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PROMOTION OF *trans*-PLATINUM *IN VIVO* EFFECTS ON RENAL HEME AND HEMOPROTEIN METABOLISM BY D,L-BUTHIONINE-*S*,*R*-SULFOXIMINE

POSSIBLE ROLE OF GLUTATHIONE

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Abstract—We have examined the toxicity of *trans*-platinum (*trans*-diamminedichloroplatinum II) to heme and hemoprotein metabolism in the kidney of glutathione (GSH)-depleted rats and compared it with that produced by *cis*-platinum. Unlike *cis*-platinum treatment (7.0 mg/kg, i.v.) which caused after 7 days significant increases in cytochromes P450 and b_5, and a marked decrease in porphyrin content of the kidney, *trans*-platinum alone (7 mg/kg, i.v.) did not elicit notable changes in these variables when measured 1 or 7 days after treatment. Also, *cis*-platinum treatment significantly altered the heme degradation pathway by increasing the activity of heme oxygenase and decreasing that of biliverdin reductase; *trans*-platinum treatment did not elicit a response in these activities. However, when rats were given the inhibitor of GSH synthesis, D,L-buthionine-*S*,*R*-sulfoximine (BSO), the subsequent administration (2 hr later) of *trans*-platinum produced, in 1 day, the spectrum of responses that were mediated by *cis*-platinum after 7 days. In the kidneys of rats treated with BSO plus *trans*-platinum the concentration of platinum measured only about 50% of that detected in the kidneys of rats treated with *trans*-platinum alone. In the liver, *trans*-platinum by itself or in combination with BSO was ineffective in altering the measured variables of heme metabolism. The possibility that similarity between *cis*-platinum and *trans*-platinum plus BSO may extend to systems other than heme metabolism, e.g. GSH synthesis and degradation, was examined. *cis*-Platinum caused significant inhibition of both renal γ-glutamyl synthetase and γ-glutamyl transpeptidase after 7 days, but not after 1 day. Twenty-four hours after treatment, BSO + *trans*-platinum caused inhibition of γ-glutamylcysteine synthetase activity, whereas this activity in animals treated with BSO alone had returned to control values. At this time point, neither oxidized glutathione (GSSG)-reductase nor γ-glutamyl transpeptidase activity was affected by *trans*-platinum + BSO treatment.

The findings suggest that GSH constitutes an important defense mechanism against *trans*-platinum alteration of heme metabolism and may play a role in cellular accumulation of the drug in an inactive complex. It is proposed that BSO treatment, despite resulting in a diminished intracellular concentration of *trans*-platinum, allows reaction of the metal complex with target molecules by virtue of its ability to deplete GSH.

cis-Platinum (*cis*-diamminedichloroplatinum II) constitutes the basis for many of the combination chemotherapy protocols currently in use in treatment of solid tumors, in particular nonseminomatous germ cell cancers [1–3]. Nephrotoxicity caused by the drug, however, causes serious dose limitations in its usage [4–6]. The cellular basis for the nephrotoxicity has not been fully elucidated, although several mechanisms have been evoked [7–11], including that which stemmed from our studies [12, 13]. The heme and porphyrin metabolism pathway is exquisitely sensitive to perturbations by various chemicals including metal complexes [14], and has been promoted as a sensitive indicator for exposure to toxic agents [15]. Using porphyrin and heme metabolism as indicators of *cis*-platinum toxicity [16–18], major perturbations in these processes have been observed in the rat kidney [12]. In this respect *cis*-platinum resembles the chlorides of platinum [19, 20]. Also, in *cis*-platinum-treated rats, a time-dependent and pronounced inhibition of activities of γ-glutamylcysteine synthetase, the rate-limiting enzyme in the production of GSH [21] has been observed.

trans-Platinum is known to be by far more reactive *in vitro* than *cis*-platinum; however, *in vivo* it is less nephrotoxic than the *cis* complex at equivalent doses [1]. The molecular properties of *trans*-platinum are believed to account for its relative lack of cytotoxicity [1]; one of the chlorides of *trans*-platinum is more easily substituted than is a chloride of *cis*-platinum. Hence, *trans*-platinum reacts more readily with side chains of amino acids including the sulfhydryl groups, and hydrates four times more rapidly than *cis*-platinum [5, 8]. The rapid rate of reactivity of *trans*-platinum is presumed to result in the inactivation of the compound in the circulation and the cytosol before reaching the cellular regulatory macromolecules. Glutathione (GSH) is the most prevalent cellular and plasma non-protein thiol. Accordingly, it could be postulated that altering the availability of

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this ligand may modify the *in vivo* activity of *trans*-platinum.

To examine whether *trans*-platinum *in vivo* can be rendered active we investigated the effects of altered kidney GSH metabolism using the specific inhibitor of GSH synthesis, D,L-buthionine-*S*,*R*-sulfoximine (BSO). We have shown that the combined treatment with BSO and *trans*-platinum causes a similar pattern of perturbations in heme and hemoprotein metabolism, albeit different in time–course, to those caused by *cis*-platinum alone; with both treatments the kidney is the target organ.

EXPERIMENTAL PROCEDURES

Materials. Porphyrin compounds were obtained from Porphyrin Products (Logan, UT). *cis*-Platinum, *trans*-platinum, and BSO were obtained from the Sigma Chemical Co. (St Louis, MO). All chemicals were of analytical grade.

Animals and tissue preparations. Male Sprague–Dawley rats (180–220 g) were purchased from Harlan Industries (Madison, WI), and maintained on Purina rat chow and water *ad lib*. *trans*- or *cis*-Platinum was injected as a single dose of 7.0 mg/kg in a vehicle of 0.9% NaCl intravenously. The time–course of effect of BSO on renal GSH concentration was assessed by measuring the concentration of the tripeptide following a single injection of 4 mmol/kg (s.c.). In subsequent studies, BSO was given subcutaneously at a dose of 4.0 mmol/kg 2 hr before *trans*-platinum. Control animals received saline or BSO, depending on the experimental protocol. All injections were made between 9:30 and 10:00 a.m. The regimen of treatments is given in the legends to the appropriate table and figures. When animals were utilized for measuring the time–course of the effect of BSO on the renal GSH level, a portion of the organ was removed immediately after sacrifice and was homogenized in a buffer of 10% trichloroacetic acid, 0.01 M hydrochloric acid and 0.1 mM EDTA (by vol.). The same protocol was followed when measuring the hepatic GSH concentration. The remainder of the kidney was processed as described below and analyzed for γ-glutamylcysteine synthetase activity. All other animals were decapitated, and the liver and kidneys were perfused *in situ* with saline. The organs were homogenized in 4 vol. of 0.01 M Tris–HCl buffer, pH 7.4, containing 0.25 M sucrose. The microsomal and the cytosol fractions were prepared by differential centrifugation as detailed previously [17]. The tissue homogenate was utilized for determinations of total porphyrin content, and the activity of γ-glutamyl transpeptidase. The microsomal fraction was used for measurements of heme oxygenase activity and the contents of cytochromes P450 and b_5. The activity of biliverdin reductase, γ-glutamylcysteine synthetase and oxidized glutathione (GSSG)-reductase was assessed in the cytosol fraction (105,000 *g* supernatant). Biliverdin reductase was purified from rat liver as described earlier [22] and used in the heme oxygenase assay system.

Assay procedures. The activity of heme oxygenase was measured as detailed previously [23] using iron-protoporphyrin (heme *b*) as the substrate. The activity of biliverdin reductase was determined as before [22], at pH 8.6, with NADPH as the cofactor. GSSG-reductase activity was assayed by the method of Massey and Williams [24]. The assay medium (1.0 mL) contained the enzyme source (80–100 μg protein), EDTA (3 mM), bovine serum albumin (2 mg), NADPH (0.1 mM), GSSG (3 mM) and potassium phosphate buffer (50 mM, pH 7.6). The reaction was started by the addition of GSSG, and enzyme activity was assessed from the disappearance of NADPH. The blank assay system did not contain GSSG. The assay was carried out at 25°; a unit of activity was defined as 1 nmol NADPH oxidized per milligram protein per minute.

The assay of γ-glutamylcysteine synthetase was carried out by a modification of the method described by Sekura and Meister [25]. The reaction mixture (1.0 mL) contained ATP (5 mM), $MgCl_2$ (20 mM), L-glutamate (10 mM), L-α-aminobutyrate (20 mM), EDTA (0.1 mM), bovine serum albumin (0.05 mg) and Tris–HCl buffer (10 mM, pH 8.2). The reaction was initiated by the addition of the enzyme source (1–2 mg protein). L-α-Aminobutyrate was not added to the blank assay mixture. Duration of incubation was 30 min at 37°. The reaction was terminated by the addition of 1 mL trichloroacetic acid (10%), and the protein was precipitated by centrifugation (5000 *g*, 10 min). The liberated phosphate (P_i) in the supernatant fraction was determined colorimetrically (720 nm) by the method of Taussky and Shorr [26]. An enzyme unit was defined as 1 nmol P_i released per milligram protein per minute.

The activity of γ-glutamyl transpeptidase was measured by a modification [27] of the procedure described by Meister *et al.* [28]. The assay system (1.0 mL) consisted of enzyme source (20–50 μg protein), glycylglycine (2 mM), L-γ-glutamyl-*p*-nitroanilide (2.5 mM), Triton X-100 (1.0%), NaCl (7.5 mM), and Tris–HCl buffer (0.05 M, pH 8.0). The reaction was initiated by the addition of L-γ-glutamyl-*p*-nitroanilide. The rate of release of *p*-nitroaniline was followed from the increase in absorption at 405 nm. An extinction coefficient of 9.9 $mM^{-1}\,cm^{-1}$ was used for the measurement of enzyme activity. The activity was expressed as the micromoles of *p*-nitroaniline formed per milligram of protein per minute. Renal and hepatic GSH content was determined fluorometrically by the method of Cohn and Lyle [29].

Spectral studies. The kidney cytochrome P450 content was measured using the procedure described by Jones *et al.* [30], which corrects for cytochrome oxidase contamination. The total porphyrin concentration was assessed spectrofluorometrically [31]; the excitation wavelength was 400 nm with a slit width of 20 nm and the emission wavelength was 654 nm with a 20-nm slit. Coproporphyrin-III was utilized as the standard. Protein was measured by the method of Lowry *et al.* [32]. Tissue platinum levels were determined by flameless atomic absorption spectrometry, using a Perkin–Elmer spectrometer, as described by Pera and Harder [33].

The spectral studies were carried out using an SLM Aminco DW-2C spectrophotometer. The results are expressed as means ± SD for three to four determinations; one rat was used for each determination.

Table 1. Comparative effects *in vivo* of *trans*- and *cis*-platinum on total porphyrin content and activities of enzymes of the heme degradation pathway in rat kidney

Treatment	Total porphyrin (pmol/mg)	Heme oxygenase (nmol/mg/hr)	Biliverdin reductase (nmol/mg/min)
Control	34.2 ± 8.7	2.66 ± 0.22	2.22 ± 0.17
trans-Platinum (1 day)	31.7 ± 9.6	3.24 ± 0.12	2.22 ± 0.17
trans-Platinum (7 days)	27.0 ± 6.7	2.68 ± 0.43	2.54 ± 0.02
cis-Platinum (1 day)	26.6 ± 3.4	4.00 ± 0.41*	1.98 ± 0.22
cis-Platinum (7 days)	12.1 ± 2.2*	5.75 ± 0.70*	1.32 ± 0.01*

Male Sprague–Dawley rats (180–220 g) were treated (i.v.) with *trans*- or *cis*-platinum (7.0 mg/kg) and killed after the indicated periods. Cell fractions were prepared as described in the text. The tissue homogenate was used for measurement of porphyrin content. The cytosol fraction (105,000 *g* supernatant) was used for the measurement of biliverdin reductase. Heme oxygenase activity was measured in the microsomal fraction. Results are the means ± SD of four determinations.

* $P \leq 0.05$ when compared with the control group.

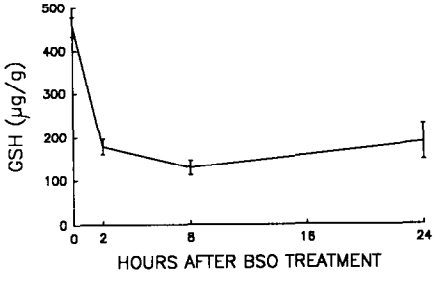

Fig. 1. BSO-mediated depletion of renal glutathione. Rats were treated with BSO (4 mmol/kg, s.c.) and killed at the indicated intervals for measurement of renal GSH levels. Results are the means ± SD of four determinations. One rat was used for each determination.

The data were analyzed by Student's *t*-test; a P value of ≤ 0.05 denoted significance.

RESULTS

Comparative effects of cis- *and* trans-*platinum on heme metabolism in the kidney.* Table 1 shows the comparative and time-dependent effects of the two platinum complexes on the kidney total porphyrin content and on heme oxygenase and biliverdin reductase activities. In *trans*-platinum-treated rats the indicated variables did not change when measured 1 or 7 days after treatment. This was unlike exposure to *cis*-platinum, which caused after 7 days pronounced decreases in the concentration of porphyrins and the activity of biliverdin reductase, and an increase in the activity of heme oxygenase. One-day treatment with *cis*-platinum did not affect biliverdin reductase activity or porphyrin concentration; however, heme oxygenase activity was somewhat increased. In the liver, *trans*-platinum treatment or *cis*-platinum treatment did not alter any of the measured variables at 1 or 7 days (data not shown).

The potent inhibitor of GSH synthesis, BSO, was used to decrease cellular GSH concentration. Initially the time–course of BSO-mediated (4 mmol/kg) depletion of the renal GSH content was determined (Fig. 1). As noted, the concentration of the tripeptide declined rapidly by 2 hr and remained substantially depressed at 24 hr post-treatment. The hepatic GSH concentration was also measured at 2 and 24 hr after BSO treatment; at those time points the values obtained were 63 and 76%, respectively, of control levels (1240 μg/g tissue). Using this information, in the subsequent experiment *trans*-platinum was given 2 hr after BSO, and 24 hr later the same variables measured in the above experiment (Table 1) were assessed. The results are shown in Fig. 2. As noted, pretreatment of rats with BSO rendered *trans*-platinum effective in altering heme metabolism variables in the kidney. The total porphyrin concentration was decreased substantially, the activity of heme oxygenase was increased significantly, and that of biliverdin reductase was decreased. In animals treated with BSO alone, significant changes were not detected when measured 24 hr after treatment.

The possibility that potentiation by BSO of the *trans*-platinum effect on kidney heme metabolism extends to the microsomal hemoprotein content of the organ was investigated. As shown in Fig. 3, significant increases in the concentration of cytochromes P450 and b_5 were produced by treatment of rats with BSO before administration of *trans*-platinum (1-day treatment). *cis*-Platinum treatment, after 7 days, also caused a significant increase in the concentration of the cytochromes. *trans*-Platinum or BSO alone did not affect significantly the microsomal content of either cytochrome. Also, 1-day *cis*-platinum treatment was ineffective. As above, in the liver, the activity of heme metabolic enzymes and the concentration of hemoproteins did not respond to *trans*-platinum, despite BSO pretreatment (data not shown).

Influence of BSO pretreatment on trans-*platinum effects on enzymes of GSH metabolism.* The response to *trans*-platinum treatment of γ-glutamyl cycle enzymes, γ-glutamylcysteine synthetase, GSSG-reductase and γ-glutamyltranspeptidase was examined. Results, presented in Fig. 4, show the comparative effects of 1-day treatment with *trans*-platinum alone or *trans*-platinum plus BSO, as well as 7-day treatment with *cis*-platinum on the activities of γ-glutamylcysteine synthetase and γ-glutamyl

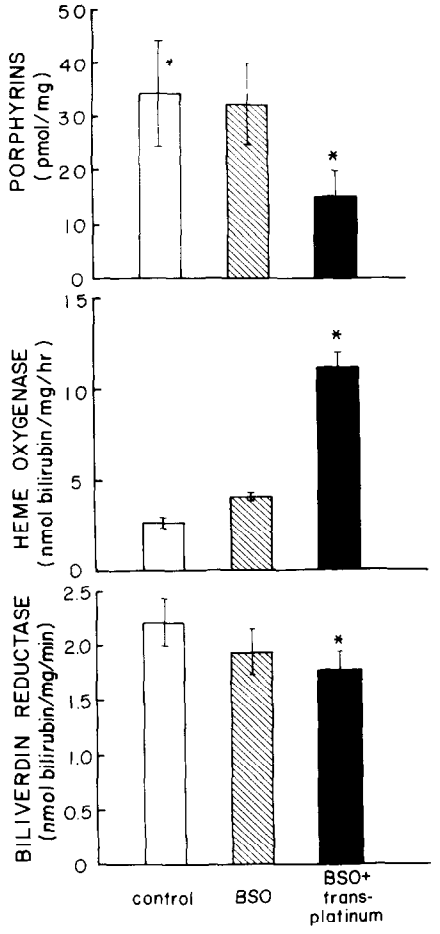

Fig. 2. Potentiation by D,L-buthionine-*S*,*R*-sulfoximine pretreatment of *trans*-platinum effects on total porphyrin concentration and activities of heme degradation enzymes. One group of four male Sprague–Dawley rats (180–220 g) was treated (s.c.) with BSO (4 mmol/kg) 2 hr before administration of *trans*-platinum (7.0 mg/kg, i.v.). The animals were killed 24 hr after the last injection. A second group of rats was given only BSO. The control group received saline. Kidneys were perfused and used for preparation of subcellular fractions. The homogenate was used for measurement of total porphyrin concentration. The microsomal and the cytosol fractions were used for assessment of heme oxygenase and biliverdin reductase activities. Experimental details are provided in the text. Data are means ± SD of four determinations. Key: (*) $P \leq 0.05$ when compared with the control group.

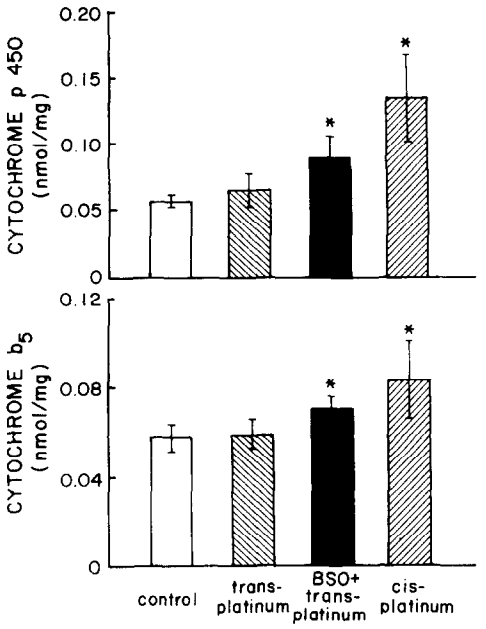

Fig. 3. Comparative effects *in vivo* of treatments with BSO plus *trans*-platinum or *cis*-platinum on kidney microsomal cytochromes P450 and b_5 concentrations. One group of male Sprague–Dawley rats was treated with BSO and *trans*-platinum as described in the legend to Fig. 2, and killed 24 hr after the last injection. Other groups were treated with *cis*-platinum (7.0 mg/kg, i.v. and killed 7 days later), *trans*-platinum (7.0 mg/kg, i.v. and killed 24 hr later), or saline. The microsomal fraction was prepared and used for the measurement of the concentrations of cytochromes P450 and b_5. Details are provided in Experimental Procedures. Data are means ± SD of four determinations. Key: (*) $P \leq 0.05$ when compared with the control group.

transpeptidase. As shown, the synthetase activity was inhibited significantly in BSO plus *trans*-platinum-treated rats as it was in the *cis*-platinum-treated animals. The decrease did not appear to be caused by BSO since, in animals treated with BSO alone, after 24 hr, the activity of the synthetase had returned to the control value. Also, the enzyme activity was not altered in the kidney of rats treated only with *trans*-platinum. The transpeptidase activity was also decreased by *cis*-platinum to about 50% of the control value; the activity, however, was not altered by either *trans*-platinum alone or in combination with BSO. The inhibition of transpeptidase activity by *cis*-platinum after 7 days, but not with *trans*-platinum plus BSO in 1 day, may reflect the turnover rate of the enzyme. Alternatively, it may reflect the structural properties of the *cis*-geometry which exerts a direct inhibitory action on the enzyme [8]. GSSG-reductase activity was not affected by any of the treatments. The activity measured 188 ± 18 units in control rats, 195 ± 30 units in rats treated with BSO plus *trans*-platinum, 210 ± 23 in *trans*-platinum-treated rats, and 213 ± 12 in BSO-treated animals.

Effect of BSO pretreatment on the concentration of platinum in the kidney. In view of the potentiation of *trans*-platinum effects that were observed with BSO pretreatment, the kidney level of platinum was measured to determine whether BSO alters tissue uptake of *trans*-platinum. As shown in Fig. 5, the platinum level in the kidney of BSO plus *trans*-platinum-treated animals was by far less than that of *trans*-platinum-treated rats; indeed, the mean value in the BSO-pretreated rats was about 50% of the *trans*-platinum-treated animals.

DISCUSSION

The structural activity studies have established that antineoplastic activity of *cis*-platinum, for the

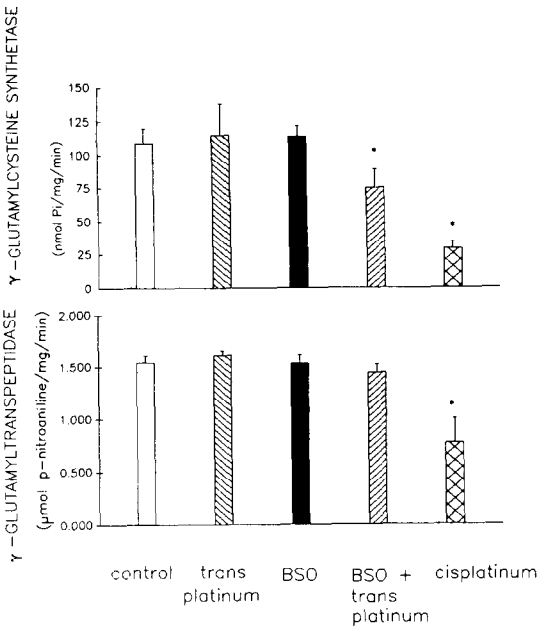

Fig. 4. Effects *in vivo* of treatments with *trans*-platinum, BSO plus *trans*-platinum or *cis*-platinum on activities of γ-glutamyl transpeptidase and γ-glutamylcysteine synthetase. Male Sprague–Dawley rats were treated with platinum complexes, as described in the legend to Fig. 3. The activity of the transpeptidase was measured in the homogenate. The activity of the synthetase was measured in the cytosol fraction. The assays were performed as described in Experimental Procedure. The results are means ± SD of four determinations. Key: (*) $P \leq 0.05$ when compared with the control group.

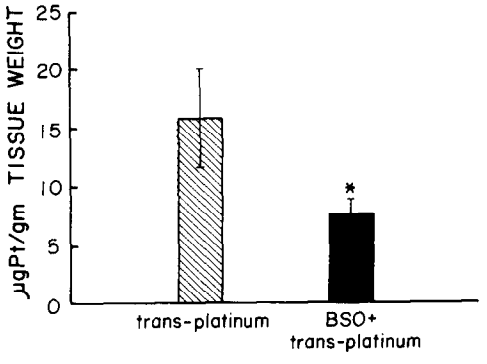

Fig. 5. Platinum concentration in kidneys of rats treated with *trans*-platinum or BSO plus *trans*-platinum. Rats were treated with the platinum compound, as described in the legend to Fig. 3. The tissue level of platinum was measured by flameless atomic absorption, as described in the text. Key: (*) $P \leq 0.05$ when compared with the control group.

most part, is dependent on the *cis*-geometry. This molecular property allows the formation of intrastrand and interstrand adducts with DNA nucleotide bases and promotes antineoplastic activity. However, recent findings from various laboratories using a variety of experimental conditions, have suggested that mechanisms other than binding to DNA nucleotide bases also may be involved in the nephrotoxicity of the complex [18, 34]. Using alterations in activities of heme metabolism pathway enzymes as indicators of cellular toxicity [14, 15], our previous studies [12, 13] have suggested that in the kidney the high level of turnover of GSH and activities of γ-glutamyl cycle enzymes may be involved in rendering the organ a target for toxicity of the platinum complex.

The *trans* configuration of dichlorodiammineplatinum II appears to enhance reactivity of the complex as is evidenced by more rapid rates of hydration and binding with a variety of potential cellular ligands; yet, despite its enhanced reactivity with cellular macromolecules *in vitro*, *trans*-platinum is less nephrotoxic *in vivo* than *cis*-platinum when given in equivalent doses [1]. The present study shows that *trans*-platinum can be rendered essentially as effective as *cis*-platinum in altering activities of heme metabolism and hemoprotein content in the kidney by pretreatment of animals with BSO, a specific inhibitor of GSH synthesis [21]. We speculate that reduction of cellular GSH levels allows *trans*-platinum to interact with regulatory molecules involved with heme, and possibly other metabolic pathways, as exemplified by the inhibition of γ-glutamyl synthetase. The observed potentiation of *trans*-platinum *in vivo* is consistent with the study of Andrews *et al.* [35] who reported that the cytotoxicity of *trans*-platinum (but not *cis*-platinum) could be enhanced by BSO treatment *in vitro*, utilizing a human ovarian carcinoma cell line.

The precise mechanism(s) of *cis*-platinum nephrotoxicity is still not fully understood, but it is evident from this study as well as previous reports [5, 12] that the onset of its perturbation of various cellular functions, including heme metabolism, is delayed by several days. In this respect, *cis*-platinum clearly differs from *trans*-platinum plus BSO treatment in which the prompt onset of effects on heme metabolism more closely resembles that noted with chlorides of platinum, as well as other heavy metal salts [19, 20]. Treatment with such metal complexes results in a rapid enhancement of heme degradation activity. Moreover, when metal complexes are given to animals whose GSH metabolism is compromised, effects on heme metabolism pathway are magnified [36]. The present study shows that the potential effects of *trans*-platinum on heme metabolism also can be unmasked by depleting GSH levels prior to injection of the platinum complex.

As noted above, although the temporal action of *trans*-platinum, when given subsequent to BSO treatment, on heme metabolism variables is similar to platinum chlorides, the nature of its effects on microsomal hemoproteins resembles that of *cis*-platinum, but with an accelerated onset. Treatment with platinum chloride and other heavy metal salts results in a significant decrease in the microsomal concentration of cytochromes in the kidney [14]. In contrast, treatment with *cis*-platinum or *trans*-platinum plus BSO resulted in an increase in renal microsomal cytochromes P450 and b_5 content. Often induction of heme oxygenase is accompanied by a decrease in cytochrome P450. This is particularly notable in the liver [14], and such observations have

implicated the role of heme oxygenase in degrading cytochrome P450 heme [14]. However, recently we have shown that in a reconstituted system not all forms of cytochrome P450 are directly degraded by heme oxygenase [37]; rather with certain species, e.g. P450c, its conversion to P420 is necessary. Hence, the present finding that induction of heme oxygenase in the kidney was not accompanied by a decrease in microsomal cytochrome P450 content may be explained by a rate of production that exceeds that of degradation and/or production of species that are resistant to direct degradation by heme oxygenase. In turn, the increased production could reflect an enhanced formation of heme from porphyrin precursors. This suggestion is consistent with the observed decrease in total porphyrin content and our previous findings demonstrating such occurrence in the kidney of *cis*-platinum-treated rats [17]. Degradation of cytochrome b_5 by heme oxygenase has not been demonstrated directly, and no comments can be offered at this time.

Previous work from our laboratory has suggested that *cis*-platinum-mediated alterations of renal cytochrome P450 appear to be mediated by systemic endocrine effects rather than direct effects on the organ [17]. The possibility that *trans*-platinum also may affect endocrine function was not presently examined, but it is conceivable that *trans*-platinum also elicits perturbations in endocrine functions under conditions of GSH depletion.

The finding that BSO treatment resulted in enhanced *in vivo* activity of *trans*-platinum, yet caused a decreased tissue concentration of platinum levels, is similar to that observed with mercury chloride. Berndt *et al.* [38] have shown that GSH depletion by diethylmaleate plus BSO not only decreases renal accumulation of mercury but also simultaneously exaggerates inhibition of *p*-aminohippurate transport by mercury. The present experiments do not allow prediction as to whether BSO treatment prevents initial uptake of *trans*-platinum and/or enhances subsequent excretion from the kidney. Nonetheless, regardless of the mechanism, it would appear that the diminished amount of *trans*-platinum accumulating in the organ following BSO treatment is in a form more effective in altering heme metabolism.

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