Transport of a neurotoxicant by molecular mimicry: the methylmercury–L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2

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Methylmercury (MeHg) readily crosses cell membrane barriers to reach its target tissue, the brain. Although it is generally assumed that this rapid transport is due to simple diffusion, recent studies have demonstrated that MeHg is transported as a hydrophilic complex, and possibly as an L-cysteine complex on the ubiquitous L-type large neutral amino acid transporters (LATs). To test this hypothesis, studies were carried out in *Xenopus laevis* oocytes expressing two of the major L-type carriers in humans, LAT1–4F2 heavy chain (4F2hc) and LAT2–4F2hc. Oocytes expressing LAT1–4F2hc or LAT2–4F2hc demonstrated enhanced uptake of [¹⁴C]MeHg when administered as the L-cysteine or D,L-homocysteine complexes, but not when administered as the D-cysteine, N-acetyl-L-cysteine, penicillamine or GSH complexes. Kinetic analysis of transport indicated that the apparent affinities (K_m) of MeHg–L-cysteine uptake by LAT1 and LAT2 (98 ± 8 and $64 \pm 8 \mu\text{M}$ respectively) were comparable with those for methionine (99 ± 9 and $161 \pm 11 \mu\text{M}$), whereas the

$V_{\max}$ values were higher for MeHg–L-cysteine, indicating that it may be a better substrate than the endogenous amino acid. Uptake and efflux of [³H]methionine and [¹⁴C]MeHg–L-cysteine were *trans*-stimulated by leucine and phenylalanine, but not by glutamate, indicating that MeHg–L-cysteine is both a *cis*- and *trans*-substrate. In addition, [³H]methionine efflux was *trans*-stimulated by leucine and phenylalanine even in the presence of an inwardly directed methionine gradient, demonstrating concentrative transport by both LAT1 and LAT2. The present results describe a major molecular mechanism by which MeHg is transported across cell membranes and indicate that metal complexes may form a novel class of substrates for amino acid carriers. These transport proteins may therefore participate in metal ion homeostasis and toxicity.

Key words: amino acid carrier, amino acid exchanger, L-cysteine, metal ion homeostasis and toxicity, methionine.

INTRODUCTION

Methylmercury (MeHg) is a powerful neurotoxicant in all animals, including humans [1–4]. The developing brain is particularly sensitive to the effects of MeHg, and there is significant concern over cognitive and developmental abnormalities that may arise from prenatal exposure to MeHg in fish-eating populations [3,4]. Fish is the primary source of human exposure to MeHg, and many populations are exposed to potentially dangerous levels of MeHg in their diet [3].

Neither the mechanism by which MeHg enters the brain nor the mechanism of cytotoxicity has been elucidated at the molecular level. Following MeHg administration by either enteral or parenteral routes, MeHg rapidly distributes to all tissues within the body, achieving a steady-state distribution within a couple of hours [1]. MeHg concentrations tend to be higher in kidney, liver and red blood cells when compared with brain, skeletal muscle and cardiac muscle, although the differences among the tissues are relatively modest. The ability of MeHg to distribute throughout the body is often attributed to the presumed lipid solubility of this organometallic compound. This explanation is untenable, however, given the physicochemical properties of MeHg [1,5,6]. The concentrations of the free MeHg cation or of the lipid-soluble chloride complex are expected to be vanishingly small in biological tissues and, indeed, lipid-soluble compounds of MeHg have never been detected in tissues of

animals dosed with MeHg [1]. Most of the MeHg in tissues is normally complexed with water-soluble sulphhydryl-containing molecules, primarily with L-cysteine, GSH, haemoglobin, albumin and other cysteine-containing polypeptides. Mercury–sulphur bonds form spontaneously (non-enzymically) under physiological conditions and they have high thermodynamic stability, although these bonds are kinetically labile [5,6]. The formation constants of glutathione and cysteine mercaptides with MeHg are exceptionally high, with $\log k_1$ values of 15.9 and 15.7 mol/l respectively [5].

These observations have forced investigators to search for an alternative explanation for the widespread tissue distribution of MeHg. One possibility is that an endogenously formed MeHg complex is a substrate for transporters that are also widely distributed throughout the body. In support of this hypothesis, studies in intact animals and with cultured cells have suggested a critical role of the ubiquitous neutral amino acid transporters in tissue uptake and transcellular movement of MeHg [7–11]. These studies indicate that MeHg–L-cysteine, which is structurally similar to methionine, is a substrate for L-type large neutral amino acid transporters (LATs), the so-called ‘L-system’ carriers. Uptake of MeHg into isolated brain capillary endothelial cells [8] and HepG2 cells [10] was found to be faster when it was present as the L-cysteine complex, as compared with the GSH, Cys-Gly, γ -Glu-Cys, D-cysteine, N-acetyl-L-cysteine, L-penicillamine or albumin complexes. Uptake of MeHg–L-cysteine

Abbreviations used: 4F2hc, 4F2 heavy chain; BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylate; LAT, L-type large neutral amino acid transporter; MeAIB, α -(methylamino)isobutyrate; MeHg, methylmercury.

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was independent of Na^+ , stereoselective and was inhibited by system-L substrates L-leucine, L-valine and L-phenylalanine. Moreover, L-[^3H]leucine uptake was inhibited by MeHg-L-cysteine, suggesting that this complex is transported by an L-type amino acid carrier.

Two of the major LATs, LAT1 and LAT2, have recently been identified at the molecular level [12–16]. These transporters are composed of a heterodimer of the 4F2 heavy chain (4F2hc) and either the LAT1 or LAT2 light chains. LAT1 and LAT2 are expressed in many tissues, including brain, and mediate uptake of large neutral amino acids (for reviews see [17–21]). Interestingly, LAT1 is expressed at high levels on both the luminal and abluminal membranes of the endothelial cells that form the blood–brain barrier [22–24], and appears to be the major uptake system for large neutral amino acids into brain [22–27]. LAT1 functions as an amino acid exchanger [12,13], whereas the mechanism by which LAT2 mediates amino acid transport was more controversial [14–16], although a recent study demonstrates that LAT2 is also an exchanger [28]. Meier et al. [28] found that LAT1 and LAT2 are obligatory amino acid exchangers with relatively symmetrical substrate selectivities, but strongly asymmetrical substrate affinities, so that the intracellular amino acid concentration controls their activity.

The present results provide the first direct evidence that a metal complex is a substrate for amino acid transporters. MeHg–L-cysteine was found to be transported with high catalytic efficiency by both LAT1 and LAT2, indicating that these proteins may provide the route of MeHg entry into brain and other tissues and may account for the rapid and widespread tissue distribution of MeHg. These observations offer important insight into molecular mechanisms of MeHg disposition, toxicity and potential therapies, and provide new understandings and approaches to biomarkers for MeHg susceptibility. Moreover, these findings suggest that other metal complexes may be substrates for amino acid carriers, and that these proteins may therefore contribute to metal ion disposition and toxicity.

EXPERIMENTAL

Materials and animals

[^3H]Methionine (85 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.), and [^{14}C]MeHg (20.2 mCi/mmol) was from American Radiolabeled (St. Louis, MO, U.S.A.). γ -Glu-Cys was a gift from the Kohjin Co. (Tokyo, Japan). Other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) or J. T. Baker (Phillipsburg, NJ, U.S.A.). Mature *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI, U.S.A.). Animals were maintained under a constant light cycle at 18 °C.

Synthesis of cRNA

The cDNAs for human 4F2hc, LAT1 and LAT2 were kindly provided by Dr Francois Verrey, Institute of Physiology, University of Zurich, Switzerland. Capped cRNA was transcribed *in vitro* using T7 or SP6 mMESSAGE mMACHINE kit (Ambion, Austin, TX, U.S.A.). cRNAs were precipitated with lithium chloride and stored at -75°C until used for oocyte microinjection.

X. laevis oocyte preparation and microinjection

Isolation of *X. laevis* oocytes was performed as described by Goldin [29] and as described previously by Li et al. [30]. Frogs were anaesthetized by immersion for 15 min in ice-cold

water containing 0.3% tricaine (Sigma Chemical Co.). Oocytes were removed from the ovary and washed with Ca^{2+} -free OR-2 solution [82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 and 20 mM Hepes/Tris (pH 7.5)] and incubated at 20–23 °C with gentle shaking for 90 min in OR-2 solution supplemented with 2 mg/ml of collagenase (type IA; Sigma Chemical Co.). Oocytes were transferred to fresh collagenase solution after the first 45 min of incubation. Collagenase was removed by extensive washing in OR-2 solution at 20–23 °C. Stage V and VI defolliculated oocytes were selected and incubated at 18 °C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.82 mM MgSO_4 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 and 20 mM Hepes/Tris (pH 7.5)] supplemented with gentamicin (0.05 mg/ml). After 3–4 h of incubation, healthy oocytes were injected with either cRNA (5–20 ng/oocyte in 50 nl of water) or 50 nl of sterile water for control. The cRNAs for 4F2hc and LAT1, or for 4F2hc and LAT2, were mixed in a 1:1 ratio and always injected simultaneously. Injected oocytes were cultured at 18 °C with a daily change of culture medium for 3 days. Healthy oocytes with a clean brown animal half and distinct equator line were selected for experiments.

Uptake and efflux studies in oocytes

Transport measurements were performed 3 days after microinjection of cRNA. For uptake measurements, six oocytes were incubated at 25 °C for 1 h with 100 μl of modified Barth's solution containing either [^3H]methionine or different [^{14}C]MeHg complexes at various concentrations. Some oocytes were injected with 50 nl of 110 mM stocks of different amino acids, to give an intracellular concentration of approx. 10 mM, just prior to executing uptake measurements. Uptake was stopped by adding 2.5 ml of ice-cold modified Barth's solution, and oocytes were washed three times each with 2.5 ml of ice-cold modified Barth's solution. Two oocytes were placed in a polypropylene scintillation vial and were dissolved with 0.2 ml of 10% (w/v) SDS. Radioisotope was counted in a Beckman model 6500 scintillation counter (Fullerton, CA, U.S.A.) after addition of 5 ml of Opti-Fluor (Packard Instruments, Downers Grove, IL, U.S.A.).

For efflux measurements, healthy oocytes were selected and loaded with either [^3H]methionine, by incubating with 10 μM [^3H]methionine for 2–3 h (2–3 μCi in 0.5 ml of modified Barth's solution for 20–40 oocytes) at 25 °C, or [^{14}C]MeHg, by microinjecting 50 nl of a solution containing 11.1 mM L-cysteine and 1.1 mM [^{14}C]MeHg (a 10:1 molar ratio, for approximate intracellular concentrations of 1 and 0.1 mM respectively). To remove residual extracellular radioactivity, the oocytes were washed three times with 2.5 ml of modified Barth's solution at 4 °C. Oocytes were placed individually in scintillation vials, the surrounding medium was removed and 0.2 ml of modified Barth's solution containing various substrates or inhibitors was added. After incubation for 1 h at 25 °C, a pipette was used to transfer the incubation medium to a different scintillation vial. The oocytes were lysed in 0.2 ml of 10% (w/v) SDS and scintillation fluid was added to both the oocyte and its incubation medium.

Statistical analyses

Data were analysed by ANOVA, and the two-tailed Student's *t* test was used for comparisons with a control. $P < 0.05$ was considered statistically significant. Kinetic data from experiments measuring initial rates of uptake of radiolabelled substrate were fitted to the Michaelis–Menten equation by non-linear least-squares regression analysis, and V_{max} and K_m values with standard errors were derived from these curves. Data are expressed as means $\pm$ S.E.M.

RESULTS

Uptake of methionine and MeHg complexes by LAT1- and LAT2-expressing oocytes

Xenopus oocytes have endogenous amino acid transporters, so that control oocytes were able to accumulate methionine from medium containing 100 μ M [3 H]methionine (Figure 1A). However, methionine uptake was markedly enhanced in oocytes injected with cRNA for LAT 1–4F2hc or LAT 2–4F2hc (Figure 1A), confirming previous findings [12–16]. LAT1- or LAT2-expressing oocytes also demonstrated higher uptake of [14 C]MeHg when administered as 100 μ M MeHg–L-cysteine (Figure 1B). L-Cysteine was present at a 5-fold molar excess (500 μ M) in these experiments, so as to maintain MeHg as the L-cysteine complex. The LAT1- and LAT2-stimulated uptake of MeHg from 100 μ M MeHg–L-cysteine was significantly higher than that from 100 μ M methionine (Figure 1), indicating that MeHg–L-cysteine may be an excellent substrate for these amino acid transporters.

To test whether uptake was selective for the L-cysteine complex, additional studies measured [14 C]MeHg uptake when added as a mercaptide with different thiol-containing compounds (Figure 2). LAT1- and LAT2-mediated MeHg uptake was also enhanced

in the presence of D,L-homocysteine, whereas none of the other compounds tested were able to stimulate uptake (Figure 2). It is of interest that D-cysteine was ineffective, indicating that transport on both LAT1 and LAT2 is stereoselective. There was also no stimulation by *N*-acetyl-L-cysteine, GSH, penicillamine (dimethylcysteine), *N*-acetyl-penicillamine, dimercaptopropane-sulphonate, γ -Glu-Cys or Cys-Gly complexes (Figure 2), confirming the specificity of these transporters for large neutral amino acids or amino acid analogues.

Kinetics of methionine and MeHg–L-cysteine uptake

A comparison of the transport kinetics of MeHg–L-cysteine and methionine uptake by LAT1 and LAT2 (Figure 3) demonstrated that the apparent affinities (K_m values) for MeHg–L-cysteine uptake by LAT1 and LAT2 (98 ± 8 and 64 ± 8 μ M respectively) were comparable with those for methionine (99 ± 9 and 161 ± 11 μ M), whereas the V_{max} values were higher for MeHg–L-cysteine (286 ± 12 and 75 ± 11 pmol/oocyte per 15 min) versus methionine (97 ± 16 and 54 ± 12 pmol/oocyte per 15 min). Indeed, because these measurements of MeHg–L-cysteine uptake had to be performed in the presence of a 3-fold molar excess of L-cysteine, which is itself a substrate for these amino acid carriers, it is likely that the actual K_m values for MeHg–L-cysteine uptake are lower and V_{max} values higher than those calculated from the data illustrated in Figure 3. Despite these limitations, the calculated apparent K_m values for methionine and MeHg–L-cysteine transport are comparable with the lowest K_m values reported for LAT1 and LAT2, which are in the 30–150 μ M range [12–16]. These low K_m and high V_{max} values for MeHg–L-cysteine indicate that this metal complex is transported with high catalytic efficiency by both LAT1 and LAT2.

Uptake of [3 H]methionine and [14 C]MeHg–L-cysteine by LAT1 and LAT2 was inhibited to the same extent by known amino acid substrates of these carriers (Figure 4). [3 H]Methionine uptake was inhibited by the L-system substrates L-leucine, 2-amino-bicyclo(2,2,1)heptane-2-carboxylate (BCH) and L-cysteine, as expected, but was also inhibited by the MeHg–L-cysteine complex and by the structurally related *S*-ethyl-L-cysteine (Figure 4A), indicating that these amino acid derivatives can also interact with these carriers. In contrast, methionine uptake was not affected by the A-system substrate α -(methylamino)isobutyrate (MeAIB). Likewise, [14 C]MeHg–L-cysteine uptake was inhibited by L-methionine, L-leucine, BCH and *S*-ethyl-L-cysteine, but not by MeAIB (Figure 4B).

MeHg–L-cysteine is both a *cis*- and *trans*-substrate for LAT1 and LAT2

To confirm that LAT1 and LAT2 function as exchangers and to establish whether MeHg–L-cysteine may be an intracellular substrate for LAT1 and LAT2, additional studies measured the effects of *trans*-amino acids on uptake (Figure 5) and efflux (Figure 6) of [3 H]methionine and [14 C]MeHg–L-cysteine.

To measure uptake of these radiolabelled substrates, control oocytes and oocytes expressing either LAT1 or LAT2 were microinjected with 50 nl of water or with 50 nl of concentrated stock solutions of L-leucine, L-phenylalanine and L-glutamate to achieve intracellular concentrations of approx. 10 mM. Uptake of 20 μ M [3 H]methionine and 20 μ M [14 C]MeHg–L-cysteine was measured over a 1 h period (Figure 5). For both LAT1- and LAT2-expressing oocytes, uptake of methionine and MeHg–L-cysteine was higher in oocytes that had been preloaded with leucine and phenylalanine, but not in those injected with

Figure 1 Time course for 100 μ M [3 H]methionine (A) and 100 μ M [14 C]MeHg–L-cysteine (B) uptake in LAT1- and LAT2-expressing *Xenopus* oocytes

Oocytes were microinjected with either water, LAT1–4F2hc or LAT2–4F2hc cRNA (5 ng of 4F2hc and 5 ng of either LAT1 or LAT2 per oocyte), cultured for 3 days at 18 °C, and uptake was measured at 25 °C over a 3 h time period. L-Cysteine was present at a 5-fold molar excess (500 μ M) to MeHg. Values are means $\pm$ S.E.M., $n = 3$ –4 independent experiments. Results for (A) and (B) were generated from the same batches of oocytes to allow a direct comparison of uptake rates.

Figure 2 Uptake of [^3H]methionine or [^{14}C]MeHg when administered as distinct mercaptide complexes into LAT1- and LAT2-expressing oocytes

Oocytes were microinjected with either water (control), LAT1-4F2hc or LAT2-4F2hc cRNA (5 ng of 4F2hc and 5 ng of either LAT1 or LAT2 per oocyte) and cultured for 3 days at 18 °C. They were incubated with 20 μM [^3H]methionine (**A**) or 20 μM [^{14}C]MeHg in the presence of 100 μM amino acids or other thiols (**B**). Uptake was measured for 1 h at 25 °C. Values are means $\pm$ S.E.M., $n = 4$ –6 separate experiments, each performed in triplicate. * $P < 0.05$ compared with control.

glutamate (Figure 5). Glutamate is a charged amino acid that is not a substrate for LAT1 or LAT2 and was therefore not expected to stimulate uptake in this experiment.

To measure efflux of [^3H]methionine, oocytes were preloaded by incubation with 10 μM [^3H]methionine for 2 h at 25 °C. After washing, efflux was measured for 1 h in medium containing 1 mM amino acids. [^3H]Methionine efflux in both LAT1- and LAT2-expressing oocytes was dramatically accelerated by extracellular L-leucine and L-phenylalanine, but was unaffected by L-glutamate (Figure 6A), indicating that both LAT1 and LAT2 function as amino acid exchangers. Interestingly, [^3H]methionine efflux was also markedly stimulated by L-cysteine and MeHg-L-cysteine, indicating that these compounds are exchanged substrates for LAT1 and LAT2.

To confirm directly that these proteins function as exchangers, efflux of [^3H]methionine from LAT1- and LAT2-expressing oocytes was measured in the presence of 200 μM unlabelled methionine in the culture medium, in order to establish an inwardly directed methionine gradient (Figure 7). Oocytes are known to contain relatively low concentrations of methionine (25 μM) [31] and the amount of [^3H]methionine incorporated during the loading and subsequent washing protocol was less than 20 μM (e.g., see Figure 3B). Because of this inwardly directed methionine gradient, any stimulation of [^3H]methionine efflux would have to occur against a chemical gradient. As illustrated in Figure 7, [^3H]methionine efflux in both LAT1- and LAT2-expressing oocytes was stimulated by extracellular L-leucine and L-phenylalanine, even in the presence 200 μM unlabelled methionine in the culture medium. Note that extracellular methionine by itself markedly stimulated [^3H]methionine efflux in both LAT1- and LAT2-expressing oocytes (compare control values in Figures 6A and 7). These data provide direct evidence for transport against a concentration gradient and demonstrate that human LAT2 functions as an amino acid exchanger. How-

ever, because these efflux measurements were made over a relatively long time interval (1 h), these data do not provide information on catalytic efficiency of the exchange process for the various substrates.

To measure efflux of [^{14}C]MeHg-L-cysteine, oocytes were preloaded by microinjection of 50 nl of a solution containing 1.1 mM [^{14}C]MeHg-L-cysteine and 10 mM excess L-cysteine to achieve initial intracellular concentrations of approx. 0.1 and 1 mM respectively. However, because of the kinetic lability of the mercury-sulphur bond, the injected MeHg will rapidly redistribute among all available intracellular ligands (principally glutathione, 2.5 mM, and protein thiols, approx. 20 mM), so that only a small fraction (probably 2–4%) will remain as the MeHg-L-cysteine complex within the cell. Although the intracellular concentration of MeHg-L-cysteine will be low under these experimental conditions, it should still permit the assessment of efflux that may be mediated by the expressed amino acid transporters. To trap any [^{14}C]MeHg that may be effluxed into the culture medium and thus prevent its re-uptake or binding to the oocytes, this experiment was performed in the presence of 0.5% BSA. The results (Figure 6B) indicate that MeHg efflux was stimulated in LAT1-expressing oocytes incubated in medium containing L-leucine and L-phenylalanine, but not in medium containing L-glutamate, suggesting that MeHg-L-cysteine is an intracellular substrate for this amino acid exchanger. On the other hand, efflux rates in LAT2-expressing oocytes were very low, and extracellular amino acids failed to increase this low level of transport activity (Figure 6B).

DISCUSSION

MeHg has very high mobility in the body [1]. It crosses diffusion barriers, such as the blood-brain and placental barriers virtually unhindered, and rapidly distributes to all tissues, both foetal and

Figure 3 Concentration-dependence of [3 H]methionine (A and B) and [14 C]MeHg–L-cysteine (C and D) uptake into LAT1- and LAT2-expressing oocytes

Oocytes were microinjected with either water, LAT1–4F2hc (A and C) or LAT2–4F2hc (B and D) cRNA (5 ng of 4F2hc and 5 ng of either LAT1 or LAT2 per oocyte). (A and B) Oocytes were exposed to 10–400 μ M methionine. (C and D) Oocytes were exposed to 10–400 μ M MeHg, with an L-cysteine/MeHg ratio of 3:1. Uptake was measured for 15 min at 25 °C. Insets illustrate Eadie–Hofstee plots of the data. Uptake values for control (water-injected) oocytes were subtracted from data obtained from LAT1- or LAT2-expressing oocytes. K_m values for MeHg–L-cysteine uptake by LAT1 and LAT2 (98 ± 8 and 64 ± 8 μ M respectively) were comparable with those for methionine uptake (99 ± 9 and 161 ± 11 μ M), whereas V_{max} values for MeHg–L-cysteine uptake (286 ± 12 and 75 ± 11 pmol/oocyte per 15 min) were higher than those for methionine (97 ± 16 and 54 ± 12 pmol/oocyte per 15 min). Values are means $\pm$ S.E.M., $n = 3$ –4 independent experiments, each performed in triplicate.

Figure 4 *Cis*-inhibition of [3 H]methionine and [14 C]MeHg–L-cysteine uptake into LAT1- and LAT2-expressing oocytes by L-amino acids, thiols and amino acid analogues

Oocytes were exposed to 10 μ M [3 H]methionine (A) or 10 μ M [14 C]MeHg–L-cysteine plus 90 μ M L-cysteine (B) in the absence (control) or presence of 1 mM of various inhibitors for 1 h at 25 °C. Values are means $\pm$ S.E.M. of three experiments, each performed in triplicate. All are significantly different from control ($P < 0.05$ by Student's *t* test), except for MeAIB.

Figure 5 Trans-stimulation of [^3H]methionine and [^{14}C]MeHg-L-cysteine uptake in LAT1- or LAT2-expressing oocytes by intracellular amino acid substrates

Oocytes were injected with 7.5 ng of 4F2hc cRNA and 7.5 ng of either LAT1 or LAT2 cRNA and cultured for 3 days. To load with 10 mM L-leucine, L-phenylalanine and L-glutamate, oocytes were injected with 50 nl of 110 mM stock solutions of these amino acids or with water 30 min before uptake measurements. Oocytes were washed in modified Barth's solution and uptake of 20 μM [^3H]methionine (A) or 20 μM [^{14}C]MeHg-L-cysteine (B) was measured at 25 °C for 1 h. Values are means $\pm$ S.E.M. of four experiments, each performed in triplicate. * $P < 0.05$ compared with control.

adult. The molecular mechanism responsible for this unusual pharmacokinetic behaviour has not yet been identified, although it has been suggested that the ubiquitously distributed amino acid transporters may play a role [7–11]. The present study tested this hypothesis by examining whether the MeHg-L-cysteine complex, a normal constituent of blood plasma and intracellular spaces, is a substrate for two of the major human neutral amino acid transporters, LAT1 and LAT2. Our results provide direct support of this hypothesis and provide the first direct evidence that amino acid carriers are involved in metal transport. Moreover, our results provide additional evidence that LAT1 and LAT2 function as amino acid exchangers.

All cells require a continuous supply of amino acids for normal functions. Uptake and release of amino acids is accomplished by several families of amino acid transporters, many of which have recently been identified at the molecular level [12–21]. These transporters are differentially expressed in multiple tissues and most are multi-specific; that is, each can accept a variety of structurally related amino acids or amino acid ana-

Figure 6 Trans-stimulation of [^3H]methionine and [^{14}C] MeHg-L-cysteine efflux in LAT1- or LAT2-expressing oocytes by extracellular amino acid substrates

Oocytes were injected with 7.5 ng of 4F2hc cRNA and 7.5 ng of either LAT1 or LAT2 cRNA and cultured for 3 days at 18 °C. (A) To load with methionine, oocytes were incubated with 10 μM [^3H]methionine for 2 h at 25 °C. Oocytes were then washed in modified Barth's solution to remove extracellular radioactivity, and efflux was measured over the next 60 min in modified Barth's solution containing various substrates or inhibitors at 1 mM. (B) To load with [^{14}C]MeHg-L-cysteine, oocytes were injected with 50 nl of 1.1 mM [^{14}C]MeHg-L-cysteine and 10 mM excess L-cysteine. Oocytes were washed in modified Barth's solution and efflux was measured over the next 60 min in modified Barth's solution containing 0.5% BSA, along with various substrates and inhibitors at 5 mM. Values are means $\pm$ S.E.M., $n = 3$ separate experiments, each performed in quadruplicate. * $P < 0.05$ compared with control.

logues as substrates. For neutral amino acids, several transporters have been identified, including the LAT1 and LAT2 proteins. LAT1 and LAT2 are expressed in many tissues and appear to be the major carriers for large neutral amino acids in multiple human tissues, including the brain [22–27]. Human LAT1 mRNA is highly expressed in the human foetal liver, bone marrow, placenta, testis and brain [18,32], whereas LAT2 mRNA is abundant in kidney and intestine [16]. Interestingly, in brain capillary endothelial cells, LAT1 is present on both the luminal and abluminal membranes [22–24], indicating that this protein contributes to transendothelial transport of amino acids.

LAT1 and LAT2 function as amino acid exchangers, so that uptake of a given amino acid is directly coupled to the efflux of an intracellular amino acid down its chemical gradient [12,13,28]. The present results support this conclusion by demonstrating that [^3H]methionine uptake and efflux rates in LAT1- and LAT2-

Figure 7 *Trans*-stimulation of [3 H]methionine efflux in LAT1- or LAT2-expressing oocytes by extracellular substrates in the presence of 200 μ M methionine

Oocytes were injected with 7.5 ng of 4F2hc cRNA and 7.5 ng of either LAT1 or LAT2 cRNA and cultured for 3 days at 18 °C. Oocytes were loaded by pre-incubating with 10 μ M [3 H]methionine for 2 h and were then washed in modified Barth's solution to remove extracellular radioactivity. Efflux was measured over the next 60 min in modified Barth's solution containing 200 μ M unlabelled methionine alone (control) or 200 μ M methionine plus 1 mM of the indicated amino acids. Values are means $\pm$ S.E.M., $n = 3$ separate experiments each performed in triplicates. * $P < 0.05$ compared with control.

expressing oocytes were markedly enhanced in the presence of imposed amino acid gradients (Figures 5 and 6). The extent of *trans*-stimulation was quite high (several-fold stimulation), indicating that transport is unlikely to be mediated by facilitated diffusion and is more consistent with coupled exchange. Both LAT1 and LAT2 were *cis*- and *trans*-stimulated by neutral amino acids (Figures 5 and 6), indicating a common mode of transport and of energy coupling. Moreover, Figure 7 demonstrates that both LAT1 and LAT2 can transport against a concentration gradient, providing direct evidence for an exchange mechanism of transport.

The present results also demonstrate that methionine and MeHg–L-cysteine are equally good substrates for LAT1 and LAT2. This is perhaps not surprising given that the molecular structure of MeHg–L-cysteine is similar to that of methionine, but it provides direct confirmation that these transporters can accept amino acid analogues, including potentially toxic analogues. Indeed, transport rates were higher for MeHg–L-cysteine than for methionine, indicating that this metal complex is transported with a higher catalytic efficiency than the endogenous amino acid by LAT1 and LAT2 (Figure 3). These findings with MeHg raise the possibility that other metals and other metal complexes may also function as substrates for amino acid transporters and possibly for other multi-specific organic solute carriers [11], although this has not yet been tested.

In addition, the present results demonstrate that MeHg–L-cysteine is both a *cis*- and *trans*-substrate for LAT1, and, most probably, for LAT2 as well. These proteins may therefore mediate uptake or efflux of MeHg–L-cysteine depending on the intracellular and extracellular concentrations of competing substrates and inhibitors and their relative affinities for the transporters. Recent work by Meier et al. [28] indicate that these transporters have a relatively symmetrical substrate selectivities, but strongly asymmetrical substrate affinities, so that the intracellular amino acid concentration controls their activity. Thus these proteins may contribute to net vectorial movement of

MeHg across epithelial or endothelial barriers, including the blood–brain barrier. As indicated above, LAT1 is expressed on both the luminal and abluminal membranes of brain capillary endothelial cells [22–24].

The molecular identification of a mechanism of MeHg transport across cell membranes opens up new understandings and approaches to biomarkers for exposure, effects and susceptibility. As in the case of genes that affect the metabolism of other toxicants, the transporter genes most probably play a key role in the response of the organism to MeHg and to its susceptibility. One example of a transporter that alters MeHg disposition and toxicity is the multidrug resistance-associated protein-2 ('MRP2'). This protein is localized to the canalicular membrane of hepatocytes and functions to transport glutathione, glutathione *S*-conjugates, glutathione mercaptides and other organic anions from hepatocytes into bile [33]. Both the activity of this transport protein and its ability to secrete MeHg into bile is low during the period of suckling, but dramatically switches on to full transport capacity at the time of weaning [34]. Likewise, animals that have a genetic defect in this transporter fail to secrete MeHg and glutathione into bile [35]. In terms of biomarkers of MeHg exposure, human scalp hair has long been used in epidemiological studies as the biomarker of choice [3]. The transport of MeHg into the hair follicle may also involve amino acid transporters, and thus the mercury in hair monitors the transportable species in blood, namely MeHg–L-cysteine.

The identification of a mechanism of MeHg transport across cell membranes may also be exploited to design more effective therapeutic strategies for removing MeHg from the body. The most effective method of protecting against MeHg toxicity is to increase elimination of the metal from cells and tissues. Current therapy for MeHg poisoning relies on chelators to enhance elimination into urine or faeces. The present results indicate that the combined use of chelators and inhibitors of neutral amino acid transporters may be even more effective in limiting tissue uptake, while at the same time enhancing excretion from the body.

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