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Molecular Homology and the Luminal Transport of Hg^{2+} in the Renal Proximal Tubule

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Abstract. The aim of this study was to define mechanisms involved in the luminal uptake of inorganic mercury in the kidney using isolated perfused straight (S_2) segments of the proximal tubule. When mercuric conjugates of glutathione (GSH), cysteinylglycine, or cysteine (containing $^{203}Hg^{2+}$) were perfused through the lumen, the rates of luminal disappearance flux (J_D) of inorganic mercury were approximately 39, 53, and 102 fmol/min per mm, respectively. Thus, the rates of luminal uptake of mercury are greater when the mercury is in the form of a mercuric conjugate of cysteine than in the form of a mercuric conjugate of cysteinylglycine or GSH. Addition of acivicin to the perfusate, to inhibit activity of the γ-glutamyltransferase, caused significant reductions in the J_D for mercury in tubules perfused with mercuric conjugates of GSH. Addition of cilastatin, an inhibitor of dehydropeptidase-1 (cysteinylglycinase) activity, caused significant reductions in the uptake of mercury in tubules perfused with mercuric conjugates of cysteinylglycine. These findings indicate that a significant amount of the luminal uptake of mercury, when mercuric conjugates of GSH are present in the lumen, is dependent on the activity of both γ-glutamyltransferase and cysteinylglycinase. Finally, the J_D for mercury in tubules perfused with mercuric conjugates of cysteine was reduced by approximately 50% when 3.0 mM L-lysine or 5.0 mM cycloleucine was added to the perfusate. It is concluded that these findings indicate that at least some of the luminal uptake of mercuric conjugates of cysteine occurs at the site of one or more amino acid transporters via a mechanism involving molecular homology.

Recent studies have implicated at least two sets of transporters in the uptake of inorganic mercury along the renal proximal tubule (1–5). Data from these studies indicate that there is at least one transport system involved in the uptake of inorganic mercury at the luminal plasma membrane and at least one at the basolateral membrane. With respect to the pool of inorganic mercury taken up at the luminal membrane, current evidence indicates that much of this mercury is first filtered at the glomerulus before gaining entry into the proximal tubular epithelial cells (2–6). Although it is not currently known which chemical forms of inorganic mercury are actually filtered at the glomerulus, experimental evidence indicates that likely candidates include mercuric conjugates of albumin, glutathione (GSH), homocysteine, and/or cysteine (4,7).

Findings from several sets of *in vivo* studies indicate that the activity of γ-glutamyltransferase (which cleaves the γ-glutamylcysteine bond in molecules of GSH) is involved significantly in the luminal uptake of inorganic mercury in the kidney (1,8–11). The data from these studies show that when this enzyme is inhibited by acivicin, there is a resulting decrease in the renal tubular uptake and accumulation of administered inorganic mercury and an increase in the urinary excretion of inorganic mercury and GSH. One of the implications of these findings is that much of the inorganic mercury taken up at the luminal membrane *in vivo* is in the form of a mercuric conjugate of GSH just before the actions of γ-glutamyltransferase that lead to the luminal uptake of inorganic mercury. Another implication of these findings is that the primary species of mercury taken up at the luminal membrane are likely mercuric conjugates of one or both of the cleavage products resulting from the enzymatic degradation of GSH, namely cysteinylglycine or cysteine.

There are recent *in vivo* findings supporting, indirectly, the hypothesis that mercuric conjugates of cysteine are transported readily into proximal tubular epithelial cells (4,12–15). These findings show that the rates of uptake and net accumulation of intravenously administered inorganic mercury are enhanced significantly when the inorganic mercury is coadministered with L-cysteine (in the form of mercuric conjugates of cysteine). Additional findings indicate that the severity of proximal tubular injury is enhanced when inorganic mercury is administered as a mercuric conjugate of cysteine (12). Moreover, recent *in vitro* evidence obtained with isolated brush-border membrane vesicles provides more direct support for the hypothesis that mercuric conjugates of cysteine are transported readily across the luminal plasma membrane of proximal tubular epithelial cells (14). As a result of several of the aforementioned studies, it has been suggested that the luminal uptake of inorganic mercury occurs via the actions of one or more of the amino acid transport systems located in the luminal plasma membrane of the proximal tubular epithelial cells

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(1,4,7,16). Unfortunately, there are insufficient data supporting this hypothesis.

Despite the significant volume of recent research, there remains a paucity of direct evidence implicating the roles of γ-glutamyltransferase, the dehydropeptidase-1 that cleaves the peptide bond in molecules of cysteinylglycine (cysteinylglycinase), and mercuric conjugates of GSH, cysteinylglycine, and cysteine in the luminal uptake of inorganic mercury in proximal tubular segments. It is for these reasons that we designed experiments to evaluate, at a cellular level, the molecular mechanisms involved in the luminal uptake of inorganic mercury in pars recta (S_2) segments of the proximal tubule. This segment of the proximal tubule is particularly important to study because it takes up and accumulates, and is vulnerable to the toxic effects of inorganic mercury (7). By using the isolated perfused tubule technique, we were able to begin testing the following three hypotheses. (*1*) The uptake of inorganic mercury at the luminal plasma membrane, when present in the lumen as a mercuric conjugate of GSH, is dependent greatly on the actions of both γ-glutamyltransferase and cysteinylglycinase. (*2*) The primary form of inorganic mercury that is taken up from the lumen of proximal tubular segments is a mercuric conjugate of cysteine (namely dicysteinylmercury). (*3*) The uptake of (at least some) inorganic mercury at the luminal membrane, when it is in the form of a mercuric conjugate of cysteine, occurs through the actions of amino acid transporter(s) involved in the absorption of cystine via a mechanism involving molecular homology or "mimicry."

Materials and Methods

Experimental Design

Three separate sets of studies were performed in the present investigation. In study 1, luminal disappearance flux data were collected to determine and compare the rates of transport of inorganic mercury in S_2 segments of the rabbit proximal tubule when the mercury is presented to the luminal membrane as a mercuric conjugate of GSH, cysteinylglycine, and cysteine. S_2 proximal tubular segments were perfused through the lumen with an electrolyte solution containing 20 μM inorganic mercury (some of which was in the form of $^{203}Hg^{2+}$) and 80 μM GSH, cysteinylglycine, or cysteine. An additional set of luminal disappearance flux experiments was carried out with S_2 segments perfused through the lumen with 20 μM inorganic mercury in the absence of any thiol. Data from these experiments were used only as a relative control, and were not used in the statistical analyses to determine differences in transport parameters among the other three perfusion groups (*i.e.*, tubules perfused with mercuric conjugates of GSH, cysteinylglycine, or cysteine).

Note: When mercuric ions and small thiol-containing molecules are present together in aqueous solution, there is a strong tendency, and a high probability, for the formation of thermodynamically stable mercuric conjugates involving each mercuric ion bonding to two molecules of the respective thiol-containing compound. Confirming evidence for this comes from ^{13}C-nuclear magnetic resonance findings, which indicate that when inorganic mercury is in aqueous solution with as little as a twofold greater concentration of GSH, each mercuric ion becomes incorporated in a linear II coordinate covalent complex with two molecules of GSH (17). Thus, addition of a fourfold higher concentration of each respective thiol (relative to the concentration of inorganic mercury) ensured the formation of thermodynamically stable linear II coordinate covalent complexes between each mercuric ion and two molecules of the respective thiol.

In study 2, we designed experiments to evaluate the role of γ-glutamyltransferase and cysteinylglycinase on the luminal uptake of inorganic mercury in S_2 segments of the proximal tubule perfused through the lumen with mercuric conjugates of GSH and cysteinylglycine, respectively. To evaluate the role γ-glutamyltransferase on the luminal uptake of inorganic mercury when in the form of a mercuric conjugate of GSH, 750 μM or 1.0 mM acivicin was added to the perfusion solution. We have demonstrated that 1.0 mM acivicin essentially inhibits 100% of the activity of γ-glutamyltransferase in isolated perfused S_2 segments of the proximal tubule (18). The 750 μM concentration of acivicin was used to determine whether near-maximal effects of acivicin (on the tubular uptake of inorganic mercury) could be reached at a concentration below 1.0 mM. In the experiments designed to evaluate the role of the cysteinylglycinase on the luminal uptake of inorganic mercury (when in the form of a mercuric conjugate of cysteinylglycine), 0.1, 0.2, or 1 mM cilastatin was added to the perfusion solution. Cilastatin has been shown to inhibit the dehydropeptidase-1 (located on the luminal membrane of proximal tubules) responsible for the catalytic cleavage of the peptide bond between the cysteinyl and glycinyl residues of cysteinylglycine (19,20). At a concentration of 1 mM, cilastatin has been shown to inhibit approximately 90% of the hydrolysis of ^{14}C-*N*-ethylmaleimide-*S*-cysteinylglycine in renal cell homogenates (20).

In the third study (study 3) of the present investigation, experiments were designed to evaluate the effects 3.0 mM L-lysine and 5.0 mM cycloleucine on the luminal uptake of mercury when the mercury is in the form of a mercuric conjugate of cysteine. Addition of these amino acids to the perfusion solution allowed us to test the hypothesis that the luminal uptake of mercuric conjugates of cysteine (in the form of dicysteinylmercury) occurs, at least in part, through the same transporters involved in the luminal uptake of amino acid cystine, as a result of molecular homology or "mimicry."

Animals

Female, New Zealand White, specific pathogen-free rabbits (purchased from Myrtle's Rabbit Farm, Thompson Station, TN) were used in the present study. Before experimentation, the rabbits were maintained on regular rabbit chow and given water *ad libitum*. All experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Procedure for Obtaining S2 Segments of the Proximal Tubule

On each day of experimentation, a rabbit was anesthetized by administering a subcutaneous injection of a cocktail containing 56 mg/ml Ketaset® (ketamine hydrochloride), 28 mg/ml xylazine, and 1.6 mg/ml acepromazine maleate, which were purchased from Butler Chemical Co. (Bedford, OH). After the rabbit was in a state of deep anesthesia (as determined by corneal reflex), the abdomen was opened and the kidneys were removed and placed in a cold (4°C) aqueous phosphate-sucrose buffer solution. The phosphate-sucrose buffer solution contained 125 mM sucrose, 13.3 mM NaH_2PO_4, and 56 mM Na_2HPO_4, and the pH of the solution was adjusted to 7.4 with 1.0 M NaOH. The kidneys were then sliced quickly into 1- to 2-mm coronal sections with a single-edged razor blade. The sections were stored in the same phosphate-sucrose buffer solution. S2 segments of the proximal tubule, which were identified as straight portions of the proximal tubule that spanned the entire thickness of the cortex, were dissected from these slices under a dissecting microscope for the next 8 to 12 h.

This particular segment of the proximal tubule was chosen for study because it can be dissected readily from the rabbit-kidney, it makes up the major portion of the pars recta of the proximal tubule, and it accumulates inorganic mercury avidly and is involved in the nephropathy induced by inorganic mercury.

Method for Perfusing S2 Segments of the Proximal Tubule

Each dissected tubule was transferred to a Lucite perfusion chamber and was suspended between two sets of pipettes. One set of pipettes was used to perfuse the suspended tubule, and the other set was used to collect the perfused fluid. Once hooked up to the pipettes, each tubular segment was allowed to warm to 37°C for 15 to 20 min before beginning an experiment. After this warm-up period, three 5-min collection periods were carried out. Perfusion rates were maintained on average between 7 to 10 nl/min with constant hydrostatic pressure. Because of differences in tip diameters of the perfusion pipettes used in the present study, the hydrostatic pressure needed to perfuse S2 segments of the proximal tubule at 7 to 11 nl/min varied between 15 to 50 mmHg. It should be stressed that most of the drop in pressure occurs at the tip of the perfusion pipette. In addition, each perfused tubule was monitored for any changes in tubular diameter resulting from abnormally high intraluminal pressures. The perfused fluid was collected from the lumen into a constant volume pipette (designed to accurately collect 30 to 50 nl). Bathing fluid surrounding the outside basolateral surface of the perfused tubule was pumped into the bathing chamber at a rate of approximately 0.33 ml/min and was continually aspirated and collected into scintillation vials at 5-min intervals. The perfusion chamber contained 0.33 ml of fluid. For additional details on the methods used to isolate and perfuse segments of the proximal tubule of the rabbit, refer to the study by Zalups *et al.* (21).

Composition of Perfusing and Bathing Solutions

In all of our experiments, the perfusing and bathing solutions consisted of simple electrolyte solutions. The perfusing solution, sometimes referred to as an artificial perfusion medium, contained the following: 145 mM Na^+, 140 mM Cl^-, 5 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 1.2 mM SO_4^{2-}, 2 mM $HPO_4^{2-}/H_2PO_4^-$, 1 mM D-glucose, and 0.5 mM glutamate, and the pH was adjusted to 7.4. To evaluate cytotoxicity of inorganic mercury, we placed the vital dye FD&C green No. 3 (molecular weight, 809 Daltons) in the perfusate at a concentration of 250 nM. Final osmolality was adjusted to 290 mosmol/kg H_2O with doubly distilled and deionized water. In all experiments, the basic composition of the bathing solution was the same as that of the perfusing solution. [^{3}H]-L-Glucose (50 μCi/ml, 58.8 mCi/mg) was used as a volume marker in all experiments and was added to the perfusing solutions. Experimental solutes, such as inorganic mercury (containing $^{203}Hg^{2+}$; 33.57 μCi/μg, 12.45 mCi/ml), acivicin, cilastatin, L-lysine, and cycloleucine were added to the perfusion solutions in particular experiments. In all experiments, the concentration of inorganic mercury in the perfusing solution was 20 μM. In addition, the concentration of any thiol-containing molecule added to the perfusion solution was always 80 μM. For additional details on perfusion conditions, see the Experimental Design section.

Chemicals

Cilastatin was purchased from Merck & Co., Inc. (Whitehouse Station, NJ). All other chemicals unless otherwise noted were obtained from Sigma Chemical (St. Louis, MO). The isotope $^{203}Hg^{2+}$ in the form of mercuric chloride was obtained from Buffalo Materials Corp. (Buffalo, NY). Two samples of the isotope ^{3}H-L-glucose (both were 14.6 Ci/mmol, 1 mCi/ml) were obtained from New England Nuclear (Boston, MA).

Collecting Samples

Samples of luminal fluid exiting from a perfused tubular segment (collectate) and bathing solution were collected for each perfused tubule to measure the rates of luminal disappearance flux (J_D [fmol/min per mm tubule length of inorganic mercury]) and the appearance flux rates (fmol/min per mm tubule length) of the volume marker. The time required to fill the constant volume pipette was used to calculate the volume collection rate (nl/min).

During the luminal disappearance flux experiments, the bathing solution was routinely collected and analyzed for the appearance of the volume marker ([^{3}H]-L-glucose) into the bathing solution. The aspirated bathing solution was collected in 20-ml scintillation vials under vacuum. Scintillation vials were changed approximately every 5 min during experiments.

Harvesting Perfused Tubular Segments at the End of an Experiment

To calculate the cellular content of inorganic mercury in the isolated perfused S2 segments of the proximal tubule, it was necessary to harvest the perfused tubule at the end of each experiment (approximately 30 to 35 min). The tubular segment was harvested while it was being perfused, and it was removed from the bathing solution and placed in 10 μl of 3% (wt/vol) TCA. The TCA precipitates all of the larger cellular proteins and releases the cytosolic contents. After a few minutes, the tubular segment was removed from the TCA solution, placed in a vial of scintillation fluid, and analyzed (by standard isotopic methods) later for the content of both inorganic mercury and the volume marker ^{3}H-L-glucose. The data obtained from the TCA-soluble and TCA-precipitable fractions permitted us to approximate the cellular content of inorganic mercury.

Assessment of Cellular and Tubular Pathology

During each experiment, the perfused tubule was observed microscopically during the entire perfusion process to detect any pathology. Typical pathologic changes detected in S2 segments of the proximal tubule exposed to inorganic mercury include cellular swelling, cytoplasmic vacuolization, shedding of brush-border membrane, blebbing of the apical plasma membrane, and cellular uptake of the vital dye FD&C green.

Calculations

The calculations used to determine rates of luminal disappearance flux (J_D), tubular content, and leak of mercuric ions ($^{203}Hg^{2+}$) are the same as those described previously (22).

Statistical Analyses

A minimum of four tubules was perfused under each experimental condition. Moreover, data for each parameter assessed were obtained from tubular segments isolated from at least two animals. In each perfused tubule, three or more flux measurements were made and averaged. The mean values from each tubule were used to compute the overall mean and standard error under each experimental condition. Data were first subjected to the Kolmogorov–Smirnov test for normality and then the Levene test of homogeneity of variance. If both tests were not statistically significant (at $P < 0.05$), a one-way ANOVA and Tukey honest significant difference *post hoc* test were

performed with a significance level set at $P < 0.05$. If a set of data failed the normality test or the test for homogeneity of variance, the nonparametric Kruskal–Wallis ANOVA by ranks, followed by a Mann–Whitney *U* test analysis, was performed with the level of significance set at $P < 0.05$.

Results

Study 1

A summary of the rates of disappearance flux (J_D) of inorganic mercury from the lumen in S_2 segments of the proximal tubule perfused with 20 μM inorganic mercury with or without 80 μM GSH, cysteinylglycine, or cysteine is presented in Figure 1. The average J_D for mercury in the tubules perfused with 20 μM inorganic mercury and 80 μM GSH was 38.6 $\pm$ 1.40 fmol/min per mm tubular length during 35 to 40 min of perfusion. In the tubules perfused with 20 μM inorganic mercury and 80 μM cysteinylglycine, the J_D for inorganic mercury was approximately 33% greater than that in the corresponding tubules perfused with inorganic mercury and GSH. In the S_2 segments perfused with 20 μM inorganic mercury and 80 μM cysteine, the J_D for inorganic mercury was approximately 96% greater than that in the tubules perfused with inorganic mercury plus cysteinylglycine and 160% greater than that in the tubules perfused with inorganic mercury plus GSH. For relative comparison purposes only, the J_D for inorganic mercury in the tubules perfused with 20 μM inorganic mercury, as a chloride salt, was 106.6 $\pm$ 7.83 fmol/min per mm tubular length. Note that this rate is similar to that in the tubules perfused with mercuric conjugates of cysteine.

In the tubular segments perfused with inorganic mercury and GSH or cysteinylglycine, the concentration of inorganic mercury in the collectate averaged 13.4 $\pm$ 0.7 or 12.9 $\pm$ 0.5 μM, respectively. Thus, the concentration of mercury in the perfusate was reduced by about 6.5 to 7.0 μM in these tubular segments. By contrast, the concentration of inorganic mercury in the collectate from the tubules perfused with inorganic mercury plus cysteine was 3.3 $\pm$ 0.5 μM, or with inorganic mercury alone was 3.0 $\pm$ 0.5 μM. This indicates that approximately 82 to 83% of the inorganic mercury in the perfusing solution was removed, along an average length of 1.0 mm, in the S_2 segments perfused with inorganic mercury alone or in combination with cysteine.

With the exception of the tubules perfused with 20 μM inorganic mercury (without any thiol-containing molecules in the perfusate), no signs of cellular pathology were detected in any of the perfused tubules. However, in the tubules perfused with 20 μM inorganic mercury, numerous pathologic features were detected during the perfusion process. We would like to stress, however, that these pathologic features occur under conditions that would never be encountered *in vivo*. As mentioned above, transport and toxicologic data were collected from tubules perfused with 20 μM inorganic mercury for relative comparison purposes only. Cellular swelling began after about 5 to 10 min into the perfusion process, but was restricted to the initial 200 to 250 μm of the tubule. Cellular blebbing and necrosis became evident along the initial 300 μm of each perfused tubule after about 15 to 20 min of perfusion. The uptake of the vital dye FD&C green was the primary marker used for detecting cellular necrosis. Necrosis rarely progressed beyond the initial 100 μm of a perfused tubule during the 35 to 40 min of perfusion. Another indicator of

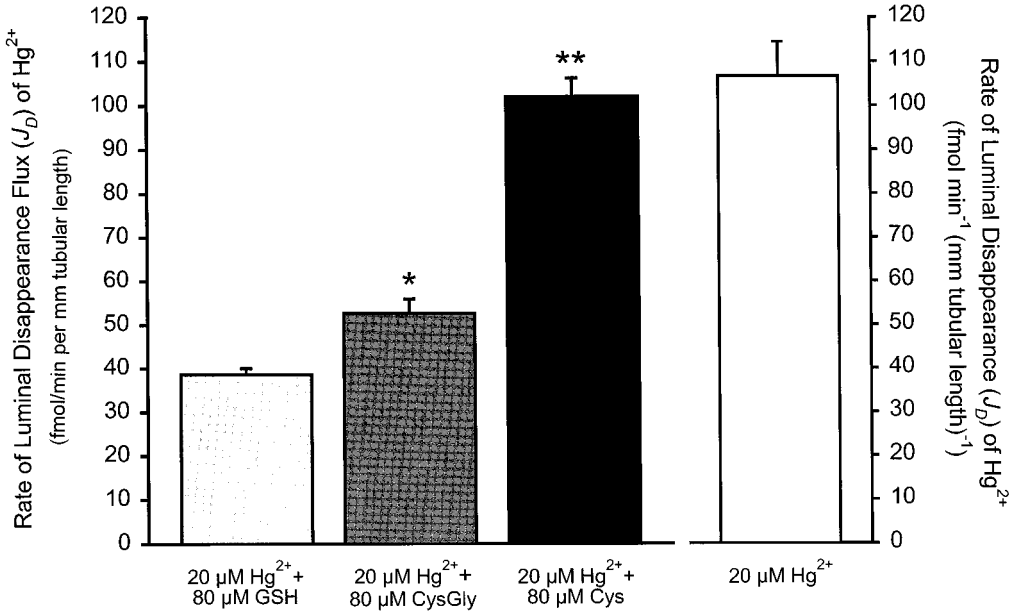

Figure 1. Rate of luminal disappearance flux (J_D) of inorganic mercury (fmol/min per mm tubular length) in isolated perfused S2 segments of the proximal tubule of the rabbit. Each value represents the mean $\pm$ SEM obtained from four to five tubular segments. *$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg and 80 μM glutathione (GSH). **$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg^{2+} and 80 μM GSH or 20 μM Hg^{2+} and 80 μM cysteinylglycine (CysGly).

tubular injury was increased tubular leak of the volume marker. Tubules were considered healthy if they had leaks below 10 fmol/min per mm tubular length.

Study 2

Effects of Acivicin in Tubules Perfused with Mercuric Conjugates of GSH. Addition of either 750 μM or 1.0 mM acivicin to a perfusate containing 20 μM inorganic mercury and 80 μM GSH caused the J_D for inorganic mercury to decrease by approximately 55 to 63% (Figure 2A). There was no significant difference in the J_D for inorganic mercury between the tubules perfused with 750 μM acivicin and those perfused with 1.0 mM acivicin.

In the tubules perfused with inorganic mercury and GSH, the concentration of mercury in the collectate averaged 13.4 $\pm$ 0.7 μM. In tubules perfused with inorganic mercury and GSH plus 750 μM or 1.0 mM acivicin, the concentration of mercury in the collectate was approximately 40% (18.8 $\pm$ 1.4 μM) or 31% (17.1 $\pm$ 0.5) greater, respectively, than that in tubules perfused without acivicin in the perfusate. There was no significant difference in the concentration of mercury in the collectate between the tubules perfused with 750 μM acivicin and those perfused with 1.0 mM acivicin.

After 35 to 40 min of perfusion, the amount of mercury detected in the tubules perfused with inorganic mercury and GSH was approximately 360 fmol/mm tubular length (Figure 2B). In the tubular segments perfused with 750 μM or 1.0 mM acivicin, the amount of mercury in the perfused segments was approximately 49 to 56% less than that in the corresponding tubules that were not perfused with acivicin. No significant difference was detected in the content of mercury between the tubules perfused with 750 μM acivicin and those perfused with 1.0 mM acivicin. No signs of tubular or cellular pathology were detected in any of the experiments described above.

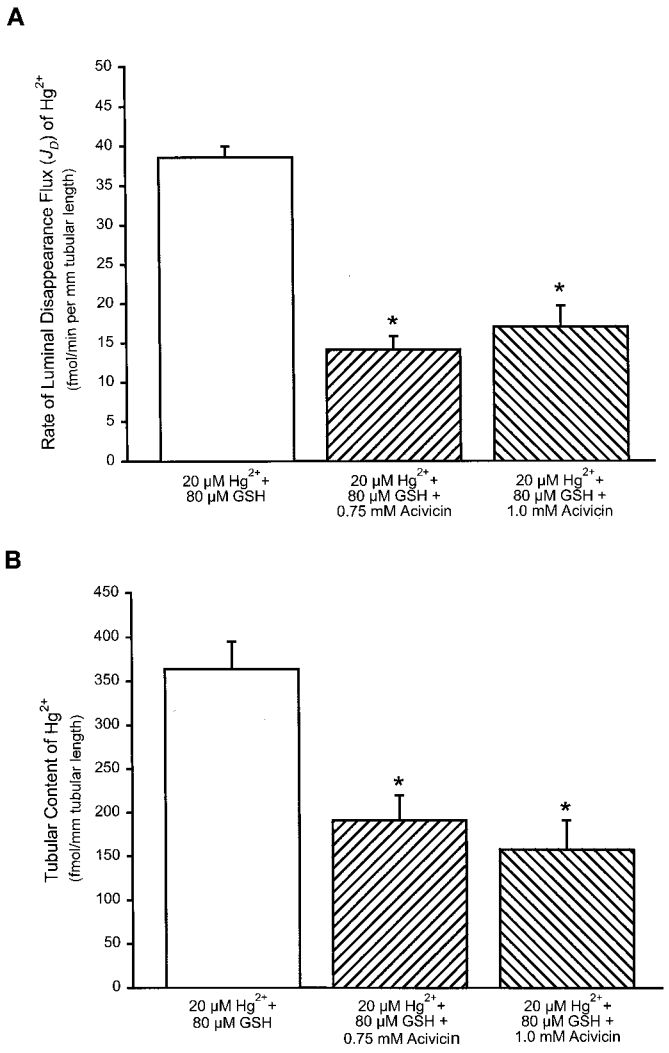

Figure 2. (A) Rate of luminal disappearance flux (J_D) of inorganic mercury (fmol/min per mm tubular length) from the luminal compartment of isolated perfused S2 segments of the proximal tubule of the rabbit. (B) Content of Hg^{2+} in the perfused tubules after approximately 30 to 35 min of perfusion. Each value represents the mean $\pm$ SEM obtained from four to five tubular segments. *$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg and 80 μM GSH.

Effects of Cilastatin in Tubules Perfused with Mercuric Conjugates of Cysteinylglycine. In S_2 segments perfused with 20 μM inorganic mercury and 80 μM cysteinylglycine, the J_D for inorganic mercury was approximately 53 fmol/min per mm tubular length (Figure 3A). In corresponding tubules in which 200 μM or 1.0 mM cilastatin was added to the perfusate, the J_D for inorganic mercury was approximately 72 and 68% less, respectively, than that in the control tubules perfused with inorganic mercury and cysteinylglycine. No significant difference in the J_D for inorganic mercury was detected between the tubules perfused with 200 μM cilastatin and those perfused with 1.0 mM cilastatin.

The concentration of inorganic mercury in the collectate from the tubules perfused with inorganic mercury and cysteinylglycine averaged 12.9 $\pm$ 0.5 μM. In the corresponding tubules perfused with 200 μM or 1.0 mM cilastatin, the concentration of mercury in the collectate was approximately 31% (16.9 $\pm$ 0.4 μM) and 42% (18.5 $\pm$ 0.4 μM) greater than that in the collectate from the tubules perfused with inorganic mercury and cysteinylglycine, respectively. A significant difference was detected in the concentration of inorganic mercury in the collectate between the tubules perfused with 200 μM and 1.0 mM cilastatin.

Tubular content of inorganic mercury in the tubules perfused with 20 μM inorganic mercury and 80 μM cysteinylglycine was about 300 fmol/mm tubular length after 35 to 40 min of perfusion (Figure 3B). Only in the tubules perfused with 1.0 mM cilastatin was the content of mercury significantly lower than that in the corresponding control tubules perfused with inorganic mercury and cysteinylglycine.

Study 3

The J_D for inorganic mercury in tubules perfused with 20 μM inorganic mercury and 80 μM cysteine was approximately 102 fmol/min per mm tubular length (Figure 4A). In the corresponding experimental tubules that were also perfused with 3.0 mM L-lysine or 5.0 mM cycloleucine, the J_D for

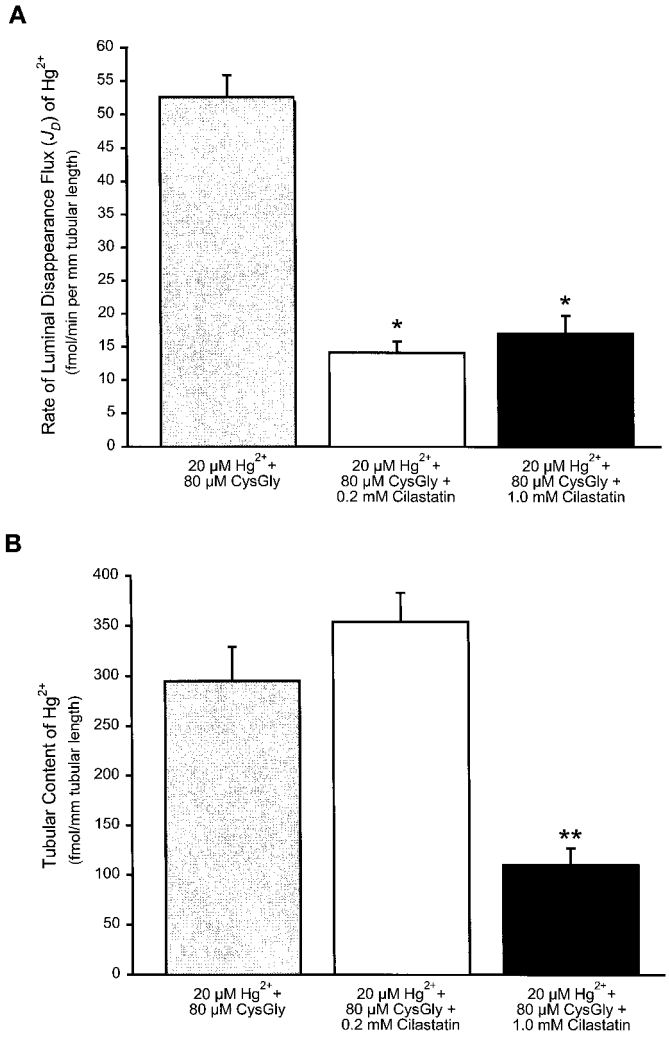

Figure 3. (A) Rate of luminal disappearance flux (J_D) of inorganic mercury (fmol/min per mm tubular length) from the luminal compartment of isolated perfused S2 segments of the proximal tubule of the rabbit. (B) Content of Hg^{2+} in the perfused tubules after approximately 30 to 35 min of perfusion. Each value represents the mean ± SEM obtained from four to five tubular segments. *$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg and 80 μM GSH. **$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg^{2+} and 80 μM CysGly or 20 μM Hg^{2+} and 80 μM CysGly in the presence of 0.2 mM cilastatin.

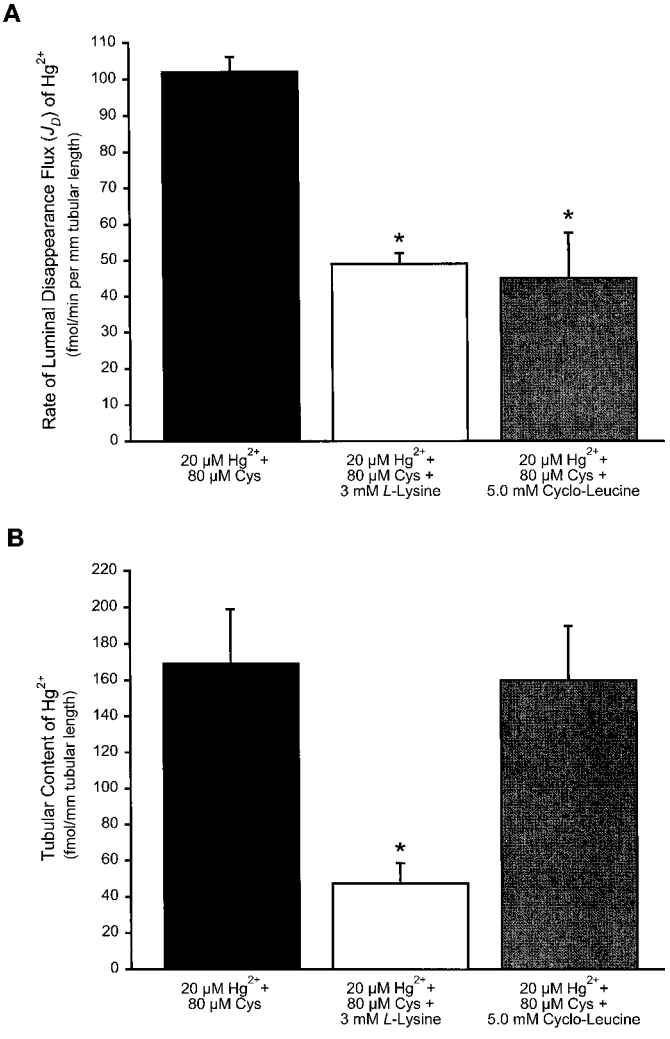

Figure 4. (A) Rate of luminal disappearance flux (J_D) of inorganic mercury (fmol/min per mm tubular length) from the luminal compartment of isolated perfused S2 segments of the proximal tubule of the rabbit. (B) Content of Hg^{2+} in the perfused tubules after approximately 30 to 35 min of perfusion. Each value represents the mean ± SEM obtained from four to five tubular segments. *$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg and 80 μM GSH. **$P < 0.05$, significantly different from the mean for the tubules perfused through the lumen with 20 μM Hg^{2+} and 80 μM CysGly or 20 μM Hg^{2+} and 80 μM CysGly in the presence of 0.2 mM cilastatin.

inorganic mercury was approximately 52 and 56% less, respectively, than that in the corresponding tubules that were not perfused with L-lysine or cycloleucine.

In the samples of collectate obtained from the tubules perfused with 20 μM inorganic mercury, 80 μM cysteine, and 3.0 mM L-lysine or 5.0 mM cycloleucine, the concentration of mercury averaged about 9.5 ± 1.6 μM or 10.8 ± 1.3 μM, respectively. The concentrations of inorganic mercury in these collectate samples were approximately threefold greater than the average concentration of mercury in the samples of collectate obtained from the corresponding control tubules not perfused with L-lysine or cycloleucine (3.26 ± 0.5 μM).

Tubular content of inorganic mercury in the tubules perfused through the lumen with mercuric conjugates of cysteine was approximately 170 fmol/mm tubular length after 35 to 40 min of perfusion (Figure 4B). In the tubules perfused with 20 μM inorganic mercury, 80 μM L-cysteine, and 3.0 mM L-lysine, the tubular content of mercury was approximately 72% less than that in the corresponding control tubules.

Discussion

The disappearance flux (J_D), collectate, and leak data from the present study indicate clearly that the rate of luminal uptake of inorganic mercury in isolated S_2 segments of the proximal

tubule is approximately twofold greater when mercuric conjugates of cysteine (dicysteinylmercury) are perfused through the lumen than when mercuric conjugates of GSH or cysteinylglycine are perfused through the lumen. Interestingly, the rates of luminal uptake of inorganic mercury in the tubules perfused with mercuric conjugates of cysteine were sufficiently high enough that they were similar to those in tubules perfused with inorganic mercury in the absence of any thiol-containing molecule in the perfusate. Comparable findings have also been reported recently using renal brush-border membrane vesicles (14). More specifically, a greater level of association of inorganic mercury has been shown to occur in these membrane vesicles when they are exposed to 1.0 μM inorganic mercury in the form of a mercuric conjugate of cysteine than in the form of mercuric chloride. It was suggested that mercuric conjugates of cysteine must have been transported across the luminal plasma membrane to account for the increased amount of association of inorganic mercury with the membranes. The association of inorganic mercury with the membrane vesicles was also significantly greater when they were exposed to mercuric conjugates of cysteine than mercuric conjugates of GSH, which is consistent with our findings in isolated perfused tubules.

One must keep in mind that the intoxication that occurred in the tubules perfused with inorganic mercury alone likely contributed to the rates of inward flux of mercury measured in those tubular segments. It should also be pointed out that the mechanisms by which inorganic mercury induces injury in pars recta segments of proximal tubules *in vivo* are likely different from those in the current setting, mainly because "free" unbound inorganic mercuric ions do not exist in plasma or extracellular fluids in any appreciable amount. In the present study, cellular intoxication was absent in isolated S2 segments perfused with a 4:1 ratio of thiol-containing molecule to mercuric ion. The concentration of mercury and the 4:1 ratio of thiol to mercury were used for two reasons: (*1*) to ensure the formation of mercuric conjugates consisting of a mercuric ion bonded to two molecules of the respective thiol molecule in a linear II coordinate covalent manner; and (*2*) to minimize induction of cellular toxicity so as to study transport processes. It is noteworthy, however, that in a previous unpublished study we demonstrate that when the ratio of cysteine to inorganic mercury is reduced to 2:1, cellular intoxication (similar to that detected with inorganic mercury alone) occurs in S2 segments. Thus, the additional amount of thiol (beyond that to ensure formation of linear II coordinate covalent complexes between each mercuric ion and two molecules of the respective thiol) in the perfusate provides protection to the tubular segment from the toxic effects of (20 μM) mercuric ions in the form of mercuric conjugates of cysteine. The mechanism for this protection is likely related to increasing the pool of ligands to which the mercuric ions can bind either inside or outside of the proximal tubular cells, thus preventing the mercuric ions from binding to critical functional thiols on, or inside, the cells. Also, when inorganic mercury is coadministered with cysteine *in vivo*, renal cellular damage induced by the mercury is enhanced significantly (12), which indicates that mercuric conjugates of cysteine are involved in both the renal tubular transport and toxicity of inorganic mercury.

The rationale for investigating the rates of luminal uptake of inorganic mercury in the form of mercuric conjugates of GSH, cysteinylglycine, and cysteine stems from findings in several previous *in vivo* studies in which the activity of the brush-border enzyme γ-glutamyltransferase on the renal uptake and accumulation of inorganic mercury had been studied (1,8–11,23). It was demonstrated in these studies that the renal uptake and accumulation of inorganic mercury decreased and the urinary excretion of inorganic mercury and GSH increased in animals in which the activity of γ-glutamyltransferase had been inhibited by pretreatment with acivicin, before exposure of inorganic or organic mercury. Collectively, these *in vivo* findings have led to the following assumptions. First, that mercuric conjugates of cysteinylglycine are formed in the proximal tubular lumen from mercuric conjugates of GSH as a result of the actions γ-glutamyltransferase (1,7,15,16,24). Second, that once formed, these mercuric conjugates of cysteinylglycine are rapidly degraded to mercuric conjugates of cysteine via the actions of cysteinylglycinase.

Inasmuch as γ-glutamyltransferase, cysteinylglycinase, and the accumulation of inorganic mercury (25–27) are all localized almost exclusively along the proximal tubule, it is logical to conclude that the majority of inorganic mercury taken up from the luminal compartment of the nephron *in vivo* occurs along the three segments of the proximal tubule. In support of this hypothesis are isolated perfused tubule data showing that inorganic mercury is transported across the luminal plasma membrane in all three segments of the proximal tubule when it is in the form of a mercuric conjugate of GSH or cysteine (21). Because of the association between the activity of γ-glutamyltransferase and the luminal uptake of inorganic mercury, it has been assumed that the majority of the mercuric ions in the lumen of proximal tubular segments, *in vivo*, are in the form of mercuric conjugates of GSH before being taken up at the luminal membrane (1,7,14,15,24). It should be pointed out, however, that it is unclear where in the body these putative conjugates are formed.

In the present study, the rates of luminal uptake and cellular accumulation of inorganic mercury in tubules perfused with mercuric conjugates of GSH were decreased by more than 50% when the γ-glutamyltransferase was inhibited by 0.75 or 1.0 mM acivicin. These findings provide the most direct support, to date, for the hypothesis that γ-glutamyltransferase plays a significant role in the luminal uptake of inorganic mercury in proximal tubular segments when mercuric conjugates of GSH are present in the lumen.

When S_2 segments were perfused through the lumen with mercuric conjugates of cysteinylglycine, the rates of uptake of inorganic mercury were decreased by more than 68% when the activity of cysteinylglycinase (and dipeptide transport) was (were) inhibited by 1.0 mM cilastatin. These findings serve as the first line of evidence implicating the role of cysteinylglycinase in the luminal uptake of inorganic mercury in proximal tubular segments, in particular when mercuric conjugates of

cysteinylglycine (or GSH) are present in the proximal tubular lumen.

It was surprising, however, that near-maximal inhibition of γ-glutamyltransferase and cysteinylglycinase did not result in complete inhibition in the luminal uptake of inorganic mercury when it was in the form of a mercuric conjugate of GSH or cysteinylglycine, respectively. This was especially the case in the tubules perfused with mercuric conjugates of cysteinylglycine, in which the dipeptide analog cilastatin reduced cellular accumulation of inorganic mercury in a concentration-dependent manner. The dose-dependent reductions in the accumulation of mercury induced by cilastatin in the tubules perfused with mercuric conjugates of cysteinylglycine likely reflect a combination of inhibition of the activity of cysteinylglycinase and one or more dipeptide or small peptide transporter(s). At the lower concentrations of cilastatin, it is likely that the activity of cysteinylglycinase was inhibited primarily, but that one or more of the peptide transporters retained sufficient activity to permit mercury gaining access to the intracellular environment and allowed mercury to accumulate in the proximal tubular epithelial cells, albeit at reduced rates. These aforementioned findings lead one to believe that some level of luminal uptake of mercury can occur via mechanisms that are capable of transporting mercuric conjugates of GSH and cysteinylglycine, providing that conditions favoring such transport are present. The current data are the first of their kind to support this hypothesis. With respect to the potential for the luminal uptake of a diglutathione mercury conjugate (GSH-Hg-GSH), it is not inconceivable that this would occur through a mechanism or transporter involved in the uptake of glutathione disulfide (GSSG), where GSH-Hg-GSH serves as a molecular homologue or "mimic" of GSSG. On the other hand, due to the tremendous amount of γ-glutamyltransferase and cysteinylglycinase lining the brush-border membrane of proximal tubular cells, it is likely that very little mercury taken up at the luminal membrane *in vivo* would occur by one of the mechanisms alluded to above.

Overall, our findings indicate that when inorganic mercury is present in the lumen of proximal tubular segments as mercuric conjugates of GSH, these conjugates are sequentially broken down to mercuric conjugates of cysteinylglycine and then to mercuric conjugates of cysteine in the tubular lumen by the actions of γ-glutamyltransferase and cysteinylglycinase, respectively. Moreover, the findings indicate that the primary form of inorganic mercury that is taken up at the luminal membrane is a mercuric conjugate of cysteine. This notion is further supported by the fact that the majority of the luminal uptake of mercury (in the kidneys) has been shown to be linked to the activity of γ-glutamyltransferase (1,5), and that increased renal uptake and renal toxicity of mercury have been detected in animals administered mercuric conjugates of cysteine (4,12,13). As an additional note, sequential enzymatic degradation of GSH to cysteine while the sulfur atom of the cysteinyl residue remains bonded to a mercuric ion has been demonstrated to occur *in vitro* using methylmercuric conjugates of GSH and purified γ-glutamyltransferase and cysteinylglycinase (28).

The structure of the mercuric conjugate of cysteine, dicysteinylmercury, resembles the structure of the amino acid L-cystine. This structural similarity has led us to postulate that the luminal uptake of dicysteinylmercury occurs, at least in part, through the actions of one or more of the amino acid transporters, perhaps those involved in the luminal uptake of cystine. The notion that molecular homology or "molecular mimicry" is involved in the transport of mercuric conjugates into specific target cells has been expounded previously. In a recent review, Clarkson (29) discusses the conceptual notion that certain toxic metals, including mercury, form complexes with endogenous biologic molecules that "mimic" other endogenous molecules. It is proposed that it is by this mechanism of "mimicry" that some of these toxic metals gain access to the intracellular environment (of various target cells in the body) via membrane transporters involved in the uptake of the molecules being "mimicked." One example of a putative "mimic" formed in the body is thought to occur when methylmercury binds to cysteine. It is purported that this complex resembles and "mimics" the amino acid methionine, and gains entry into cells via the amino acid transporter(s) involved in the transport of methionine. Evidence supporting this hypothesis comes from studies on the uptake and/or transport of methylmercury by astrocytes (30) and the endothelial cells of the blood–brain barrier (31,32). The hypothesis that inorganic mercury enters renal proximal tubular cells by a mechanism of what we prefer to call molecular homology has not been tested until the present study.

As a first attempt to test the hypothesis that dicysteinylmercury can be taken up by one or more of the transporters involved in the luminal uptake of cystine, we coperfused 3.0 mM L-lysine or 5.0 mM cycloleucine along with the mercuric conjugates of cysteine (in the form of dicysteinylmercury at concentration of approximately 20 μM). Ideally, one would have preferred to use cystine itself as a competitive inhibitor. However, this is not possible due to the poor solubility of cystine in aqueous solution. The rationale for using these two amino acids at their respective concentrations comes mainly from the study of Schafer and Watkins (33), who demonstrated (in isolated perfused proximal tubular segments) that 3.0 mM L-lysine or 5.0 mM cycloleucine inhibits the luminal uptake of cystine (300 μM) by approximately 45 to 55%. In the present study, addition of either 3.0 mM L-lysine or 5.0 mM cycloleucine to a perfusate containing dicysteinylmercury (20 μM) also caused the luminal uptake (disappearance) of inorganic mercury to be reduced by approximately 50%. We interpret these findings as the first line of evidence implicating directly at least one (or more) amino acid transporter(s) in the uptake of inorganic mercury in the form of dicysteinylmercury. Moreover, these data support the notion that at least one mechanism involving molecular homology (or "mimicry") is involved in the luminal absorptive transport of the mercuric conjugate dicysteinylmercury.

We would like to point out, however, that in contrast to the data obtained with the use of lysine, cycloleucine caused significant reductions in J_D, while it did not have a significant effect on the net tubular accumulation of inorganic mercury.

Although these data may seem to be contradictory, it may be that cycloleucine had a negative effect on the net basolateral efflux of mercury. Additional work is clearly required to substantiate the role of specific transporters in both the luminal and basolateral uptake of inorganic mercury along the proximal tubule *in vivo*.

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