

Homocysteine and the Renal Epithelial Transport and Toxicity of Inorganic Mercury: Role of Basolateral Transporter Organic Anion Transporter 1

RUDOLFS K. ZALUPS and SARFARAZ AHMAD

Division of Basic Medical Sciences, Mercer University, School of Medicine, Macon, Georgia

Abstract. The epithelial cells that line the renal proximal tubule have been shown to be the primary cellular targets where mercuric ions gain entry, accumulate, and induce pathologic effects *in vivo*. Recent data have implicated at least one of the organic anion transport systems in the basolateral uptake of inorganic mercury (Hg). With the use of a line of type II MDCK cells transfected stably with the human organic anion transporter 1 (hOAT1), the hypothesis that hOAT1 can transport mercuric conjugates of homocysteine (Hcy) was tested. Indeed, MDCK II cells expressing a functional form of hOAT1 gained the ability to transport the mercuric conjugate 2-amino-4-(3-amino-3-carboxy-propylsulfanylmercurisulfanyl) butyric acid (Hcy-S-Hg-S-Hcy). In addition, *p*-aminohippurate and the dicarboxylates adipate and glutarate

(but not succinate or malonate) inhibited individually the uptake of Hcy-S-Hg-S-Hcy in a concentration-dependent manner. Furthermore, a direct relationship between the uptake of Hcy-S-Hg-S-Hcy and the induction of cellular injury and death was demonstrated in the hOAT1-expressing MDCK II cells only. These data represent the first line of direct evidence implicating one of the organic anion transporters in the uptake of a mercuric conjugate of Hcy in a mammalian cell. Thus, mercuric conjugates of Hcy are potential transportable substrates of OAT1. More important, the findings from the present study implicate the activity of OAT1 in the uptake and toxicity of Hg (when in the form of Hcy-S-Hg-S-Hcy in the extracellular compartment) in proximal tubular epithelial cells *in vivo*.

In humans and other mammals, the largest fraction of mercuric ions that enter systemic circulation after exposure to inorganic mercury (Hg) is taken up in the kidneys by proximal tubular epithelial cells, which are the primary cellular targets where Hg exerts its toxic effects *in vivo*. In fact, as much as 50% of a nontoxic dose can be taken up by the kidneys within several hours after exposure (1). Until recently, however, the mechanisms by which mercuric ions gain entry into proximal tubular epithelial cells have been unclear. Part of the problem in understanding how mercuric ions gain entry into their primary cellular targets relates to a lack of knowledge about the chemical form of Hg in different compartments in the body, especially when it is presented to and taken up by these target cells. On the basis of their physical and thermodynamics properties, we know that mercuric ions are strong electrophiles that have a particularly strong affinity for reduced sulfhydryl groups in biologic aqueous environments. Accordingly, mercuric ions are carried around in the plasma primarily as conjugates of thiol-containing biomolecules, such as albumin, cysteine (Cys), and glutathione (GSH) (1–4).

During each passage through the renal circulatory system,

only a relatively small fraction (~20 to 30%) of blood is actually filtered at the glomeruli. The majority of the blood that enters the kidneys traverses through the peritubular capillaries and vasa recta and then exits through the renal veins. Consequently, plasma solutes such as thiol-S conjugates of Hg both can be filtered at the glomerulus, into the luminal compartment of the nephron (assuming the compounds are ultrafiltrable), and can be delivered *via* peritubular blood (at approximately the same time) to the basolateral surface of the nephron. It is interesting that current evidence indicates that there are indeed both luminal and basolateral mechanisms involved in the proximal tubular uptake and accumulation of Hg (1).

Several lines of evidence have emerged indicating that mercuric conjugates of cysteine (Cys), in the form of 2-amino-3-(2-amino-2-carboxy-ethylsulfanylmercurisulfanyl) propionic acid (Cys-S-Hg-S-Cys), are substrates that can be taken up from the blood and extracellular fluid by proximal tubular epithelial cells at their basolateral membrane by one or more organic anion transport systems (5–14). The most conclusive evidence implicating the organic anion transporters has come recently from two studies that used type II MDCK cells transfected stably with the human isoform of *oat1*. The findings from these studies show clearly that the mercuric conjugate Cys-S-Hg-S-Cys is indeed a transportable substrate of the organic anion transporter 1 (OAT1; and perhaps OAT3) (14). What remains unclear, however, is whether there are other chemical species of Hg in the blood that are transportable substrates of one or more of the organic anion transporters.

It is interesting that the sulfhydryl-containing amino acid homocysteine (Hcy) is also present in the extracellular com-

Received March 30, 2004. Accepted May 18, 2004.

Correspondence to Dr. Rudolfs K. Zalups, Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College Street, Macon, GA 31207. Phone: 478–301–2559; Fax: 478–301–5489; E-mail: zalups_rk@mercer.edu

1046-6673/1508-2023

Journal of the American Society of Nephrology

Copyright © 2004 by the American Society of Nephrology

DOI: 10.1097/01.ASN.0000135115.63412.A9

partment *in vivo* along with Cys and GSH. Hcy has received much attention recently, largely because elevations in the plasma concentration of this amino acid (known as hyperhomocysteinemia) can result from abnormalities in the metabolism of Hcy, which in turn can lead to pathologic conditions favoring the induction of atherosclerotic cardiovascular disease. Under normal conditions, plasma levels of Cys and GSH range between 5 and 10 μM (15). Normal levels of Hcy in plasma are generally similar to those of Cys and GSH. However, under certain pathophysiologic conditions, the plasma levels of Hcy can be as much as 20- to 30-fold greater than those of Cys or GSH (16–18). Therefore, not only is Hcy a potential plasma thiol to which mercuric ions can bind, but also mercuric conjugates of Hcy may prove to be very important transportable substrates that are taken up by proximal tubular epithelial cells *in vivo*.

Indirect evidence from an *in vivo* study in our laboratory provides some support for this hypothesis. The findings indicate that when the mercuric conjugate of Hcy, 2-amino-4-(3-amino-3-carboxy-propylsulfanylmercurisulfanyl) butyric acid (Hcy-S-Hg-S-Hcy) is administered intravenously to rats, the basolateral and luminal uptake of administered Hg is enhanced in renal (proximal) tubular epithelial cells (9). Although the mechanism for the basolateral uptake has not yet been defined, the participation of organic anion transporters in the uptake of Hcy-S-Hg-S-Hcy seems to be a logical possibility.

Therefore, we designed experiments to test the hypothesis that the mercuric conjugate Hcy-S-Hg-S-Hcy is a transportable substrate of the organic anion/dicarboxylate exchanger OAT1 (specifically hOAT1) and may behave as a molecular homolog of the disulfide homocystine (Hcy-S-S-Hcy). We tested this hypothesis by evaluating the transport of Hcy-S-Hg-S-Hcy in type II MDCK cells transfected stably with hOAT1. MDCK cells were chosen for study because they are derived from the distal nephron, where OAT1 is not expressed. We also tested the hypothesis that OAT1-mediated uptake of Hcy-S-Hg-S-Hcy is linked in a causal manner to concentration- and time-dependent induction of renal epithelial cell pathology. The findings from the present study provide the first line of direct evidence implicating Hcy-S-Hg-S-Hcy as a transportable substrate of OAT1.

Materials and Methods

Transfection of MDCK II Cells with hOAT1

A mycoplasma-free subclone of type II MDCK cells was provided by Dr. Daniel Balkovetz (University of Alabama at Birmingham, Birmingham, AL). This line was originally developed in the laboratory of Dr. Kai Simmons (EMBL, Heidelberg, Germany). As described previously by Aslamkhan *et al.* (14,19), these cells were transfected with the cDNA for hOAT1 ligated to pcDNA3.1 (Invitrogen, Carlsbad, CA) using Qiagen's (Chatsworth, CA) SuperFect Reagent according to the manufacturer's protocol (5 μl of SuperFect/ μg of DNA). Subclones of hOAT1-expressing (and wild-type control) MDCK II cells used in the present investigation were provided as a gift by Dr. John Pritchard (National Institute of Environmental Health Sciences, Research Triangle Park, NC). In our laboratory, cells expressing hOAT1 were maintained in culture media with 200 $\mu\text{g}/\text{ml}$ geneticin (G418; Invitrogen) and were screened regularly for gaining

the ability to transport the organic anions by assaying the uptake of [^3H]-*p*-aminohippurate (PAH) as described below.

Cell Culture

All transfected and wild-type control MDCK II cells were grown at 37°C in Eagle's minimum Essential medium (EMEM; Life Technologies BRL, Rockville, MD) supplemented with 1 mM sodium pyruvate and 10% FBS (Life Technologies BRL) as outlined previously (20).

Uptake of PAH in hOAT1-Transfected and Nontransfected MDCK II Cells

At the time of experimentation, each well that contained cells was first rinsed with Hank's buffered saline solution (HBSS) supplemented with 10 mM HEPES (pH 7.4) for three consecutive 5-min periods. At the beginning of each experiment, cells were incubated with 333 μl of the aforementioned HBSS containing 5 μM PAH, with or without of 200 μM probenecid, for 30 min. Some of the PAH was in the form of ^3H -PAH (4.54 mCi/ μmol ; Perkin Elmer Life and Analytical Sciences, Shelton, CT). At the end of a particular exposure, the cells in each well were rinsed with cold (4°C) "stop" buffer (HBSS supplemented with 10 mM HEPES [pH 7.4]). Cellular content of ^3H -PAH was determined after adding 1 ml of 1 N NaOH to each well and shaking (in an orbital shaker at 500 rpm) for 24 h. Seven hundred microliters of cellular lysate from each well was neutralized with 700 μl of 1 N HCl. The total volume of neutralized solution was added to 15 ml of OPTI-FLOUR high flash-point liquid scintillation fluid (Parkard Bioscience). The radioactivity of each sample was determined using a Beckman Scintillation Counter. Fifty microliters of the remaining cellular lysate from each well were used to determine the total amount of protein per well using the Bradford protein assay (21). Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

Uptake of Hg in hOAT1-Transfected and Nontransfected MDCK II Cells

Insertion of hOAT1 protein occurs at both apical and basolateral plasma membranes in the MDCK cells transfected with the cDNA encoding hOAT1. Apical expression of hOAT1 permits one to study hOAT1-dependent transport using cells grown on a solid surface. Accordingly, cells were grown to confluence in 24-well (2.0 cm^2) cell-culture cluster plates (Costar Corning, New York, NY) that contained supplemented EMEM.

During the assessment of transport activity, media were aspirated from the wells and cells were rinsed three times with 3 ml of HBSS supplemented with 10 mM HEPES (pH 7.4). A total of 333 μL of transport buffer (specific to each experiment) that contained radioactive ^{203}Hg was added to each well. In selective experiments, competitive inhibitors of OAT1, such as PAH or the dicarboxylates adipate or glutarate, were added to the transport buffer. Hcy was added in a 4:1 molar ratio to the concentration of mercuric cations to ensure that each mercuric ion in solution formed a thermodynamically stable, linear-II, coordinate-covalent complex with two molecules of the respective thiol. The association constant between mercuric ions and the sulfur atom of low-molecular-weight thiols is >10 orders of magnitude than that between mercuric ions and any other biologically occurring nucleophilic groups (1).

At the end of a particular exposure to one of the mercuric species studied, the cells in each well were rinsed with cold (4°C) "stop" buffer (HBSS supplemented with 10 mM HEPES [pH 7.4] that contained 1 mM 2,3-dimercaptopropane-1-sulfonic acid [DMPS] and 200 μM probenecid). DMPS is a very effective dithiol chelator of

mercuric ions. It was used to reduce the pool of mercuric ions bound to outer surfaces of the plasma membrane to negligible levels. Because DMPS oxidizes rapidly in aqueous solutions, it was mixed into solution within the first 15 min of its use. Probenecid was used in the stop buffer as an added measure to inhibit the activity of OAT1 at the termination of each experiment.

Cellular content of Hg was determined using both γ -spectrometry and liquid scintillation spectroscopy. When liquid scintillation counting was used, 1 ml of 1 N NaOH was added to each well. After the NaOH was added, the 24-well plates were shaken in an orbital shaker at 500 rpm for 24 h. A total of 700 μ l of cellular lysate from each well was neutralized with 700 μ l of 1 N HCl. The total volume of neutralized solution was added to 15 ml of Opti-Flour (Packard Biosciences) scintillation fluid. The radioactivity of each sample was determined using a Beckman LS6500 Liquid Scintillation Analyzer (Beckman Instruments, Fullerton CA; ^{203}Hg counting-efficiency ~80 to 90%). Fifty microliters of the remaining cellular lysate from each well was used to determine the total amount of protein per well using the Bradford protein assay (21). Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

Effect of Hcy-S-S-Hcy on the Transport of Hg as Hcy-S-Hg-S-Hcy

In a separate set of experiments, the influence of 1 mM Hcy-S-S-Hcy on the uptake of Hg in the form of 5 μ M Hcy-S-Hg-S-Hcy was assessed in both wild-type control and hOAT1-transfected MDCK II cells. Uptake of Hg was assessed in both cell types during a period of 1 h.

Assessment of Toxicity and Cellular Viability

The effects of Hcy-S-Hg-S-Hcy on cellular viability were measured using a methylthiazolotetrazolium (MTT)-based toxicology assay (Sigma Chemical Co., St. Louis, MO). This assay measures the activity of mitochondrial dehydrogenase by the conversion of the yellow tetrazolium dye MTT to purple formazan crystals. Cells were plated in supplemented EMEM at a density of 5.0×10^4 cells/well (added as 200 μ l/well) in sterile 96-well microtiter plates (Costar Corning) and allowed to grow for 48 h in a humidified atmosphere of 5% CO_2 , 95% air at 37°C. Supplemented EMEM was changed after the first 24 h by inversion. Excess medium that adhered to the plate was blotted off with sterile gauze (Johnson & Johnson, Arlington, TX). After 48 h, wells were again washed two times with 200 μ l of HBSS per well. After washing, test compounds were added to individual wells (200 μ l/well) in unsupplemented EMEM, and cells were grown for 24 h (unless otherwise specified) in a humidified atmosphere of 5% CO_2 , 95% O_2 at 37°C. At the conclusion of the exposure period, medium was removed by inversion and blotting, wells were washed with 200 μ l of HBSS, and 100 μ l of 0.5 mg/ml (1.2 mM) MTT in HBSS was added to each well. Cells were incubated for 2 h, and 100 μ l of solubilization buffer (10% Triton X-100, 0.1 N HCl in isopropyl alcohol) was added to each well. This buffer both lysed the cells (releasing the formazan) and dissolved the water-insoluble formazan crystals. After an overnight incubation at room temperature, full solubilization had occurred and plates were read at 595 nm in a Titertek Multiskan MKII plate reader (Fisher Scientific, Suwanee, GA).

Pathologic changes in the transfected and wild-type control MDCK II cells that were exposed to different concentrations of Hcy-S-Hg-S-Hcy were also characterized and semiquantified microscopically. Wild-type control and transfected MDCK cells were seeded in chambered (eight chambers per coverslip) coverslips (Nalge Nunc, Naperville, IL) at a density of 0.2×10^6 cells/ml (0.5 ml/chamber). Cells were treated with various concentrations of Hcy-S-Hg-S-Hcy in mod-

ified media for 24 h at 37°C. Microscopic images were captured with a Nikon DXM 1200 digital camera mounted on an Olympus IX-70 inverted biologic microscope (Melville, NY) equipped with Normarsky optics. All photomicrographic observations of cells were made using $\times 10$ eyepieces and a $\times 20$ plan-fluor objective.

Production of $^{203}\text{Hg}^{++}$

^{203}Hg was produced by exactly the same method outlined in Zalups *et al.* (20).

Statistical Analyses

Results are presented as representative data from at least two sets of experiments. Data are expressed as the mean \pm SE for $n = 3$ or 4 per treatment group. Assuming that each sample was mutually independent, statistical analysis for each parameter assessed was performed by first using a two-way ANOVA followed by either Tukey's or Dunnett's *post hoc* test. Data expressed as a percentage were first normalized using the arcsine transformation before applying parametric statistical analyses. This transformation takes the arcsine of the square root of the decimal fraction of the percentage score. Differences among means were considered statistically significant at $P < 0.05$.

Results

Uptake of PAH

Confirmation that a functional hOAT1 transporter was being inserted into the plasma membranes of the MDCK II cells transfected with hOAT1 was achieved by assessing the uptake of ^3H -PAH (a high-affinity competitive substrate transported by this organic anion/dicarboxylate exchanger) from the apical membrane of cells grown on solid support. At an extracellular concentration of 5 μ M, PAH was taken up from the extracellular environment into the cytosolic compartment at a rate that was approximately fourfold greater than that in the corresponding wild-type control cells (Figure 1). Addition of 200 μ M probenecid, 1 mM glutarate, or 1 mM adipate to the extracellular media reduced significantly the uptake of PAH in the transfected cells only. It is interesting that addition of 1 mM Hcy to the extracellular media also caused the uptake of PAH in the hOAT1-transfectants to be reduced significantly but not to the level of PAH uptake/binding detected in the wild-type control MDCK II cells.

Uptake Hg in MDCK Cells Exposed to Hcy-S-Hg-S-Hcy

At all times studied, the rate of uptake of Hg was greater in the MDCK cells that were transfected with the cDNA for hOAT1 than in the corresponding wild-type control cells when they were exposed to 5 μ M Hcy-S-Hg-S-Hcy, with the greatest differences detected at both 4 and 8 h of exposure (Figure 2A). There was a slight linear increase in the content of Hg associated with the wild-type control MDCK cells over time.

Extracellular to intracellular transport of Hg, presumably in the form Hcy-S-Hg-S-Hcy, in the hOAT1-transfected cells was determined to have a K_m of 128 ± 16 μ M and a V_{max} of 169 ± 9 pmol/min per mg of protein (Figure 2B). In the wild-type control MDCK II cells, there was a much smaller change in the rate of uptake of Hg over the range of concentrations of Hcy-S-Hg-S-Hcy used than in the OAT1-expressing

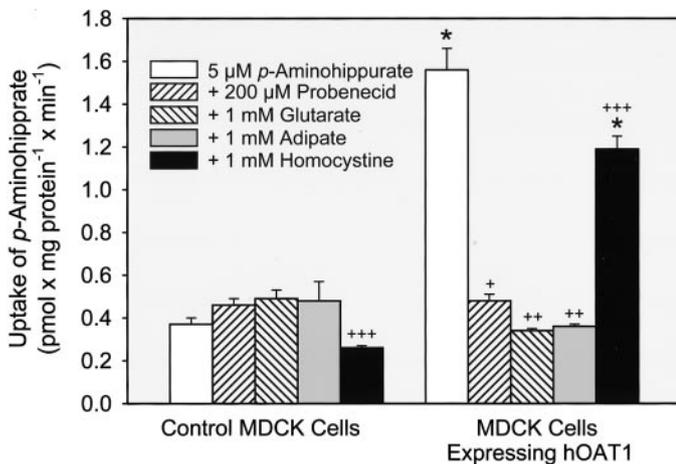


Figure 1. Uptake of *p*-aminohippurate (PAH; pmol/mg protein per min) in wild-type control and human organic anion transporter 1 (hOAT1)-expressing type II MDCK cells exposed to 5 μ M PAH in the presence and absence of 200 μ M probenecid, 1 mM glutarate, 1 mM adipate, or 1 mM homocysteine. Both transfectants and wild-type control cells were exposed to PAH for 60 min. Values are mean \pm SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells; + = significantly different ($p < 0.05$) from the mean for the corresponding group exposed to 5 μ M *p*-aminohippurate. ++ = significantly different ($p < 0.05$) from the mean for the corresponding group exposed to 5 μ M *p*-aminohippurate with or without 200 μ M probenecid. +++ = significantly different ($p < 0.05$) from each of the other means for the corresponding type of MDCK II cells.

MDCK cells. Uptake of Hcy-S-Hg-S-Hcy in the transfected cells was time and concentration dependent, indicating that the cells gained and retained the ability to transport the mercuric conjugate.

Effect of PAH on the Uptake of Hg in MDCK Cells

When PAH was added to the extracellular compartment, it inhibited the uptake of Hg^{2+} (in the form of Hcy-S-Hg-S-Hcy) in a concentration-dependent manner in the hOAT1-expressing MDCK II cells only (Figure 3). PAH did not affect significantly the uptake of Hg in the wild-type control cells when they were exposed to Hcy-S-Hg-S-Hcy.

Effect of Dicarboxylates on the Uptake of Hg in MDCK Cells

Addition of 1 mM adipate or glutarate (which are exchangeable dicarboxylates at OAT1) to the extracellular medium caused the uptake of Hg (in the form of Hcy-S-Hg-S-Hcy) in the hOAT1-expressing MDCK II cells to decrease to levels detected in corresponding wild-type control MDCK cells (Figure 4). No significant reductions in the uptake of Hg were detected in the transfected MDCK cells that were exposed to either 1 mM malonate or 1 mM succinate (neither of which is an exchangeable substrate at OAT1). No significant differences in the mean levels of uptake or accumulation of Hg^{2+} were detected among any of the groups of wild-type control MDCK II cells.

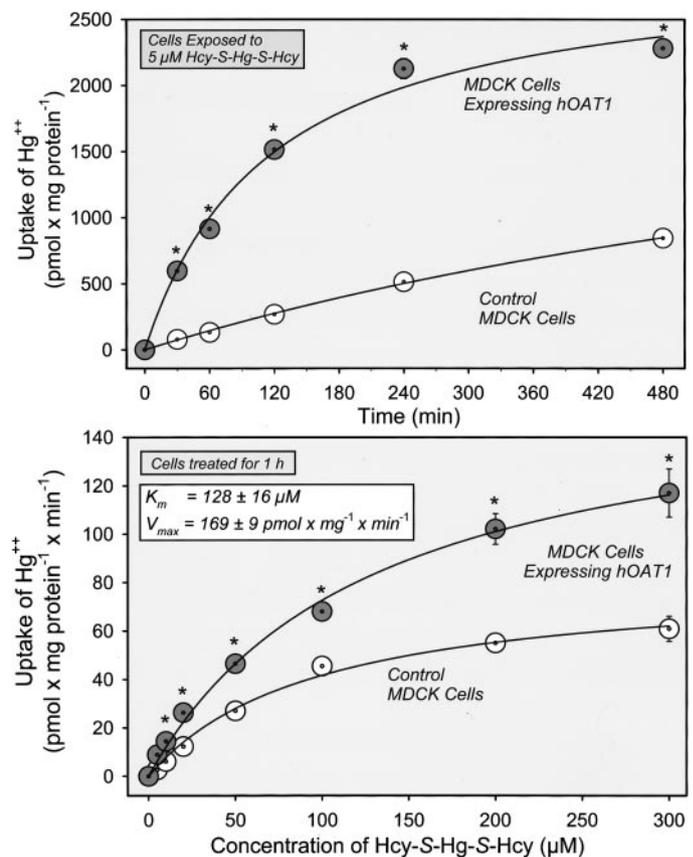


Figure 2. Effect of time (A) and concentration (B) on the uptake of mercury (Hg) in wild-type control and hOAT1-expressing MDCK II cells exposed to the mercuric conjugate of homocysteine Hcy-S-Hg-S-Hcy (at 37°C). Values are mean \pm SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells.

Effect of Temperature on the Uptake of Hg

At 37°C, uptake of Hg in the hOAT1-expressing MDCK cells that were exposed to 5 μ M Hcy-S-Hg-S-Hcy for 1 h was more than twofold greater than that in corresponding wild-type control MDCK cells (Figure 5). Transport activity in the hOAT1-expressing cells was for the most part abolished when the temperature of the extracellular medium was reduced to either 21 or 4°C. Moreover, reducing the temperature of the extracellular medium revealed a temperature-sensitive component in the uptake of Hg in the control MDCK cells that were exposed to Hcy-S-Hg-S-Hcy (Figure 5).

Influence of Hcy-S-S-Hcy on the Uptake of Hg

Uptake of Hg in the OAT1-transfected MDCK cells that were exposed to 5 μ M Hcy-S-Hg-S-Hcy for 1 h was more than fivefold greater than that in corresponding wild-type control cells (Figure 6). In the hOAT1-transfected cells that were exposed to 5 μ M Hcy-S-Hg-S-Hcy plus 1 mM Hcy-S-S-Hcy, the rate of transport of Hg was significantly lower than that in the corresponding hOAT1-transfected cells that were not exposed to 1 mM homocysteine (Figure 6). It is interesting that the

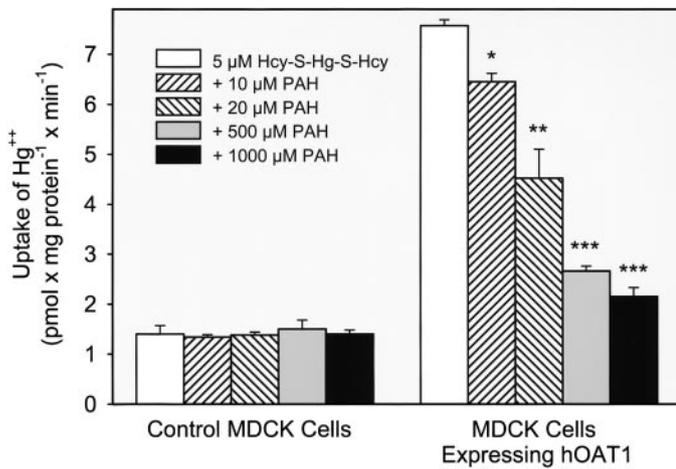


Figure 3. Effect of PAH on the uptake of Hg in wild-type control and hOAT1-expressing MDCK II cells exposed for 60 min to the mercuric conjugate of homocysteine, Hcy-S-Hg-S-Hcy (at 37°C). Values are mean ± SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells exposed to only 5 μM Hcy-S-Hg-S-Hcy; **significantly different ($P < 0.05$) from the means for the corresponding groups of MDCK cells exposed only to 5 μM Hcy-S-Hg-S-Hcy or exposed to 5 μM Hcy-S-Hg-S-Hcy in the presence of 10 μM PAH; ***significantly different ($P < 0.05$) from the means for the corresponding groups of MDCK cells exposed only to 5 μM Hcy-S-Hg-S-Hcy or exposed to 5 μM Hcy-S-Hg-S-Hcy in the presence of 10 or 20 μM PAH.

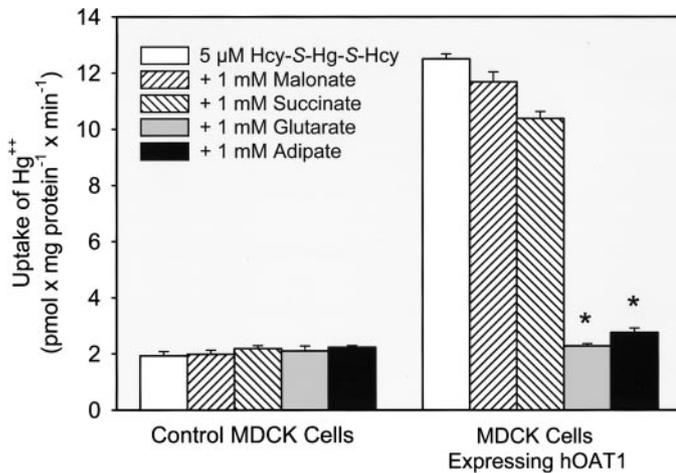


Figure 4. Influence of 1 mM malonate, succinate, glutarate, or adipate on the uptake of Hg (pmol/mg protein per min) in wild-type control and hOAT1-expressing MDCK II cells exposed for 60 min to the mercuric conjugate of homocysteine, Hcy-S-Hg-S-Hcy (at 37°C). Values are mean ± SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells not exposed to a dicarboxylic acid or the corresponding groups exposed to 1 mM malonate or succinate.

rate of uptake of Hg in the wild-type control cells that were exposed to 5 μM Hcy-S-Hg-S-Hcy plus 1 mM Hcy-S-S-Hcy was significantly greater than that in the corresponding wild-type control cells that were not exposed to Hcy-S-S-Hcy.

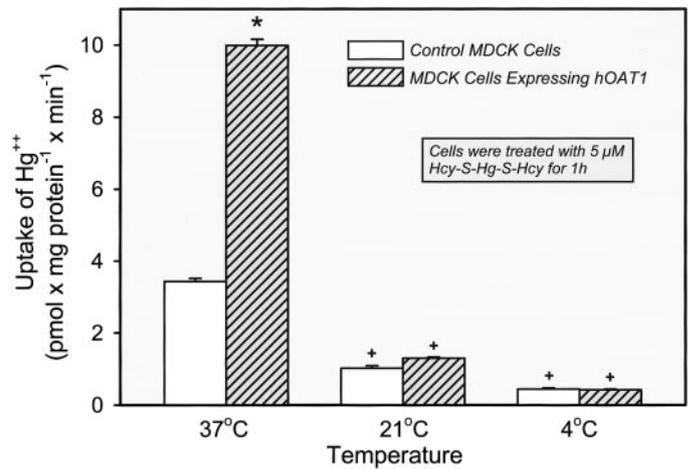


Figure 5. Influence of decreasing temperature on the uptake of Hg (pmol/mg protein per min) in wild-type control and hOAT1-expressing MDCK II cells exposed to the mercuric conjugate of homocysteine, Hcy-S-Hg-S-Hcy. Both transfectants and wild-type control cells were exposed to 5 μM Hcy-S-Hg-S-Hcy for 60 min. Values are mean ± SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells; +significantly different ($P < 0.05$) from the mean for the corresponding group of cells studied at 37°C.

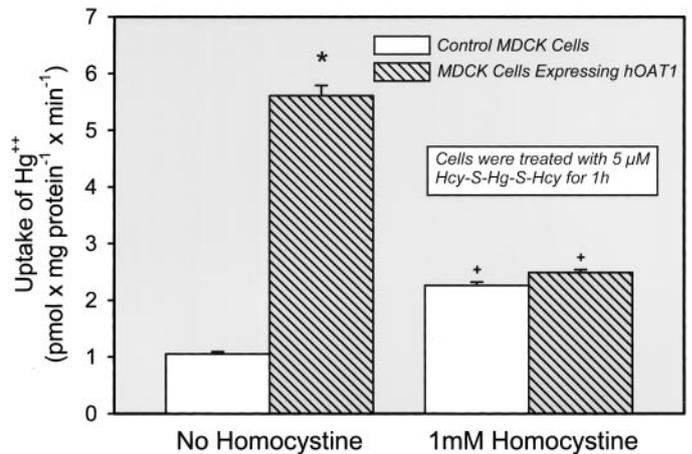


Figure 6. Effect of 1 mM homocysteine on the uptake of Hg (pmol/mg protein per min) in wild-type control and hOAT1-expressing MDCK II cells exposed to the mercuric conjugate of homocysteine, Hcy-S-Hg-S-Hcy. Both transfectants and wild-type control cells were exposed to 5 μM Hcy-S-Hg-S-Hcy with or without 1 mM homocysteine for 60 min. Values are mean ± SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells; +significantly different ($P < 0.05$) from the mean for the corresponding group exposed to 5 μM Hcy-S-Hg-S-Hcy without 1 mM homocysteine.

Assessment of Toxicity and Cellular Viability

Cellular viability, as determined by the MTT assay, was unaffected among the wild-type control MDCK cells that were exposed to Hcy-S-Hg-S-Hcy (from 5 to 200 μM) for 24 h at 37°C (Figure 7). By contrast, cellular viability diminished in a concentration-dependent manner in the MDCK cells express-

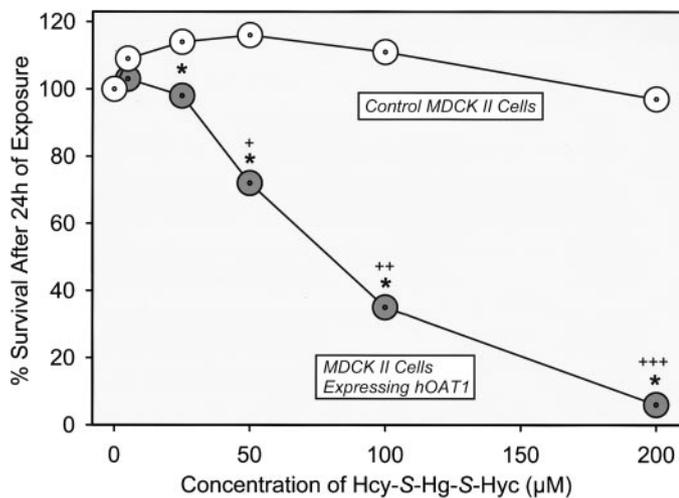


Figure 7. The percentage of wild-type control and hOAT1-expressing type II MDCK cells that survived a 24-h exposure to various concentrations of inorganic Hg in the form of the mercuric conjugate of homocysteine, Hcy-S-Hg-S-Hcy. Values are mean \pm SEM. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells; +significantly different ($P < 0.05$) from the preceding mean for corresponding group of MDCK II cells exposed to the same form of Hg; ++significantly different ($P < 0.05$) from the means for the groups of the corresponding type of MDCK II cells exposed to the same form of Hg at the lowest three concentrations studied; +++significantly different ($P < 0.05$) from the means for the preceding four groups of corresponding MDCK II cells exposed to the same form of Hg; ++++significantly different ($P < 0.05$) from the means for the preceding four groups of corresponding MDCK II cells exposed to the same form of Hg.

ing hOAT1. In fact, the concentration (dose)-response curve for the hOAT1-expressing cells was steep. To illustrate this, ~72% of the hOAT1-expressing MDCK II cells survived 24 h of exposure to 50 μ M Hcy-S-Hg-S-Hcy, whereas <10% survived 24 h of exposure to 200 μ M Hcy-S-Hg-S-Hcy.

Morphologically, the wild-type control MDCK II cells remained in confluent monolayers after exposure to Hcy-S-Hg-S-Hcy for 24 h, even at concentrations as high as 500 μ M (Figure 8A). Occasional or small clusters of wild-type control MDCK cells displayed some pathologic features. One of the more prevalent pathologic features displayed involved the cells' taking on a round or spherical morphology. Occasional cells displaying evidence of nuclear fragmentation or dissolution were detected microscopically among the wild-type control cells that were exposed to the highest concentrations of Hcy-S-Hg-S-Hcy. Stark differences in the cellular morphology were detected among the hOAT1-expressing cells and the wild-type control cells over a range of concentrations of Hcy-S-Hg-S-Hcy evaluated. At concentrations >200 μ M, profound pathologic changes were induced in the hOAT1-expressing cells. As displayed in Figure 8B, there were areas where cells had detached from the plastic surface of the 24-well plates. Overall, the morphologic features and changes detected microscopically among the wild-type control and hOAT1-expressing MDCK cells that were exposed to Hcy-S-Hg-S-Hcy were con-

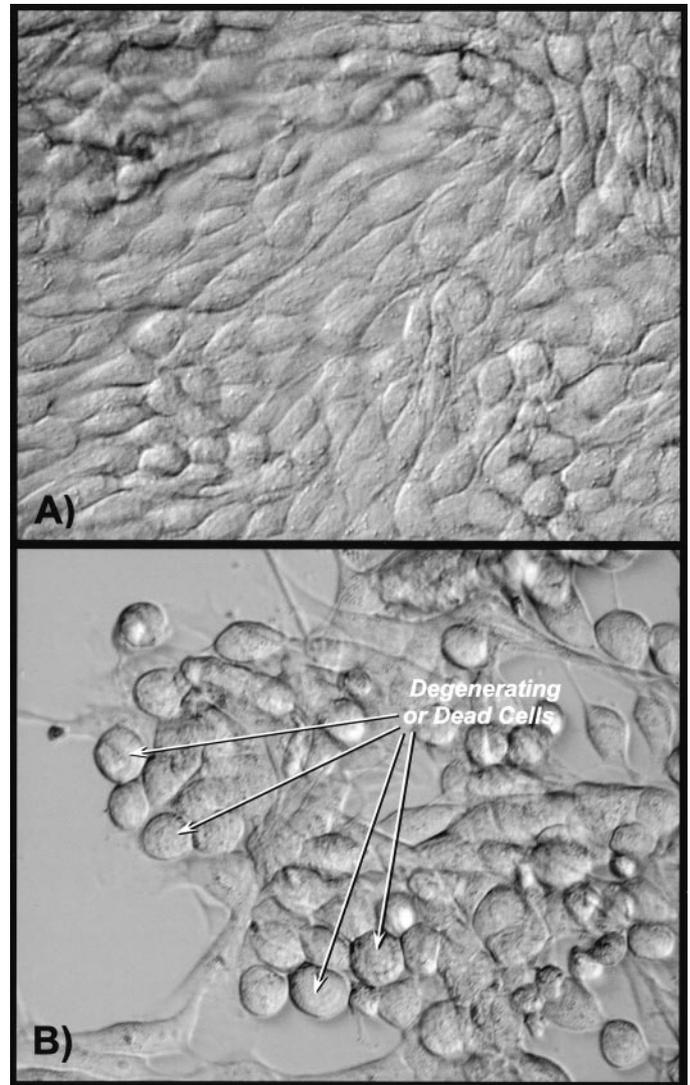


Figure 8. Photomicrographs of wild-type control (A) and hOAT1-transfected (B) MDCK II cells exposed to 500 μ M Hcy-S-Hg-S-Hcy for 24 h in phenol-free and serum-free media. Note that the wild-type control cells are confluent and display fairly normal cellular morphology. However, confluence is absent in the hOAT1-transfected cells, and many of the detached cells have taken on a round, spherical morphology. Variants of these cells include those that display patterns of nuclear fragmentation and cellular polymorphism.

sistent and in agreement with the pathophysiologic data obtained with the MTT assays.

Discussion

Contamination of the environment with Hg continues to be a global problem, mainly because it increases the risk of humans' being exposed to various forms of Hg (1). As more humans become exposed to Hg, it becomes important for scientists and health care providers to understand how the various chemical forms of this metal are handled after they gain entry into systemic circulation.

It is well documented that the kidneys are the primary sites in the body where Hg is taken up and accumulated, with the

preponderance of this accumulation occurring in proximal tubular epithelial cells (1,22–24). At least one luminal mechanism and at least one basolateral mechanism have been shown to be responsible for the uptake of mercuric ions by proximal tubular epithelial cells (5–13,25,26).

At the basolateral membrane, one or more PAH-sensitive transporter(s) have been implicated in the uptake of Hg *in vivo* (5,7,10,11,13). Because PAH is a high-affinity substrate of the OAT1 (27,28), we postulated that OAT1 is involved in the basolateral uptake of Hg *in vivo* (5–13,25,26).

In the present study, we tested the hypothesis that the organic anion/dicarboxylate exchanger protein 1 is capable of transporting the mercuric conjugate of Hcy, Hcy-S-Hg-S-Hcy, into renal epithelial cells that express this protein. This hypothesis was tested by evaluating and characterizing the transport of Hcy-S-Hg-S-Hcy in type II MDCK cells that were or were not transfected stably with the cDNA encoding the human isoform of the renal dicarboxylate/organic anion exchanger 1 (hOAT1). We assert that the mercuric conjugate of Hcy studied in the present investigation is a biologically relevant form of Hg that can be present in plasma after exposure to various forms of Hg, especially in individuals who have hyperhomocysteinemia, in whom plasma levels of Hcy can be as much as 20- to 30-fold greater than normal plasma concentrations of Cys or GSH (which have been implicated as important thiol ligands involved in the proximal tubular uptake and handling of Hg) (16–18).

Data from our transport experiments demonstrate clearly that type II MDCK cells transfected stably with hOAT1 gained the ability to transport Hg²⁺ from the extracellular fluid into their intracellular compartment when the mercuric ions are bonded to Hcy in a linear coordinate covalent manner (Hcy-S-Hg-S-Hcy). Our data show that the uptake of Hcy-S-Hg-S-Hcy in the hOAT1-expressing cells was dependent on substrate concentration, time, and temperature, which confirms that a carrier-mediated process is involved in this transport. Consequently, our transport data provide strong molecular evidence supporting the hypothesis that Hcy-S-Hg-S-Hcy is a transportable substrate of OAT1. It is interesting that some level of carrier-mediated uptake of Hcy-S-Hg-S-Hcy seemed to occur in the wild-type control MDCK cells, which may indicate that an additional transport system present in the type II MDCK cells is capable of transporting Hcy-S-Hg-S-Hcy. Clearly, additional studies are required to test this hypothesis.

The morphologic and pathophysiologic (MTT) data from the transfectants also show that there is a direct dose- and time-dependent relationship between the uptake of Hcy-S-Hg-S-Hcy (by hOAT1) and the induction of cellular injury and death. By contrast, wild-type control MDCK cells that were exposed to the Hcy-S-Hg-S-Hcy displayed little or no pathologic changes. Only after 24 h of exposure to 500 μ M or 1 mM Hcy-S-Hg-S-Hcy were some pathologic changes detectable morphologically. As indicated in Figure 7, the concentration–effect relationship for the induction of cellular pathology and death in the transfected cells is considerably more steep and to the left of that in the wild-type control cells.

Dicarboxylates represent an important class of inhibitors of

OAT1, because they exchange for organic anions at this transporter. *In vivo*, generation (and maintenance) of a downhill, intracellular-to-extracellular gradient of α -ketoglutarate serves to facilitate the transport of organic anions into the cytosolic compartment of proximal tubular cells by a molecular exchange mechanism mediated by OAT1 (27–31). Other dicarboxylates, especially molecular homologs of α -ketoglutarate, such as adipate and glutarate, have been shown to compete for OAT1. In contrast, the four-carbon dicarboxylate succinate is not an effective inhibitor of OAT1 (32). In a recent study, it was demonstrated that both adipate and glutarate but not succinate or malonate (three-carbon atoms long), inhibited (in a dose-dependent manner) the uptake of intravenously administered Hg²⁺ in rats (8). These findings are in complete agreement with those of the present investigation. The current findings also establish that the uptake of PAH or Hcy-S-Hg-S-Hcy in cells expressing hOAT1 are inhibited by adipate or glutarate but not by succinate or malonate (Figures 1 and 4). These findings, therefore, serve as additional evidence implicating mercuric S-conjugates of Hcy as transportable substrates of OAT1.

Homocystine (Hcy-S-S-Hcy) forms in plasma during the oxidation of Hcy. Normally, a fraction of this disulfide formed in plasma is excreted into the urine, by mechanisms that have not yet been defined. Inasmuch as Hcy-S-S-Hcy and Hcy-S-Hg-S-Hcy are structurally similar, we examined whether Hcy-S-Hg-S-Hcy could be acting as a molecular mimic or homolog of Cys-S-Hg-S-Cys at OAT1. Because Hcy-S-S-Hcy was capable of inhibiting the extracellular-to-intracellular transport of Hcy-S-Hg-S-Hcy in the MDCK expressing hOAT1, two conclusions can be made: (1) Hcy-S-S-Hcy itself may be a transportable substrate of OAT1, and (2) the mechanism by which Hcy-S-Hg-S-Hcy is transported by OAT1 may involve some form of molecular mimicry. Unfortunately, radioactive forms of Hcy-S-S-Hcy or Hcy are not readily available commercially, which makes it difficult to determine directly whether Hcy-S-S-Hcy is indeed a substrate of OAT1. Additional support for these conclusions comes from the data showing that Hcy-S-S-Hcy was able to inhibit partially the uptake of PAH in the OAT1-expressing cells.

As is the case with other membrane transporters, the basis for transport at OAT1 is dependent on molecular structure–function relationships between the substrate and the transporter (33). Molecular homology among substrates likely plays an important role in the activity of OAT1, as is alluded to by the data obtained with Hcy-S-S-Hcy. Moreover, molecular mimicry or homology is implicated in the transport of certain molecular homologs as result of their having similar structural and/or functional characteristics common to other known substrates of OAT1 (34).

The findings from the present and two previous studies indicate that mercuric conjugates of cysteine (Cys) and two of its homologs N-acetylcysteine (NAC) and Hcy share enough common structural and chemical properties permitting them to be transportable substrates of hOAT1. Figure 9 provides a diagrammatic representation of the transport of these three mercuric conjugates at hOAT1. It is interesting that a previous

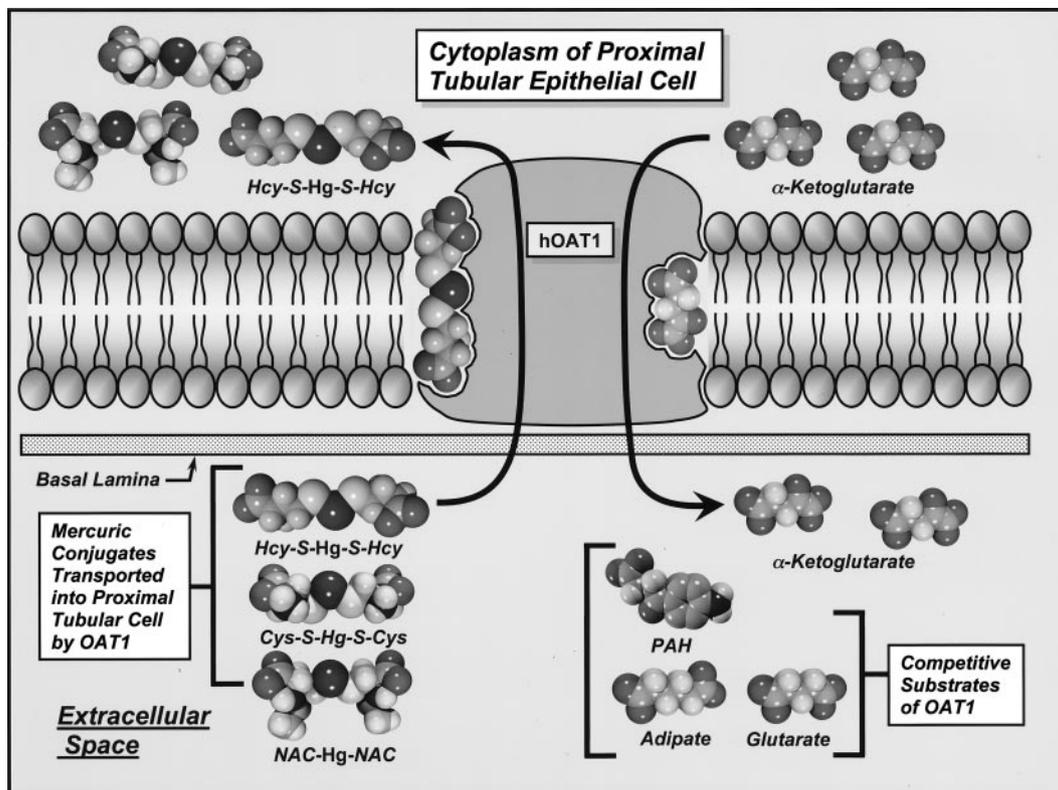


Figure 9. Diagram of the mechanisms by which OAT1 is involved in the basolateral uptake of inorganic Hg, in the form of mercuric conjugates of cysteine (Cys), homocysteine (Hcy), and *N*-acetylcysteine (NAC), in a proximal tubular epithelial cell. In this representation, the intracellular concentration of α -ketoglutarate and the extracellular concentrations of mercuric conjugates (or other substrates of OAT1) provide the energy to allow α -ketoglutarate to be transported out of the proximal tubular epithelial cell in exchange for a mercuric conjugate of Cys, Hcy, or NAC by OAT1. This diagram also illustrates that known substrates of OAT1 serve as competitive inhibitors for the uptake of mercuric conjugates of Cys, Hcy, or NAC at the site of OAT1. Note the homology among the three mercuric conjugates. The three-dimensional, space-filled, molecular models were generated in part with MOLPOV2 and Persistence of Vision (version 3.5).

in vivo study, in which the renal disposition of Hg^{2+} was studied in rats that were administered one of these three mercuric species, provided the first line of evidence supporting the above-mentioned notion. Moreover, not only does hOAT1 have the ability to transport mercuric conjugates of Cys, NAC, or Hcy, but also its activity in transporting these conjugates is linked directly to the induction of renal cellular injury and death. This suggests that once any one of these mercuric species gains access to the cytosolic compartment of these cells, there must be a series of complex thiol-exchange reactions that occur, which result in the binding of mercuric ions to one or more critical nucleophilic sites. With enough mercuric ions binding to these critical nucleophiles, the cell eventually becomes incapable of carrying out normal homeostatic functions and dies.

In summary, the present findings represent the first line of direct evidence implicating OAT1 in the transport and cellular intoxication of a mercuric conjugate of Hcy in a mammalian cell. More important, these data add to the growing body of evidence indicating that OAT1 likely plays an important role in the basolateral uptake of mercuric ions along the renal proximal tubule and that molecular homology/mimicry is involved in this process. Finally, they also provide a link between the

basolateral uptake of mercuric conjugates of Hcy by OAT1 and the proximal tubular nephropathy induced by inorganic forms of mercury *in vivo*.

Acknowledgments

R.K.Z. is supported, in part, by grants ES05157, ES05980, and ES11288 from the National Institute of Environmental Health Science (NIEHS). We thank Dr. Delon Barfuss (Georgia State University) for assistance in supplying radioactive mercury for the experiments described in this article. The laboratory of R.K.Z. thanks Dr. John Pritchard (NIEHS) for assistance in providing MDCK II cells expressing OAT1 and Dr. Christy Bridges for reviewing this manuscript.

References

- Zalups RK: Molecular interactions with mercury in the kidney. *Pharmacol Rev* 52: 113–143, 2000
- Cember H, Gallagher P, Faulkner A: Distribution of mercury among blood fractions and serum proteins. *Am Ind Hyg Assoc J* 29: 233–237, 1968
- Fuhr BJ, Rabenstein DL: Nuclear magnetic resonance studies of the solution chemistry of metal complexes. IX. The binding of cadmium, zinc, lead, and mercury by glutathione. *J Am Chem Soc* 95: 6944–6950, 1973

4. Lau S, Sarkar B: Inorganic mercury(II)-binding components in normal human blood serum. *J Toxicol Environ Health* 5: 907–916, 1979
5. Zalups RK, Barfuss DW: Pretreatment with p-aminohippurate inhibits the renal uptake and accumulation of injected inorganic mercury in the rat. *Toxicology* 103: 23–35, 1995
6. Zalups RK, Minor KH: Luminal and basolateral mechanisms involved in the renal tubular uptake of inorganic mercury. *J Toxicol Environ Health* 46: 73–100, 1995
7. Zalups RK: Organic anion transport and action of gamma-glutamyl transpeptidase in kidney linked mechanistically to renal tubular uptake of inorganic mercury. *Toxicol Appl Pharmacol* 132: 289–298, 1995
8. Zalups RK, Barfuss DW: Small aliphatic dicarboxylic acids inhibit renal uptake of administered mercury. *Toxicol Appl Pharmacol* 148: 183–193, 1998
9. Zalups RK, Barfuss DW: Participation of mercuric conjugates of cysteine, homocysteine, and N-acetylcysteine in mechanisms involved in the renal tubular uptake of inorganic mercury. *J Am Soc Nephrol* 9: 551–561, 1998
10. Zalups RK: Basolateral uptake of mercuric conjugates of N-acetylcysteine and cysteine in the kidney involves the organic anion transport system. *J Toxicol Environ Health A* 55: 13–29, 1998
11. Zalups RK: Basolateral uptake of inorganic mercury in the kidney. *Toxicol Appl Pharmacol* 151: 192–199, 1998
12. Zalups RK, Parks LD, Cannon VT, Barfuss DW: Mechanisms of action of 2,3-dimercaptopropane-1-sulfonate and the transport, disposition, and toxicity of inorganic mercury in isolated perfused segments of rabbit proximal tubules. *Mol Pharmacol* 54: 353–363, 1998
13. Zalups RK, Barfuss DW: Renal organic anion transport system: A mechanism for the basolateral uptake of mercury-thiol conjugates along the pars recta of the proximal tubule. *Toxicol Appl Pharmacol* 182: 234–243, 2002
14. Aslamkhan AG, Han YH, Yang XP, Zalups RK, Pritchard JB: Human renal organic anion transporter 1-dependent uptake and toxicity of mercuric-thiol conjugates in Madin-Darby canine kidney cells. *Mol Pharmacol* 63: 590–596, 2003
15. Lash LH, Jones DP: Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch Biochem Biophys* 240: 583–592, 1985
16. Malinow MR: Hyperhomocyst(e)inemia. A common and easily reversible risk factor for occlusive atherosclerosis. *Circulation* 81: 2004–2006, 1990
17. Selhub J: Homocysteine metabolism. *Annu Rev Nutr* 19: 217–246, 1999
18. Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG, Lindenbaum J: Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. *J Clin Invest* 81: 466–474, 1988
19. Aslamkhan A, Han YH, Walden R, Sweet DH, Pritchard JB: Stoichiometry of organic anion/dicarboxylate exchange in membrane vesicles from rat renal cortex and hOAT1-expressing cells. *Am J Physiol Renal Physiol* 285: F775–F783, 2003
20. Zalups RK, Aslamkhan AG, Ahmad S: Human organic anion transporter 1 mediates cellular uptake of cysteine-S conjugates of inorganic mercury. *Kidney Int* 2004, in press
21. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976
22. Zalups RK, Barfuss D: Accumulation of inorganic mercury along the renal proximal tubule of the rabbit. *Toxicol Appl Pharmacol* 106: 245–253, 1990
23. Zalups RK: Autoradiographic localization of inorganic mercury in the kidneys of rats: Effect of unilateral nephrectomy and compensatory renal growth. *Exp Mol Pathol* 54: 10–21, 1991
24. Zalups RK: Method for studying the in vivo accumulation of inorganic mercury in segments of the nephron in the kidneys of rats treated with mercuric chloride. *J Pharmacol Methods* 26: 89–104, 1991
25. Zalups RK, Lash LH: Binding of mercury in renal brush-border and basolateral membrane-vesicles. *Biochem Pharmacol* 53: 1889–1900, 1997
26. Zalups RK: Enhanced renal outer medullary uptake of mercury associated with uninephrectomy: Implication of a luminal mechanism. *J Toxicol Environ Health* 50: 173–194, 1997
27. Pritchard JB: Intracellular alpha-ketoglutarate controls the efficacy of renal organic anion transport. *J Pharmacol Exp Ther* 274: 1278–1284, 1995
28. Pritchard JB, Miller DS: Renal secretion of organic anions and cations. *Kidney Int* 49: 1649–1654, 1996
29. Dantzler WH, Evans KK, Wright SH: Kinetics of interactions of para-aminohippurate, probenecid, cysteine conjugates and N-acetyl cysteine conjugates with basolateral organic anion transporter in isolated rabbit proximal renal tubules. *J Pharmacol Exp Ther* 272: 663–672, 1995
30. Dantzler WH: Renal organic anion transport: A comparative and cellular perspective. *Biochim Biophys Acta* 1566: 169–181, 2002
31. Kim YK, Dantzler WH: Specificity of basolateral organic cation transport in snake renal proximal tubules. *Am J Physiol* 270: R1025–R1030, 1996
32. Chatsudthipong V, Dantzler WH: PAH/alpha-KG countertransport stimulates PAH uptake and net secretion in isolated rabbit renal tubules. *Am J Physiol* 263: F384–F391, 1992
33. Burckhardt G, Bahn A, Wolff NA: Molecular physiology of renal p-aminohippurate secretion. *News Physiol Sci* 16: 114–118, 2001
34. Pombrio JM, Giangreco A, Li L, Wempe MF, Anders MW, Sweet DH, Pritchard JB, Ballatori N: Mercapturic acids (N-acetylcysteine S-conjugates) as endogenous substrates for the renal organic anion transporter-1. *Mol Pharmacol* 60: 1091–1099, 2001