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Human organic anion transporter 1 mediates cellular uptake of cysteine-*S* conjugates of inorganic mercury

RUDOLFS K. ZALUPS,[1] AMY G. ASLAMKHAN, and SARFARAZ AHMAD[1]

Division of Basic Medical Sciences, Mercer University, School of Medicine, Macon, Georgia; and Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

Human organic anion transporter 1 mediates cellular uptake of cysteine-*S* conjugates of inorganic mercury.

Background. The epithelial cells lining 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^{2+}).

Methods. Using a line of Madin-Darby canine kidney (MDCK) II cells transfected stably with the human organic anion transporter 1 (hOAT1), and oocytes from *Xenopus laevis* microinjected with cRNA for hOAT1, we tested the hypothesis that hOAT1 can transport biologically relevant mercuric conjugates of cysteine (Cys).

Results. Indeed, MDCK II cells expressing a functional form of hOAT1 gained the ability to transport the mercuric conjugate 2-Amino-3-(2-amino-2-carboxy-ethylsulfanyl-mercuricsulfanyl)-propionic acid (Cys-*S*-Hg-*S*-Cys), but not the corresponding di-glutathione *S*-conjugate of Hg^{2+} (G-*S*-Hg-*S*-G). Moreover, *p*-aminohippurate (PAH), adipate, and glutarate (but not succinate or malonate) inhibited individually the uptake of Cys-*S*-Hg-*S*-Cys in a dose-dependent manner. Uptake of Cys-*S*-Hg-*S*-Cys, but not G-*S*-Hg-*S*-G, was also documented in *Xenopus* oocytes expressing hOAT1.

Conclusion. These data represent ostensibly the most direct line of evidence implicating a specific membrane protein (i.e., hOAT1) in the transport of a biologically relevant molecular species of Hg^{2+} in a mammalian cell. Moreover, these data indicate that the organic anion transporter(s) likely play a prominent role in the basolateral transport of mercuric ions by proximal tubular cells and in the nephropathy induced by Hg^{2+}.

Divalent cations of many heavy metals, such as mercury, lead, cadmium, uranium, and others, have no known nutritive or beneficial value in any mammalian cell. Yet these metal-ions manage to gain entry into the cytosolic compartment of various target cells in the body under certain conditions and induce a host of deleterious effects, largely by mechanisms that are poorly understood [1, 2].

A number of investigators have proposed that unbound cationic species of some of the smaller toxic heavy metals can behave as ionic mimics (under the appropriate conditions) at the sites of membrane-transporters and/or channels involved in the cytosolic entry of essential elements (such as calcium, zinc, iron, etc.). It has also been proposed that some of the more electrophilic cations form stable conjugates or complexes (with various endogenous biomolecules) that can behave as molecular "mimics" or functional homologs of essential biomolecules (such as amino acids, organic acids, dicarboxylates, etc.) at the sites of membrane transporters involved in their uptake [2–4].

Mercuric ions are one group of cations that are believed to gain entry into target epithelial cells in mammalian organ systems by mechanisms involving molecular "mimicry" or homology [2–4]. In the kidneys, for example, it has been proposed that the mercuric conjugate of the amino acid cysteine (Cys) 2-Amino-3-(2-amino-2-carboxy-ethylsulfanylmercuricsulfanyl)-propionic acid (Cys-*S*-Hg-*S*-Cys) acts as a molecular homolog or "mimic" of the amino acid cysteine (Cys-*S*-*S*-Cys) at the site(s) of one or more amino acid transporters involved in the luminal absorption of this amino acid along proximal tubular segments [2, 5, 6], which, coincidentally, are primary regions of the nephron that take up and accumulate inorganic forms of mercury [7–9]. Support for this hypothesis comes mainly from various indirect in vivo findings and competitive inhibition data from isolated perfused proximal tubular segments.

Interestingly, uptake of inorganic mercury (Hg^{2+}) by proximal tubular epithelial cells is not restricted to the luminal plasma membrane. Various lines of evidence (mainly from in vivo studies) indicate that mercuric ions are also taken up from the blood and extracellular fluid at their basolateral membrane, primarily in the form of one or more mercuric conjugates of low-molecular-weight thiols present in plasma. Moreover, a number of indirect lines of evidence coming from recent in vivo and

[1]Drs. Zalups and Ahmad are affiliated with Mercer University, School of Medicine.

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in vitro studies have implicated the organic anion transport-system(s) in this basolateral transport of mercuric ions [10–16].

Within the kidneys, these transport-systems are localized exclusively along the proximal tubule (and particularly along the *pars recta*), and normally provide a secretory mechanism for the elimination of many xenobiotics and endogenous organic anions that escape filtration during their passage through the kidney(s) [17]. One of the best characterized organic anion transport-systems mediates the basolateral uptake of a wide range of organic anions by a dicarboxylate/organic anion exchanger, which has been denoted as organic anion transporter 1 (OAT1). This membrane protein transports organic anions into the cytosolic compartment in exchange for molecules of α-ketoglutarate. The intracellular-to-extracellular gradient of α-ketoglutarate is maintained by both intracellular metabolism and reclamation of α-ketoglutarate from the extracellular compartment by the sodium-coupled dicarboxylate transporter present in the basolateral membrane of proximal tubular cells [18].

Recent cloning studies have provided the sequence of OAT1 in both rats [19, 20] and humans [21–24], and have shown that OAT1 can mediate the uptake of a host of low-molecular-weight and structurally dissimilar organic acids and neutral compounds. Although OAT1 has been implicated indirectly in the uptake of inorganic forms of mercury, direct molecular evidence supporting a functional role of this membrane protein in the transport of Hg^{2+} in the form of a biologically relevant mercuric conjugate, such as Cys-*S*-Hg-*S*-Cys, is lacking.

Therefore, we designed experiments in the present study to test the hypothesis that the dicarboxylate/organic anion exchanger OAT1 (specifically the human isoform hOAT1) is capable of transporting the biologically relevant mercuric conjugate Cys-*S*-Hg-*S*-Cys into the intracellular compartment of renal epithelial cells or *Xenopus laevis* oocytes altered to express a functional form of this membrane-protein. We also tested the hypothesis that OAT1-mediated uptake of Cys-*S*-Hg-*S*-Cys 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 most comprehensive, direct line of evidence implicating a specific mammalian transporter in the cellular uptake of, and induction of cellular injury by, a biologically relevant molecular species of Hg^{2+}.

METHODS

Transfection of MDCK II cells with hOAT1

A Mycoplasma-free, subclone of type II Madin-Darby canine kidney (MDCK) cells was provided by Dr. Daniel Balkovetz (University of Alabama at Birmingham). This line was originally developed in the laboratory of Dr. Kai Simmons (EMBL, Heidelberg). As described previously by Aslamkhan et al [25, 26], these cells were transfected with the cDNA for hOAT1 ligated to pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) using Qiagen's (Chatsworth, CA, USA) SuperFect Reagent according to the manufacturer's protocol (5 μL SuperFect/μg DNA). Subclones of hOAT1-expressing (and wild-type) MDCK II cells used in the present investigation were provided as a gift by Dr. John Pritchard at National Institute of Environmental Health Sciences.

In our laboratory, cells expressing hOAT1 were maintained in culture media with 200 μg/mL geneticin (G418; Invitrogen), and were screened regularly for gaining the ability to transport organic anions by assaying uptake of [^{3}H]-PAH, as described below. OAT1-expressing cells displaying the greatest level of [^{3}H]-PAH uptake (greater than 20× above that in nontransfected MDCK cells) were used experimentally in the present study.

Cell culture

All transfected and wild-type MDCK II cells were grown at 37°C in Eagle's minimum essential medium (EMEM; Gibco BRL, Rockville, MD, USA) supplemented with 1 mmol/L sodium pyruvate and 10% fetal bovine serum (FBS; Gibco BRL). The EMEM used to grow the MDCK cells will hereafter be referred to as "supplemented EMEM." While in culture, the MDCK cells were grown and maintained in a humidified atmosphere consisting of 5% CO_2 and 95% O_2. Cells were split every 3 to 7 days, and 1/10th to 1/20th of the culture was inoculated into new flasks. For each experiment, cells were plated in 24-well (2.0 cm^2 per well) cell-culture cluster-plates (Costar Corning, NY, USA) containing supplemented EMEM, at a density of 0.5×10^6 cells per well (added as 2 mL). All wild-type and hOAT1-expressing cells used for transport and toxicity experiments were first grown in culture for 48 hours to allow the cells to grow to confluence. During this period of growth, the media was changed after the first 24 hours of culture. Cell-to-cell attachments afforded a low transepithelial resistance in trans-well plates (12 mm in diameter having a pore-size of 0.4 μm; Costar, Cambridge, MA, USA).

Uptake of PAH in hOAT1-transfected and nontransfected MDCK II cells

At the time of experimentation, each well containing cells was first rinsed with Hank's balanced salt solution (HBSS) supplemented with 10 mmol/L 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.4) for three consecutive five-minute periods. At the beginning of each experiment, cells were incubated with 333 μL of the aforementioned Hank's buffer containing 5 μmol/L PAH, with or without of 200 μmol/L

probenecid, for 30 minutes. Some of the PAH was in the form of ^{3}H-PAH (4.54 mCi/μmol). At the end of a particular exposure, the cells in each well were rinsed with cold (4°C) "stop" buffer [HBSS supplemented with 10 mmol/L HEPES (pH 7.4)]. Cellular content of ^{3}H-PAH was determined after adding 1 mL of 1N NaOH to each well and shaking (in an orbital shaker at 500 rpm) for 24 hours. Seven hundred μL of cellular lysate from each well were neutralized with 700 μL of 1N HCl. The total volume of neutralized solution was added to 15 mL of OPTI-FLOUR high flash-point liquid scintillation fluid (Parkard Bioscience Co., Meriden, CT, USA). The radioactivity of each sample was determined using a Beckman Scintillation Counter (Beckman Instruments, Fullerton, CA, USA). Fifty μL of the remaining cellular lysate from each well were used to determine the total amount of protein per well using the Bradford protein assay [3]. Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

Uptake of Hg^{2+} 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 [1]. 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) containing supplemented EMEM.

During the assessment of transport activity, media was aspirated from the wells, and cells were rinsed three times with 3 mL of HBSS supplemented with 10 mmol/L HEPES (pH 7.4). Three hundred thirty-three μL of transport buffer (specific to each experiment) containing radioactive $^{203}Hg^{2+}$ were 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. Cys or GSH (which are both present in the plasma of blood) 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 more than 10 orders of magnitude greater than that between mercuric ions and any other biologically occurring nucleophilic groups [2].

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 mmol/L HEPES (pH 7.4) containing 1 mmol/L 2,3-dimercaptopropane-1-sulfonic acid (DMPS) and 200 μmol/L 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 minutes of its use. Probenecid was used in the stop-buffer as added measure to inhibit the activity of OAT1 at the termination of each experiment.

Cellular content of Hg^{2+} was determined using both gamma-spectrometry and liquid scintillation spectroscopy. When liquid scintillation counting was employed, 1 mL of 1N NaOH was added to each well. After adding the NaOH, the 24-well plates were shaken in an orbital shaker at 500 rpm for 24 hours. Seven hundred μL of cellular lysate from each well were neutralized with 700 μL of 1N HCl. The total volume of neutralized solution was added to 15 mL of OPTI-FLOUR high flash-point liquid scintillation fluid (Packard Bioscience Co.). The radioactivity of each sample was determined using a Beckman LS6500 Liquid Scintillation Analyzer (^{203}Hg counting-efficiency ≃80% to 90%; Beckman Instruments). Fifty μL of the remaining cellular lysate from each well were used to determine the total amount of protein per well using the Bradford protein-assay [27]. Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

To determine if Cys-*S*-Hg-*S*-Cys is a transportable substrate of hOAT1, dose-response data for the uptake of Hg^{2+} (in the form of Cys-*S*-Hg-*S*-Cys) were fitted to the Michaelis-Menten equation

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

In this equation, V represents velocity, V_{max} is the maximal velocity for transport of the substrate being studied, [S] is the concentration of the substrate being transported, and K_m is the Michaelis-Menten constant.

Uptake of Hg^{2+} in oocytes expressing hOAT1

Female *Xenopus laevis* obtained from Xenopus One (Ann Arbor, MI, USA) were used in the present study. After a period of acclimation, the ovaries were removed from a randomly selected number of frogs after they had been anesthetized with triacin. Oocytes were then isolated and defolliculated as described previously [25]. Briefly, this process employs digestion of connective tissue with collagenase A (Roche Molecular Biochemicals, Indianapolis, IN, USA), followed by incubation in a K_2HPO_4-containing buffer. After isolation, the oocytes were stored overnight in an incubator at 18°C using an oocyte Ringer's 2 solution (OR-2; 82.5 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L Na_2HPO_4, 3 mmol/L NaOH,

1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L Na-pyruvate, 5 mmol/L HEPES, pH 7.6) supplemented with 5% horse serum and 50 μg/mL gentamicin. After overnight storage, stage IV and V oocytes were microinjected with 16.1 nL of either high-performance liquid chromatography (HPLC)-grade water or capped RNA (encoding hOAT1) at a concentration of 1.93 μg/μL.

Transport experiments with the oocytes were conducted three days after injection. Before use, the oocytes were separated into groups of 10 in 24-well plates and were washed three times with 1 mL of unsupplemented OR-2. Then, as with cellular transport-assays, 333 μL of OR-2 containing 5 μmol/L Hg^{2+}(containing $^{203}Hg^{2+}$; 8.5 mCi/mg) and 20 μmol/L Cys or GSH were added to each well and incubated for 1 hour at room temperature. After incubation, oocytes were rinsed three times with 1 mL of ice-cold "stop buffer" composed of OR-2 supplemented with 200 μmol/L probenecid and 1 mmol/L DMPS (added to the buffer no more than 1 hour before use). Oocytes were then lysed individually in 200 μL of 10% sodium dodecyl sulfate (SDS). After complete lysis, 4 mL of EcoLume™ scintillation fluid were added to each vial, and the radioactivity of the samples was determined using by liquid scintillation spectroscopy using a Beckman LS 6500 scintillation counter.

Assessment of toxicity and cellular viability

The effects of mercuric chloride ($HgCl_2$) and Cys-*S*-Hg-*S*-Cys on cellular viability were measured using a methylthiazoletetrazolium (MTT)-based toxicology assay (Sigma Chemical Co., St. Louis, MO, USA). 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 μL/well) in sterile 96-well microtiter plates (Costar Corning), and allowed to grow for 48 hours in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Supplemented EMEM was changed after the first 24 hours by inversion. Excess media adhering to the plate was blotted off with sterile gauze (Johnson & Johnson, Arlington, TX, USA). After 48 hours, wells were again washed two times with 200 μL of HBSS per well. After washing, test compounds were added to individual wells (200 μL/well) in unsupplemented EMEM, and cells were grown for 24 hours (unless otherwise specified) in a humidified atmosphere of 5% CO_2, 95% O_2 at 37°C. At the conclusion of the exposure period, media was removed by inversion and blotting, wells were washed with 200 μL HBSS, and 100 μL of 0.5 mg/mL (1.2 mmol/L) MTT in HBSS was added to each well. Cells were incubated for 2 hours, and 100 μL of solubilization buffer (10% Triton X-100, 0.1 N HCl in isopropyl alcohol) were 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 occurred, and plates were read at 595 nm in a Titertek Multiskan MKII plate reader (Fisher Scientific, Suwanee, GA, USA).

Production of $^{203}Hg^{2+}$

Three mg of mercuric oxide (HgO) containing the stable isotope $^{200}Hg^{2+}$ and enriched $^{202}Hg^{2+}$ (target) were weighed and double-sealed in quartz tubing (actual mercuric oxide isotopic composition: <0.05% ^{196}Hg, 1.5% ^{198}Hg, 2.82% ^{199}Hg, 4.24% ^{200}Hg, 3.11% 201, 86.99% ^{202}Hg, and 1.34% ^{204}Hg). The double encapsulated target was sent to the Missouri University Research Reactor (MURR) facility to be irradiated (by neutron activation) for four weeks. The irradiated target was placed in protected storage for 10 days to allow for the isotopic decay of the newly formed $^{197}Hg^{2+}$. The target was removed from the quartz tubing with four 50-μL rinses of 1N HCl. All four rinses were placed and sealed in a single 1.7-mL polypropylene vial. A sample of the solution was then used to determine the precise solid content of Hg using plasma-coupled elemental mass spectrometry. The radioactivity of the solution was determined with a Wallac Wizard 3″ 1480 Automatic Gamma Counter (^{203}Hg counting-efficiency ≃50%; Gaithersburg, MD, USA). The specific activities of the $^{203}Hg^{2+}$ used in the present study ranged between 8 to 12 mCi/mg Hg.

Statistical analysis

Results are presented as representative data from at least two sets of experiments. Data are expressed as the mean ± standard error. For uptake studies, a sample size of $N = 3$ or 4 was used. Assuming that each sample was mutually independent, statistical analysis for each parameter assessed was performed by first using a two-way analysis of variance (ANOVA) followed by either Tukey's or Dunnett's post-hoc test. Data expressed as a percent 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 percent 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 uptake of ^{3}H-PAH (a high-affinity substrate of this organic anion/dicarboxylate exchanger) from the apical membrane of cells grown on solid support. At an extracellular concentration of 5 μmol/L, PAH was taken up from

the extracellular environment into the cytosolic compartment by the hOAT1-transfected MDCK cells at a rate of 2.1 ± 0.6 pmol × min^{-1} × mg protein^{-1}. Moreover, addition of 200 μmol/L probenecid (a well established inhibitor OAT1) to the extracellular media caused the uptake of PAH in the transfected cells to be reduce to 0.21 ± 0.01 pmol × min^{-1} × mg protein^{-1}, which is the same level of PAH uptake/binding detected in the control or nontransfected MDCK II cells. Furthermore, the very low level of uptake/binding of PAH in the control cells was not affected by the addition of probenecid to the extracellular medium. Additional experiments utilizing transwell-plates confirmed that uptake of PAH occurred at both the luminal and basolateral membranes in confluent monolayers of the transfected MDCK II cells, and that this uptake could be inhibited by probenecid.

Based on these gain-of-function findings, it is clear that a functional form of hOAT1 was indeed being inserted into the plasma membranes of the transfected MDCK cells.

Uptake Hg^{2+} in MDCK cells exposed to Cys-*S*-Hg-*S*-Cys

At all times studied, the rate of uptake of Hg^{2+} was greater in the MDCK cells transfected with the cDNA for hOAT1 than in the corresponding control cells when they were exposed to 5 μmol/L Cys-*S*-Hg-*S*-Cys, with the greatest differences detected at both 4 and 8 hours of exposure (Fig. 1A). There was a slight increase in the content of Hg^{2+} associated with the control MDCK cells over time, but this increase likely represents time-dependent increases in nonspecific binding of Hg^{2+}.

Uptake of Hg^{2+} as Cys-*S*-Hg-*S*-Cys in the hOAT1-transfected cells had a K_m of 91 ± 7 μmol/L and a V_{max} of 137 ± 4 pmol × min^{-1} × mg protein^{-1} (Fig. 1B). Very little change in the uptake of Hg^{2+} was detected in the control MDCK II cells over the range of concentrations of Cys-*S*-Hg-*S*-Cys used.

It is clear from the assessment of both the time course and the concentration dependence for the uptake of Hg^{2+}, in the form of Cys-*S*-Hg-*S*-Cys, that the MDCK II cells gained the ability to transport Cys-*S*-Hg-*S*-Cys subsequent to being transfected with hOAT1.

Effect of PAH on the uptake of Hg^{2+} in MDCK cells

When PAH was added to the extracellular compartment, it inhibited the uptake of Hg^{2+}, in the form of Cys-*S*-Hg-*S*-Cys, in a concentration-dependent manner in the hOAT1-transfected cells only (Fig. 2). By contrast, PAH did not affect significantly the uptake of Hg^{2+} in the control cells when they were exposed to Cys-*S*-Hg-*S*-Cys.

Effect of dicarboxylates on the uptake of Hg^{2+} in MDCK cells

Addition of 1 mmol/L adipate or glutarate (which are exchangeable dicarboxylates at OAT1) to the extracellular medium caused the uptake of Hg^{2+} (in the form of Cys-*S*-Hg-*S*-Cys) in the transfected MDCK II cells to decrease to levels detected in corresponding control cells (Fig. 3). By contrast, no significant reductions in the uptake of Hg^{2+} were detected in the transfected MDCK cells exposed to either 1 mmol/L malonate or 1 mmol/L succinate (neither of which are exchangeable substrates at OAT1).

No significant differences in the mean levels of uptake or accumulation of Hg^{2+} were detected among the groups of control MDCK II cells.

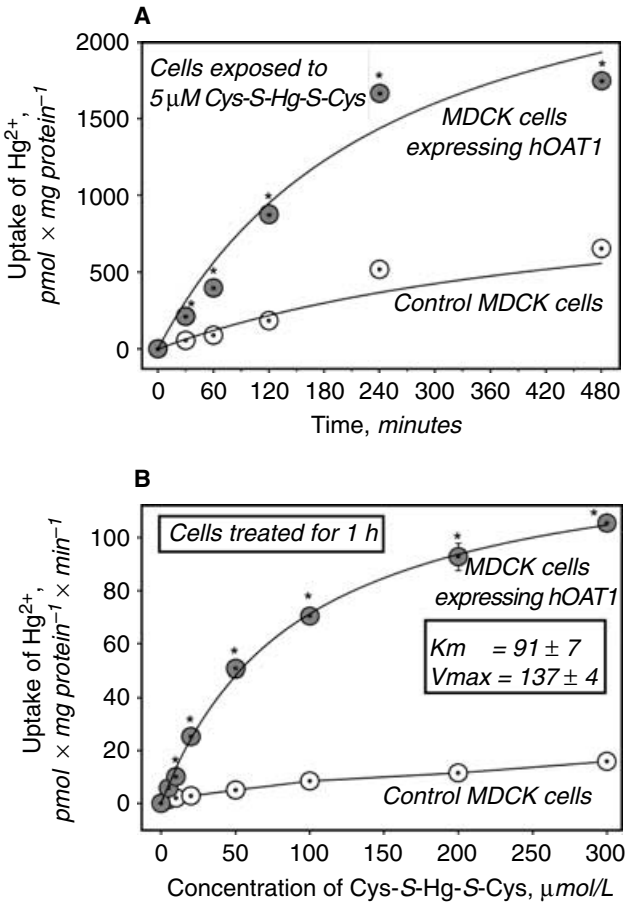

Fig. 1. Effect of time (*A*) and concentration (*B*) on the uptake of Hg^{2+} in control and hOAT1-expressing MDCK II cells exposed to the mercuric conjugate of cysteine Cys-*S*-Hg-*S*-Cys (at 37°C). Values are mean ± SE. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control MDCK cells.

Uptake of Hg^{2+} in MDCK cells exposed to G-*S*-Hg-*S*-G

When either the transfected or control MDCK cells were exposed to Hg^{2+}, in the form of G-*S*-Hg-*S*-G, the level of uptake of Hg^{2+} in both cell types was very low (Fig. 4). More importantly, there was no significant difference in the uptake of Hg^{2+} between the two types of cells exposed to this mercuric conjugate. Furthermore, addition of 1 mmol/L PAH to extracellular medium did not

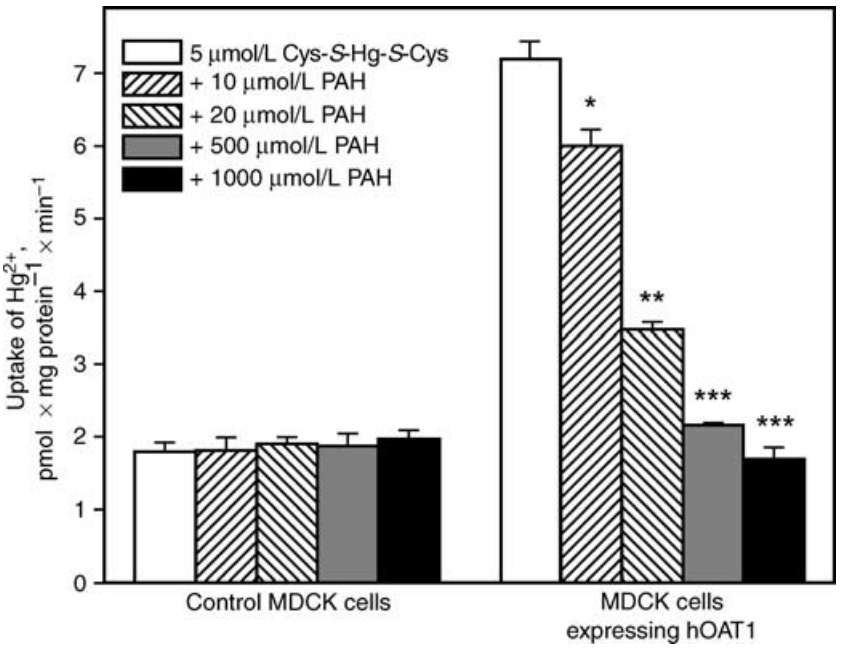

Fig. 2. Effect of *p*-aminohippurate (PAH) on the uptake of Hg^{2+} in control and hOAT1-expressing MDCK II cells exposed for 60 minutes to the mercuric conjugate of cysteine Cys-*S*-Hg-*S*-Cys (at 37°C). Values are mean ± SE. *Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells exposed to only 5 µmol/L Cys-*S*-Hg-*S*-Cys. **Significantly different ($P < 0.05$) from the means for the corresponding groups of MDCK cells exposed only to 5 µmol/L Cys-*S*-Hg-*S*-Cys or exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys in the presence of 10 µmol/L PAH. ***Significantly different ($P < 0.05$) from the means for the corresponding groups of MDCK cells exposed only to 5 µmol/L Cys-*S*-Hg-*S*-Cys or exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys in the presence of 10 or 20 µmol/L PAH.

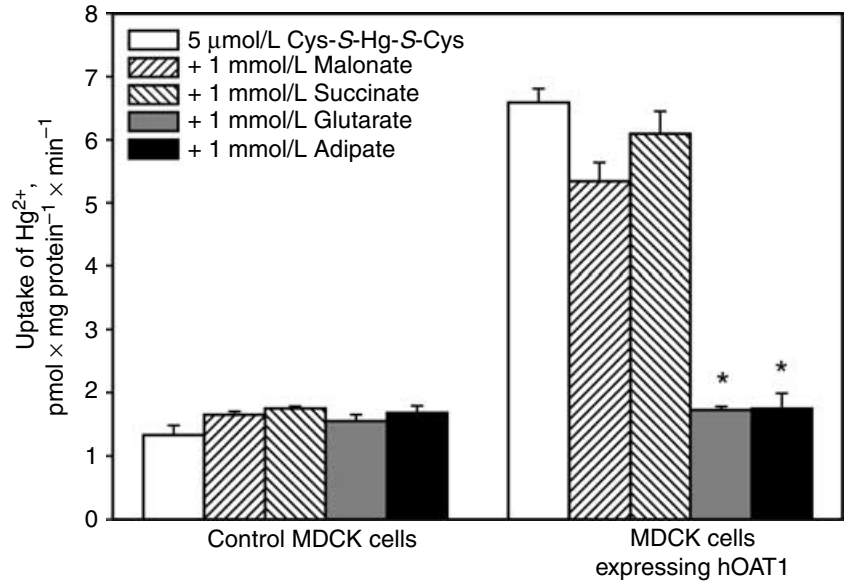

Fig. 3. Influence of 1 mmol/L malonate, succinate, glutarate, or adipate on the uptake of Hg^{2+} (pmol × mg protein^{-1} × min^{-1}) in control and hOAT1-expressing MDCK II cells exposed for 60 minutes to the mercuric conjugate of cysteine Cys-*S*-Hg-*S*-Cys (at 37°C). Values are mean ± SE. *Significantly 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 mmol/L malonate or succinate.

significantly affect the level of uptake of Hg^{2+} in either cell type.

Effect of temperature on the uptake of Hg^{2+}

At 37°C, uptake of Hg^{2+} in the transfected MDCK cells exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys for one hour was more than twofold greater than that in corresponding control cells (Fig. 5). Transport activity in the hOAT1-transfected cells was for the most part abolished when the temperature of the extracellular medium was reduced to either 21°C or 4°C. Moreover, reducing the temperature of the extracellular medium revealed a slight level of temperature-sensitive uptake of Hg^{2+} in the control MDCK cells (Fig. 5).

Uptake of Hg^{2+} in xenopus oocytes exposed to Cys-*S*-Hg-*S*-Cys or G-*S*-Hg-*S*-G

Uptake of Hg^{2+} in the two groups of oocytes injected with just water was very low during the one hour of exposure to 5 µmol/L Cys-*S*-Hg-*S*-Cys or 5 µmol/L G-*S*-Hg-*S*-G (Fig. 6). By contrast, the uptake of Hg^{2+} by the oocytes injected with 30 ng of hOAT1-cRNA that were exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys was more than 17-fold greater than that in the corresponding group of

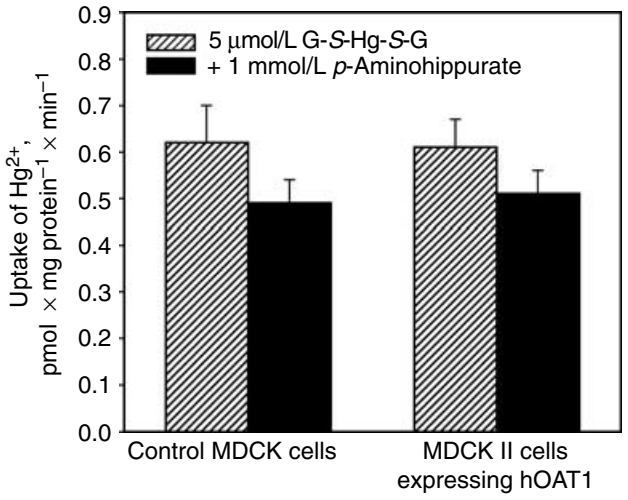

Fig. 4. Uptake of Hg^{2+} (pmol × mg protein^{-1} × min^{-1}) in control and hOAT1-expressing MDCK II cells exposed for 60 minutes to the mercuric conjugate of glutathione G-*S*-Hg-*S*-G (at 37°C). Both transfectants and control cells were exposed to 5 µmol/L G-*S*-Hg-*S*-G for 60 minutes in the presence or absence of 1 mmol/L *p*-aminohippurate (PAH). Values are mean ± SE.

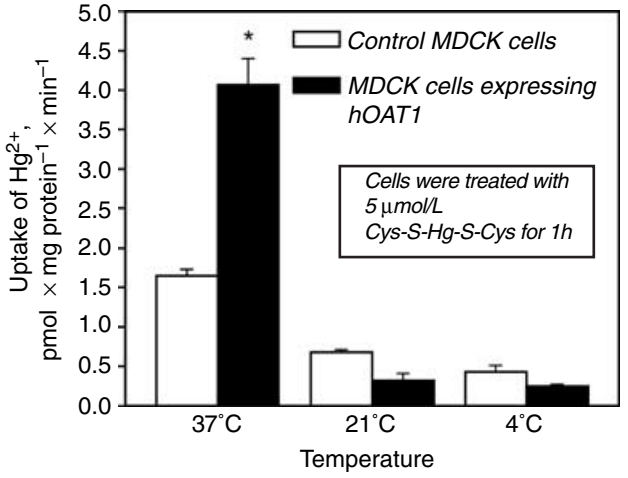

Fig. 5. Influence of decreasing temperature on the uptake of Hg^{2+} (pmol × mg protein^{-1} × min^{-1}) in control and hOAT1-expressing MDCK II cells exposed to the mercuric conjugate of cysteine Cys-*S*-Hg-*S*-Cys. Both transfectants and control cells were exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys for 60 minutes. Values are mean ± SE. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control (nontransfected) MDCK cells.

water-injected oocytes exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys or the group of oocytes injected with hOAT1-cRNA that were exposed to 5 µmol/L G-*S*-Hg-*S*-G. Moreover, no significant difference was detected between the means for the two groups of oocytes exposed to G-*S*-Hg-*S*-G for one hour.

Assessment of toxicity and cellular viability

Significant decreases in survival of wild-type cells were documented only in the cells exposed to mercuric chloride. At 100 and 200 µmol/L, less than 5% of the wild-type cells survived 24 hours of exposure to the chloride salt form of Hg^{2+} (Fig. 7A). Cellular viability was unaffected among the wild-type cells exposed for 24 hours to mercuric conjugates of Cys (from 5 to 200 µmol/L) for 24 hours.

By contrast, both forms of mercury diminished cellular viability in the transfectants expressing hOAT1 (Fig. 7B). Among the groups of transfectants exposed to mercuric conjugates of Cys, significant changes in cellular viability were detected at concentrations as low as 50 µmol/L. At 200 µmol/L, both forms of Hg^{2+} caused cellular survival in the transfectants to decrease to below 20% during the 24 hours of exposure.

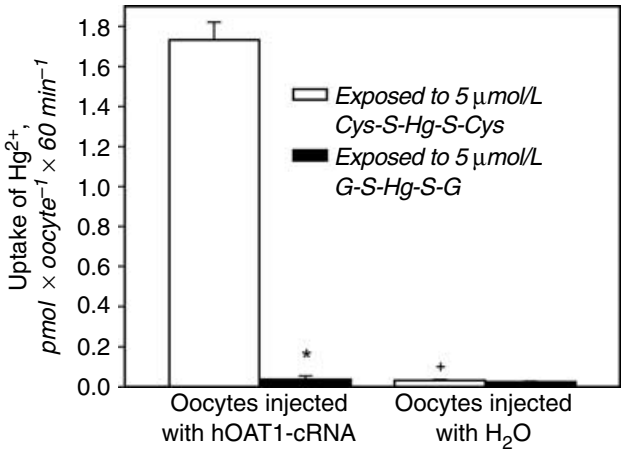

Fig. 6. Uptake of Hg^{2+} (pmol × oocyte^{-1} × 60 min^{-1}) in oocytes (isolated from *Xenopus laevis*) microinjected with hOAT1-cRNA or water three days before being exposed to the mercuric conjugate of cysteine, Cys-*S*-Hg-*S*-Cys, or the mercuric conjugate of glutathione, G-*S*-Hg-*S*-G. Oocytes were either exposed to 5 µmol/L Cys-*S*-Hg-*S*-Cys or 5 µmol/L G-*S*-Hg-*S*-G at room temperature for 60 minutes. Values are mean ± SE. *Significantly different ($P < 0.05$) from the mean for the corresponding group of oocytes exposed to Cys-*S*-Hg-*S*-Cys. +Significantly different ($P < 0.05$) from the mean for the corresponding group of oocytes microinjected with cRNA for hOAT1 and exposed to the same mercuric conjugate.

DISCUSSION

Because of the continuing worldwide contamination of the environment with mercury and the increasing risk of humans being exposed to various forms of mercury [31], there is a growing need to better understand how the various chemical forms of this metal are handled by humans and other species of mammals. In order for one to truly comprehend how mercury induces its deleterious effects in a particular organism, it is paramount for one to understand how mercury (in its many forms) gains entry into the target cells it affects.

Over the past decade, a number of studies have been carried out in an attempt to characterize the disposition and transport of mercury in target organs. The

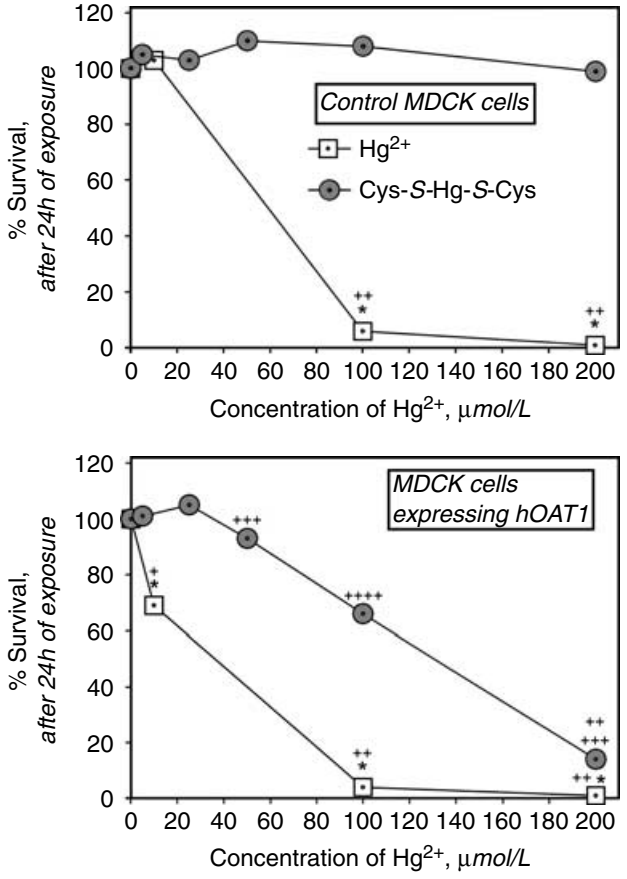

Fig. 7. The percent (%) of control and hOAT1-expressing MDCK II cells surviving exposure to various concentrations of inorganic mercury (Hg^{2+}) in the form of $HgCl_2$ or the mercuric conjugate of cysteine, Cys-*S*-Hg-*S*-Cys for 24 hours. Values are mean ± SE. *Significantly different ($P < 0.05$) from the mean for the corresponding group of (control or transfected) MDCK cells exposed to Cys-*S*-Hg-*S*-Cys. +Significantly different ($P < 0.05$) from the preceding mean for corresponding group of MDCK II cells exposed to the same form of Hg^{2+}. ++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^{2+} at the lowest two concentrations studied. +++Significantly different ($P < 0.05$) from the means for the preceding three groups of corresponding MDCK II cells exposed to the same form of Hg^{2+}. ++++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^{2+}. +++++Significantly different ($P < 0.05$) from the means for the preceding five groups of corresponding MDCK II cells exposed to the same form of Hg^{2+}.

findings from a number of these studies have conclusively documented that the kidneys are the primary sites in the body where Hg^{2+} is taken up and accumulated [32], and that the preponderance of this accumulation is linked to the uptake of mercuric species by proximal tubular epithelial cells [2, 7–9]. More importantly, these findings indicate that at least one luminal mechanism and at least one basolateral mechanism are involved in the uptake of mercury by proximal tubular epithelial cells [10–16, 28–31].

With the discovery that Hg^{2+} is taken up at the basolateral membrane of proximal tubular epithelial cells in vivo, it was determined that much of this uptake could be inhibited by PAH [10, 11, 13, 15, 31]. Because PAH is a high-affinity substrate of the dicarboxylate/organic anion exchanger OAT1 [18, 32], it seemed logical for us to postulate that organic anion transporter(s) is/are involved in the basolateral uptake of mercury [10–16, 28–31].

In the present study, we directly tested the hypothesis that the membrane protein OAT1 (specifically hOAT1) is capable of transporting biologically relevant molecular species of mercury. We tested this hypothesis by characterizing the transport of specific mercuric conjugates in a line of MDCK II cells transfected stably with the cDNA encoding the human isoform of the renal dicarboxylate/organic anion exchanger 1 (hOAT1). However, before experimentation with mercuric conjugates, we first established that expression and membrane insertion of a fully functional hOAT1-protein was occurring in the transfected MDCK II cells. This was accomplished by demonstrating concentration- and time-dependent transport of PAH, and by confirming the inhibitory effects of probenecid or small dicarboxylates on the uptake of PAH [1]. After we had established that a functional form of hOAT1 was being inserted into the luminal plasma membrane of the transfectants, we began to characterize the transport of specific mercuric conjugates.

We discovered that when the MDCK II cells are transfected with hOAT1, they not only gain the ability to transport PAH, but they also gain the ability to transport selective biomolecular species of Hg^{2+}. More specifically, they gain the ability to transport Hg^{2+} in the form of Cys-*S*-Hg-*S*-Cys (and NAC-*S*-Hg-*S*-NAC [26]), from the extracellular fluid into their intracellular compartment. In addition, we discovered that when oocytes from *Xenopus laevis* are microinjected with cRNA encoding hOAT1, they also gain the ability to transport Hg^{2+} in the form of Cys-*S*-Hg-*S*-Cys into their cytosolic compartment. Collectively, these findings clearly and convincingly demonstrate that the mercuric conjugate Cys-*S*-Hg-*S*-Cys is indeed a transportable substrate of hOAT1. Furthermore, the toxicologic findings from the transfectants show that there is a direct, dose-dependent relationship between the uptake of Cys-*S*-Hg-*S*-Cys by hOAT1 and the induction of cellular injury and death. The findings obtained from the transfectants are particularly interesting inasmuch as MDCK cells (which are derived from the distal nephron) are normally not cellular targets affected adversely by biologically relevant forms of Hg^{2+}. It should be pointed out, however, that when either transfected or control MDCK cells were exposed to the chloride salt form of Hg^{2+}, they became intoxicated, owing to the tremendously toxic effects of mercuric ions in culture (when they are not covalently linked to the sulfur atom of a thiol ligand). Overall, these data represent the

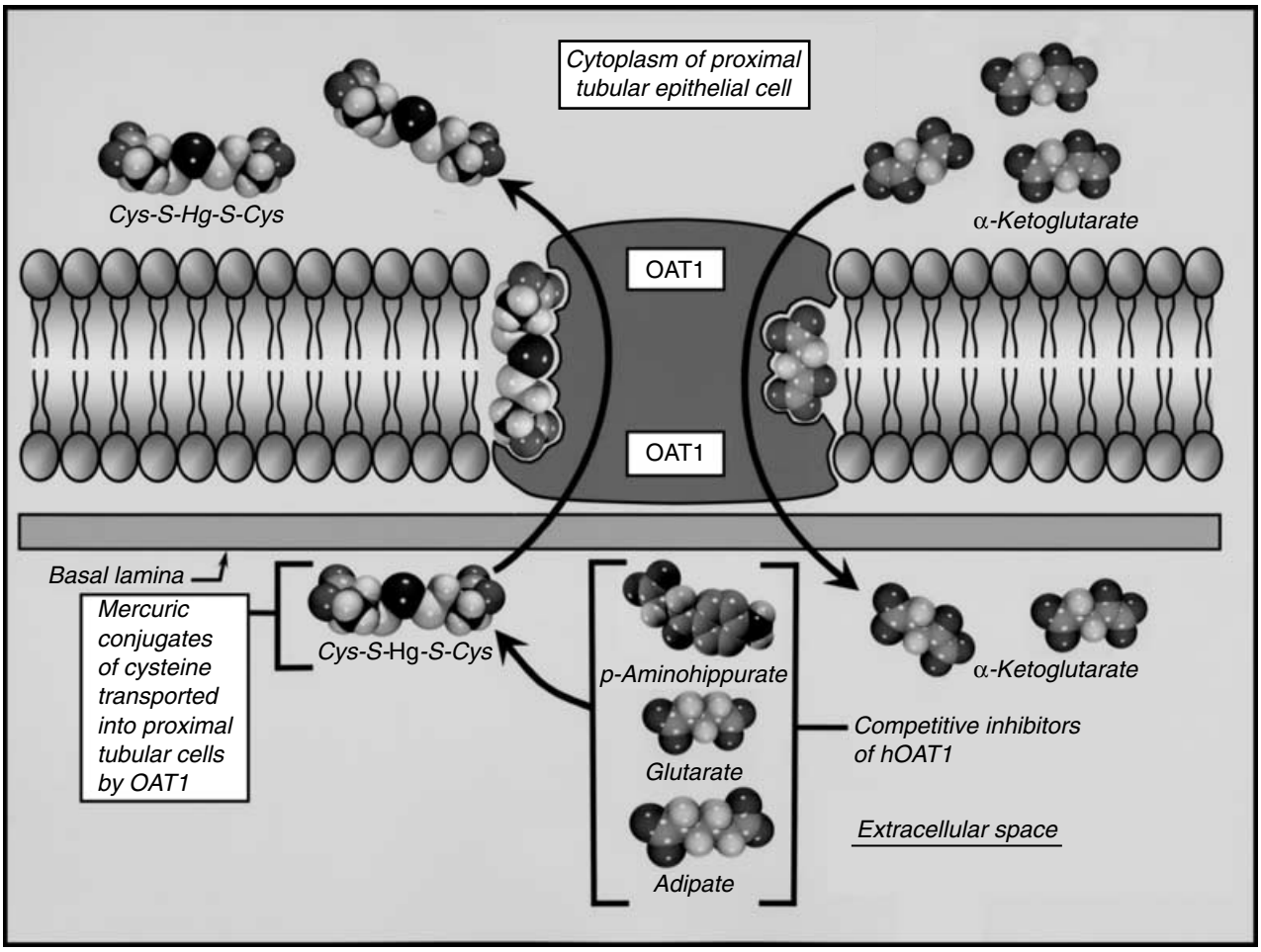

Fig. 8. Diagrammatic representation of the mechanism by which OAT1 is involved in the basolateral uptake of Hg^{2+}, in the form of mercuric conjugates of cysteine (Cys), in a proximal tubular epithelial cell. In this representation, the intracellular concentration of α-ketoglutarate and the extracellular 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 by OAT1. This diagram also illustrates that known substrates of OAT1 serve as competitive inhibitors for the uptake of mercuric conjugates of Cys at the site of OAT1. The three-dimensional, space-filled, molecular models were generated in part with MOLPOV2 and Persistence of Vision (POV, version 3.5).

most direct line of evidence, to date, implicating a specific mammalian protein (namely, hOAT1) in the transport of biologically relevant molecular species of Hg^{2+}.

Interestingly, only minimal levels of mercury (similar to control levels) were associated with, or taken up by, hOAT1-expressing MDCK II cells or oocytes when these cells were exposed to mercuric *S*-conjugates of GSH. These transport findings indicate that mercuric conjugates in the form of G-*S*-Hg-*S*-G are not transportable substrates of OAT1. Normally, negatively charged molecules containing 5 to 6 carbons appear to have a particularly high affinity for OAT1 [18, 32–35]. Because G-*S*-Hg-*S*-G is much larger and bulkier than the typical substrates of OAT1, the present findings are not that surprising. What does make the findings perplexing, though, are the various lines of evidence implicating mercuric conjugates of GSH in the in vivo basolateral uptake of Hg^{2+} by proximal tubular cells [15, 31, 36]. These findings are also surprising because certain organic *S*-conjugates of GSH have been implicated as substrates of the organic anion transport system(s) [37–39]. It may be that although mercuric *S*-conjugates of GSH are species of mercury present in blood, the actual forms of Hg^{2+} transported by OAT1 (and other organic anion transporters) are smaller, less sterically hindering forms of mercury, such as mercuric conjugates of Cys.

A second class of inhibitors of OAT1 includes dicarboxylates that 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 [18, 32–35]. 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 [37]. In a recent study, [16] it was demonstrated that both adipate and glutarate, but not succinate or malonate (3-carbon atoms long), inhibited (in a dose-dependent manner) the uptake of intravenously administered Hg^{2+} in rats. These findings are in complete agreement with those of the present investigation. The current findings also establish that the uptake of PAH or Cys-*S*-Hg-*S*-Cys in cells expressing hOAT1 are inhibited by adipate or glutarate, but not by succinate or malonate (Figs. 3 and 8). These findings, therefore, serve as additional evidence implicating mercuric *S*-conjugates of Cys as transportable substrates of OAT1.

Much like any membrane transporter, the basis for transport at hOAT1 is greatly dependent on molecular structure-function relationships that exist between the transporter and substrate [17]. Molecular homology among substrates likely plays an important role in the activity of OAT1. Molecular homology is implicated in the transport of these conjugates because they share structural and/or functional characteristics common to other mercapturic acids that are known to be substrates of hOAT1 [40]. The present findings also implicate molecular homology in that Cys-*S*-Hg-*S*-Cys is a molecular homolog of NAC-*S*-Hg-*S*-NAC, which we have shown recently to be a transportable substrate of hOAT1 [26]. We were also able to show a correlation between the rates of uptake of NAC-*S*-Hg-*S*-NAC and the induction of cellular injury and death in the hOAT1-expressing cells [26]. Even though Cys has a net neutral charge in blood, certain organic *S*-conjugates of Cys have been implicated as transportable substrates of OAT1 [33, 39, 41]. Thus, based on the aforementioned findings, mercuric conjugates of Cys and its homolog NAC make up a class of homologous molecules that are substrates of hOAT1.

Because Cys and GSH are present in the blood of humans and other mammals at low μmol/L concentrations [42–45], mercuric conjugates of these low-molecular-weight thiols are believed to be the most biologically relevant forms of Hg^{2+} (in addition to mercuric conjugates of albumin) that are presented to proximal tubular epithelial cells in vivo. It is during the initial hours after exposure that as much as 50% of a dose of Hg^{2+} is taken up by the proximal tubular segments through luminal and basolateral mechanisms. There is presently insufficient evidence to speculate whether mercuric conjugates of NAC are present in the blood before and/or after exposure to the various forms of Hg^{2+}.

CONCLUSION

The present findings represent the first line of direct evidence implicating a specific membrane protein (i.e., hOAT1) in the transport and cellular intoxication of specific, biologically relevant, molecular species of Hg^{2+} in a mammalian cell. More importantly, these data indicate 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. Figure 8 provides a diagrammatic summary of the putative role of OAT1 in the basolateral uptake of Cys-*S*-Hg-*S*-Cys in a proximal tubular cell. Furthermore, they also indicate that the basolateral uptake of mercuric conjugates of Cys by OAT1 is linked directly to the proximal tubular nephropathy induced by inorganic forms of mercury in vivo.

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Reprint requests to Dr. Rudolfs K. Zalups, Division of Basic Medical Sciences, Mercer University School of Medicine, 1550 College Street, Macon, GA 31207.
E-mail: zalups_rk@mercer.edu

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