Accelerated Urinary Excretion of Methylmercury following Administration of Its Antidote N-Acetylcysteine Requires Mrp2/Abcc2, the Apical Multidrug Resistance-Associated Protein

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ABSTRACT

N-Acetylcysteine (NAC) is a sulfhydryl-containing compound that produces a dramatic acceleration of urinary methylmercury (MeHg) excretion in poisoned mice, but the molecular mechanism for this effect is poorly defined. MeHg readily binds to NAC to form the MeHg-NAC complex, and recent studies indicate that this complex is an excellent substrate for the basolateral organic anion transporter (Oat)-1, Oat1/Slc22a6, thus potentially explaining the uptake from blood into the renal tubular cells. The present study tested the hypothesis that intracellular MeHg is subsequently transported across the apical membrane of the cells into the tubular fluid as a MeHg-NAC complex using the multidrug resistance-associated protein-2 (Mrp2/Abcc2). NAC markedly stimulated urinary [14C]MeHg excretion in wild-type Wistar rats, and a second dose of NAC was as effective as the first dose in stimulating MeHg excretion. In contrast with the normal Wistar rats, NAC was much less effective at stimulating urinary MeHg excretion in the Mrp2-deficient (TR/H11002) Wistar rats. The TR-rats excreted only ~30% of the MeHg excreted by the wild-type animals. To directly test whether MeHg-NAC is a substrate for Mrp2, studies were carried out in plasma membrane vesicles isolated from livers of TR- and control Wistar rats. Transport of MeHg-NAC was lower in vesicles prepared from TR-rats, whereas transport of MeHg-cysteine was similar in control and TR-rats. These results indicate that Mrp2 is involved in urinary MeHg excretion after NAC administration and suggest that the transported molecule is most likely the MeHg-NAC complex.

The main human exposure to the common environmental toxicant methylmercury (MeHg) is from eating fish in which the compound has bioaccumulated. Once ingested, MeHg is absorbed in the intestine and readily distributes to all tissues of the body, including its target organ, the brain, and the developing fetus. The fetal brain is much more susceptible to the toxic effects of methylmercury than is the mature brain (Clarkson, 1972, 2002).

Once absorbed, MeHg has a half-life of around 45 to 70 days in humans (Clarkson, 2002). In the case of exposure to high levels of MeHg, the only way to counteract its deleterious effects is to accelerate its excretion. Past and current therapies for removing MeHg from the body include hemodialysis, exchange transfusion, thiol resin, and chelation therapy, with the latter seen as the least invasive and most commonly used method (Clarkson et al., 1973, 1981; Al-Abbasi et al., 1978; Elhassani, 1982; Lund et al., 1984). The most widely used chelating agents are dimercaptosuccinic acid and dimercaptopropanesulfonate (DMPS). Both are water-soluble, anionic, sulfhydryl-containing compounds to which MeHg readily bind. However, another agent, the amino acid derivative N-acetyl-L-cysteine (NAC), has more recently been shown to be effective in increasing urinary MeHg excretion in mice (Ballatori et al., 1998a). Mice administered NAC in their drinking water after methylmercury exposure excreted 47 to 54% of the MeHg dose in 48 h compared with 4 to 10% in control animals.

Although NAC produces a profound acceleration of urinary MeHg excretion, the mechanism for this effect is unknown. NAC itself is excreted in urine at high concentrations (Borgström et al., 1986; Aposhian et al., 1995; Ballatori et al.,...
1998a), suggesting that the MeHg-NAC complex may also be excreted into urine. The MeHg-NAC complex appears to be an excellent substrate for the renal organic anion transporter-1, Oat1/Slc22a6, with an apparent $K_m$ value of 31 μM (Koh et al., 2002). Because Oat1/Slc22a6 is localized to the basolateral membrane (Tojo et al., 1999; Sekine et al., 2000), it is most likely a major route of entry for the MeHg-NAC complex into renal tubule cells. The present study tested the hypothesis that MeHg is transported from the cells into renal tubular fluid as the anionic MeHg-NAC complex, using the apical multidiure resonance-associated protein-2 (Mrp2/Abcc2) protein. Mrp2 belongs to subfamily C of the ATP binding cassette (ABC) superfamily of transporters (gene symbol Abcc).

It is an organic anion transport pump with a broad range of substrates. In hepatocytes, Mrp2 functions to transport glutathione, glucuronide, and sulfate conjugates, as well as glutathione itself across the canalicular membrane into bile (Ballatori, 1994, 2002; Borst et al., 2000; Kruh and Belinsky, 2003). Mrp2 has been localized to the renal brush-border membrane (Schaub et al., 1997) and may have a similar excretory function in the kidney. The present results demonstrate that NAC is highly effective in accelerating urinary MeHg excretion in rats, that Mrp2 plays a major role in urinary MeHg excretion after NAC administration, and that the transported molecule is most likely the MeHg-NAC complex.

Materials and Methods

Materials. Male and female wild-type Wistar rats (200–250 g) were purchased from Charles River Laboratories (Kingston, NY). The animals were housed in our on-site animal care facility with access to food and water ad libitum until the time of experimentation. Transport-deficient (TR−) Wistar rats were taken from our breeding colony, which was established with animals kindly provided by Dr. Mary Vore at the University of Kentucky (Lexington, KY). Experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Urine Collection from Rats in Metabolic Cages. Female wild-type and TR− Wistar rats were placed individually in stainless steel metabolic cages (Lab Products Inc., Rochelle Park, NJ) and were allowed to acclimate to the cages for 3 days. All rats were injected i.p. with $[^{14}C]$MeHg (2 ml/kg of 0.5 μmol of MeHg/kg with 5 μCi/kg $^{14}$C). After 48 h, some of the wild-type and TR− Wistar rats were given NAC in their drinking water (10 mg/ml), whereas others served as control. Urine and feces were collected every 24 h, and $[^{14}C]$MeHg content was analyzed. At the end of the experiments, tissues were removed and weighed as described above.

Determination of $[^{14}C]$MeHg Content. A small cross-section of each tissue was taken for analysis of $[^{14}C]$MeHg content. Tissue, fecal, and blood samples (approximately 0.2–0.4 g of tissue, 1.0 ml of blood) were placed in preweighed 20-ml glass vials. Solvable (Packard Instrument Company, Meriden, CT) was added to each vial (1.0 ml/0.1 g tissue or 2.0 ml/1.0 ml blood), which was then heated to 60°C for 2 to 3 h. The vials were allowed to cool to room temperature, and 0.1 ml of 0.1 M EDTA/1.0 ml of Solvable was added to each vial of blood. $H_2O_2$ (30%) was then added in 0.1-ml aliquots (0.3 ml/1.0 ml Solvable for blood or 0.1 ml/1.0 ml Solvable for tissues), with swirling between additions. After standing at room temperature for 15 to 30 min, the vials were capped tightly and heated to 60°C for 1 h and allowed to cool back to room temperature before counting. For urine, 5.0 ml of Opti-Fluo (Packard Instrument Company) was added to 50 to 100 μl of urine. For the other tissues, 5.0 ml of Opti-Fluo was added to 200 μl of the solubilized solution. After the addition of scintillation fluid, the samples were allowed to sit overnight at room temperature before counting. Samples were counted in a Beckman LS 6500 scintillation counter (Beckman Coulter, Fullerton, CA). Total blood volume was estimated as 6% of body weight.

Preparation of Liver Plasma Membrane Vesicles. The methods for isolating plasma membranes were modified from Blitzer and Donovan (1984) and Smith and Ploch (1991). In brief, tissue was homogenized in sterile, ice-cold 5 mM HEPES/Tris buffer, pH 7.5, with a Dounce homogenizer and loose-fitting pestle. Membranes were pelleted by centrifugation (10,000g, 20 min), and the mixed plasma membranes were resuspended and homogenized with a tight-fitting (type B) pestle. The homogenate was then spun through a two-step sucrose gradient (16 and 35% (w/v) sucrose density) in an SA-600 rotor (30,000g, 40 min). The discrete band formed at the 16/35% interface was collected and washed twice by diluting to 40 ml with transport buffer (250 mM sucrose, 10 mM HEPES/Tris, pH 7.5, 20 mM KCl, and 0.20 mM CaCl$_2$) and collected at 15,000g for 10 min. The final pellet was resuspended in 0.5 to 1 ml of transport buffer and was passed through a 25-gauge needle (10×) and stored in aliquots at −80°C. Protein was determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

Transport Measurements. Frozen membrane suspensions were quickly thawed by immersion in a 37°C water bath, diluted to the desired protein concentration, and passed repeatedly (10×) through a 25-gauge needle. To prevent the membrane potential from influencing the transport of substrates, the experiments were voltage-clamped using the potassium ionophore valinomycin (10 μg/ml protein) in the presence of 20 mM KCl in the incubation and vesicle suspension media. In all experiments, an excess of NAC was used to ensure that MeHg was present as the NAC complex. Specific details regarding the NAC/MeHg ratio are given in the legends. Uptake into membrane vesicles was measured by a rapid Millipore filtration technique (Millipore Corporation, Billerica, MA; Meier et al., 1984; Ballatori et al., 1986). Membrane suspensions (5–16 μg of protein in 20 μl) were preincubated at 37°C for 10 min. Uptake studies were initiated by the addition of 80 μl of transport buffer, also prewarmed, containing the respective radiolabeled substrates. Transport was terminated by the addition of 0.9 ml of ice-cold stop solution. Unless otherwise indicated, the stop solution consisted of 250 mM sucrose, 10 mM HEPES/Tris, pH 7.5, 20 mM KCl, and 500 μM DMPS. DMPS,
a water-soluble compound containing two sulphydryl groups, was used to remove MeHg nonspecifically bound to the outside of the vesicles. Membrane vesicle-associated ligand was separated from free ligand by rapid filtration through a 0.45-μm filter (Millipore, HAWP). Vesicles were then washed with an additional 4 ml of ice-cold stop solution. Filters containing radioisotopes were placed in 5 ml of Opti-Fluor (PerkinElmer Life and Analytical Sciences, Boston, MA) and counted in a Beckman LS 6500 scintillation counter.

**Statistical Analysis.** Differences between NAC doses were analyzed by analysis of variance, followed by Student’s *t* test. Statistical significance was assigned at *p* < 0.05.

**Results**

NAC Markedly Stimulates Urinary MeHg Excretion in Normal Wistar Rats but Is Largely Ineffective in Mrp2/Abcc2-Deficient Rats. Previous studies demonstrated that NAC is efficient at stimulating urinary excretion of MeHg in mice (Ballatori, 1998a,b), but other animal species were not examined. To test whether NAC is also effective in rats, wild-type male and female Wistar rats were injected i.v. with a nontoxic dose of [14C]MeHg (0.5 μmol/kg) and subsequently received NAC (0.42 or 0.84 mmol/kg i.v.). Urine was collected at 0.5-h intervals throughout the experiment and analyzed for [14C]MeHg. Control rats excreted trace amounts of [14C]MeHg in urine, whereas NAC was able to stimulate [14C]MeHg excretion in a dose-dependent fashion (Fig. 1, A and C). Males were able to excrete a slightly larger amount with a single equivalent dose of NAC (Fig. 1; Table 1). This gender difference became significant at the higher dose of NAC (Table 1). Urine flow did not appear to be significantly effected by NAC (Fig. 1, B and D). When a second dose of NAC was administered at 4 h, an equivalent amount of [14C]MeHg was excreted as after the first NAC dose (Fig. 2). In these two dose experiments, males and females were able to excrete comparable amounts of MeHg (5.9 ± 1.2 and 15.1 ± 3.2 for males versus 3.5 ± 1.2 and 14.8 ± 2.2 for females at low and high doses of NAC, respectively).

TR– Wistar rats were used to investigate the role of Mrp2 in the urinary excretion of MeHg after NAC administration. TR– rats have a mutation in their Mrp2 gene that introduces
Male rats and \([14C]MeHg (0.5\, \text{mmol/kg})\) was measured after NAC doses of 0.42 or 0.84 mmol/kg. Minimal in both wild-type and TR rats was observed among the different groups of rats. In the absence of NAC in the drinking water, urinary MeHg excretion was consumed among the different groups of rats. In the absence of NAC, the TR rats also excreted trace amounts of MeHg into urine (Fig. 1, E–H). However, in contrast with wild-type Wistar rats, NAC was much less effective at stimulating urinary MeHg excretion in the Mrp2-deficient animals. Cumulative MeHg dose excreted in urine over this time interval was only 11 to 34% of that observed in wild-type rats (Table 1).

To confirm the effects noted in this short-term study in anesthetized rats, a longer term study was performed in which the rats received an injection of \([14C]MeHg (0.5\, \text{mmol/kg i.p.})\), and after 48 h, some animals were given NAC in their drinking water (10 mg/ml) for the next 10 days. No significant differences were observed in the amount of water consumed among the different groups of rats. In the absence of NAC in the drinking water, urinary MeHg excretion was minimal in both wild-type and TR– rats. However, NAC administration in drinking water resulted in a dramatic increase in urinary MeHg excretion in wild-type rats but had no effect on MeHg excretion in TR– rats (Fig. 3). Fecal excretion of MeHg was unaffected by NAC in both the wild-type and TR– rats (data not shown).

NAC administration efficiently mobilized MeHg from the blood, liver, and kidney in wild-type rats, whereas it had no effect on tissue levels of MeHg in TR– rats (Fig. 4). Brain MeHg levels were also lower in wild-type rats that received NAC, but this difference was not statistically significant.

**ATP-Dependent Transport of the MeHg-NAC Complex in Liver Plasma Membrane Vesicles.** Liver plasma membrane vesicles isolated from wild-type and TR– rats were employed to further demonstrate the involvement of Mrp2 in the transport of the MeHg-NAC complex. ATP-dependent transport of MeHg-NAC was significantly higher in liver membrane vesicles prepared from wild-type rats versus TR– rats, whereas there was no difference in MeHg transport when given as the L-cysteine complex (Fig. 5).

**Discussion**

The neurotoxicant MeHg is a highly reactive compound, which readily reaches an equilibrium state as complexes with free thiol groups in the body. It is likely that these MeHg-thiol complexes are the main forms by which MeHg is transported. It has been shown that MeHg can be transported as an L-cysteine complex via amino acid transporters such as the L-type large neutral amino acid carriers LAT1 and LAT2 (Simmons-Willis et al., 2002). The MeHg-L-cysteine complex is structurally similar to methionine, an endogenous substrate of these transporters, and it is likely that other MeHg complexes may also mimic the substrates of other transporters. The present study demonstrates that the complex formed

**TABLE 1**

<table>
<thead>
<tr>
<th>Control</th>
<th>0.42 mmol/kg NAC</th>
<th>0.84 mmol/kg NAC</th>
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<tbody>
<tr>
<td>WT male</td>
<td>0.09 ± 0.01</td>
<td>2.82 ± 0.88</td>
</tr>
<tr>
<td>TR– male</td>
<td>0.09 ± 0.02</td>
<td>0.31 ± 0.04b</td>
</tr>
<tr>
<td>WT female</td>
<td>0.06 ± 0.01</td>
<td>1.68 ± 0.26</td>
</tr>
<tr>
<td>TR– female</td>
<td>0.07 ± 0.01</td>
<td>0.30 ± 0.05b</td>
</tr>
</tbody>
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* Significantly different from wild-type animals of the same gender, \(p < 0.05\).
* Significantly different between genders of the same type, \(p < 0.05\).
with the exogenous compound NAC is a substrate for another transporter, the apically localized Mrp2 organic anion pump. MeHg has a half-life for elimination in humans of 45 to 70 days, which allows this toxicant to accumulate. Under normal conditions, the main route of MeHg elimination is via excretion through bile and the intestine into feces, whereas very little is detectable in the urine. In cases in which there is a high body burden, the only way to minimize toxicity is by increasing its elimination rate. Current therapy for MeHg poisoning is via the administration of a chelating agent and its subsequent elimination from the body. The currently used chelating agents dimercaptosuccinic acid and DMPS are anionic, dithiol-containing compounds that can complex with free metals, including essential metals, and are excreted. Previous work has demonstrated that NAC is also able to increase the urinary excretion of MeHg, at least in mice (Ballatori et al., 1998a). NAC is used clinically in the treatment of acetaminophen overdose and possesses a number of desirable properties as a potential MeHg antidote; it is nontoxic, can be administered orally, it selectively forms a stable complex with MeHg via its thiol group, NAC itself is readily toxic to other intracellular thiols, especially glutathione. Under our experimental conditions, however, it is likely that a portion will stay as a complex with NAC, due to the high NAC concentration. Glutathione complexes have been shown to be substrates for Mrp2, the apically localized organic anion and conjugate pump (Ballatori, 2002), and this may also be true of the MeHg-NAC complex. This hypothesis was tested using TR− Wistar rats. TR− rats have a natural point mutation in their Mrp2 gene causing the formation of an early stop codon and a nonfunctional, truncated protein. NAC administration to these rats had minimal effects at the lower dose tested and only a very modest effect at the higher dose. Again, the male rats were somewhat more responsive than female rats. The observation that there is still a modest increase in MeHg excretion in the TR− rats indicates that although Mrp2 is the major contributor to MeHg-NAC excretion, it is not the only mechanism involved. Simple glomerular filtration probably accounts for much of this increase; however, other transporters may also be involved. Mrp4, another member of the MRP/CFTR/ABCC family, has also been localized to the apical membrane of renal tubular cells, and a compensatory increase in the expression of Mrp4 in the liver and kidney of TR− rats has been reported (Chen et al., 2005). Mrp4 has some overlapping substrate specificity with Mrp2 and may also be able to transport the MeHg-NAC complex. However, such a possible compensatory role of Mrp4 was not evident in the study in which animals were placed on drinking water containing NAC for 10 days (Fig. 3). There was no significant difference in urinary MeHg excretion in wild-type rats without NAC or in TR− rats with or without NAC in drinking water.

One potential complicating factor in these studies is the demethylation of the $^{14}$CMeHg. However, demethylation rates for MeHg are relatively slow (Norseth and Clarkson, 1970; Friberg and Mottet, 1989). In rats, demethylation rates are estimated to be about 3 to 5% per day (Norseth and Clarkson, 1970) and, thus, should not influence the present short-term studies. In the long-term study (Figs. 3 and 4), the conversion of MeHg to inorganic Hg might lead to an underestimate of the total amount of Hg that is excreted. However,
NAC is relatively selective for MeHg and does not affect the elimination rate of inorganic mercury (Ballatori et al., 1998a). Therefore, the majority of the excreted [14C] over the 12-day experiment should reflect the amount of MeHg excreted.

As anticipated, the body burden of MeHg in wild-type rats that received NAC in drinking water was lower in all tissues studied, when compared with TR− rats that received similar treatment or wild-type and TR− rats without NAC in drinking water. The level of MeHg in the brain of wild-type rats that received NAC in drinking water was lower than those of all other groups, but this difference was not statistically significant. The first line of action of NAC is probably by chelating MeHg in blood. Because the distribution of a single-compartment model, reduction of blood MeHg level invariably lowers those of other tissues. Due to a lower exchange rate between blood and brain, only a sustained fall in blood level of MeHg would result in a significant difference for this tissue.

The proposed model that Mrp2 is the apical transporter of MeHg-NAC was further investigated by examining its role in the transport of the MeHg-NAC complex in membrane vesicles derived from livers of TR− and wild-type rats. The findings illustrated in Fig. 5 support the hypothesis that the MeHg-NAC complex is a substrate for Mrp2. Therefore, based on the previous finding that MeHg-NAC complex is a substrate for Oat1, which has been localized to the basolateral membrane of the renal tubular cells, it is highly probable that when Oat1 transports the MeHg-NAC complex into the renal tubular cells, Mrp2 is mainly responsible for its transport into the tubular lumen for excretion via the urine (Fig. 6). In addition, the MeHg-cysteine complex may be formed as the highly labile MeHg redistributes to other intracellular thiol. This complex has been shown to be transported via both system L amino acid exchanger (Simmons-Willis et al., 2002) and the B(−) carrier (Bridges and Zalups, 2006) and possibly by other amino acid carriers; thus, these carriers may play a role in the excretion and reuptake of MeHg in renal tubule cells. Although these data provide insight into the mechanism of MeHg-NAC transport, additional studies are needed to establish the NAC dose that may be therapeutically relevant for potential use as an antidote in poisoned individuals and to assess the use of NAC as a potential biomonitoring agent of MeHg exposure.

References


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