OH^{\cdot}$ and $O^{\cdot -}$ radicals [39–41]. A similar mechanism might alleviate CDDP nephrotoxicity, since peroxidative damage has been claimed to be involved in the toxicity induced by CDDP [42, 43].

The purpose of the present investigations was to evaluate the effect of pretreatment with Bi^{3+}-salts on CDDP-induced nephrotoxicity in correlation with the changes in renal GSH, SOD and the induction of MT.

MATERIALS AND METHODS

Chemicals

cis-Diamminedichloroplatinum (II) (CDDP) was a gift from Dr D. de Vos (Pharmachemie B.V., Haarlem, The Netherlands). Bis-2,3-dibromopropylphosphate was a gift of Dr S. D. Nelson (Dept. of Med. Chem., Univ. of Washington, Seattle, WA, U.S.A.). *S*-(1,1-Difluoro-2,2-dichloroethyl)-L-cysteine (DCDFE-Cys) was prepared as described previously [44, 45]. Other chemicals were obtained as follows: [U-^{14}C]-α-methylglucopyranoside (α-MG, α-methylglucose) (150 mCi/mmol) from Amersham (Amersham, U.K.), $^{109}CdCl_2$ (2.65 mCi/mg Cd^{2+}) from New England Nuclear (Dreieich, F.R.G.); collagenase (from *Clostridium histolyticum*) and xanthine oxidase (from cow milk) from Boehringer Mannheim (Mannheim, F.R.G.); rabbit hepatic metallothionein (75.2 mg Cd/g, 9.3 mg Zn/g), crystalline bovine haemoglobin, bovine serum albumin (fraction V) (BSA) and bovine renal superoxide dismutase from the Sigma Chemical Co. (St Louis, MO, U.S.A.); Nycodenz® (iohexol) from Nycomed (Oslo, Norway); bismuth nitrate pentahydrate from Merck (Darmstadt, F.R.G.); monobromobimane from Calbiochem (La Jolla, CA, U.S.A.). All chemicals were of the highest purity available.

Animal experiments

For all experiments male Wistar rats (180–220 g) from the strain of the Sylvius Laboratory, University of Leiden were used. The animals had free access to a commercial diet (SRM-A, Hope Farms, Woerden, The Netherlands) and tap water and were kept on a 12 hr light/dark cycle in a temperature controlled room.

Bismuth nitrate (485 mg) was dissolved in 50 mL glycerol under gentle heating, an equal volume of deionized water was added and the pH of the solution was adjusted to 7.0 with 4 N sodium hydroxide. Upon addition of the hydroxide bismuth subnitrate precipitates to yield a suspension (10 mmol Bi^{3+}/L).

By oral gavage 50 μmol Bi^{3+}/kg/day was administered during 8 consecutive days. Control animals received 5 mL 50% glycerol daily. For the *in vitro* experiments PTC were isolated from the pretreated rats on day 9. For the *in vivo* experiments the pretreated animals received, on day 9, an i.p. injection of CDDP (12 mg/kg; CDDP was dissolved at 2 mg/mL in saline supplemented with 100 mg mannitol/mL). Control animals received the vehicle (mannitol/saline) in which the CDDP was dissolved. Animals were killed 24 hr later, under diethylether anaesthesia, by exsanguination via the aorta. Kidneys and liver were rapidly perfused with ice-cold 10 mM Tris–HCl buffer supplemented with 1 mM mercaptoethanol (pH 7.4) (Tris–ME buffer). The left kidney was cut in two halves and a sample of the left liver lobe (approximately 300 μg) was taken; tissue was immediately frozen in liquid nitrogen and stored at −80° until assayed.

Proximal tubular cells (PTC) were isolated from rat kidney by collagenase digestion and purified by isopycnic centrifugation using Nycodenz® as density medium as previously described [46, 47]. Cells were incubated in Hanks' buffer supplemented with 25 mM HEPES and 2.5% (w/v) BSA (HH–BSA buffer) at a concentration of approximately 3×10^6 cells/mL, at 37° under 95% O_2/5% CO_2, on a rotatory shaker (160 cycles/min). In some studies 0.3 mM L-cystine, 0.5 mM glycine and 0.5 mM L-glutamine were added to the HH–BSA buffer. After isolation, PTC were preincubated for 15 min before the experiments were started. The chemicals under

investigation were added in a volume of 100 μL to the cell suspension.

Toxicity was determined by the extent of inhibition of α-MG uptake by the PTC [46].

Gel-chromatography

The right kidney and half the left kidney of each animal were homogenized in 2 volumes Tris–ME buffer in a Potter–Elvehjem glass homogenizer by six strokes with a Teflon pestle (0°, 1200 rpm). The homogenate was then centrifuged in a Beckmann ultracentrifuge ($10^5 g$, fixed angle rotor, 120 min, 4°). A 1.5-mL aliquot of the supernatant was applied to a Sephadex G-75 column (80 × 1.9 cm) and eluted with Tris–ME buffer. Forty 5-mL fractions were collected, frozen and stored at −80° until assayed.

Atomic absorption spectroscopy (AAS)

Bi. Aliquots (1.5 mL) of all fractions were lyophilized. The lyophilized samples were dissolved in 500 μL 36 mM HNO_3 containing 50 μg Pt/mL. Concentrations of Bi were determined by AAS (Perkin–Elmer, model 3030). Atomic absorption was measured as peak height with Zeeman background correction at $\lambda = 223.1$ nm after electrothermal atomization (2100°) in pyrolytically coated graphite furnaces using Ar as carrier gas. $BiCl_3$ was used for calibration.

Pt. Pt was determined in all fractions (without any further treatment) by AAS at $\lambda = 265.9$ nm, after electrothermal atomization (2600°) in graphite furnaces (Perkin–Elmer, model 4000).

Assays

Protein. Protein was measured according to Lowry *et al.* [48], using BSA for calibration.

MT. Aliquots (1.0 mL) of all fractions were lyophilized and the cadmium binding potential was determined by the Cd/haem affinity assay according to Eaton and Toal [49]. Rabbit liver MT was used for calibration.

SOD. The kidney homogenate was prepared according to Chung *et al.* [50]. After centrifugation ($1.5 \times 10^4 g$, 30 min, 4°), the supernatant was lyophilized and dissolved in 50 mM Na_2CO_3 containing 17 μM EDTA. Superoxide anions were generated by addition of a xanthine (0.1 mM)/xanthine oxidase (8.3 μg/mL) system. The SOD activity was determined spectrophotometrically by measuring the inhibition of the oxidation of adrenaline (0.3 mM) to adenochrome at $\lambda = 480$ nm [51]. Bovine renal SOD was used as a standard.

GSH. Aliquots (250 μL) of the cell suspensions were added to an equal volume of 0.4 M trichloroacetic acid supplemented with 50 mM L-serine.borate (TCA-SB). The frozen tissue (left kidney half, that was not homogenized) was thawed in 1 mL TCA-SB and homogenized. GSH was assayed by HPLC after derivatization with monobromobimane using fluorometric detection [52].

Statistics

Student's *t*-test (unpaired, two-tailed) was used for all statistical comparisons; probability values (P) greater than 0.05 were considered statistically insignificant. All values are expressed as means ± SE.

RESULTS

Effect of Bi^{3+}-pretreatment on toxicity by nephrotoxicants in isolated PTC

In order to investigate the influence of Bi^{3+}-pretreatment on the sensitivity of PTC to toxicants, six nephrotoxins were tested at concentrations that previously had been shown to be toxic within a 1.5 hr incubation in this cell system: $HgCl_2$, $CdCl_2$, CDDP, *p*-aminophenol, bis-(2,3-dibromopropyl)phosphate and the cysteine-*S*-conjugate of 1,1-dichloro-2,2-difluoroethylene (DCDFE-Cys) [47].

PTC were isolated from rats that had been fed a bismuth subnitrate-suspension by oral gavage for 8 days and from control rats. There was no difference in viability, α-MG uptake or yield of cells prepared from control animals or Bi^{3+}-pretreated rats.

Pretreatment with Bi^{3+} protected against toxicity induced by Hg^{2+}, Cd^{2+}, CDDP and *p*-aminophenol but had no protective effect against the toxicity of bis-2,3-dibromopropylphosphate and DCDFE-Cys (Table 1). Inclusion of $Bi(NO_3)_3$ in the incubation medium at concentrations that had no effect on α-MG uptake by themselves (<100 μM) had no protective effect against the toxicity induced by $HgCl_2$, $CdCl_2$, CDDP or *p*-aminophenol.

GSH in isolated PTC

Since GSH protects cells against damage by heavy metals and oxidative stress, the effect of Bi^{3+}-treatment on the GSH-status of the PTC was studied. In preliminary studies with PTC it was established that the recovery of GSH, determined by standard addition was only 70 ± 6%. Presumably, deproteination by addition of 6.5% (w/v) TCA does not completely prevent GSH hydrolysis, due to γGT activity. The recovery was not improved by using 10% $HClO_4$ or 15% HPO_3. Addition of 50 mM L-serine.borate to the TCA solution [54] inhibited GT completely, resulting in a GSH recovery of 102 ± 3%.

PTC incubated in HH-BSA buffer had an initial GSH concentration of 20–25 nmol/mg protein, which declined gradually to approximately 70% of the initial value in 3 hr, without loss of viability. If the precursors for GSH (cystine, glutamine and glycine) [53] were added to the HH-BSA buffer, the intracellular GSH levels remained essentially constant during this period. Exposure of the PTC to 250 μM CDDP did not affect GSH for the first 15 min, but thereafter GSH was rapidly decreased by over 75% in another 15 min. Within 1 hr virtually all GSH was lost; however, the PTC had not lost their ability to synthesize GSH and recovered partially in the next hour (Fig. 1). If precursors for GSH were added to the incubation medium, exposure to 250 μM CDDP resulted initially in the same rapid loss of GSH as was observed during the incubation without precursors, but now the cells were able to recover completely within the next 30 min, and maintained their intracellular GSH concentrations at control levels during subsequent incubation. No differences were found in the effect of CDDP on GSH in PTC isolated from rats that had been pretreated with Bi^{3+} as compared to control rats (Fig. 2).

Table 1. Effect of pretreatment with $Bi(NO)_3$ *in vivo* on toxicity of nephrotoxicants in isolated renal PTC

Nephrotoxicant	Concentration (mM)	α-MG uptake (%) after 3 hr exposure	
		Untreated	Bi^{3+} pretreated
None (control)	—	95 ± 8	97 ± 4
Mercury(II) chloride	0.25	27 ± 1	49 ± 3
Cadmium chloride	0.25	40 ± 3	72 ± 4
cis-Diamminedichloroplatinum(II)	0.25	53 ± 6	98 ± 13
S-(1,1-Dichloro-2,2-difluoroethyl)-L-cysteine	0.25	24 ± 6	20 ± 4
p-Aminophenol	0.25	67 ± 1	101 ± 15
	1.0	10 ± 3	35 ± 6
Bis-(2,3-dibromopropyl)phosphate	1.0	60 ± 8	68 ± 11

Each value represents the mean ± SE of at least three separate cell preparations.

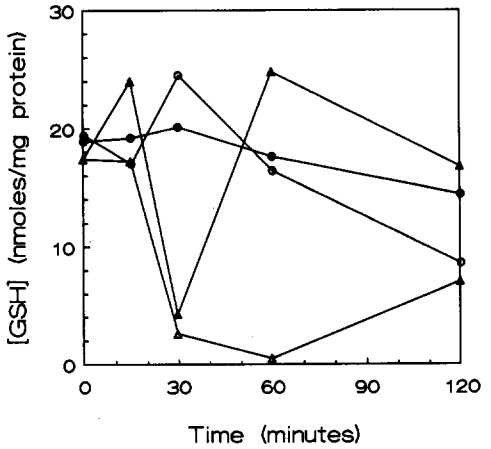

Fig. 1. Effect of CDDP on intracellular GSH concentrations in isolated PTC. Filled symbols (●, ▲) indicate PTC incubated in HH-BSA buffer supplemented with GSH-precursors (0.3 mM L-cystine, 0.5 mM L-glutamine and 0.5 mM glycine), open symbols (○, △) in normal HH-BSA buffer. (○, ●): controls; (△, ▲): 250 μM CDDP. Values represent the means of two separate cell isolations and differed less than 15%.

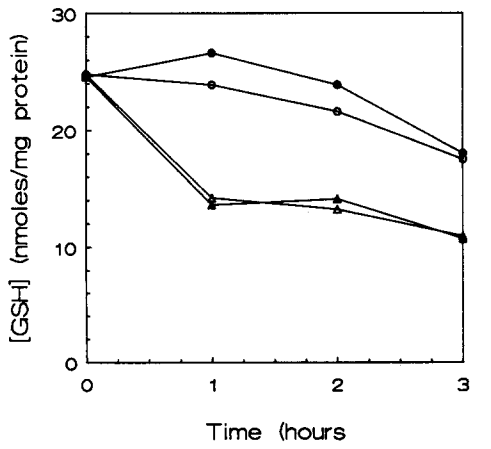

Fig. 2. Effect of CDDP on intracellular GSH concentrations in PTC isolated from control rats (open symbols) and in PTC isolated from rats pretreated with Bi^{3+} (filled symbols). (○, ●): controls (△, ▲): 250 μM CDDP. Values represent the means of two separate cell isolations and differed less than 15%.

Renal MT

In all fractions that eluted from the Sephadex G-75 column Cd binding protein was assayed, expressed as MT. Peaks found at a relative retention volume (V/V_0) of 1.8–2.3, with negligible UV absorption at $\lambda = 280$ nm, were considered to be MT proteins. Bi^{3+}-pretreatment resulted in a significant increase of these MT proteins. In animals that had only been treated with CDDP no increase was found (Table 2).

Renal metal distribution

Bi^{3+} pretreatment reduced the total amount of Pt present in the renal cytosol by 30% (Table 3). Subsequently Bi and Pt were determined in all fractions when the postmicrosomal supernatant was fractionated across a Sephadex G-75 column. The elution pattern shows that the decrease is a result of less binding to both high molecular weight proteins and compounds with a molecular weight less than approximately 1500. The absolute amount of Pt bound to MT protein fraction had not changed, although the total amount of MT protein had more than doubled. CDDP seemed to displace Bi^{3+}, since both total and MT-bound Bi was lowered, although the decrease in MT-bound Bi was not statistically significant.

Renal SOD activity

SOD activity was assayed in the kidney homogenates, which were lyophilized in order to remove mercaptoethanol, which may interfere with the SOD assay when present at concentrations higher than 1 mM [54]. Bi^{3+} pretreatment did not alter renal SOD activity. In contrast, SOD was significantly increased (34%) in rats 24 hr after they had received CDDP. Bi^{3+}-treatment abolished this rise in activity due to CDDP (Table 2).

DISCUSSION

The *in vitro* experiments with isolated PTC demonstrate that Bi^{3+}-pretreatment provides protection against CDDP-induced toxicity, similar to the protection by Bi^{3+}-pretreatment observed *in vivo*. As was to be expected if administration of Bi^{3+}-induced MT, the toxicity of Hg^{2+} and Cd^{2+} was reduced as well. DCDFE-Cys, *p*-aminophenol and bis-2,3-

Table 2. Renal SOD activity and MT content

Treatment*	SOD activity mUnits/g protein (% of control)	Metallothionein in cytosol mg/g protein (% of control)
Controls	4.60 ± 0.14 (100)	0.69 ± 0.01 (100)
Bi^{3+}	4.75 ± 0.20 (101)	1.78 ± 0.45† (258)
CDDP	6.18 ± 0.44† (134)	0.72 ± 0.06 (105)
Bi^{3+} and CDDP	5.01 ± 0.19 (108)	1.70 ± 0.14‡ (246)

* Animals were treated as described in Materials and Methods; each group consisted of eight animals for SOD determinations and of four animals for MT assays.
† Significantly different from control ($P < 0.05$).
‡ Significantly different from control ($P < 0.005$).

Table 3. Renal Bi and Pt concentrations

	Pt concentration (natom/g protein)		Bi concentration (natom/g protein)	
Treatment*	Total homogenate	MT-fractions	Total homogenate	MT-fractions
Control	ND	ND	ND	ND
Bi^{3+}	ND	ND	115 ± 43	12 ± 6
CDDP	774 ± 113	215 ± 21	ND	ND
Bi^{3+} and CDDP	548 ± 31†	220 ± 21	45 ± 15	2.9 ± 0.5

ND, not detectable.
* Same animals as in Table 2.
† Significantly different from Bi^{3+}-pretreated group.

dibromopropylphosphate were anticipated to be negative controls. Surprisingly, Bi^{3+} pretreatment also afforded protection against *p*-aminophenol toxicity. *p*-Aminophenol is readily converted into its quinoneimine, which may induce toxicity via a redox-cycling mechanism. Thus, the protection by Bi^{3+} pretreatment might be related to the antioxidant properties of MT, similar to the observation by Satoh *et al.* [15, 36] that the cardiotoxicity of Adriamycin®, which is due to redox-cycling as well, can be alleviated by Bi^{3+}-treatment.

CDDP induces lipid peroxidation *in vitro* and *in vivo* [42, 55] which may be reduced by antioxidants [43, 56] and aggravated by GSH depletion [35]. In isolated PTC CDDP (250 μM) induced a rapid and virtually complete depletion of GSH. The effect of CDDP on GSH was the same in control cells and in PTC isolated from Bi^{3+}-pretreated rats. Since the initial GSH concentration was the same in controls and Bi^{3+}-pretreated rats, it is not likely that the protection against CDDP induced by Bi^{3+}-pretreatment is mediated by GSH.

Although GSH was replenished if a sufficient supply of precursor was present, substantial peroxidative damage may be inflicted by CDDP to the cells during the absence of GSH, which may lead to cell toxicity *in vitro*. Conditions that lead to peroxidative damage often result in an increased expression of SOD [50, 57]. We found that treatment of rats *in vivo* with CDDP increased the renal SOD activity by 34%; this increase was not observed in rats that had received Bi^{3+} prior to CDDP. This may be a result of the rise of MT in Bi^{3+}-pretreated rats, because free $OH^{\cdot}$ and $O^{\cdot -}$ radicals, that lead to an increase of SOD, can be scavenged by MT.

A substantial induction of MT upon Bi^{3+}-pretreatment was observed, but the total amount of Pt bound in the kidney was reduced by Bi^{3+}-pretreatment. The absolute amount of Pt bound to proteins in the MT fraction had not changed. Although there was a slight increase in Pt found in the MT fraction relative to the total amount of Pt (40% vs 27% in the untreated rats), the amount of Pt bound per mg of MT is decreased. The Sephadex G-75 elution pattern showed that both in the fractions containing higher as in those containing lower molecular weight components than MT, less Pt was bound in the kidney of Bi^{3+}-pretreated rat as compared with the controls. Therefore, the reduction of CDDP-induced nephrotoxicity by Bi^{3+}-pretreatment seems to be due to a lower overall Pt binding in the kidney and not to a higher binding of Pt to renal MT.

In conclusion, these experiments confirm the induction of renal MT upon Bi^{3+}-pretreatment. The protection against CDDP toxicity appears a renal effect, as demonstrated by the experiments with isolated PTC. It seemed not due to an increased binding of Pt to MT. Bi^{3+}-pretreatment altered the Pt binding in the kidney, which resulted in a lower overall renal Pt concentration. It has been suggested that oxygen radicals might play a role in the nephrotoxicity of CDDP [35, 42, 43, 55, 56], the present results support this view. The reported antioxidant properties of MT may be responsible for the reduction in CDDP-induced nephrotoxicity since the effects on renal SOD suggest that there is less oxidative stress due to CDDP exposure after Bi^{3+}-pretreatment.

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