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Handling of cysteine *S*-conjugates of methylmercury in MDCK cells expressing human OAT1

RUDOLFS K. ZALUPS and SARFARAZ AHMAD

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

Handling of cysteine *S*-conjugates of methylmercury in MDCK cells expressing human OAT1.

Background. The activity of the organic anion transporter 1 (OAT1) has been implicated recently in the basolateral uptake of thiol conjugates of inorganic mercury in renal proximal tubular cells. However, very little is known about the role of OAT1 in the renal epithelial transport of organic forms of mercury, such as methylmercury (CH_3Hg^+), especially when it is in the form of the cysteine (Cys) *S*-conjugate of methylmercury (CH_3Hg-Cys), which is believed to be a biologically relevant form of mercury.

Methods. Accordingly, the present study, was designed to characterize the transport of CH_3Hg-Cys in Madin-Darby canine kidney (MDCK) cells transfected stably with the human isoform of OAT1 (hOAT1) and in proximal tubular–derived NRK-52E cells.

Results. Data on saturation kinetics, time dependency, substrate specificity, and temperature dependency demonstrate that CH_3Hg-Cys is transported by hOAT1. Substrate-specificity data from the control cells also show that CH_3Hg-Cys is a substrate of one or more transporter(s) that is/are not hOAT1. Additional findings indicate that at least one amino acid transport system is involved in the uptake of CH_3Hg-Cys in MDCK cells. Furthermore, in the presence of cytotoxic concentrations of CH_3Hg-Cys, rates of survival were lower in hOAT1-transfected cells than in wild-type control cells.

Conclusion. The present data demonstrate clearly that CH_3Hg-Cys is indeed a transportable substrate of OAT1. Moreover, the collective findings from the MDCK cells and NRK-52E cells infer that CH_3Hg-Cys is a likely transportable mercuric species in proximal tubular epithelial cells that is taken up in vivo by both OAT1 and amino acid transporters.

Methylmercury is the most prevalent species of mercury found in the environment. It poses a potential health risk to humans, especially to pregnant women and young children, who consume contaminated fish or water. When humans and other mammals are exposed to organic forms of mercury, mercuric ions gain access to a number of target compartments in the body, including the brain, liver, and kidneys. Within the kidneys, mercuric ions primarily gain entry into the epithelial cells lining proximal segments of the nephron, especially the pars recta of proximal tubules [1, 2]. As the burden of mercuric ions in these target epithelial cells becomes too great for the cells to handle, cellular intoxication and death occurs.

It is well established that mercuric ions have a predilection to bind to both protein and nonprotein thiols. Moreover, there is evidence indicating that under certain conditions, the organometal complexes formed by the coordinate covalent binding of an inorganic mercuric ion to the reduced sulfur atom of two molecules of an endogenous nonprotein thiol, can behave chemically as a molecular "mimic" or functional homologue of an essential biomolecule (such as an amino acid or organic anion) [1–7]. In fact, recent cellular and molecular evidence from our laboratory indicates that molecular "mimicry" is a mechanism involved in the luminal absorption of thiol *S*-conjugates of inorganic mercury by renal epithelial cells. More specifically, we have shown that the linear II, coordinate-covalent mercuric conjugate of cysteine (Cys), Cys-Hg-Cys, can serve as a molecular mimic of the amino acid cystine (Cys-*S*-*S*-Cys) at the site of the amino acid transporter system $b^{0,+}$, which is an important absorptive amino acid transporter found on the luminal plasma membrane of proximal tubular epithelial cells [1, 8–11].

Interestingly, uptake of inorganic mercury by proximal tubular epithelial cells is not restricted to transport proteins located in the luminal plasma membrane. Numerous lines of evidence indicate that mercuric ions are also taken up (from the blood and extracellular fluid) at the basolateral membrane by one or more organic anion transport-system(s) [12–17]. Within the kidneys, these transport systems are localized exclusively along the proximal tubule, where they provide a secretory mechanism for the elimination of many xenobiotics and endogenous organic anions that escape filtration during their passage through the kidney(s).

Key words: methylmercury, cysteine S-conjugates, hOAT1, amino acid transporters, MDCK cells, proximal tubules.

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One of the best characterized organic anion transport (OAT) systems mediates the basolateral uptake of a wide range of organic anions by a dicarboxylate/organic anion exchanger. This transport protein has been denoted as organic anion transporter 1 (OAT1), and is responsible for transporting a wide variety of organic anions into the cytosolic compartment of proximal tubular epithelial cells 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.

As alluded to above, OAT1 can mediate the uptake of a host of low molecular weight and structurally dissimilar organic acids and neutral compounds. Recent findings from our laboratory provide the first lines of molecular evidence indicating that Cys, homocysteine (Hcy), and *N*-acetylcysteine (NAC) *S*-conjugates of inorganic mercury can serve as transportable substrates of OAT1 [6, 7, 18].

Although there is considerable evidence from mammalian renal epithelial cells implicating thiol *S*-conjugates of inorganic mercury as substrates of OAT1, little is known about the potential role of this membrane transporter in the basolateral uptake of thiol *S*-conjugates of organic forms of mercury in the kidney. Inasmuch as methylmercury (CH_3Hg^+) is the primary form of mercury present in the environment, it is of paramount importance to understand the mechanisms involved in the transport and handling of this species of mercury in target cells affected by this toxicant.

In the present study, we designed experiments to characterize and compare the transport of Cys *S*-conjugates of CH_3Hg^+ (CH_3Hg-Cys) in Madin-Darby canine kidney (MDCK) cells transfected stably with the human isoform of OAT1 (hOAT1). Inasmuch as MDCK cells are derived from the distal nephron, direct molecular evidence for the participation of OAT1 in the transport of specific mercuric species could be obtained from a line of mammalian renal epithelial cells that do not express organic anion transporters. The rationale for studying the transport of Cys *S*-conjugates of methylmercury is that this mercuric species has been implicated in the transport of CH_3Hg^+ in endothelial cells and astrocytes of the blood-brain barrier [3, 19]. We also studied the transport of CH_3Hg-Cys in NRK-52E cells, which are derived from the renal proximal tubule of the rat. These cells were chosen since they represent an established in vitro model of proximal tubular epithelial cells in culture. Using the NRK-52E cells permitted us to assess similarities in the mechanism in the luminal uptake of CH_3Hg-Cys between cells derived from the proximal tubule and distal regions of the nephron. Collectively, the findings from the present study provide the first line of molecular evidence from mammalian renal epithelial cells indicating that CH_3Hg-Cys can serve as a transportable substrate of OAT1 and one or more amino acid transport systems.

METHODS

Transfection of MDCK-II cells with hOAT1

A subclone of Mycoplasma-free type II MDCK cells was used in the present investigation. These cells were originally developed in the laboratory of Dr. Kai Simmons (EMBL, Heidelberg, Germany). As described previously, [3, 18–20] 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 Science (NIEHS). These subclones were maintained in culture media with 200 μg/mL geneticin (G418) (Invitrogen), and were screened regularly for gaining the ability to transport organic anions. This was accomplished by assaying uptake of [^{3}H]-para-aminohippuric acid (PAH) as described below.

Cell culture

When wild type and hOAT1-expressing MDCK cells were grown in a confluent monolayer, the cell-to-cell attachments afforded a low to moderate transepithelial resistance. All 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). While in culture, the MDCK cells were grown and maintained in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. Cells were split every 3 to 7 days, and 1/10th to 1/20th of the culture was inoculated into new flasks.

NRK-52E cells were grown to confluency in Vitacell Dulbecco's modified Eagle's medium (DMEM) (ATCC, Manassas, VA, USA) supplemented with 4 mmol/L L-glutamine, 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, and 10% bovine calf serum (BCS). Cells were grown and maintained in a humidified atmosphere consisting of 95% O_2 and 5% CO_2. Cells were split every 5 to 7 days and were subcultured into new flasks.

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

Dr. Pritchard's laboratory had established that 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. Therefore, uptake of [^{3}H]-PAH (as well as other substrates) was assessed in cells plated in 24-well (2.0 cm^2 per well) cell-culture cluster-plates (Costar Corning, New York, NY, USA), containing supplemented EMEM, at a density of 0.5×10^6 cells per well (added as 2 mL). The cells were grown to confluency in a humidified atmosphere of 95% air and 5% CO_2 for 2 days at 37°C, with media being changed after the first 24 hours.

Immediately prior to assessing transport parameters, each well containing cells was first rinsed with Hank's buffered saline solution (HBSS) supplemented with 10 mmol/L 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) (pH 7.4) for three consecutive 5-minute periods. At the beginning of each experiment, 350 μL of the aforementioned HBSS containing 5.0 μmol/L PAH, with or without 200 μmol/L probenecid, were added to each well. Some of the PAH was in the form of [^{3}H]-PAH (4.54 mCi/μmol) (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA). Following 60 minutes of exposure to PAH, the cells in each well were rinsed with cold (4°C) "stop" buffer [HBSS supplemented with 10 mmol/L Hepes (pH 7.4)]. To determine the cellular content of [^{3}H]-PAH, cells were first lysed by adding 1 mL of 1 N NaOH to each well. The plates containing NaOH were shaken overnight (for at least 12 hours) in an orbital shaker operating at a rate of 500 rpm. Subsequently, 700 μL of cellular lysate from each well were neutralized with 700 μL of 1 N HCl. The total volume of neutralized solution was added to 15 mL of Opti-Fluor high-flash point liquid scintillation fluid (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The radioactivity in each sample was determined using a Beckman LS6000IC Liquid Scintillation Analyzer (Beckman Instruments, Fullerton, CA, USA). 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 and rates of cellular uptake of [^{3}H]-PAH were expressed as pmol $\times$ mg protein^{-1} $\times$ min^{-1}.

Experimental design

Concentration-dependence, time-dependence, temperature-dependence, and substrate specificity for the extracellular to intracellular transport of CH_3Hg-Cys were characterized and compared in both hOAT1-transfectants and corresponding wild-type control MDCK cells. The hOAT1 component for the uptake of CH_3Hg^+ (in the form of CH_3Hg-Cys) from the hOAT1-transfected cells and the concentration dependent data from the control cells were fitted to the Michaelis-Menten equation. In addition, linear regression analysis was applied to transport data plotted by the Eadie-Hofstee method, where velocity (V) is plotted against the quotient of velocity divided by the concentration of substrate (V/[S]). The slope of each regression line determined by this method is equal to the negative of the Michaelis-Menton constant ($-K_m$). By using these equations; we were able to characterize the kinetics of the transport of CH_3Hg-Cys.

Transport of CH_3Hg^+ in hOAT1-transfected and nontransfected MDCK-II cells and NRK-52E cells

In the experiments where the transport of Cys *S*-conjugates of CH_3Hg^+ were studied, both the MDCK cells and NRK-52E cells (which are derived from the rat proximal tubule) were also plated in 24-well (2.0 cm^2) cell-culture-cluster-plates (Costar Corning), containing supplemented DMEM, at a density of 0.5×10^6 cells/well (added as 2 mL). They were then grown in a humidified atmosphere of 95% air and 5% CO_2 for 3 days at 37°C with media being changed after the first 24 hours. 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 mmol/L Hepes (pH 7.4). Three hundred and fifty microliters of transport buffer (specific to each experiment) containing radioactive methylmercury ([^{14}C]-CH_3Hg^+) (20 mCi/mmol) (American Radiolabeled Chemical Inc., St. Louis, MO, USA) 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. In additional experiments, the influence of various L-type amino acids (such as leucine, isoleucine, methionine, alanine, and phenylalanine) on the transport of CH_3Hg-Cys was assessed.

L-Cys was added in a 2:1 mol ratio with CH_3Hg^+ to ensure that each methyl mercuric ion in solution formed a thermodynamically stable, linear-I, coordinate-covalent complex with a molecule of Cys. The association constant for the bonding of mercuric ions to the sulfur atom of low-molecular-weight thiols is more than ten orders of magnitude greater than that for the bonding of mercuric ions to any other biologically occurring nucleophilic group [1].

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) 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. Since 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, at the termination of each experiment, as an added measure to inhibit the activity of OAT1.

Cellular content of [^{14}C]-labeled CH_3Hg^+ was determined by liquid scintillation spectrometry after adding 1 mL of 1 N NaOH to each well. After adding the NaOH, the 24-well plates were shaken in an orbital shaker at 500 rpm for at least 12 hours. Seven-hundred microliters of cellular lysate from each well were neutralized with 700 μL of 1 N HCl. The total volume of neutralized solution was added to 15 mL of Opti-Fluor (Perkin Elmer Life and Analytical Sciences) scintillation fluid. The radioactivity of each sample was determined using a Beckman LS6000IC Liquid Scintillation Analyzer) (^{203}Hg counting efficiency $\underline{\sim}$ 80% to 90%). Fifty microliters of the remaining cellular lysate from each well were used to determine the total amount of protein per well by the Bradford protein assay. Transport data obtained from each well of cells were normalized to the corresponding concentration of cellular protein.

Assessment of toxicity and cellular viability

The effects of the mercuric conjugates CH_3Hg-Cys on cellular viability were measured using a methylthiazoletetrazolium (MTT)-based toxicity 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 three 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 6 hours in a humidified atmosphere of 5% CO_2, 95% O_2 at 37°C. At the conclusion of the exposure period, media were 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 and 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 had occurred, and plates were read at 595 nm in a Titertek Multiskan MKII plate reader (Fisher Scientific, Suwanee, GA, USA).

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 in the MDCK cells was performed by first using a two-way analysis of variance (ANOVA), followed by either Tukey's or Dunnett's post hoc test. The data obtained from the NRK-52E cells were analyzed by using a one-way ANOVA, followed by the Tukey's multiple comparison, post hoc test. Data expressed as a percent were first normalized using the arcsine transformation prior to 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

Insertion of a functional hOAT1 transporter into the plasma membranes of the MDCK-II cells transfected with hOAT1 was confirmed by assessing uptake of [^{3}H]-PAH (a high-affinity substrate of this organic anion/dicarboxylate exchanger). 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 9.4 ± 0.7 pmol × mg protein^{-1} × min^{-1}. Addition of 200 μmol/L probenecid to the extracellular media caused the rate of uptake of PAH in the transfected cells to decrease to an average level 0.8 ± 0.1 pmol × mg protein^{-1} × min^{-1}. By contrast, uptake of PAH in the wild-type MDCK cells was insignificant, averaging only about 0.10 ± 0.01 pmol × mg protein^{-1} × min^{-1}. Probenecid did not have a significant effect on the uptake of PAH in the wild-type control cells (0.10 ± 0.01 pmol × mg protein^{-1} × min^{-1}).

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 hOAT1-transfected MDCK cells.

Concentration-dependent uptake of CH_3Hg^+

Assessment of the concentration dependence and saturation kinetics for the uptake of CH_3Hg^+ revealed that the uptake of this species of mercury, when presented as CH_3Hg-*S*-Cys, was greater in hOAT1-transfected MDCK-II cells than in corresponding control cells

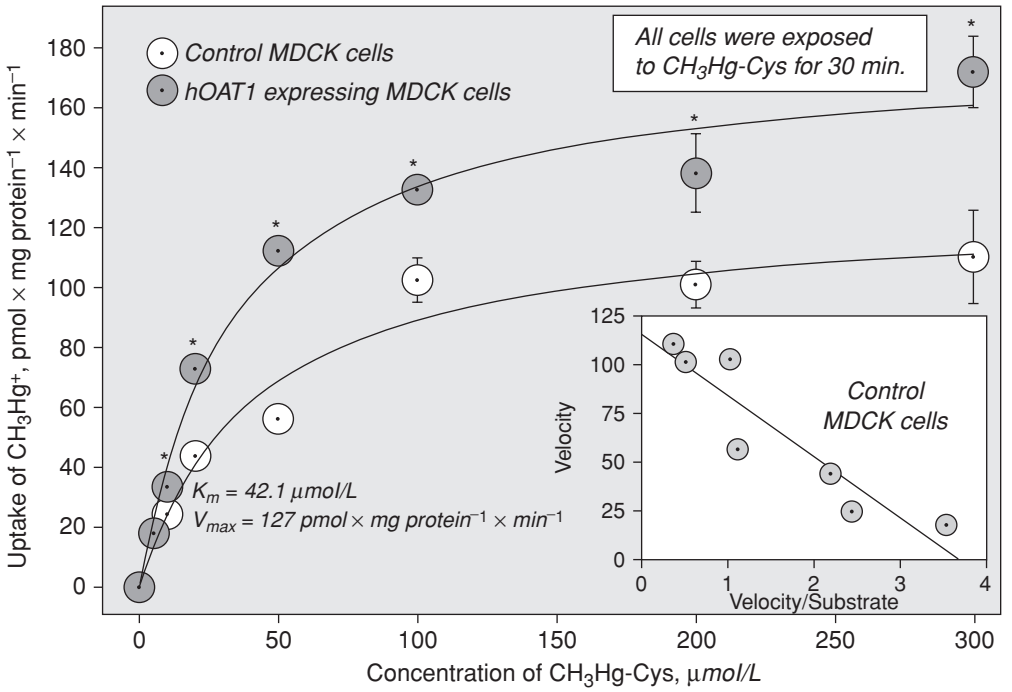

Fig. 1. Concentration-dependent uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1)-expressing Madin-Darby canine kidney (MDCK)-II cells exposed to cysteine (Cys) CH_3Hg (CH_3Hg-Cys). Cellular uptake was studied for 30 minutes (at 37°C). Inset, Eadie-Hofstee plot of the uptake of CH_3Hg^+ in the control MDCK-II cells exposed to CH_3Hg-Cys. Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control MDCK cells.

(Fig. 1). Interestingly, the kinetic data indicate that carrier mediated processes were involved in the uptake of CH_3Hg^+ in both the hOAT1-transfected and wild-type control MDCK-II cells, although the levels of uptake were overall greater in the hOAT1-transfected cells.

Analysis of kinetic parameters revealed that the apparent K_m for the uptake of CH_3Hg^+ in the control MDCK cells was 42 ± 10 µmol/L, while the V_{max} was 127 ± 9 pmol × mg protein^{-1} × min^{-1} (Fig. 1). In the hOAT1-transfected MDCK cells, the apparent K_m for the uptake of CH_3Hg^+ that could be attributed to hOAT1 (hOAT1 minus control) was 36 µmol/L and the V_{max} was 50 pmol × mg protein^{-1} × min^{-1} (Fig. 2).

Time-dependent uptake of CH_3Hg^+

Time-dependent uptake of CH_3Hg^+, when presented as 5 µmol/L CH_3Hg-Cys, was detected in both the hOAT1-transfected and wild-type control MDCK cells (Fig. 3). However, the rates of uptake of CH_3Hg^+ were significantly greater in the MDCK cells transfected with the cDNA for hOAT1 than in the corresponding control MDCK cells at almost all of the times studied. The greatest differences in the transport of CH_3Hg^+ between the hOAT1-transfected and wild-type control cells were detected at 60 and 120 minutes of exposure.

Effect of PAH and probenecid on the uptake of CH_3Hg^+

Addition of 1 mmol/L PAH or 200 µmol/L probenecid to the extracellular compartment containing 5 µmol/L CH_3Hg-Cys did not affect significantly the uptake of CH_3Hg^+ in the control MDCK cells during 60 minutes of exposure (Fig. 4). By contrast, addition of PAH or probenecid to the extracellular medium caused the uptake of CH_3Hg^+ to decrease significantly in the hOAT1-transfected MDCK cells (Fig. 4).

Effect of dicarboxylates on the uptake of CH_3Hg^+

Uptake of CH_3Hg^+ was unaffected in the control MDCK cells when 1 mmol/L glutarate or adipate was added to the extracellular medium containing 5 µmol/L CH_3Hg-Cys during 60 minutes of exposure (Fig. 5). However, both of these dicarboxylates did cause the uptake of CH_3Hg^+ to be decreased significantly in the hOAT1-transfected MDCK cells (Fig. 5).

Effect of temperature on the uptake of CH_3Hg^+

Significant temperature-dependent differences in the extracellular to intracellular transport of CH_3Hg^+ were detected among the groups of corresponding hOAT1-transfected and wild-type control cells.

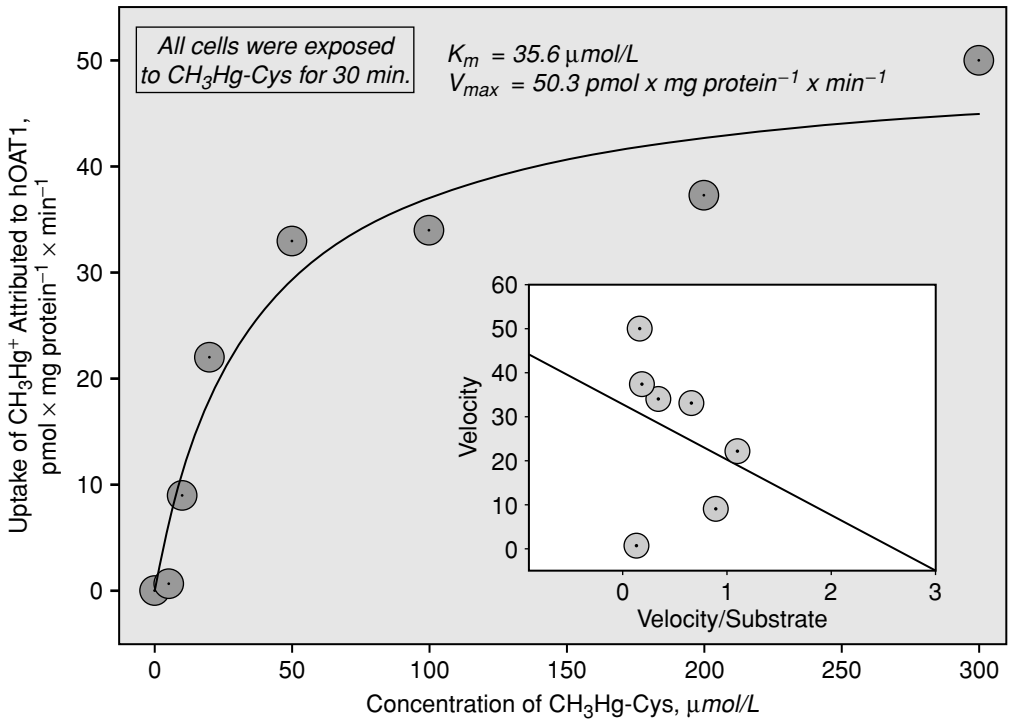

Fig. 2. Concentration-dependent uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) that can be attributed to the activity human organic anion transporter-1 (hOAT1) in the transfected Madin-Darby canine kidney (MDCK)-II cells exposed to cysteine (Cys) CH_3Hg (CH_3Hg-Cys). Cellular uptake was studied for 30 minutes (at 37°C). Inset, Eadie-Hofstee plot of the hOAT1-attributable component for the uptake of CH_3Hg^+ in the transfected MDCK-II cells exposed to CH_3Hg-Cys. Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control MDCK cells.

At 37°C, uptake of CH_3Hg^+ was significantly greater in the hOAT1-transfected MDCK cells than in the corresponding wild-type control MDCK cells when the cells were exposed to 5 µmol/L CH_3Hg-Cys during 1 hour of exposure (Fig. 6).

When the extracellular temperature was reduced to 21°C, uptake of CH_3Hg^+ in the hOAT1-transfected and wild-type control MDCK cells was significantly lower than that detected at 37°C (Fig. 6). In addition, there was no significant difference in the rate of uptake of CH_3Hg^+ between the two corresponding groups of hOAT1-expressing cells and wild-type control cells.

Transport of CH_3Hg^+ was for the most part abolished in both the hOAT1-expressing cells and the wild-type control cells when the temperature of the extracellular medium was reduced to 4°C. The level of CH_3Hg^+ detected in both cell types at this temperature likely represents nonspecific binding.

Assessment of toxicity and cellular viability

During 6 hours of exposure to CH_3Hg-Cys, significant decreases in the cellular viability were detected (Fig. 7). Greater decreases in survival were detected in the hOAT1-expressing cells than in the corresponding control cells at each concentration of CH_3Hg-Cys studied (Fig. 7).

Effect of L-amino acids on the uptake of CH_3Hg^+

Uptake of CH_3Hg^+ was significantly greater in the hOAT1-transfected MDCK cells exposed to 5 µmol/L CH_3-Hg-Cys than in the corresponding wild-type control MDCK cells (Fig. 8). Addition of 1 mmol/L various L-isomers of amino acids to the extracellular medium induced significant effects on the transport of CH_3Hg^+ in both control and hOAT1-transfected MDCK cells. Among the control MDCK cells exposed to CH_3Hg-Cys, significant effects on the uptake of CH_3Hg^+ were detected in all groups exposed to an amino acid except for the group of cells exposed to 1 mmol/L L-methionine. Among the hOAT1-expressing cells exposed to amino acids, the levels of uptake of CH_3Hg^+ were reduced significantly in all groups of cells exposed to an amino acid.

In both the control and hOAT1-expressing cells, addition of 1 mmol/L L-cysteine to the extracellular compartment had the greatest effect on the uptake of CH_3Hg^+. Addition of 1 mmol/L L-leucine had the second greatest effect on the uptake of CH_3Hg^+, but only in the control cells.

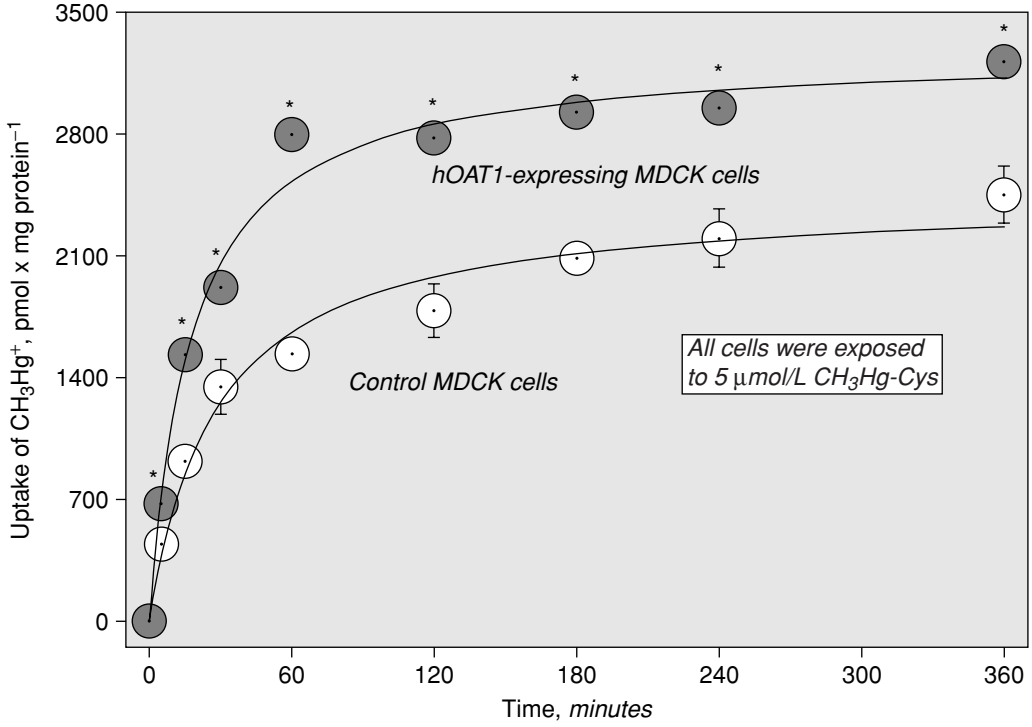

Fig. 3. Time-dependent uptake (pmol × mg cellular protein^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys). Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control MDCK cells.

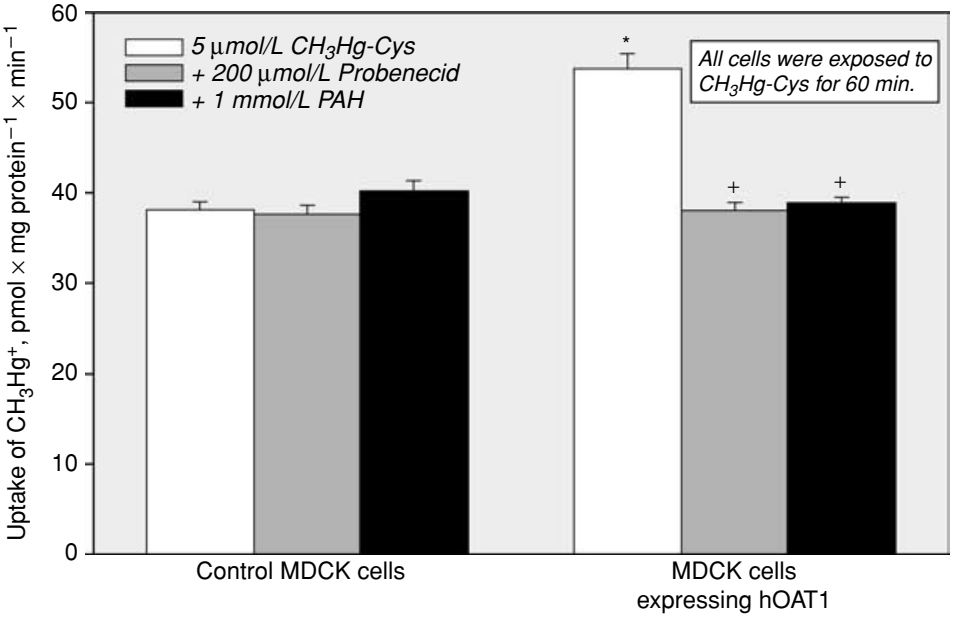

Fig. 4. Influence of 200 μmol/L probenecid or 1 mmol/L [^{3}H]-paraaminohippuric acid (PAH) on the uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine CH_3Hg (CH_3Hg-Cys) for 60 minutes (at 37°C). Each value represents the mean ± SE for a sample size of three or four. *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 hOAT1 expressing MDCK cells not exposed to either PAH or probenecid.

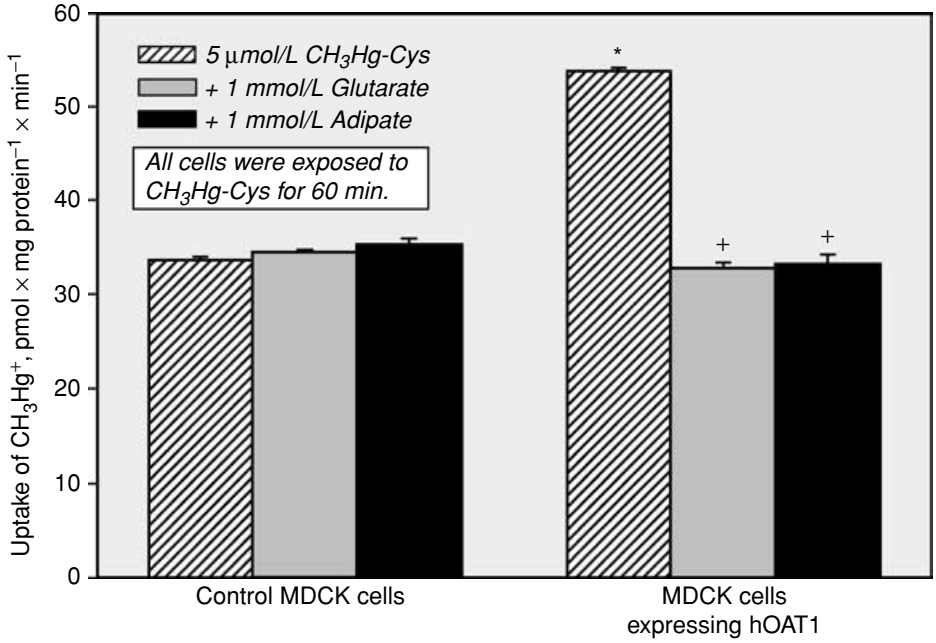

Fig. 5. Influence of 1 mmol/L glutarate or adipate on the uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anionic transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys) for 60 minutes (at 37°C). Each value represents the mean ± SE for a sample size of three or four. *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 hOAT1 expressing MDCK cells not exposed to either dicarboxylic acid.

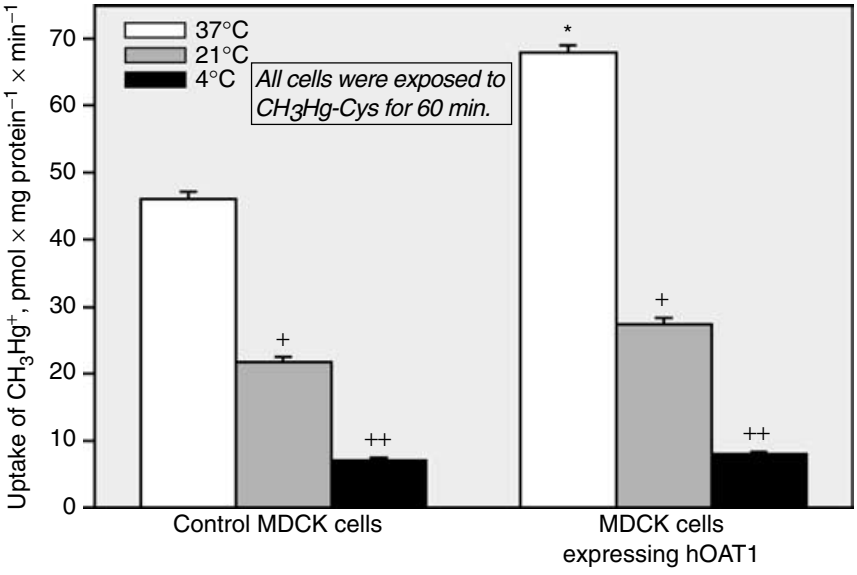

Fig. 6. Effect of temperature on the uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys) for 60 minutes. Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner; +Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells treated at 37°C; ++Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells treated at 37°C or 21°C.

Effect of L-leucine and/or PAH on the uptake of CH_3Hg^+

Significant differences in the uptake of CH_3Hg^+ were again detected between the control and hOAT1-expressing MDCK cells exposed to 5 μmol/L CH_3Hg-Cys (Fig. 9). Among the control and hOAT1-expressing MDCK cells, uptake of CH_3Hg^+ in the group exposed to 1 mmol/L L-leucine was significantly lower than that in the corresponding group treated with only 5 μmol/L CH_3Hg-Cys during 30 minutes of exposure.

Addition of 1 mmol/L PAH to the extracellular compartment did not have a significant effect on the uptake of CH_3Hg^+ in the control MDCK cells, but did have a significant effect on the uptake of CH_3Hg^+ in the hOAT1-expressing cells (Fig. 9). In fact, the effect of PAH was just as great as L-leucine in the hOAT1-expressing

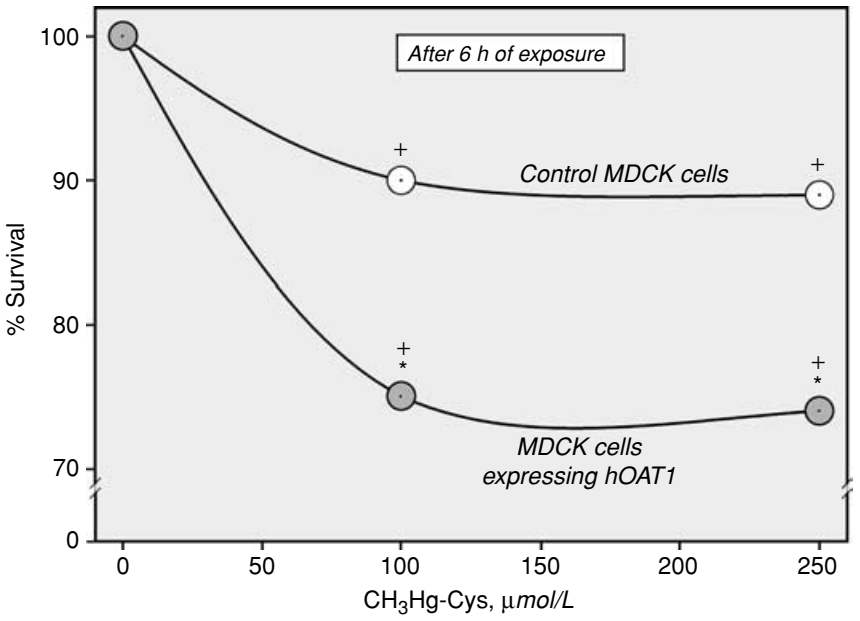

Fig. 7. The percent (%) of control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells surviving 6 hours of exposure to various concentrations of cysteine (Cys) CH_3Hg (CH_3Hg-Cys). Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control MDCK cells; +Significantly different ($P < 0.05$) from the mean for the corresponding group of MDCK cells not treated with CH_3Hg-Cys.

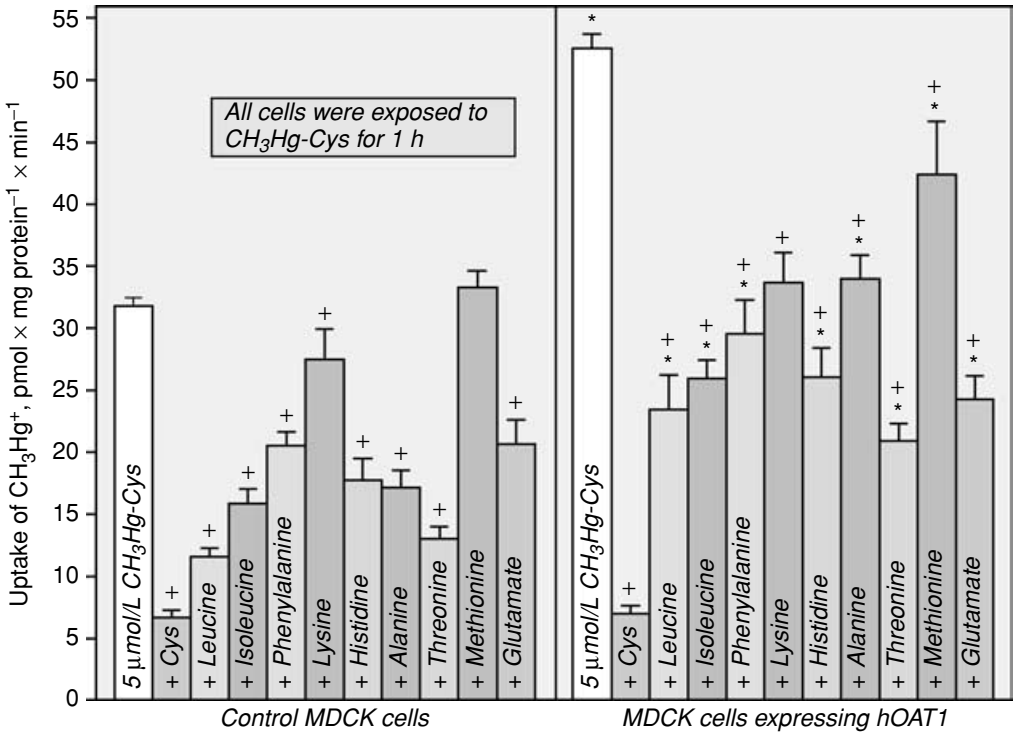

Fig. 8. Influence of extracellular amino acids (1 mmol/L) on the uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys) for 60 minutes (at 37°C). All amino acids used were L isomers. Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner; +Significantly different ($P < 0.05$) from the mean for the corresponding group of control or hOAT1 expressing MDCK cells exposed to 5 μmol/L CH_3Hg-Cys without any additional amino acids added.

cells, inasmuch as no significant difference in the uptake of CH_3Hg^+ was detected between the group of cells treated with L-leucine and the group of cells exposed to PAH.

When both PAH and L-leucine were added to the extracellular medium, an additive inhibitory effect on the uptake of CH_3Hg^+ was detected only among the groups of hOAT1-expressing MDCK cells (Fig. 9). No

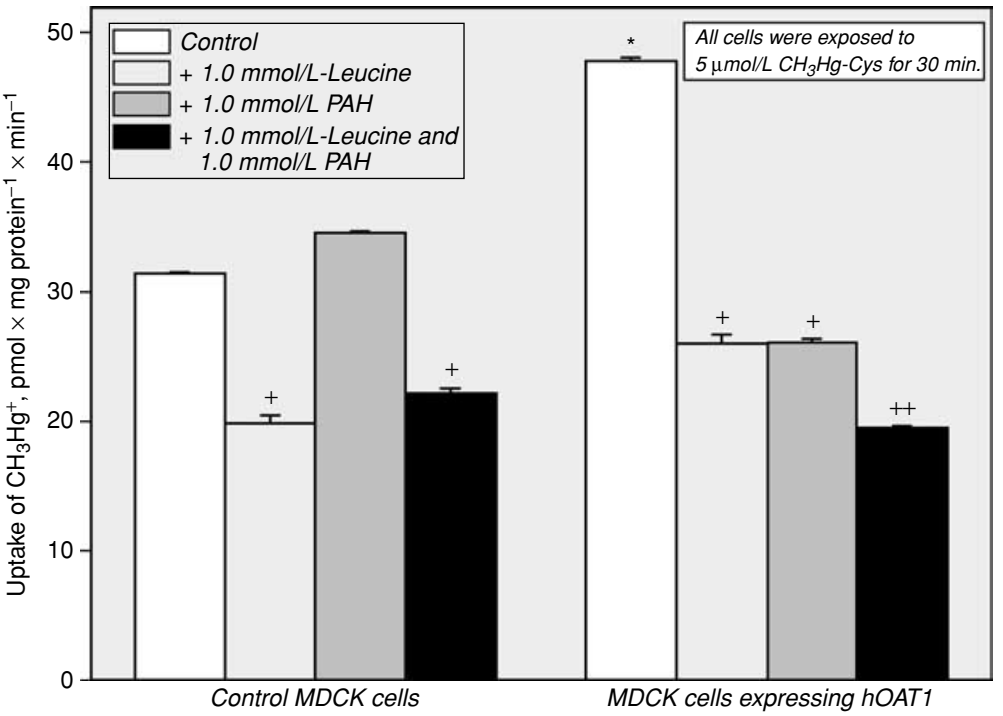

Fig. 9. Effect of adding 1 mmol/L L-leucine and/or 1 mmol/L [^{3}H]-paraaminohippuric acid (PAH) on the uptake (pmol × mg cellular protein^{-1} × min^{-1}) of methylmercury (CH_3Hg^+) in control and human organic anion transporter-1 (hOAT1) expressing Madin-Darby canine kidney (MDCK)-II cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys) for 30 minutes (at 37°C). Each value represents the mean ± SE for a sample size of three or four. *Significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type control MDCK cells treated in the same manner; +Significantly different ($P < 0.05$) from the mean for the corresponding group of control or hOAT1 expressing MDCK cells exposed to 5 μmol/L CH_3Hg-Cys without any additional amino acids added; ++Significantly different ($P < 0.05$) from the means for all of corresponding groups of control or hOAT1 expressing MDCK cells.

significant difference in the uptake of CH_3Hg^+ was detected between the group of control MDCK cells exposed to 5 μmol/L CH_3Hg-Cys and 1 mmol/L L-leucine and the corresponding group of control MDCK cells exposed to 5 μmol/L CH_3Hg-Cys, 1 mmol/L L-leucine, and 1 mmol/L PAH.

Effect of L-amino acids on the uptake of CH_3Hg^+ in NRK-52E cells

In the absence of 140 mmol/L extracellular Na^+, uptake of CH_3Hg^+ during 15 minutes of exposure, was significantly lower in all of the groups exposed to both 5 μmol/L CH_3Hg-Cys and 1 mmol/L of one of the amino acids studied than in the control group of cells exposed to only 5 μmol/L CH_3Hg-Cys (Fig. 10A). Addition of 1 mmol/L cysteine had the greatest inhibitory effect on the uptake of CH_3Hg^+. The next greatest level of inhibition in the uptake of CH_3Hg^+ was detected in the groups of cells exposed to L-leucine, L-isoleucine, or L-phenylalanine.

In the presence of 140 mmol/L extracellular Na^+, a similar pattern for uptake of CH_3Hg^+ was detected among the same groups of cells studied under Na^+-dependent conditions during 15 minutes of exposure (Fig. 10B). However, the overall levels of uptake tended to be lower under Na^+-independent conditions, indicating that much of the transport detected was mediated primarily by one or more Na^+-independent transporters.

Effect of PAH, probenecid, and glutarate on the uptake of CH_3Hg^+ in NRK-52E cells

Uptake of CH_3Hg^+ by NRK-52E cells exposed to 5 μmol/L CH_3Hg-Cys for 15 minutes (in the presence of 140 mmol/L Na^+) was unaffected by the addition of 1 mmol/L PAH, 0.2 mmol/L probenecid, or 1 mmol/L glutarate to the extracellular compartment.

DISCUSSION

Due to the continuing risk of humans being exposed to various forms of mercury [1], there is a growing need to better understand how the various chemical species of this metal are handled by humans and other species of mammals. In order for one to comprehend how these mercuric species induce their deleterious effects in a particular organism, it is paramount for one to understand how mercuric ions (in their varied forms) gain entry into the target cells they affect.

Over the past decade, a number of studies have been carried out in an attempt to characterize the disposition

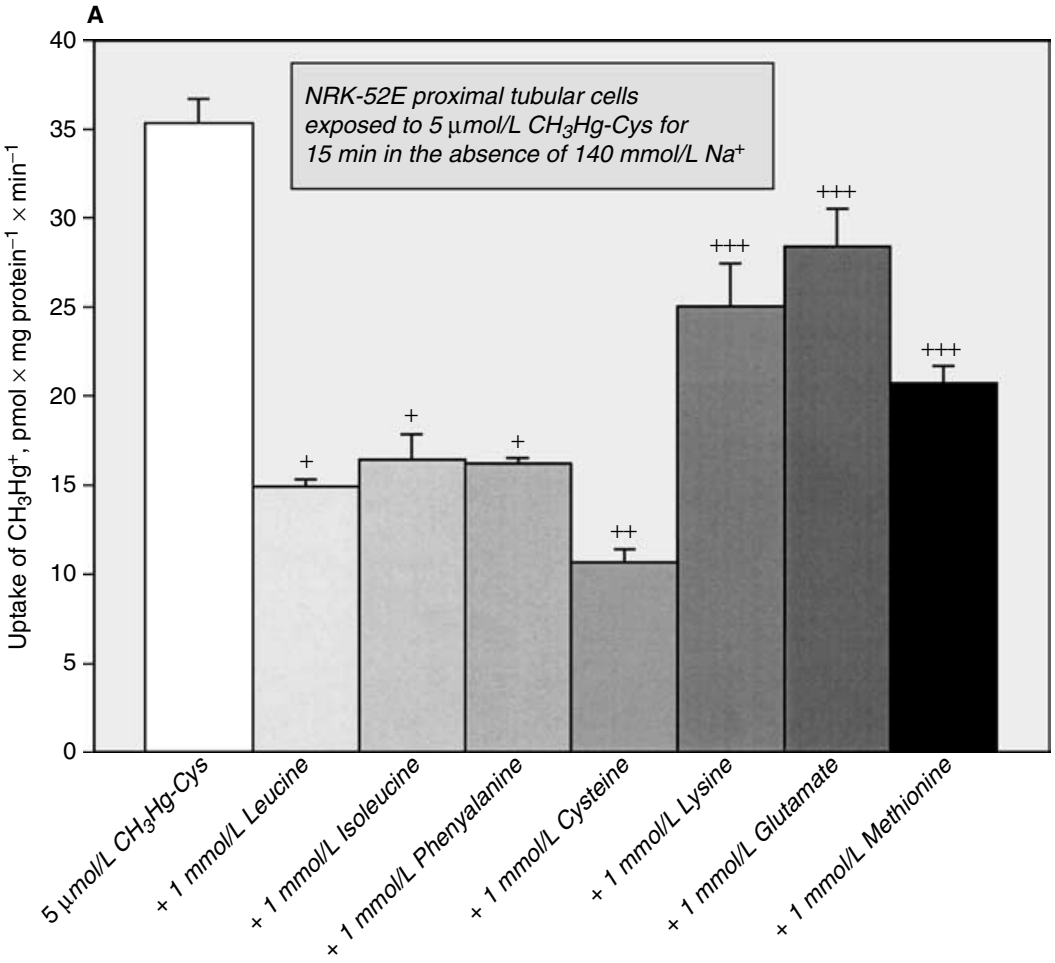

Fig. 10. Uptake of methylmercury (CH_3Hg^+) (pmol × mg cellular protein^{-1} × min^{-1}) in NRK-52E cells exposed to 5 μmol/L cycsteine (Cys) CH_3Hg (CH_3Hg-Cys) for 15 minutes (at 37°C) in the absence and presence of 1 mmol/L leucine, isoleucine, phenylalanine, cysteine, lysine, glutamate, or methionine and in the presence (*A*) or absence (*B*) of 140 mmol/L extracellular Na^+. All amino acids used were L isomers. Each value represents the mean ± SE for a sample size of three or four. +Significantly different ($P < 0.05$) from the mean for the corresponding group of control NRK-52E cells exposed to 5 μmol/L CH_3Hg-Cys; ++Significantly different ($P < 0.05$) from the preceding means for all of corresponding groups of NRK-52E cells exposed to 5 μmol/L CH_3Hg-Cys with or without 1 mmol/L leucine, isoleucine, or phenylalanine; +++Significantly different ($P < 0.05$) from all of the other corresponding groups of NRK-52E cells.

and transport of inorganic and organic forms of mercury in target organs [1]. The findings from a number of these studies have established that the kidneys are the primary sites in the body where Hg^{2+} and CH_3Hg^+ are taken up and accumulated, and that the preponderance of this accumulation is linked to uptake of thiol *S*-conjugates of these mercuric species by proximal tubular epithelial cells [1, 22–24]. More important, 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 [12–17, 25–29].

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 the systemic administration of PAH [12, 13, 15, 16, 29]. Since PAH is a high-affinity substrate of the dicarboxylate/organic anion exchanger OAT1, it seemed logical for us to postulate that organic anion transporter(s) is/are involved in the basolateral uptake of Hg^{2+} [12–17, 25, 28–30]. However, prior to the present study, very little was known about the potential role of OAT1 in the renal tubular uptake of CH_3Hg^+ [1], especially in the form of thiol *S*-conjugates.

Thus, in the present study, we tested directly the hypothesis that the Cys *S*-conjugate of CH_3Hg^+ is a transportable substrate of the membrane transport protein, OAT1 (specifically hOAT1). We tested this hypothesis by characterizing and comparing the transport of CH_3Hg-Cys 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).

We first established that expression and membrane-insertion of a fully functional hOAT1 protein was occurring in the transfected MDCK-II cells. This was

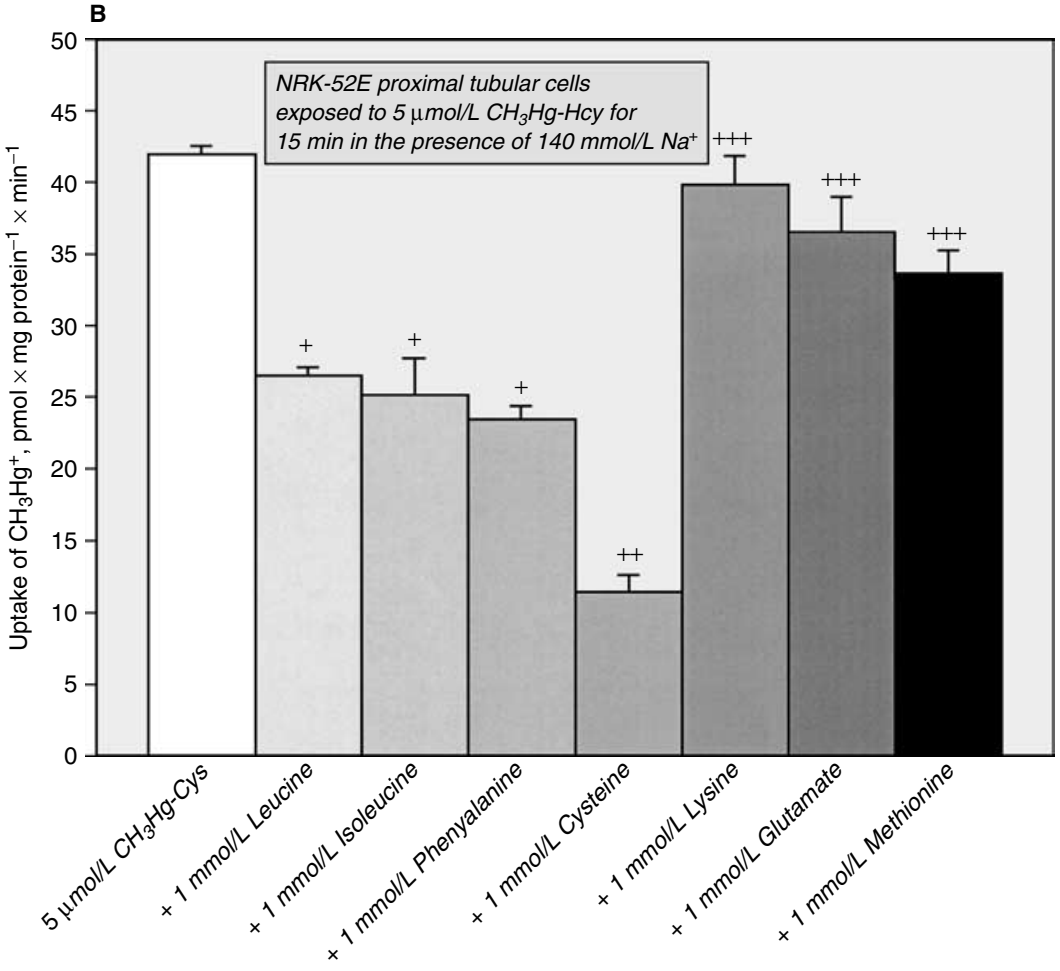

Fig. 10. (Continued.)

accomplished by demonstrating concentration- and time-dependent transport of PAH in only the hOAT1-transfected cells, and by confirming the inhibitory effects of probenecid or small dicarboxylates on the uptake of PAH [6, 7, 15–17, 28]. Only then did we begin to characterize the transport of CH_3Hg^+ in the form of CH_3Hg-Cys.

Analysis of saturation kinetics and time-dependency for the transport of CH_3Hg-Cys in both the hOAT1-transfected and wild-type MDCK cells demonstrate clearly that CH_3Hg-Cys is taken up to a greater extent in MDCK cells expressing hOAT1 than in corresponding control MDCK cells. Moreover, both analysis of the saturations kinetics and assessment of temperature dependency of the transport of CH_3Hg-Cys indicate that the enhanced uptake of CH_3Hg-Cys in the hOAT1 expressing cells is due to a carrier-mediated process (i.e., the activity of the hOAT1 transport protein).

In proximal tubular segments in vivo, the downhill, intracellular-to-extracellular chemical gradient for α-ketoglutarate serves to facilitate the transport of organic anions into the cytosolic compartment by a molecular exchange mechanism mediated by OAT1. Other dicarboxylates, such as adipate and glutarate, also compete for OAT1 [6, 7, 15–17, 28]. In the present study, we demonstrate that the uptake of CH_3Hg-Cys in MDCK cells expressing hOAT1 is not only inhibited by PAH and probenecid, but by adipate or glutarate (Figs. 4 and 5). These findings, therefore, serve as additional evidence implicating Cys *S*-conjugates of CH_3Hg^+ as transportable substrates of hOAT1.

OAT1 ostensibly has the ability to transport a broad spectrum of functionally and/or structurally similar molecules. This transport is greatly dependent on molecular structure-function relationships that exist between the transporter and substrate. Molecular homology among substrates appears to play an important role in the activity of OAT1. In support of this hypothesis are recent data implicating OAT1 in the transport of inorganic mercuric conjugates of NAC (NAC-*S*-Hg-*S*-NAC), Cys (Cys-*S*-Hg-*S*-Cys), and Hcy (Hcy-*S*-Hg-*S*-Hcy) [6, 7, 15–17, 28].

In contrast to the present findings, Koh et al [31] provide data showing that uptake of CH_3Hg-Cys in *Xenopus*

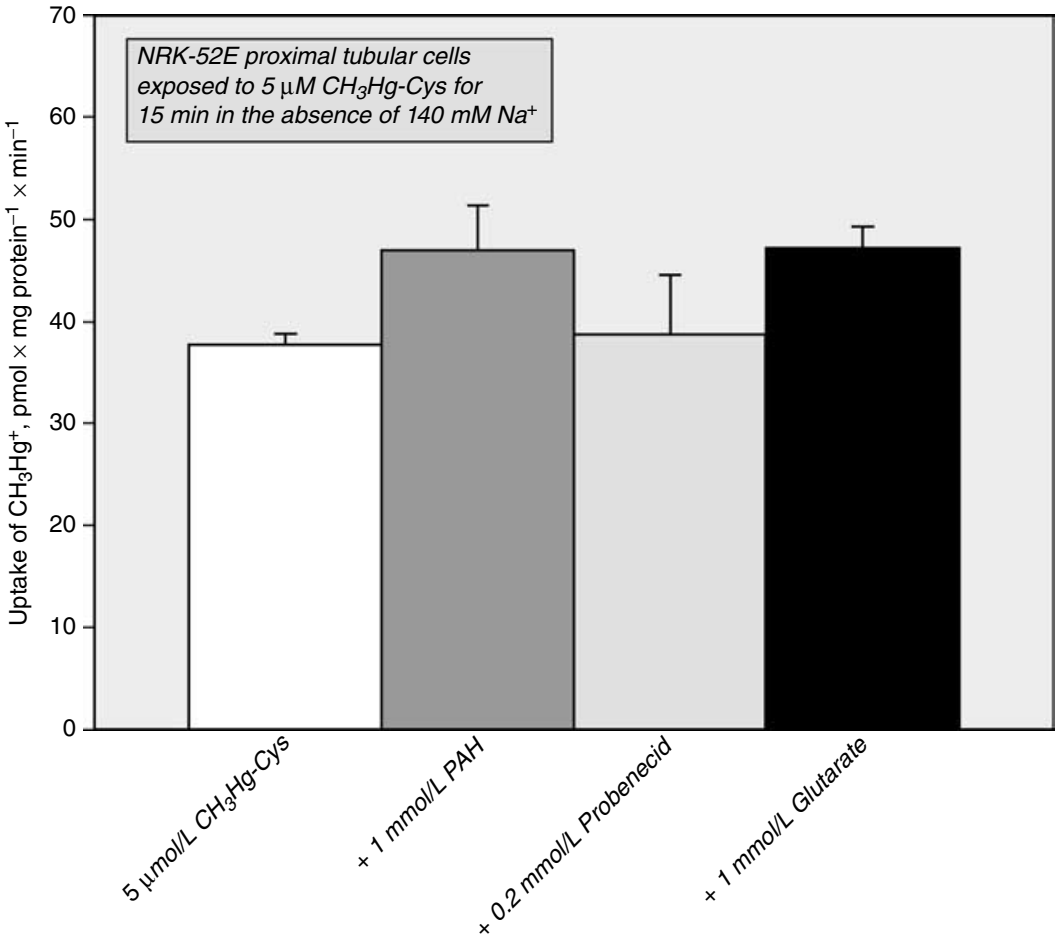

Fig. 11. Apical (luminal) uptake of methylmercury (CH_3Hg^+) (pmol × mg cellular protein^{-1} × min^{-1}) in NRK-52E cells exposed to 5 μmol/L cysteine (Cys) CH_3Hg (CH_3Hg-Cys) or 15 minutes (at 37°C) in the presence and absence of 1.0 mmol/L [^{3}H]-paraaminohippuric acid (PAH), 0.2 mmol/L probenecid, or 1 mmol/L glutarate. Each value represents the mean ± SE for a sample size of three or four. Mean values were not statistically different from one another.

(*laevis*) oocytes expressing rat OAT1 or OAT3 was not significantly different from that in corresponding control oocytes. It is not clear why rOAT1-dependent uptake of CH_3Hg-Cys was not detected in the oocytes studied by Koh et al, although species-variants in OAT1 may have played a role. It should also be pointed out that among the control groups of oocytes, uptake of CH_3Hg-Cys was at least threefold greater than the uptake of methylmercuric conjugates of NAC, glutathione, cysteinylglycine, penicillamine, DMPS, or dimercaptosuccinic acid (DMSA). Thus, an inherent mechanism in the control oocytes appears to have been responsible for a significant level of uptake of CH_3Hg-Cys. This inherent mechanism of uptake could have overshadowed or diluted the activity of rOAT1 or rOAT3.

OAT3 is an additional organic transporter localized in the basolateral plasma membrane of proximal tubular epithelial cells in vivo. Some of the same substrates transported by OAT1 also appear to be taken up by OAT3. For example, we recently obtained unpublished findings showing that the inorganic mercuric conjugate Cys-Hg-Cys is not only a substrate in hOAT1-transfected MDCK cells and hOAT1-expressing *X. laevis* oocytes [7], but is also a transportable substrate in *Xenopus* oocytes expressing rOAT3. However, further experiments are needed to determine if OAT3 participates in the transport of CH_3Hg-Cys in mammalian cells expressing this protein.

Interestingly, two distinct carrier-mediated mechanisms for the uptake of CH_3Hg-Cys were implicated between the two populations of MDCK cells studied. The first mechanism was the PAH- and dicarboxylate-sensitive component afforded exclusively to the hOAT1-transfected MDCK cells. Unexpectedly, though, a significant level of uptake of CH_3Hg-Cys also occurred in the wild-type control MDCK cells. However, this uptake was not linked to a mechanism sensitive to PAH, probenecid, adipate, or glutarate, but was found to be concentration, time, and temperature dependent and was substrate specific. Clearly, a disparate mechanism, not

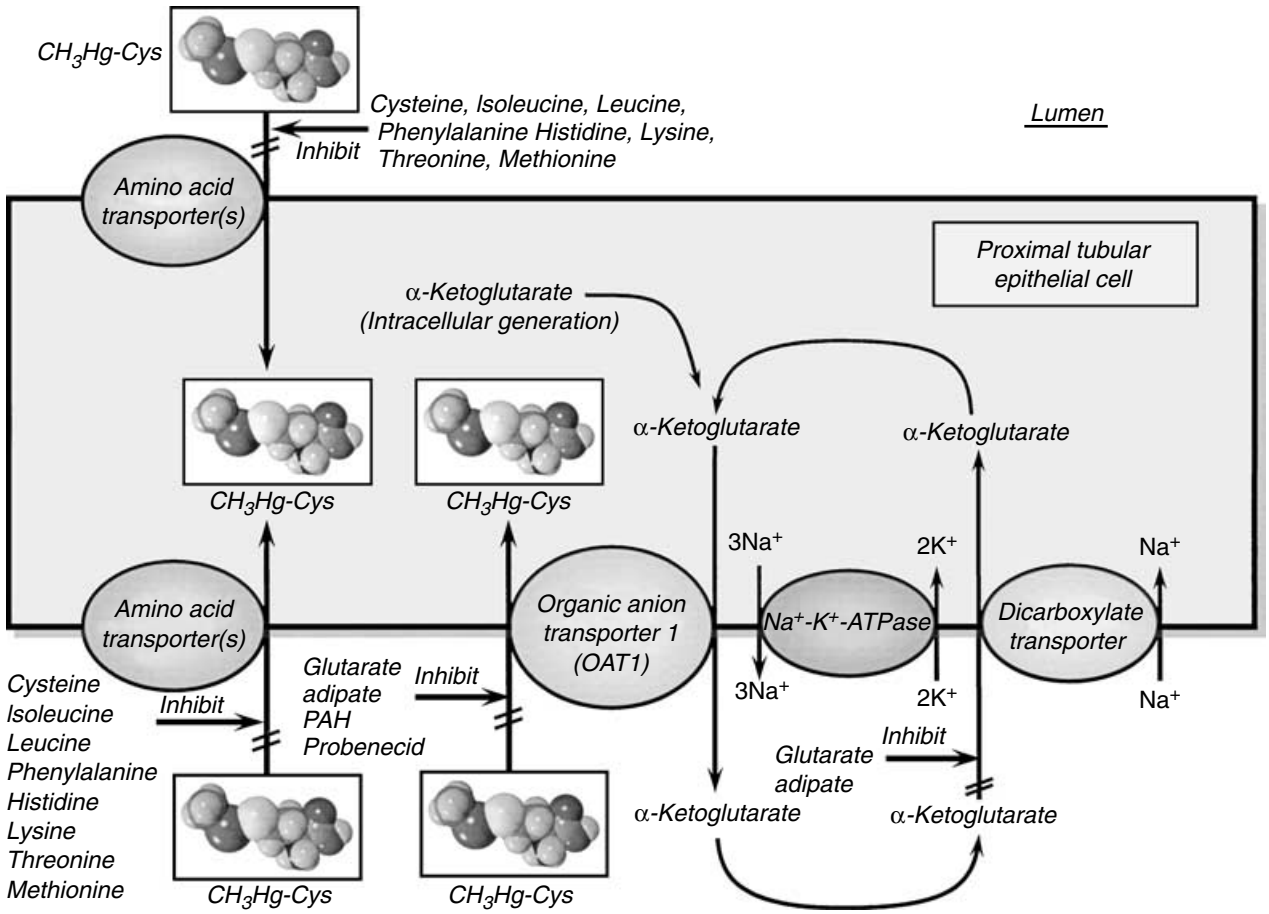

Fig. 12. Diagrammatic representation of the role of organic anion transporter-1 (OAT1) and amino acid transporters in the basolateral uptake of the cysteine (Cys) *S*-conjugate of methylmercury (CH_3Hg^+), CH_3Hg-Cys, in a proximal tubular epithelial cell. In this representation, the intracellular concentration of α-ketoglutarate and the extracellular concentration CH_3Hg-Cys provide the energy to allow α-ketoglutarate to be transported out of the proximal tubular epithelial cell in exchange for CH_3Hg-Cys by OAT1. This diagram also illustrates that amino acid transport proteins likely participate in the luminal and/or basolateral uptake of CH_3Hg-Cys. The three-dimensional, space-filled, molecular model was generated in part with MOLPOV2 and persistence of vision (POV), version 3.5.

involving hOAT1, was involved in this transport. The data obtained from the study of the effects of amino acids on the transport of CH_3Hg-Cys show that this mechanism is likely mediated by one or more amino acid transporters native to the MDCK cells. In both types of MDCK cells, exposure to 1 mmol/L L-Cys, L-leucine, L-isoleucine, L-phenylalanine, L-threonine, L-alanine, or L-glutamate had the greatest inhibitory effects on the uptake of CH_3Hg-Cys. Of note, however, is the fact that L-methionine did not have a significant effect on the uptake of CH_3Hg-Cys in the control MDCK cells. Among the MDCK cells expressing hOAT1, methionine did have a significant effect on the uptake of CH_3Hg-Cys, although it was the least effective in inhibiting the uptake of CH_3Hg-Cys.

Additional evidence for a second mechanism being involved in the uptake of CH_3Hg-Cys in the hOAT1-expressing cells was revealed by the additive effects that PAH and L-leucine (Fig. 9). The findings from these experiments indicate that the overall transport of CH_3Hg-Cys in the hOAT1-transfected cells was mediated by both hOAT1 and one or more amino acid transporters.

For the sake of comparison, we also studied the apical (luminal) uptake of CH_3Hg-Cys in NRK-52E cells, which are derived from the proximal tubule of the rat. Assessment of the effects of amino acids on the uptake of CH_3Hg-Cys revealed that the apical uptake of CH_3Hg-Cys in the NRK-52E cells was due primarily to one or more Na^+-independent amino acid transporters. Although L-cysteine, L-leucine, L-isoleucine, and L-phenylalanine were most effective in inhibiting the uptake of CH_3Hg-Cys, L-methionine also had a significant inhibitory effect. Apical uptake of CH_3Hg-Cys was confirmed by the data obtained with PAH, probenecid, and glutarate. These substrates did not affect the uptake of CH_3Hg-Cys when it was presented to the apical plasma

membrane. This lack of effect is consistent with the fact that OAT1 is located predominantly on the basolateral membrane, which was apparently not accessible to a significant degree in the monolayer cultures of NRK-52E cells.

It is noteworthy to mention that molecular mimicry has been suggested to be involved in the uptake of Cys S-conjugates of CH_3Hg^+ in glial and endothelial cells [3, 19, 32]. CH_3Hg-*S*-Cys has been hypothesized to serve as a molecular mimic of the amino acid methionine at one or more neutral amino acid transporters (Clarkson, 1993). Recent data obtained from *Xenopus* oocytes expressing the neutral amino acid transporters LAT1 or LAT2 provide evidence supporting this hypothesis [4].

Although this form of molecular mimicry is supported in part by findings obtained from the NRK-52E cells, the findings obtained from the MDCK cells in the present study do not. Unfortunately, the present data do not provide sufficient evidence for one to identify the specific amino acid transporter(s) involved in the uptake CH_3Hg-Cys in either the MDCK cells or NRK-52E cells.

Decreased survival of hOAT1-expressing and control MDCK cells occurred following exposure to various toxic concentrations of CH_3Hg-Cys. However, the percentage of cells surviving 6 hours of exposure was significantly greater in the wild-type control cells than in the hOAT1-expressing cells. This pattern of increased severity of cellular injury in the hOAT1-expressing cells correlates with the fact that an additional mechanism (i.e., the activity hOAT1) contributed to the over level of uptake of CH_3Hg-Cys.

CONCLUSION

The findings from the present study represent the first line of direct molecular evidence from hOAT1-expressing renal epithelial cells implicating the basolateral organic anion/dicarboxylate transporter OAT1 in the renal epithelial uptake of, and intoxication with, Cys *S*-conjugates of CH_3Hg^+. These data support the hypothesis that one of the mechanisms by which proximal tubule take up CH_3Hg^+ involves the basolateral uptake of CH_3Hg-Cys via OAT1. Moreover, the present findings also indicate that the mercuric conjugate CH_3Hg-Cys can be transported by Na^+-independent amino acid transporters present in the plasma membranes of epithelial cells lining both proximal and distal segments of the nephron. Figure 12 presents a diagrammatic representation of the putative roles of OAT1 and amino acids transporters in the proximal tubular uptake of CH_3Hg^+ as the Cys *S*-conjugate, CH_3Hg-Cys.

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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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