

MRP2 and the DMPS- and DMSA-Mediated Elimination of Mercury in TR⁻ and Control Rats Exposed to Thiol S-Conjugates of Inorganic Mercury

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Cysteine (Cys) and homocysteine (Hcy)-S-conjugates of inorganic mercury (Hg²⁺) are transportable species of Hg²⁺ that are taken up readily by proximal tubular cells. The metal chelators, 2,3-dimercaptopropane-1-sulfonic acid (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA), have been used successfully to extract Hg²⁺ from these cells, presumably via the multidrug resistance protein (Mrp2). In the current study, we tested the hypothesis that Mrp2 is involved in the DMPS- and DMSA-mediated extraction of Hg²⁺ following administration of Hg²⁺ as an S-conjugate of Cys or Hcy. To test this hypothesis, control and TR⁻ (Mrp2-deficient) rats were injected with 0.5 μmol/kg HgCl₂ (containing ²⁰³Hg²⁺) conjugated to 1.25 μmol/kg Cys or Hcy. After 24 and 28 h, rats were treated with saline or 100 mg/kg DMPS or DMSA. Tissues were harvested 48 h after Hg²⁺ exposure. The renal and hepatic burden of Hg²⁺ was greater in saline-injected TR⁻ rats than in corresponding controls. Accordingly, the content of Hg²⁺ in the urine and feces was less in TR⁻ rats than in controls. Following treatment with DMPS or DMSA, the renal content of Hg²⁺ in both groups of rats was reduced significantly and the urinary excretion of Hg²⁺ was increased. In liver, the effect of each chelator appeared to be dependent upon the form in which Hg²⁺ was administered. *In vitro* experiments provide direct evidence indicating that DMPS and DMSA-S-conjugates of Hg²⁺ are substrates for Mrp2. Overall, these data support our hypothesis that Mrp2 is involved in the DMPS and DMSA-mediated extraction of the body burden of Hg²⁺.

Key Words: mercury; transport; chelators; multidrug resistance protein.

Within biological systems, inorganic mercury (Hg²⁺) does not exist in an unbound ionic state (Hughes, 1957). Inorganic mercury has a very high affinity for thiol-containing biomolecules, such as cysteine (Cys) and homocysteine (Hcy), and binds to these molecules in a linear II coordinate covalent manner (Canty *et al.*, 1994; Fuhr and Rabenstein, 1973; Rubino *et al.*, 2004). This binding apparently increases the

delivery of Hg²⁺ to target organs, such as the kidney. *In vivo* studies in rats have demonstrated that the renal burden of Hg²⁺ is greater when Hg²⁺ is administered (i.v.) as an S-conjugate of Cys (Cys-S-Hg-S-Cys) than when Hg²⁺ is administered as HgCl₂ (Zalups and Barfuss, 1996, 1998). Recent studies have shown that thiol conjugates of Hg²⁺ are taken up readily at both, luminal and basolateral plasma membranes of proximal tubular epithelial cells, which are the primary targets in the kidneys where mercuric ions accumulate (Zalups, 2000). At the luminal membrane, the amino acid transporter, system b^{0,+}, participates in the uptake of Cys and Hcy S-conjugates of Hg²⁺ (Bridges and Zalups, 2005). On the other hand, at the basolateral membrane of proximal tubular cells, the organic anion transporter 1 (OAT1) (and perhaps OAT3) mediate(s) the uptake of a number of Hg²⁺-thiol conjugates from plasma (Bridges and Zalups, 2005). The ability of these carriers to transport Hg²⁺ is thought to be dependent upon the conjugation of low molecular weight thiols (such as Cys and Hcy) with Hg²⁺.

The affinity of mercuric ions for the reduced sulfur atom of thiol-containing molecules also serves as the basis of therapeutic interventions following exposure to mercuric species. 2,3-Dimercaptopropane-1-sulfonic acid (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA) are dithiol metal chelators used frequently throughout the world. Although these chelators are distinct structurally, they both possess vicinal thiol groups that facilitate the formation of highly stable DMPS and DMSA S-conjugates with mercuric ions (Aposhian, 1983). Surprisingly, only DMSA has been approved for use in humans by the U.S. Food and Drug Administration. Given the structural differences between DMPS and DMSA, and the differences in their use throughout the world, it is important to understand the effect of each chelator on the disposition of Hg²⁺.

DMPS and DMSA act primarily by promoting the unidirectional extraction of mercuric ions from within renal proximal tubular cells into the luminal compartment of the tubule (Diamond *et al.*, 1988; Zalups, 1993; Zalups *et al.*, 1998). DMPS is taken up from blood via OAT1 (Bahn *et al.*,

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2002; Islinger *et al.*, 2001), whereas DMSA appears to be taken up by the sodium dependent dicarboxylate transporter (Burckhardt *et al.*, 2002). We hypothesize that DMPS and DMSA, each, forms complexes with intracellular Hg^{2+} , which are subsequently transported into the lumen of the proximal tubule for excretion in urine. Although it is clear that each chelator facilitates a profound decrease in the renal burden of Hg^{2+} (Aposhian, 1983), some of the cellular mechanisms involved in this process remain unclear.

Our laboratory has suggested that the multidrug resistance protein 2 (Mrp2) is a likely candidate for the secretion of DMPS- and DMSA-*S*-conjugates of Hg^{2+} across the luminal membrane of proximal tubular cells. In the proximal tubule, Mrp2 is localized exclusively in the luminal membrane (Schaub *et al.*, 1997) and it has been hypothesized to play a role in the excretion of metabolic wastes and xenobiotics (Leslie *et al.*, 2005). In liver, Mrp2 has been implicated in the transport of Hg^{2+} from hepatocytes into the biliary tree (Sugawara *et al.*, 1998). Therefore, we suggest that this carrier may also be involved in the renal elimination of Hg^{2+} . Indeed, previous studies from our laboratory using Wistar and Mrp2-deficient (TR^-) rats and inside-out membrane vesicles containing MRP2 provide strong evidence supporting a role for Mrp2 in the DMPS- and DMSA-mediated extraction of Hg^{2+} from proximal tubules (Bridges *et al.*, 2008).

In the current study, we utilized Wistar (control) and TR^- rats to examine the role of Mrp2 in the DMPS- and DMSA-mediated extraction of Hg^{2+} following intravenous exposure to either, Cys-*S*-Hg-*S*-Cys or the Hcy *S*-conjugate of Hg^{2+} (Hcy-*S*-Hg-*S*-Hcy). This is in contrast to our previous study, in which control and TR^- rats were exposed to HgCl_2 alone. In addition, the current study examined ability of Mrp2 to transport DMPS and DMSA *S*-conjugates of Hg^{2+} by using inside-out membrane vesicles prepared from Sf9 cells transfected with MRP2. The collective results of the present experiments provide strong support for the hypothesis that Mrp2 facilitates the transport of mercuric-chelator complexes from within proximal tubular cells into the lumen of the tubule.

METHODS

Manufacture of $^{203}\text{Hg}^{2+}$. The production of $^{203}\text{Hg}^{2+}$ has been described previously (Belanger *et al.*, 2001). Briefly, three milligrams of mercuric oxide were doubly sealed in quartz tubing with an acetylene torch. The encapsulated mercury was irradiated by neutron activation for 4 weeks at the Missouri University Research Reactor (MURR) facility. After removing the outer quartz tube, the inner tube was crushed and rinsed with four 50- μl washes of 1N HCl. All rinses were placed in a single polypropylene vial. In order to determine the exact solid content of mercury, a sample of the solution was taken and subjected to plasma-coupled elemental mass spectrometry. The radioactivity of the solution was determined by using a PerkinElmer Wallac Wizard 3 automatic gamma counter (Gaithersburg, MD). The specific activities of the $^{203}\text{Hg}^{2+}$ ranged from 6 to 12 mCi/mg.

Animals. Male Wistar (control) and Mrp2-deficient (TR^-) rats weighing 200–225 g were purchased from Harlan Laboratories (Indianapolis, IN). In

TR^- rats, the *mrp2* gene possesses an early stop codon, which results in the production of a truncated, nonfunctional Mrp2 protein (Mayer *et al.*, 1995; Paulusma *et al.*, 1996). These rats have been used reliably to study the hepatic and renal secretion of various Mrp2 substrates (de Vries *et al.*, 1989; Leslie *et al.*, 2007; Masereeuw *et al.*, 2003; Smeets *et al.*, 2004). Furthermore, our laboratory has shown that these rats are reliable models in which to study the effects of DMPS and DMSA on the disposition of Hg^{2+} (Bridges *et al.*, 2008).

The body weights of the animals used in these studies were not significantly different. All rats were provided a commercial laboratory diet (Tekland 6% rat diet, Harlan Laboratories) and water *ad libitum* throughout all aspects of animal experimentation. Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health.

Experimental design. The following sets of experiments were carried out as detailed in the paragraphs below: (1) injection with HgCl_2 and Cys followed by treatment with DMPS or saline; (2) injection with HgCl_2 and Cys followed by treatment with DMSA or saline; (3) injection with HgCl_2 and Hcy followed by treatment with DMPS or saline; (4) injection with HgCl_2 and Hcy followed by treatment with DMSA or saline.

The injection solution was made by mixing HgCl_2 , containing $^{203}\text{Hg}^{2+}$, with either Cys or Hcy in a 1:2.5 ratio in a polypropylene vial. This mixture was incubated for at least 10 min at room temperature to ensure complete conjugation of mercuric ions to Cys or Hcy. Control and TR^- rats (eight per strain) were each injected intravenously with a non-nephrotoxic dose of the Hg-Cys or Hg-Hcy mixture (0.5 $\mu\text{mol/kg}$ Hg and 1.25 $\mu\text{mol/kg}$ Cys or Hcy in 2 ml of normal saline), according to a previously published protocol (Bridges *et al.*, 2008; Zalups, 1993; Zalups *et al.*, 1992). Briefly, each animal was anesthetized lightly with ether (Sigma, St Louis, MO), a small incision was made in the skin, and a calculated dose of HgCl_2 (conjugated to Cys or Hcy) was administered into the femoral vein. The wound was closed using two 9-mm stainless steel wound clips and animals were placed in individual metabolic cages.

Twenty-four hours after the injection of HgCl_2 , four control rats and four TR^- rats were injected intraperitoneally with a 100 mg/kg (0.39 $\mu\text{mol/kg}$) dose of DMPS (in 2 ml/kg normal saline; Sigma) or with 100 mg/kg (0.55 $\mu\text{mol/kg}$) DMSA. At the same time, another group of four control rats and a group of four TR^- rats were injected intraperitoneally with normal saline (2 ml/kg). Four hours later (28 h after injection of Hg^{2+}), rats were administered a second intraperitoneal dose of DMPS/DMSA (100 mg/kg) or saline (2 ml/kg). Twenty-four hours after the initial dose of DMPS/DMSA or saline (48 h after injection with Hg^{2+}), rats were sacrificed by exsanguination.

Collection of tissues, organs, urine and feces. Forty-eight h after the initial injection of Hg^{2+} , rats were anesthetized with an intraperitoneal overdose of sodium-pentobarbital (100 mg/kg in 2 ml of saline; Sigma). Blood, liver, kidneys, urine and feces were collected as described previously (Bridges *et al.*, 2008). Urine and feces were collected at 24-h intervals. The content of $^{203}\text{Hg}^{2+}$ in each sample was determined by counting the samples in a Wallac Wizard 3 automatic gamma counter.

Membrane vesicle transport assays. Inside-out membrane vesicles were purchased from Xenotech (Lenexa, KS). Control vesicles were prepared from normal Sf9 cells, whereas MRP2 vesicles were prepared from Sf9 cells transfected with human MRP2. Prior to use, vesicles were centrifuged at 100,000 \times g for 40 min at 4°C to remove the storage buffer. Vesicles were resuspended in ice-cold incubation buffer (250mM sucrose, 10mM Tris/HCl, 10mM MgCl_2 , pH 7.4) by passing the suspension through a 27-gauge needle 10 times.

Membrane vesicle transport assays were performed using a rapid filtration method as described previously (Bridges *et al.*, 2008; Cui *et al.*, 1999). Mercuric conjugates were formed by mixing 5 μM HgCl_2 , containing $^{203}\text{Hg}^{2+}$ with 12.5 μM Cys or Hcy in incubation buffer supplemented with 10mM creatine phosphate (Sigma) and 100 $\mu\text{g/ml}$ creatine phosphokinase (Sigma) in the presence or absence of 5mM ATP (Sigma). Prior to the experiment,

membrane vesicles and mercuric conjugates were incubated separately for 15 minutes at 37°C. Transport was initiated by the addition of 40 µl of membrane vesicle solution (20 µg) to 160 µl of buffer containing mercuric conjugate. Transport was allowed to proceed for 15 s, after which, vesicles were collected on prewet Tuffym filter discs (pore size, 0.45 µm; Pall, East Hills, NY). Filters were washed with two changes (8 ml each) of ice-cold stop buffer (250mM sucrose, 10mM Tris/HCl, 100mM NaCl, 1mM DMPS, pH 7.4). Filters were placed in scintillation vials and the radioactivity present was determined by counting in a Wallac Wizard 3 gamma counter.

Data analyses. Data for each rat experiment were analyzed first with the Kolmogorov-Smirnov test for normality and then with Levene's test for homogeneity of variances. Data were then analyzed using a 2 × 2 two-way ANOVA to assess differences among the means. When statistically significant *F* values were obtained with ANOVA, the data were analyzed using Tukey's *post hoc* multiple comparison test. A *p* value of < 0.05 was considered statistically significant.

Data from membrane vesicle assays were analyzed by first calculating ATP-dependent transport. The resulting data were analyzed by the Student's *t* test. A *p* value of < 0.05 was considered statistically significant.

RESULTS

Effect of DMPS and DMSA on the Renal Burden of Hg²⁺ after Exposure to Cys-S-Hg-S-Cys

Figure 1 shows the renal burden of Hg²⁺ in control and TR⁻ rats treated with saline, DMPS, or DMSA 24 and 28 h after the injection of Cys-S-Hg-S-Cys. The content of Hg²⁺ in the total renal mass of TR⁻ rats injected with saline was approximately 69% of dose, and was significantly greater in than that in corresponding saline-injected controls (approximately 58% of

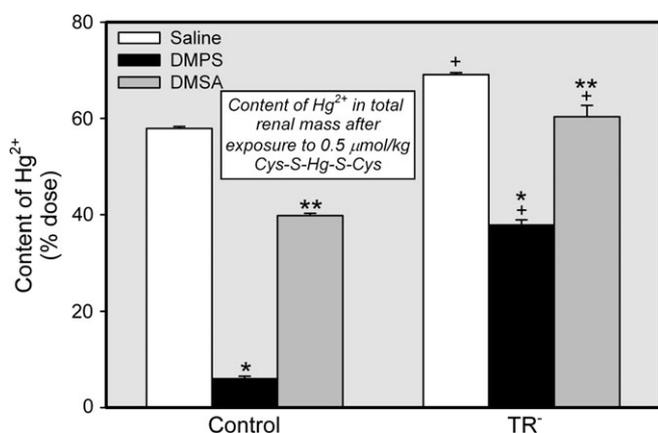


FIG. 1. Content of Hg²⁺ in the total renal mass (% of administered dose) of control and TR⁻ rats injected (i.v.) with 0.5 µmol/kg HgCl₂ and 1.25 µmol/kg Cys. Rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline 24 and 28 h after injection of HgCl₂. Kidneys were harvested for determination of Hg²⁺ content 48 h exposure to HgCl₂. Data represent mean ± SE of four rats. *Significantly different (*p* < 0.05) from the corresponding mean for the saline-injected rats of the same strain. **Significantly different (*p* < 0.05) from the corresponding mean for each of the other groups of rats of the same strain. +Significantly different (*p* < 0.05) from the corresponding mean for control rats treated in the same manner.

dose). The renal content of Hg²⁺ was reduced significantly in both strains of rats treated with DMPS. In controls, treatment with DMPS lowered the content of Hg²⁺ in the total renal mass by approximately 90%, whereas in the TR⁻ rats, DMPS lowered the content of Hg²⁺ in the total renal mass by only about 45%. The content of Hg²⁺ in the total renal mass of TR⁻ rats remained sixfold greater than that of corresponding DMPS-treated controls.

Treatment of rats with DMSA also reduced the renal burden of Hg²⁺ in both strains of rats (Fig. 1). Interestingly, DMSA was less effective than DMPS at reducing the amount of Hg²⁺ in kidneys. In control rats, DMSA reduced the renal burden of Hg²⁺ by approximately 31%. In TR⁻ rats, treatment with DMSA resulted in only a 13% reduction in the renal content of Hg²⁺. The renal burden of Hg²⁺ in TR⁻ rats remained significantly greater than that in control rats.

The effect of DMPS on the disposition of Hg²⁺ in the four zones of the kidney is shown in Figure 2A. The majority of Hg²⁺ was detected in the cortex and outer stripe of the outer medulla. The concentration of Hg²⁺ in the renal cortex was significantly greater in saline-injected TR⁻ rats than in corresponding controls. Interestingly, the concentration of mercury in the outer stripe of the outer medulla was similar in both strains of rats injected with saline. Treatment of rats with DMPS reduced significantly the concentrations of Hg²⁺ in the cortex and in the outer stripe of the outer medulla in both, control and TR⁻ rats. This reduction was more profound in control rats than in TR⁻ rats. There were no differences in Hg²⁺ content of the inner stripe of the outer medulla and the inner medulla among the groups of rats.

Figure 2B shows the effect of DMSA on the disposition of Hg²⁺ in the kidney. In control rats, exposure to DMSA yielded a significant reduction in the concentration of Hg²⁺ in the cortex and in the outer stripe of the outer medulla. In contrast to DMPS, DMSA did not significantly reduce the concentration of Hg²⁺ in any of the renal zones of TR⁻ rats.

Effect of DMPS and DMSA on the Urinary Excretion of Hg²⁺ after Exposure to Cys-S-Hg-S-Cys

The amount of Hg²⁺ excreted in the urine after injection with saline was significantly greater in the control rats than in the corresponding TR⁻ rats (Fig. 3). Following injection of controls with DMPS, the urinary excretion of Hg²⁺ increased approximately fivefold. In TR⁻ rats, DMPS treatment yielded nearly a 12-fold increase in the urinary excretion of Hg²⁺. Even so, the Hg²⁺ in the urine of these rats remained significantly lower than that of corresponding controls.

When control rats were injected with DMSA the urinary excretion of Hg²⁺ increased approximately fourfold (Fig. 3). In TR⁻ rats, treatment with DMSA resulted in an eightfold increase in the amount of Hg²⁺ excreted in the urine. The amount of Hg²⁺ in urine of control rats remained significantly greater than that of TR⁻ rats even after treatment with DMSA.

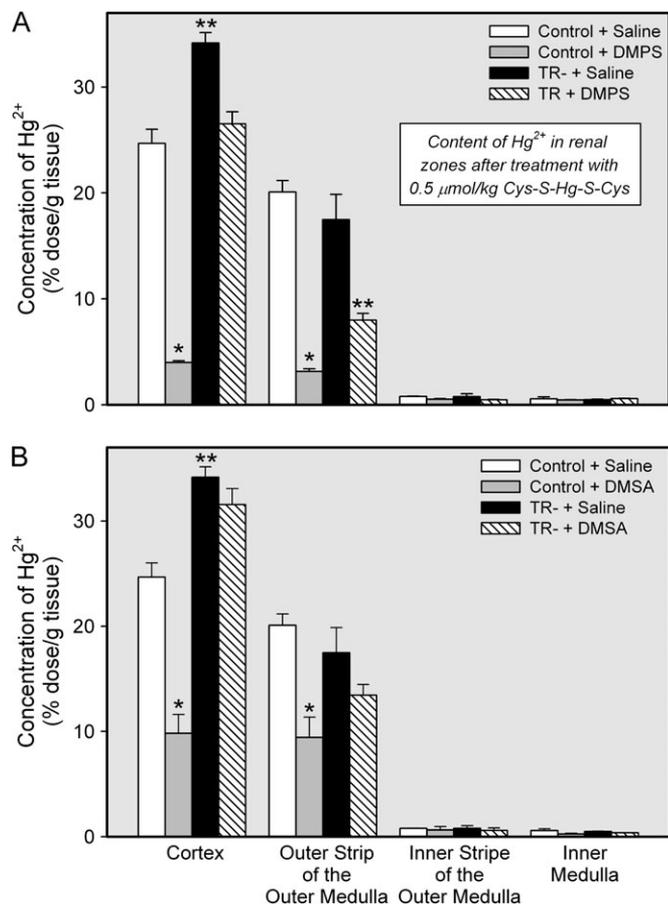


FIG. 2. Concentration of Hg^{2+} (% of administered dose/g tissue) in the various zones of kidney of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Cys. Rats were injected (i.p.) with 100 mg/kg DMPS (A), 100 mg/kg DMSA (B), or 2 ml/kg saline 24 and 28 h after injection of HgCl_2 . Kidneys were harvested for determination of Hg^{2+} content 48 h exposure to HgCl_2 . Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain.

Effect of DMPS and DMSA on the Fecal Excretion of Hg^{2+} after Exposure to Cys-S-Hg-S-Cys

The fecal elimination of Hg^{2+} was significantly greater in each group of control rats than in each corresponding group of TR^- rats (Fig. 4). The amount of Hg^{2+} excreted into the feces by the control rats treated with saline was approximately 2.5-fold greater than that of corresponding TR^- rats. In both, control and TR^- rats, treatment with DMPS resulted in a modest, yet significant, increase in the amount of Hg^{2+} excreted in the feces. Exposure of control and TR^- rats to DMSA also resulted in an increase in the fecal excretion of Hg^{2+} . The amount of Hg^{2+} excreted in the feces after treatment with DMSA was not significantly different from that excreted in feces after treatment with DMPS.

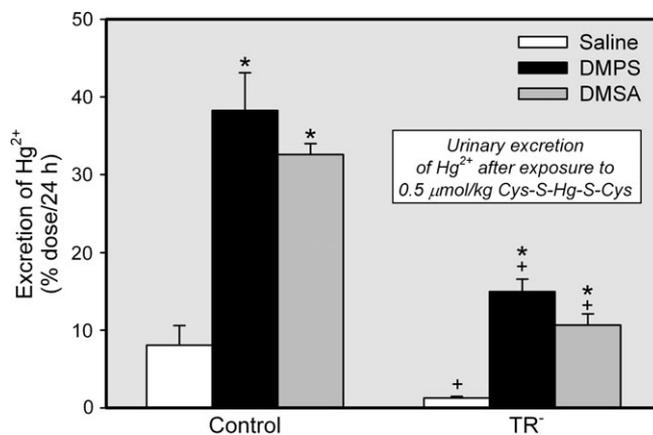


FIG. 3. Amount of Hg^{2+} excreted in urine (% of administered dose/24 h) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Cys. Twenty-four and 28 h later, rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or saline (2 ml/kg). Data represent the amount of Hg^{2+} in urine collected during the 24 h following treatment with DMPS, DMSA, or saline. Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. +Significantly different ($p < 0.05$) from the corresponding mean for control rats treated in the same manner.

Effect of DMPS and DMSA on the Burden of Hg^{2+} in Liver after Exposure to Cys-S-Hg-S-Cys

Approximately 5% of the administered dose of Hg^{2+} was detected in the livers of saline-injected TR^- rats (Fig. 5). This amount was significantly higher than that detected in livers of corresponding control rats (approximately 2% of the dose). Treatment of TR^- rats with DMPS or DMSA did not alter significantly the disposition of Hg^{2+} in the livers of these animals. In contrast, when control rats were treated with DMPS, the hepatic burden of Hg^{2+} was reduced by 87%. DMSA was less effective; it reduced the hepatic burden of Hg^{2+} in control rats by only 25%.

Effect of DMPS and DMSA on the Renal Burden of Hg^{2+} after Exposure to Hcy-S-Hg-S-Hcy

Separate groups of control and TR^- rats were treated with saline, DMPS, or DMSA 24 and 28 h after the intravenous injection of Hcy-S-Hg-S-Hcy (Fig. 6). The total renal burden of Hg^{2+} in saline-injected TR^- rats (approximately 70% of the dose) was significantly greater than that of corresponding control rats (approximately 57% of dose). Treatment with DMPS reduced the total renal content of Hg^{2+} in control rats by approximately 85%. DMSA reduced the renal burden of Hg^{2+} by only 60%. In the TR^- rats, DMPS reduced the renal burden of Hg^{2+} by approximately 35%, whereas treatment with DMSA resulted in a reduction of only 15%.

The effect of DMPS on the concentration of Hg^{2+} in the various zones of the kidney was also examined (Fig. 7A). The concentration of Hg^{2+} in the renal cortex was significantly

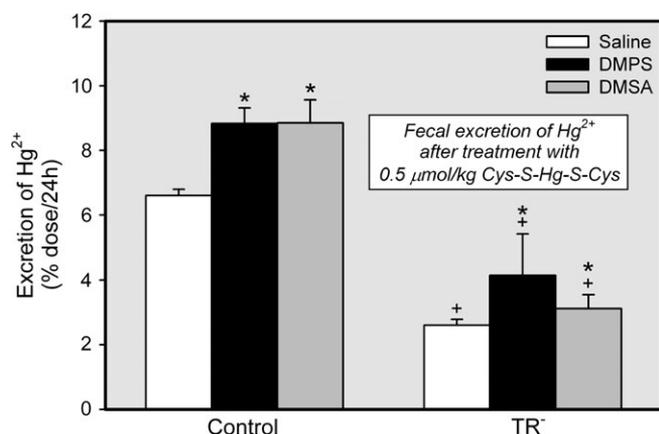


FIG. 4. Content of Hg^{2+} in feces (% of administered dose/24 h) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Cys. Rats were injected (i.p.) 24 and 28 h later with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline. Data represent the amount of Hg^{2+} in feces collected during the 24 h following treatment with DMPS, DMSA, or saline. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. +Significantly different ($p < 0.5$) from the corresponding mean for control rats treated in the same manner.

greater in saline-injected TR^- rats than in the corresponding control rats. In contrast, the concentration of Hg^{2+} in the outer stripe of the outer medulla of the TR^- rats was not significantly different from that of corresponding controls. Following treatment with DMPS, the concentration of Hg^{2+} in the cortex and in the outer stripe of the outer medulla was reduced

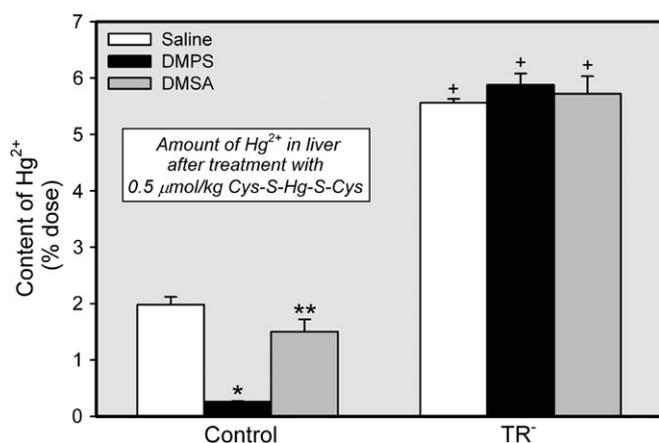


FIG. 5. Content of Hg^{2+} in liver (% of administered dose) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Cys. Rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline 24 and 28 h after injection of HgCl_2 . Kidneys were harvested for determination of Hg^{2+} content 48-h exposure to HgCl_2 . Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain. +Significantly different ($p < 0.05$) from the corresponding mean for control rats treated in the same manner.

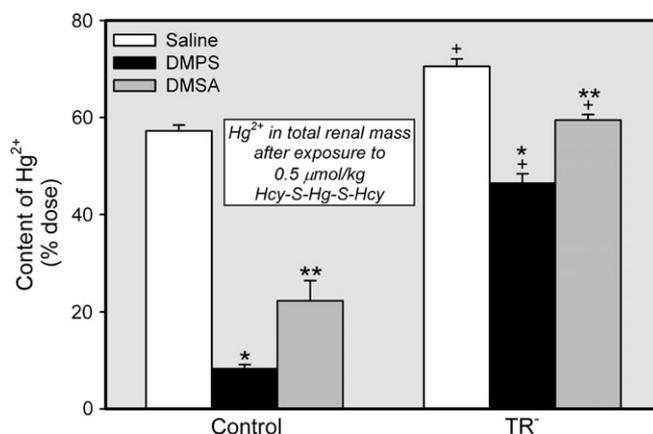


FIG. 6. Content of Hg^{2+} in the total renal mass (% of administered dose) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Hcy. Rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline 24 and 28 h after injection of HgCl_2 . Kidneys were harvested for determination of Hg^{2+} content 48 h exposure to HgCl_2 . Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain. +Significantly different ($p < 0.05$) from the corresponding mean for control rats treated in the same manner.

significantly in both strains of rats. The concentration of Hg^{2+} in these zones remained greater in TR^- animals than in controls.

Treatment of rats with DMSA reduced the concentration of Hg^{2+} in the cortex of both, control and TR^- rats (Fig. 7B). In control rats, treatment with DMSA reduced the concentration of Hg^{2+} in the cortex only. In contrast, treatment of TR^- rats with DMSA reduced significantly the concentration of Hg^{2+} in the cortex and in outer stripe of the outer medulla.

Effect of DMPS and DMSA on the Urinary Excretion of Hg^{2+} after Exposure to Hcy-S-Hg-S-Hcy

The urinary excretion of Hg^{2+} in saline-injected control rats was fivefold greater than that in corresponding TR^- rats (Fig. 8). After injection of control rats with DMPS, the urinary excretion of Hg^{2+} increased fivefold. Similarly, treatment of control rats with DMSA resulted in a 4.5-fold increase in the urinary excretion of Hg^{2+} . When TR^- rats were treated with DMPS, the urinary excretion of Hg^{2+} increased 10-fold; however, this excretion remained approximately half of that observed in corresponding control rats. Treatment of TR^- rats with DMSA resulted in a sixfold increase in the urinary excretion of Hg^{2+} . Even so, the urinary excretion of Hg^{2+} was significantly lower in TR^- rats than in corresponding control rats.

Effect of DMPS and DMSA on the Fecal Excretion of Hg^{2+} after Exposure to Hcy-S-Hg-S-Hcy

Fecal excretion of Hg^{2+} in the saline-injected control rats (approximately 9% of the dose) was significantly greater than

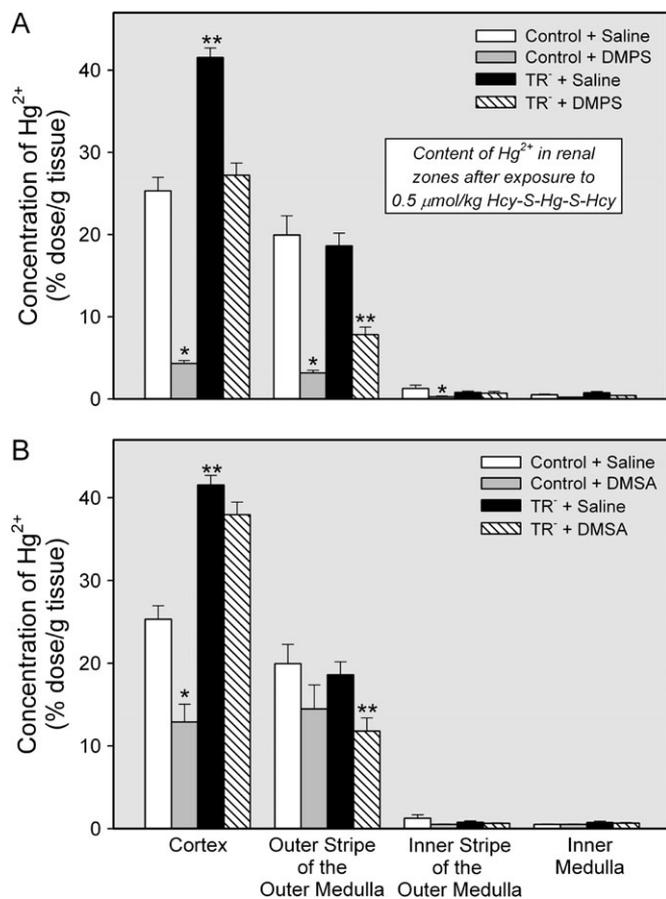


FIG. 7. Concentration of Hg^{2+} (% of administered dose/g tissue) in the various zones of kidney of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Hcy. Rats were injected (i.p.) with 100 mg/kg DMPS (A), 100 mg/kg DMSA (B), or 2 ml/kg saline 24 and 28 h after injection of HgCl_2 . Kidneys were harvested for determination of Hg^{2+} content 48 h exposure to HgCl_2 . Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain.

that in corresponding TR^- rats (about 3% of the dose) (Fig. 9). Neither DMPS nor DMSA significantly altered the fecal content of Hg^{2+} in control rats. In TR^- rats, treatment with DMPS resulted in a slight, but significant, increase in the fecal excretion of Hg^{2+} . Injection with DMSA did not alter significantly the amount of Hg^{2+} excreted in the feces of these rats.

Effect of DMPS and DMSA on the Burden of Hg^{2+} in Liver after Exposure to Hcy-S-Hg-S-Hcy

The hepatic burden of Hg^{2+} in saline-injected TR^- rats was approximately twofold that of corresponding control rats (Fig. 10). Interestingly, treatment of control and TR^- rats with DMPS did not reduce significantly the disposition of Hg^{2+} in livers of either strain of rat. In contrast, DMSA reduced

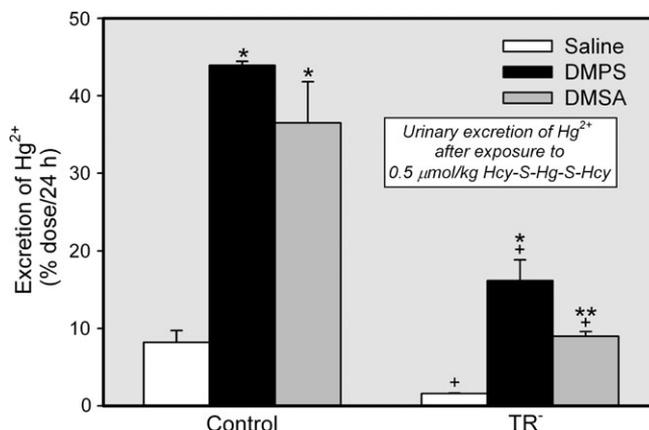


FIG. 8. Amount of Hg^{2+} excreted in urine (% of administered dose/24 h) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Hcy. Twenty-four and 28 h later, rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or saline (2 ml/kg). Data represent the amount of Hg^{2+} in urine collected during the 24 h following treatment with DMPS, DMSA, or saline. Data represent mean \pm SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain. +Significantly different ($p < 0.05$) from the corresponding mean for control rats treated in the same manner.

significantly the hepatic burden of Hg^{2+} in both, control and TR^- rats. Interestingly, treatment of rats with DMSA reduced the hepatic burden of Hg^{2+} in each strain of rat to similar levels despite the fact that the initial amount of Hg^{2+} in the liver was twofold greater in TR^- rats than in controls.

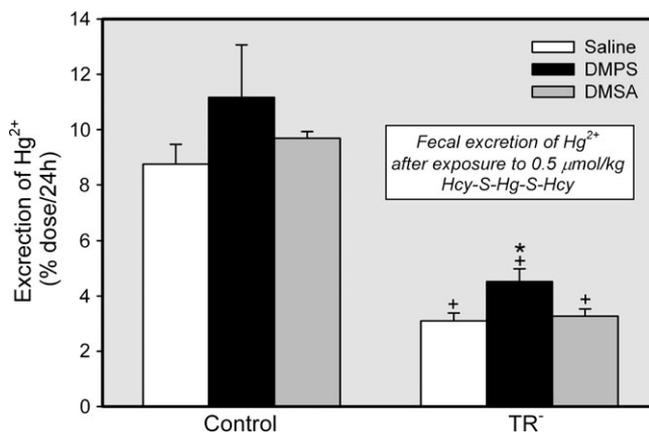


FIG. 9. Content of Hg^{2+} in feces (% of administered dose/24 h) of control and TR^- rats injected (i.v.) with $0.5 \mu\text{mol/kg}$ HgCl_2 and $1.25 \mu\text{mol/kg}$ Hcy. Rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline 24 and 28 h after the initial injection with HgCl_2 . Data represent the amount of Hg^{2+} in feces collected during the 24 h following treatment with DMPS, DMSA, or saline. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. +Significantly different ($p < 0.5$) from the corresponding mean for control rats treated in the same manner.

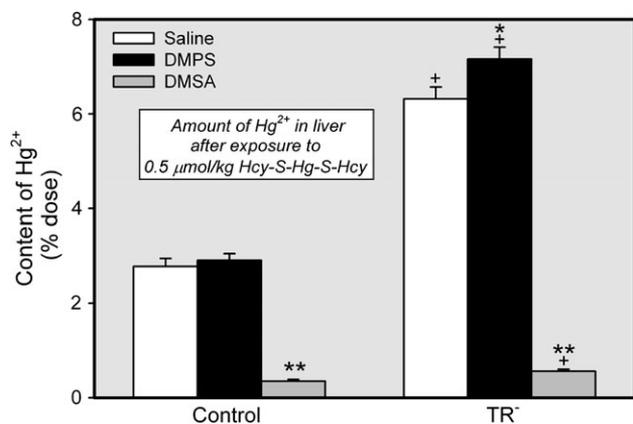


FIG. 10. Content of Hg²⁺ in liver (% of administered dose) of control and TR⁻ rats injected (i.v.) with 0.5 μmol/kg HgCl₂ and 1.25 μmol/kg Hcy. Rats were injected (i.p.) with 100 mg/kg DMPS, 100 mg/kg DMSA, or 2 ml/kg saline 24 and 28 h after injection of HgCl₂. Kidneys were harvested for determination of Hg²⁺ content 48-h exposure to HgCl₂. Data represent mean ± SE of four rats. *Significantly different ($p < 0.05$) from the corresponding mean for the same strain of rats treated with saline. **Significantly different ($p < 0.05$) from the corresponding mean for each of the other groups of rats of the same strain. +Significantly different ($p < 0.05$) from the corresponding mean for control rats treated in the same manner.

Transport of Hg²⁺ in Inside-Out Membrane Vesicles with and without MRP2

ATP-dependent transport of DMPS and DMSA-S-conjugates of Hg²⁺ was measured in inside-out membrane vesicles prepared from either control Sf9 cells or Sf9 cells transfected with human MRP2 (Fig. 11). The uptake of each mercuric conjugate was significantly greater in membrane vesicles with MRP2 than in corresponding control vesicles.

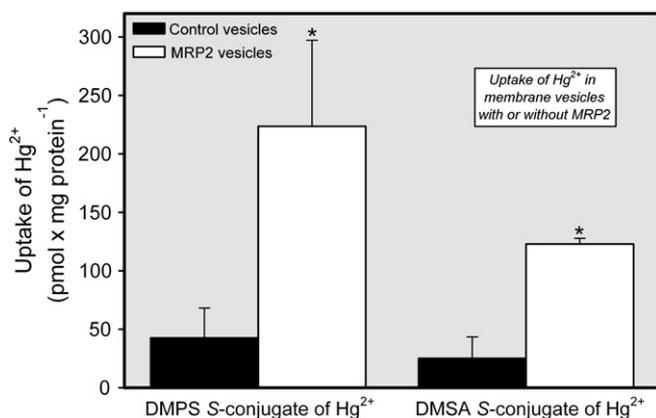


FIG. 11. Uptake of Hg²⁺ into inside-out membrane vesicles prepared from either, control Sf9 cells or Sf9 cells transfected with human MRP2. Control and MRP2 vesicles were exposed to HgCl₂ (5 μM) conjugated to DMPS (12.5 μM) or DMSA (12.5 μM) for 15 min at 37°C. Data represent two experiments, each performed in triplicate. *Significantly different ($p < 0.05$) from the corresponding mean for control vesicles treated with the same conjugate.

DISCUSSION

An important premise underlying the present study is that administration of mercuric ions, as Cys or Hcy S-conjugates, results in an increase in the availability of species of Hg²⁺ that can be transported into proximal tubular cells. The foundation for this premise comes from a series of whole animal studies in rats, in which the renal tubular accumulation of Hg²⁺ was shown to be greater in animals exposed to Cys S-conjugates of Hg²⁺ (Cys-S-Hg-S-Cys) than in corresponding rats exposed to HgCl₂ (Lash *et al.*, 1998, 1999; Zalups and Barfuss, 1998, 1996). Data from a recent study, in which we used TR⁻ and control rats exposed to HgCl₂ (Bridges *et al.*, 2008), combined with the findings from the present study confirm the aforementioned premise. In the present study, we showed that when TR⁻ and control Wistar rats were exposed to Cys-S-Hg-S-Cys or Hcy-S-Hg-S-Hcy, the renal burden of Hg²⁺ was approximately 20% greater than it was in corresponding TR⁻ and control rats (from our recent study) that were exposed to the same dose of Hg²⁺ in the form of HgCl₂ (Bridges *et al.*, 2008). We have also demonstrated recently (Bridges *et al.*, 2008) that the uptake of mercuric ions into cultured renal tubular epithelial cells is facilitated by exposure to Cys-S-Hg-S-Cys or Hcy-S-Hg-S-Hcy (Bridges *et al.*, 2008).

Of significant clinical importance is the fact that the majority of mercuric ions that accumulate in the epithelial cells lining the proximal tubule can be extracted by the vicinal, dithiol chelating agents, DMPS and DMSA (Aposhian, 1983). Experimental evidence suggests that these chelators mediate the extraction of mercuric ions from proximal tubular cells via a direct secretory process, whereby mercuric ions are extracted from the intracellular compartment of these cells and are delivered into the lumen of the proximal tubule. The negative charge on the DMPS or DMSA molecule prevents re-entry of the mercuric-chelator complex into proximal tubular cells, and thus facilitates the excretion of mercuric ions in the urine (Diamond *et al.*, 1988; Zalups *et al.*, 1998). Accordingly, we have hypothesized that DMPS and DMSA, which are both taken up at the basolateral plasma membrane of proximal tubular epithelial cells (Bahn *et al.*, 2002; Burckhardt *et al.*, 2002; Islinger *et al.*, 2001), form stable DMPS or DMSA S-conjugates with intracellular mercuric ions. Subsequently, these mercuric-DMPS or -DMSA complexes appear to be transported into the lumen of the proximal tubule by a membrane transporter, which we have hypothesized to be Mrp2. Mrp2 is an ATP-binding cassette protein localized in the luminal plasma membrane of proximal tubular cells, and is thought to be involved in the elimination of metabolic waste products (Leslie *et al.*, 2005).

As part of the current study, we examined the disposition of Hg²⁺ in TR⁻ rats (which possess truncated, nonfunctional Mrp2 proteins) and in control rats, which have normal Mrp2 proteins. When the disposition of Hg²⁺ was compared between control and TR⁻ rats exposed to Cys-S-Hg-S-Cys or Hcy-S-Hg-S-Hcy

(without subsequent chelation treatment), the renal and hepatic contents of Hg^{2+} were greater in the TR^- rats than those in corresponding control rats. Within the kidneys, the greatest amounts of Hg^{2+} were found in the cortex and outer stripe of the outer medulla. Interestingly, all three segments of the proximal tubule, within which Mrp2 is localized (Peng *et al.*, 1999; Schaub *et al.*, 1997), are present in these two zones. In addition, the urinary and fecal excretion of Hg^{2+} was greater in the control rats than in the TR^- rats. Collectively, these data indicate that the absence of a functional Mrp2 protein contributes to the inability of proximal tubular cells in TR^- rats to secrete and subsequently excrete mercuric ions. Thus, the dispositional findings from the control and TR^- rats, without chelation treatment, indicate that Mrp2 plays an important role in the proximal tubular elimination of Hg^{2+} .

The findings from the current study also show that in rats exposed to Cys-S-Hg-S-Cys or Hcy-S-Hg-S-Hcy, the renal burden of Hg^{2+} is reduced significantly following treatment with DMPS or DMSA, with the reduction being greater in control rats than in TR^- rats. A corresponding increase in the urinary excretion of Hg^{2+} was also observed in both strains of rats. Not surprisingly, the amount of Hg^{2+} excreted by control rats was greater than that of TR^- rats. Together, these findings indicate that Mrp2 likely plays a role in the DMPS- and DMSA-mediated extraction of mercuric ions from the kidney. We hypothesize that this extraction process involves the transport of DMPS or DMSA-S-conjugates of Hg^{2+} via Mrp2.

The *in vitro* data of the current study provide strong evidence implicating Mrp2 in the transport of DMPS and DMSA-S-conjugates of Hg^{2+} . When inside-out membrane vesicles containing MRP2 were incubated with Hg^{2+} , as a conjugate of DMPS or DMSA, uptake of these mercuric species into the MRP2-containing vesicles was significantly greater than that in control vesicles (not containing MRP2). These data provide the most direct support for our hypothesis that DMPS and DMSA-S-conjugates of Hg^{2+} are transportable substrates for Mrp2.

Because the Mrp2 protein present in TR^- rats is a non-functional protein, we would not expect DMPS or DMSA to affect the disposition of mercuric ions in these animals if Mrp2 was the only carrier capable of transporting DMPS and DMSA-S-conjugates of Hg^{2+} . However, our data show that both DMPS and DMSA-mediated significant extraction of Hg^{2+} from within the kidneys of the TR^- rats. A corresponding increase in urinary excretion was also observed. These findings indicate that, at least one transporter, in addition to Mrp2, is involved in the proximal tubular elimination of Hg^{2+} (at least in TR^- rats). We have hypothesized previously that Mrp4 may play a role in the export of mercuric ions from within proximal tubular cells (Bridges *et al.*, 2008). As with Mrp2, Mrp4 is localized in the luminal plasma membrane of proximal tubular cells (Van Aubel *et al.*, 2002) and has been shown to be involved in the elimination of various other compounds (Deeley *et al.*, 2006).

In liver, the effects of DMPS and DMSA on the disposition of Hg^{2+} were not as straightforward as those in the kidney. Treatment with DMPS reduced the hepatic burden of Hg^{2+} in the control rats, but not in TR^- rats. This observation was true of both, rats exposed to Cys-S-Hg-S-Cys, as well as those exposed to Hcy-S-Hg-S-Hcy. These data suggest that in the liver of rats exposed to mercuric-thiol complexes, DMPS S-conjugates of Hg^{2+} are transported primarily, if not solely, by Mrp2. Surprisingly, in both strains of rats, treatment with DMPS increased the fecal excretion of Hg^{2+} even though the hepatic burden of Hg^{2+} was not altered in either strain. One possible explanation for these findings is that Hg^{2+} may be secreted into the intestinal lumen from the enterocytes (Zalups, 1998), and subsequently excreted in the feces. In control rats, upregulation of Mrp2 on the canalicular membrane of hepatocytes may also play a role in the fecal elimination of mercuric ions.

The effect of DMSA on the hepatic burden of Hg^{2+} appeared to be dependent upon the mercuric species administered. In rats exposed to Cys-S-Hg-S-Cys, DMSA reduced the hepatic burden of Hg^{2+} only slightly in control rats and had no effect in TR^- rats. Fecal elimination of Hg^{2+} was increased in both strains of rats, and was greater in control rats than in TR^- rats. Interestingly, in rats exposed to Hcy-S-Hg-S-Hcy, treatment with DMSA resulted in a dramatic reduction in the hepatic burden of Hg^{2+} in both, control and TR^- rats. Surprisingly, treatment with DMSA did not alter the fecal elimination of Hg^{2+} in either strain of rat. The precise reasons for these discrepancies are unclear at present.

It is important to note that the actual species of Hg^{2+} extracted from proximal tubular cells has/have not been determined. Studies in Sperber chickens have shown that DMPS and DMSA each form S-conjugates with Hg^{2+} intracellularly, and promote the extraction of mercuric ions from intracellular ligands via a mechanism involving thiol competition (Diamond *et al.*, 1988; Klotzbach and Diamond, 1988; Stewart and Diamond, 1987, 1988). Subsequently, the mercuric-chelator complex can be detected in urine (Maiorino *et al.*, 1991; Ruprecht, 1997), indicating that these complexes are likely transported from within proximal tubular cells into the tubular lumen. The actual chemical structures of the complexes are unclear currently.

To our knowledge, the results of the current study are the first to implicate a role for Mrp2 in the DMPS- or DMSA-mediated elimination of Hg^{2+} from animals exposed initially to a transportable form of Hg^{2+} . These results complement our previous study in which TR^- rats were injected with HgCl_2 , which in itself, is not a readily transportable form of Hg^{2+} . The current *in vitro* data provide direct evidence that DMPS and DMSA-S-conjugates of Hg^{2+} are transportable substrates of Mrp2. The data from the present study support our hypothesis that DMPS and DMSA extract mercuric ions by forming mercuric-chelator conjugates, which are transported out of proximal tubular cells via Mrp2. It is important to note that mechanisms in addition to Mrp2 are likely involved in this

process. Even so, the results of the current study provide significant insight into the handling of mercuric ions in the proximal tubule.

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