Monomethylmercury (MeHg) is a potent neurotoxicant that is rapidly taken up by organisms living in aquatic environments and is biomagnified through the food chain, reaching concentrations in some fish 10,000–100,000 times greater than in the surrounding water (Schwehammer et al. 2007). In humans, exposure to high concentrations of MeHg causes central nervous system dysfunction because MeHg readily crosses the blood–brain barrier via the L-type large neutral amino acid transporter and accumulates in the brain (Simmons-Willis et al. 2002). In the past there have been accidental MeHg poisonings such as those in Minamata, Japan, and Iraq (Bakir et al. 1973); however, recent concerns focus on the risk to human health from the accumulation of MeHg through daily consumption of large predatory fish such as tuna and swordfish (Grandjean et al. 2010). The mechanism of MeHg toxicity is, in part, thought to involve the covalent interaction of MeHg with the reactive thiols of certain proteins (Kanda et al. 2008; Shinyashiki et al. 1996; Vogel et al. 1985). Unbound MeHg undergoes conjugation with glutathione (GSH), which is synthesized by glutamate-cysteine ligase (GCL) to form a polar MeHg–GSH adduct (Rabenstein and Fairhurst 1975) through nonenzymatic processes and possibly enzymatic processes involving GSH S-transferase (GSTs). The MeHg–GSH adduct is thought to be excreted into the extracellular space via multidrug resistance–associated proteins (MRPs) (Ballatori 2002; Madejczyk et al. 2007; Vollrath et al. 2006).

The current consensus is that GCL (the rate-limiting enzyme for GSH synthesis), phase II detoxification enzymes such as GSTs, and phase III MRP transporters are coordinately regulated by the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) (Itoh et al. 1997, 2004; Maher et al. 2007). Under basal conditions, Nrf2 is bound to Kelch-like ECH-associated protein 1 (Keap1), the negative regulator of Nrf2, and it undergoes degradation by the ubiquitin/proteasome system in the cytoplasm (Itoh et al. 2004). When the reactive thiol groups of Keap1 are modified by electrophiles and/or reactive oxygen species, Nrf2 is readily translocated into the nucleus, where it stimulates the antioxidant-responsive element (ARE) in the promoter region. We previously reported that activation of Nrf2 may be a key factor in detoxification of MeHg because Nrf2 facilitates the excretion of MeHg into the extracellular space in human neuroblastoma SH-SY5Y cells and primary mouse hepatocytes (Toyama et al. 2007). This observation has been further confirmed in primary rat astrocytes and microglial cells (Ni et al. 2010; Wang et al. 2009).

Evidence indicates that the activation of Nrf2 by chemopreventive agents is effective against various stresses and diseases (Kensler et al. 2007), and studies have shown that Nrf2 activators are capable of protecting against carcinogenesis in an Nrf2-dependent manner (Kwak and Kensler 2010; Ramos-Gomez et al. 2001). Isothiocyanates (ITCs) are among the most potent Nrf2 activators. For example, sulforaphane (SFN), an ITC found in broccoli sprouts, activates Nrf2, and up-regulates detoxifying enzymes, resulting in the reduction of arsenic accumulation and cytotoxicity in primary mouse hepatocytes (Shinkai et al. 2006). We hypothesize, therefore, that ITC-mediated activation of Nrf2 and up-regulation of the genes downstream of Nrf2 reduce cellular and organ mercury levels after exposure to MeHg, thereby diminishing the toxicity of this substance. We report here that SFN and 6-methylsulfinylhexyl isothiocyanate (6-HITC), an analogue of SFN isolated from wasabi (Japanese horseradish) that is also a potent Nrf2 inducer (Morimitsu et al. 2002), effectively suppress mercury accumulation after exposure to MeHg both in vitro and in vivo, leading to decreased cytotoxicity and intoxication through Nrf2 activation.
Materials and Methods

Materials. MeHg was purchased from Nacalai Tesque (Kyoto, Japan), and SFN was obtained from LKT Laboratories (St. Paul, MN, USA). We purchased anti-GCL modifier subunit (GCLM), anti-GCL catalytic subunit (GCLC), anti-MRP2, and anti-5′-nucleotidase (5′NT) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We obtained anti-MRP1 from Alexis Biochemicals (San Diego, CA, USA) and anti-actin from Sigma (St. Louis, MO, USA). Anti-GSTA1 was purchased from Oxford Biomedical Research (Oxford, MI, USA). 6-HITC was prepared as described by Shibata et al. (2008). All other reagents and chemicals used were of the highest grade available.

Cells and cell culture. Primary hepatocytes were isolated from 6- to 10-week-old C57BL/6J male mice by two-step collagenase perfusion as described by Shinkai et al. (2009). Parenchymal hepatocytes were separated from nonparenchymal cells by differential centrifugation 50 × g for 3 min. Dead parenchymal hepatocytes were removed by density gradient centrifugation in Percoll. Final preparations were suspended at 4 × 10^6 cells/mL in Williams medium E supplemented with 10% fetal bovine serum, 2 mM L-alanyl-L-glutamine, and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin) and then seeded at a density of 8 × 10^4 cells/cm² on a 24-well culture plate. Cultured cells were maintained at 37°C in a humidified incubator under an atmosphere of 5% CO₂/95% ambient air.

Cells were removed by density gradient centrifugation of complexes. The complexes were then washed twice with ice-cold PBS and solubilized with sodium dodecyl sulfate (SDS) sample buffer [50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol] to obtain total cellular protein. A crude membrane fraction was prepared by differential centrifugation, as described by Shinkai et al. (2009). Brieﬂy, cells were scraped into PBS, resuspended in hypotonic lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM MgCl₂], and incubated on ice for 15 min. Swollen cells were ruptured with 20 strokes in a tightly ﬁttting Dounce homogenizer, and the nuclei were removed by centrifugation at 400 × g for 10 min at 4°C. The pellet obtained by subsequent centrifugation at 30,000 × g for 30 min at 4°C was used as the crude membrane fraction. Protein concentration was determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard. Proteins were separated by SDS/PAGE. The blots were blocked for 1 hr with 5% skim milk in Tween-Tris-buffered saline [TTBS; 20 mM Tris (pH 8.0), 150 mM NaCl, 0.5% Tween 20]. Blots were incubated with the indicated primary antibodies, washed with TTBS, and incubated with horseradish peroxidase–conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence (Chemix-Lumi One; Nacalai Tesque) and scanned using an LAS-4000 imaging system (Fujifilm, Tokyo, Japan).

Luciferase assay. We performed DNA transfections using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Brieﬂy, cells were cultured in 12-well plates. Two micrograms of ARE-luciferase cDNA and 0.2 μg pRL-TK cDNA or 4 μL transfection reagent were mixed with serum-free media. After addition to the cells, the DNA solution and transfection reagent solution were mixed together and incubated for 20 min at room temperature to allow the formation of complexes. The complexes were then mixed with the culture media and incubated for 24 hr to transfect. After transfection, the cells were treated with 6-HITC or SFN for 12 hr, and then luciferase activity was measured in cellular extracts (Dual-Luciferase reporter assay system; Promega, Madison, WI, USA).

Measurement of intracellular GSH. Intracellular GSH content was measured as described by Vignaud et al. (2004), with slight modification. Brieﬂy, we used an HPLC system (Simadzu, Kyoto, Japan) linked to a coulometric detector (Coulonch II; ESA, Chelmsford, MA, USA). Cells were washed twice with PBS and collected in 1 mM EDTA. After sonication, protein concentrations were measured by bicinchoninic acid (BCA) protein assay. Cell lysates were ﬁltered by using an Ultrafree-MC.
were subjected to Western blot analysis with anti-Nrf2. \( \ast \text{p}<0.01 \) compared with control. \( \ast\ast \text{p}<0.05 \), and \( \ast\ast\ast \text{p}<0.01 \).

Total cell lysates from cells incubated with 6-HITC (5 or 10 μM) for 6, 12, or 24 hr were subjected to Western blot analysis with antibodies to MRP1 and MRP2; anti–5´NT was used as the internal control. Values shown are mean ± SE of three determinations.

Statistical analysis. Statistical significance was assessed by Student’s t-test or chi-square test, and \( \text{p}<0.05 \) was considered statistically significant.

Results

Effects of inhibitors on MeHg accumulation and toxicity. The major detoxification pathway for MeHg is conjugation with GSH derived from GCL in the presence or absence of GSTs, followed by excretion of the MeHg–GSH adduct into the extracellular space via MRPs. To confirm this, we first examined whether inhibition of GSH production, or GST or MRP activity, affects intracellular levels of MeHg (evaluated by determination of total mercury content) and MeHg-induced cytotoxicity in vitro. Pretreatment with BSO (a specific GCL inhibitor), EA (a GST inhibitor), or MK-571 (an MRP antagonist) resulted in significant enhancement of mercury accumulation in primary mouse hepatocytes exposed to MeHg (Figure 1A–C). Under these conditions, the cells were significantly more sensitive to MeHg, and cell viability decreased (Figure 1D–F). These results indicate that GCL, GST, and MRP contribute to cellular protection against MeHg toxicity through the reduction of mercury accumulation.

ITCs activate Nrf2 in primary mouse hepatocytes. Because GCL, GSTs, and MRPs are regulated by Nrf2, we investigated the effects of the ITCs 6-HITC and SFN (Figure 2) on activation of Nrf2 and up-regulation of GCL, GSTs, and MRPs (Figure 3). Exposure

Figure 3. Effect of the ITCs 6-HITC and SFN in primary mouse hepatocytes. (A) Total cell lysates from cells incubated with 6-HITC (5 or 10 μM) for 6, 12, or 24 hr were subjected to Western blot analysis with anti-Nrf2. (B) Luciferase activity in primary mouse hepatocytes transfected with ARE-luciferase and pRL-TK cDNA and treated with ITCs (10 μM) for 12 hr. (C) Total cell lysates from cells incubated with 6-HITC (5 or 10 μM) for 6, 12, or 24 hr were subjected to Western blot analysis with antibodies to GCLC, GCLM, and GSTA1. For A and C, anti-actin was used as the internal control. (D) Crude membrane fractions of cells incubated with 6-HITC (10 μM) for 6, 12, or 24 hr were subjected to Western blot analysis using the antibodies to MRP1 and MRP2; anti-5´NT was used as the internal control. Values shown are mean ± SE of three determinations.

\( \ast \text{p}<0.05 \), and \( \ast\ast \text{p}<0.01 \) compared with control.
of primary mouse hepatocytes to 6-HITC for 6 hr resulted in a significant increase in Nrf2 accumulation. The extent of the increase in Nrf2 accumulation was less at 12 and 24 hr than at 6 hr (Figure 3A). ARE luciferase activity was enhanced by exposure to 6-HITC or SFN for 12 hr (Figure 3B). Under these conditions, 6-HITC significantly increased the expression of GCLM, GCLC, and GSTA1 (Figure 3C) and MRP1 and MRP2 (Figure 3D) in primary mouse hepatocytes.

**ITCs inhibit MeHg-induced mercury accumulation and cytotoxicity.** Because GCL is a rate-limiting enzyme for GSH synthesis, we measured intracellular GSH levels after exposure to ITCs in primary hepatocytes from wild-type and Nrf2-deficient mice. Both 6-HITC and SFN significantly increased the intracellular GSH level in wild-type cells, whereas GSH levels were not changed by ITCs in Nrf2-deficient cells (Figure 4A). This suggests that ITCs facilitate MeHg-GSH adduction formation and excretion into the extracellular space via an Nrf2-dependent pathway. To explore this possibility, we examined the effect of ITCs on mercury accumulation and cytotoxicity induced by MeHg exposure in primary hepatocytes from wild-type and Nrf2-deficient mice. Pretreatment with 6-HITC or SFN before exposure to MeHg resulted in a significant decrease in mercury accumulation (Figure 4B) and cytotoxicity (Figure 4C) in the wild-type cells, whereas no such protective effect was observed in Nrf2-deficient cells. To measure mercury accumulation, we exposed 6-HITC-pretreated or SFN-pretreated cells to MeHg for 1 hr and then incubated them in MeHg-free medium for an additional 1 hr. 6-HITC and SFN increased mercury accumulation and MeHg-induced cytotoxicity in the Nrf2-deficient cells. Because the Nrf2-deficient cells expressed lower basal levels of GCL, GST, and MRP than did the wild-type cells (data not shown), the basal level of intracellular GSH was also lower than that in the wild-type cells (Figure 4A). In nonpretreated primary hepatocytes from wild-type mice, mercury accumulation and cytotoxicity were increased by Nrf2 deficiency after MeHg exposure (Figure 4B, C).

**Nrf2 suppresses MeHg intoxication in vivo.** To examine the contribution of Nrf2 to protection against MeHg toxicity in vivo, we counted the number of mice with flaccid hind limbs each day; this flaccidity is a typical sign of MeHg intoxication in rodents (Su et al. 1998). Oral administration of MeHg (5 mg/kg/day for 8 days) to Nrf2-deficient mice resulted in the induction of hind-limb flaccidity (Figure 5A), whereas wild-type mice administered MeHg (50 mg/kg) did not show hind-limb flaccidity. To determine whether Nrf2 deficiency increased MeHg toxicity in vivo, we counted the number of mice that died after administration of MeHg (5 mg/kg/day for 8 days) to Nrf2-deficient mice. Oral administration of MeHg (50 mg/kg) to wild-type mice resulted in increased mortality after MeHg exposure (Table 1). In contrast, oral administration of MeHg (5 mg/kg/day for 8 days) to Nrf2-deficient mice did not result in increased mortality compared with wild-type mice (Table 1). These results suggest that Nrf2 deficiency exacerbates MeHg toxicity in vivo.

**Table 1. Hind-limb flaccidity and mortality of wild-type mice given MeHg.**

| Treatment | Hind-limb flaccidity (%) | Mortality (%) |
|-----------|--------------------------|--------------|
| SFN (20 mg/kg) | 0/15 | 0/15 |
| MeHg (50 mg/kg) | 14/25** | 17/25 |
| SFN pretreatment + MeHg | 8/25** | 10/25* |

Wild-type mice received a single injection of SFN 16 hr before a single administration of MeHg. Experiments were carried out 2 days (hind-limb flaccidity) and 3 days (mortality) after injection of MeHg into mice. *p < 0.05 and **p < 0.01 compared with MeHg (50 mg/kg) alone.
mice did not show any abnormalities within 22 days after termination of MeHg treatment. The body weight of Nrf2-deficient mice 5 days after MeHg administration was approximately 30% less than that of wild-type mice (data not shown). All of the Nrf2-deficient mice and none of the wild-type mice had died from progression of MeHg intoxication within 3 weeks of the first administration of MeHg (Figure 5B).

SFN inhibits mercury accumulation in vivo.

If Nrf2 plays a role in the elimination of MeHg from the body, the accumulation of mercury in the organs should be increased by Nrf2 deficiency and decreased by Nrf2 activation. As expected, oral administration of MeHg (1 mg/kg) resulted in significantly greater accumulation of mercury in the cerebrum, cerebellum, and liver of Nrf2-deficient mice than in those of wild-type mice (Figure 5C–E). To examine the protective effect of ITC-mediated Nrf2 activation on MeHg accumulation in vivo, we administered SFN (5 mg/kg) to wild-type and Nrf2-deficient mice by intraperitoneal injection as previously described (Zhang et al. 2007). Injection of SFN before MeHg administration resulted in significant suppression of the accumulation of mercury in the cerebrum, cerebellum, and liver of wild-type mice (Figure 5C–E). In Nrf2-deficient mice, however, SFN did not affect the accumulation of mercury in the cerebrum and cerebellum and actually increased the accumulation of mercury in the liver (Figure 5C–E). Interestingly, pretreatment with SFN (20 mg/kg) significantly reduced not only acute MeHg intoxication as determined by hind-limb flaccidity but also mortality after oral administration of MeHg (50 mg/kg) to wild-type mice (Table 1).

Discussion

Our findings indicate that both 6-HITC and SFN activate Nrf2, resulting in up-regulation of GCL and the GSTs and MRPs responsible for conjugation of MeHg with GSH and excretion of the MeHg–GSH adduct into the extracellular space. Increased Nrf2 activation is associated with a reduction in cellular and organ levels of mercury and substantial suppression of MeHg-induced cytotoxicity and intoxication in primary mouse hepatocytes and in mice. Previous findings (Toyama et al. 2007) suggest that Nrf2 is a critical transcription factor in the reduction of MeHg-induced cytotoxicity and the excretion of MeHg into the extracellular space, because Nrf2 deletion significantly enhances MeHg accumulation and cytotoxicity in primary mouse hepatocytes. Our findings support this notion.

A common characteristic of MeHg-resistant cell lines is the reduced accumulation of MeHg compared with that in nonresistant parent cells (Miura and Clarkson 1993), indicating that protection against MeHg intoxication is, at least in part, associated with decreased influx and/or increased efflux of MeHg. Several researchers have reported that the GSH transport system is closely associated with MeHg efflux (Ballatori and Clarkson 1983, 1985; Fujiiyama et al. 1994). Kaur et al. (2006) reported that diethylmaleate, used to deplete GSH levels, increased MeHg accumulation and enhanced MeHg-induced oxidative stress in primary cell cultures of neurons and astrocytes. Also, the GSH transport inhibitor phenol-3,6-dibromophthalic inhibits MeHg efflux from PC12/TM cells (a MeHg-resistant rat pheochromocytoma cell line), thereby increasing MeHg accumulation (Miura and Clarkson 1993). Consistent with these results, pretreatment with BSO (a GCL inhibitor), EA (a GST inhibitor), or MK-571 (an MRP antagonist) before MeHg exposure increased mercury accumulation and cytotoxicity in primary mouse hepatocytes (Figure 1). A reduction of steady-state levels of MeHg in cells is associated with diminished chemical modification of cellular proteins, confirming the importance of formation of the MeHg–GSH adduct, and its excretion into the extracellular space, in the detoxification of MeHg.

Even if cellular proteins are covalently modified by MeHg, MeHg–protein adducts are reversibly exchanged by GSH (Dallas 1978). This suggests that GSH protects against MeHg toxicity by increasing MeHg efflux and subsequently decreasing the levels of proteins modified by MeHg in the cell. Wirtzloth et al. (1981) reported that the MeHg–protein adduct is a major form of MeHg in brain of monkeys administered MeHg. When GSH concentrations are low, the excess accumulation of MeHg–protein adducts may be the trigger for cell death. Consistent with this, Nrf2 deletion enhanced MeHg–protein adduct formation during exposure of primary mouse hepatocytes to MeHg, as evaluated by the biotin-maleimide labeling assay (Toyama T, unpublished data).

As shown in Figure 3, 6-HITC activated Nrf2 and up-regulated downstream proteins associated with the detoxification and excretion of MeHg, such as GCLM, GCLC, GSTA1, MRP1, and MRP2, in primary mouse hepatocytes. Similar results have been obtained using SFN (Shinkai et al. 2006). The mechanisms of action of these ITCs are thought to involve reversible modification of the cysteine residues of Keap1 by the carbon in the –N=C=S motif (Figure 2); this leads to the stabilization of Nrf2 and the subsequent activation of ARE in the promoter region (Nakamura and Miyoshi 2010). In the present study, both 6-HITC and SFN suppressed mercury accumulation and cytotoxicity in primary mouse hepatocytes. Our findings support this notion.

A common characteristic of MeHg-resistant cell lines is the reduced accumulation of MeHg compared with that in nonresistant parent cells (Miura and Clarkson 1993), indicating that protection against MeHg decrease MeHg accumulation in several cell lines; however, the precise mechanism of this effect is not known (Kaur et al. 2007, 2008). In the present study, we demonstrated that ITCs such as 6-HITC and SFN are potentially useful chemopreventive agents for MeHg accumulation and toxicity and that they exert their action through an Nrf2-dependent mechanism. This finding may provide helpful information for risk management strategies to reduce the potential health risk of MeHg exposure.

Conclusion

The present study indicates that ITCs are effective agents for the reduction of MeHg accumulation via an Nrf2-dependent mechanism in vitro and in vivo.

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