Increased Hepatic Accumulation of Ingested Cd is Associated with Upregulation of Several Intestinal Transporters in Mice Fed Diets Deficient in Essential Metals

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Received April 18, 2008; accepted July 13, 2008

Essential metals (EMs) can affect the metabolism of non-essential metals. It has been suggested that Fe deficiency increases intestinal absorption of Cd via divalent metal transporter 1 (DMT1). To investigate whether EM nutritional status is a host risk factor for Cd accumulation, we studied the effect of nutritional status of Ca, Cu, Mg, Zn, and Fe that most often ingested by humans at levels below recommended dietary allowances on tissue accumulation of orally administered Cd. Mice were divided into groups and given different EM-deficient (EMDF) diets (CaDF, CuDF, MgDF, ZnDF, or FeDF) for 4 weeks. EMDF mice had significantly (p < 0.05) lower plasma or hepatic concentrations of the deficient EM than did mice receiving control diets. Hepatic Cd accumulation was significantly (p < 0.05) increased after oral Cd administration in all EMDF mice, but not in any EM-supplemented mice. Intestinal expression of mRNAs for the Fe-transporters DMT1 and ferroportin was increased in FeDF mice, but not in other EMDF mice, causing an increase in hepatic Fe concentration. Similarly, intestinal expression of mRNA for calcium transporter 1 was significantly increased in CaDF mice, but not in other EMDF mice. These results suggest that DMT1 is not the sole transporter of Cd, and that Cd is absorbed and accumulated through multiple pathways that maintain EM homeostasis in EMDF condition. Therefore, EM nutritional status is a risk factor for increasing hepatic accumulation of ingested Cd.

Key Words: essential metals; accumulation of cadmium; nutritional status; deficiency; divalent metal transporter 1; metal transporter.

Cadmium is a ubiquitous contaminant of the environment and dietary products. Food is the major source of Cd exposure in the general population, and dietary Cd intake is considered to be the main contributor to the body burden of Cd. The mammalian gastrointestinal tract absorbs approximately 1–6% of ingested Cd, and Cd accumulates preferentially in the liver and kidneys (Lehman and Klaassen, 1986). Chronic exposure to Cd can cause renal damage in humans and experimental animals (Friberg et al., 1974). However, the details of the pathway by which Cd is absorbed from the intestinal lumen and subsequently redistributed to the liver and kidneys are as yet unknown. Many factors, including other components of the diet and the overall nutritional condition of the subject, can influence the absorption and distribution of Cd.

Although required for numerous cellular metabolic functions, essential metals (EMs) are potentially toxic to cells when present in excess. The ability of mammals to excrete EMs is limited, and therefore ion acquisition by the absorptive epithelium of the small intestine must be regulated carefully (Cousins et al., 2006; Papanikolaou and Pantopoulos, 2005; Sharp, 2003). Each EM has an individual metabolic pathway through which its homeostasis is maintained. However, the status of some EMs affect the metabolism of other EMs. For example, Cu deficiency reduces the relative duodenal concentration of the Cu-dependent ferroxidase protein, hephaestin, which in turn reduces Fe absorption and causes anemia (Reeves and Demars, 2005), and iron supplements inhibit Zn but not Cu absorption in vivo (Troost et al., 2003).

Further, EMs can affect the metabolism of not only other EMs, but also nonessential metals. Cd interacts strongly with high concentrations of dietary EMs, such as Zn, Fe, and Ca, which reduce the rate of Cd absorption from various food sources (Brzoska and Moniuszko-Jakoniuk, 2001; Brzoska et al., 2001; Fox, 1983). Reeves and Chaney (2004) demonstrated that rats simultaneously fed marginal amounts of Zn, Fe, and Ca retained as much as eight times more $^{109}$Cd than rats fed sufficient amounts. Fe deficiency increases the concentration of Cd in the duodenal mucosa because of the Fe transporters upregulation, the transfer of Cd from the intestinal tract to the tissues through the circulation, and the deposition of absorbed Cd in the kidneys (Flanagan et al., 1978). In these experiments, Cd may compete with EMs for binding to transporter molecules on the apical side of the small intestinal epithelium when animals received diets or drinking water.
containing Cd and the EM at various concentrations. However, the effect of intestinal expression of metal transporters regulated by the nutritional status of EM on intestinal absorption of Cd remains unclear.

Recently, intestinal transporters of EMs, including Zn transporters, divalent metal transporter 1 (DMT1), ferroportin (FPN), and calcium transporter 1 (CaT1), have been identified. These transporters are upregulated or downregulated according to the host’s EM nutritional status (Dupic et al., 2002; McMahon and Cousins, 1998a, b; Peng et al., 1999). These transporters are upregulated or downregulated according to the host’s EM nutritional status (Dupic et al., 2002; Ford, 2004; Gunshin et al., 2001; Song et al., 2003). Park et al. (2002) demonstrated that Fe depletion likely upregulates mRNA encoding functional DMT1, an apical Fe\(^{2+}\) transporter, in the small intestine in order to acquire more Fe; this upregulation increases Cd uptake from the gastrointestinal tract, with subsequent transfer of Cd to the circulation and body tissues. Expression of DMT1 and the basolateral Fe exporter FPN are correlated with Cd body burden in rats, suggesting important roles for DMT1 and FPN in Cd absorption (Ryu et al., 2004). Furthermore, DMT1 has a broad substrate specificity in vitro, favoring divalent metals, including Fe\(^{2+}\), Mn\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), and Pb\(^{2+}\) (Gunshin et al., 1997). Both EMs and nonessential metals may compete for binding to transporter molecules on the apical side of the small intestinal epithelium.

Reported Cd concentrations in the human renal cortex range from an average of 10–30 \(\mu\)g/g in Europeans, Americans, and Africans, but from 65 to 115 \(\mu\)g/g in Japanese (Kawada and Suzuki, 1998). Daily Cd intake is reported to be higher in Japan (20–30 \(\mu\)g/day/person) than in Germany and Sweden (10 \(\mu\)g/day/person) because cadmium-rich soil generally results in cadmium-rich food (Kawada and Suzuki, 1998). These values show that the ratio of renal Cd concentration to daily Cd intake is much higher in Japanese than in Europeans. Therefore, it suggests that a determinant in renal Cd accumulation may not be only dietary intakes of Cd in Japanese. Some of EMs (Ca, Cu, Fe, Mg, and Zn) most often ingested by Japanese at levels below recommended dietary allowances (Dietary Reference Intakes For Japanese, 2005; National Health and Nutrition Survey in Japan, 2003). In 30–49 years old male and female, ratio of insufficient populations were high in minerals such as iron, calcium and magnesium (Itokawa, 2006). It is possible that the host’s EM nutritional status is a risk factor for body Cd accumulation. We therefore investigated whether EM nutritional status (Ca, Cu, Fe, Mg, or Zn) affects tissue accumulation of ingested Cd in mice via the action of intestinal metal transporters.

**MATERIALS AND METHODS**

**Animals.** Male mice (ddY strain, 5 weeks old) were purchased from Nihon SLC (Shizuoka, Japan). Mice were housed in plastic cages and maintained on a 12:12-h light–dark cycle. All mice were given control (mineral concentration; Ca 5.0 g/kg, Cu 6.3 mg/kg, Fe 38.8 mg/kg, Mg 0.5 mg/kg, Zn 33.7 mg/kg) or EM-deficient (EMDF) diets based on the AIN-93M diet (Orienta Yeast, Osaka, Japan) and water ad libitum. All animal experiments were performed under the control of the Animal Research Committee of Osaka Ohtani University, in accordance with the Guidelines for Animal Experiments at Osaka Ohtani University and the Japanese Animal Protection and Management Law (No. 105).

**EMDF diets and mice.** Individual EMDF diets were separately prepared and sucrose powder was substituted for each of calcium carbonate, copper carbonate, ferric citrate, magnesium oxide, and zinc carbonate in mineral mixture of the AIN-93M diet (Orienta Yeast). All of the individual EMDF diets were wet-ashed in concentrated nitric acid at 80°C for 5 days, after which the concentration of each metal in each was determined by atomic absorption spectrophotometry (AAS; Z2300 Polarized Zeeman Atomic Absorption spectrophotometer, Hitachi, Tokyo, Japan; Fig. 1). Each five mice were fed each of the EMDF diets (CaDF, CuDF, FeDF, MgDF, or ZnDF) for 4 weeks. Mice were euthanized under anesthesia with ether. The blood and tissues were collected, and then 0.3 ml of plasma prepared from each mouse was wet-ashed in an equal volume of concentrated nitric acid at 80°C for 6 h. Each liver (0.2 g) sample was digested with 3 ml of nitric acid for 3 days. The concentrations of EMs in each sample were determined by AAS.

![FIG. 1.](https://academic.oup.com/toxsci/article-abstract/106/1/284/1705725)

**FIG. 1.** Concentration of each EM in the EMDF diets (A), in the plasma of mice fed EMDF diets (B), and in the livers of EMDF or EMSP mice (C). EMDF diets were prepared such that each EM salt was removed from the base diet, AIN-93M. Plasma and liver samples were obtained from mice fed each of the EMDF diets for 4 weeks and from EMSP mice. All samples were digested with nitric acid, and then metal concentrations were determined by AAS. Data points represent percentages (means ± SD; \(n = 5\) mice) relative to control values. *\(p < 0.05\), **\(p < 0.01\) versus control group.
**EM-supplemented mice.** As one mouse consumed 2 g of the control diet for 24 h, daily dietary intakes of each EM was calculated from analytical mineral data on the AIN-93M diet (Oriental Yeast). CaCl₂, CuSO₄, FeSO₄, MgCl₂, or ZnSO₄ was dissolved in deionized water to make up each supplemental solution of EM (the EM content equivalent to that found in 1 g of control diet was present in each 0.2 ml of supplement). Individual EM solution (0.2 ml daily) was administered orally to each of five twenty-five mice that fed control diet for 10 days to get 1.5-fold of dietary intakes of each EMs. Twenty-four hours after the administration of EM solution, all mice were exsanguinated under anesthesia with ether and the liver was removed. Each tissue sample (0.2 g of liver) was wet-ashed in 3 ml of concentrated nitric acid, and the concentration of EMs in each tissue was determined by AAS.

**Oral administration of ⁰⁹CdCl₂ to control, EMDF, and EM-supplemented mice.** Each milliliter of ⁰⁹CdCl₂ injection solution had a radioactivity of 0.4 MBq and contained 2 mg Cd. The solution was prepared by combining ⁰⁹CdCl₂ (0.3 mg/37 MBq, PerkinElmer, Boston, MA) and CdCl₂ solution (10 mg/ml). The ⁰⁹CdCl₂ solution was administered orally by gavage as a single dose of 5 mg Cd/kg to the controls and to the five EMDF groups (CaDF, CuDF, FeDF, MgDF, and ZnDF; n = 15 or 16 in each group) and five EM-supplemented (EMSP) groups (CaSP, CuSP, FeSP, MgSP, and ZnSP) (n = 5 in each group); the mice were fasted for 3 h beforehand. Food was replaced in the cages 3 h after administration of ⁰⁹CdCl₂. Twenty-four hours after dosing, all mice were exsanguinated under ether and their livers were removed. ⁰⁹Cd radioactivity was assayed with an Auto-Well scintillation counter (Aloka, Tokyo, Japan), and tissue concentrations of Cd were calculated from the specific radioactivity of ⁰⁹Cd (37,296 cpm/µg). ⁰⁹CdCl₂ was injected i.p. as a single dose of 0.5 mg Cd/kg to the controls and the five EMDF groups (CaDF, CuDF, FeDF, MgDF, ZnDF) (n = 5 in each group) to get a similar concentration of hepatic Cd to the p.o. injection of Cd. All mice were exsanguinated under ether anesthesia and their livers were removed 24 h after dosing. ⁰⁹Cd radioactivity was assayed and tissue concentrations of Cd were calculated.

**Intraperitoneal injection of ⁰⁹CdCl₂ in control and EMDF mice.** ⁰⁹CdCl₂ solution was injected i.p. as a single dose of 0.5 mg Cd/kg to the controls and the five EMDF groups (CaDF, CuDF, FeDF, MgDF, ZnDF) (n = 5 in each group) to get a similar concentration of hepatic Cd to the p.o. injection of Cd. All mice were exsanguinated under ether anesthesia and their livers were removed 24 h after dosing. ⁰⁹Cd radioactivity was assayed and tissue concentrations of Cd were calculated.

**RNA isolation from small intestine of EMDF and FeSP mice, and reverse transcription–polymerase chain reaction analysis for Fe transporter.** Five groups of mice on each of the EMDF diets (n = 5 in each group) and five groups of mice of each on the FeSP diets (n = 5 in each group) were exsanguinated under anesthesia with ether. The small intestine was removed and homogenized in TRIzol reagent (Invitrogen Japan, Osaka, Japan), and total RNA was isolated as specified in the manufacturer’s instructions. cDNA was synthesized and reverse-transcribed to cDNA by using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The various mRNAs were quantified by real-time PCR using RT-PCR Master Mix (Toyobo) and TaqMan Universal PCR Master Mix in a thermocycler (DNA Engine Opticon 2 Real-Time PCR Detection System; Bio-Rad Laboratories, Tokyo, Japan). The levels of DMT1, FPN, CaT1, and β-actin mRNAs were assessed by real-time PCR with primers that had been described previously (Dupic et al., 1998; Hubert and Hentze, 2002; Song et al., 2003) and were obtained from Gene Design (Osaka, Japan).

**Statistics.** Results are expressed as means ± SD. Data were analyzed for significance by Student’s t-test or by ANOVA with the post hoc Dunnett’s test (Prism; GraphPad Software, CA). Differences were considered significant when p < 0.05.

### RESULTS

We examined the concentrations of EMs in individual EMDF diets and in the plasma and livers of individual EMDF and EMSP mice (Fig. 1). The Fe concentration in the FeDF diet was about 20% of that in the control diet, and the concentrations of EMs in the other EMDF diets were less than 10% of those in the control diet (Fig. 1A). The concentrations of EMs in the plasma of EMDF mice fed these diets for 4 weeks were less than 50% of those in control mice, except in the case of CaDF mice (Fig. 1B). EMSP did not affect the concentration of EM in the plasma compared with that in control mice (data not shown). Hepatic Fe concentration was significantly (p < 0.05) decreased in FeDF mice and increased in FeSP mice compared with that in controls. The hepatic concentration of each target EM was also significantly (p < 0.01) lower in mice receiving individual diets deficient in Ca, Mg, or Zn than in controls, but it was not affected in EMSP mice, apart from FeSP mice (Fig. 1C).

We examined the effects of individual EM nutritional status on tissue Cd accumulation after oral or i.p. Cd administration (Fig. 2). Oral Cd administration led to significant increases in hepatic Cd accumulation in all EMDF mice compared with the controls (p < 0.01; Fig. 2A). However, i.p. injection of Cd in EMDF mice did not result in any increase in hepatic Cd levels compared with the controls (Fig. 2A). This indicates that increased intestinal transport of Cd from the intestinal lumen may be the mechanism involved in the increase in tissue Cd accumulation. Moreover, no significant increase in hepatic Cd accumulation was observed after an oral Cd administration to EMSP mice (Fig. 2B). Thus, individual EMDF, but not individual EMSP, may be a host risk factor for increasing body Cd accumulation. These data suggest that intestinal metal transporters affect intestinal Cd absorption.

We then investigated the relationship between increased hepatic Cd accumulation and Fe metabolism, because Fe-related transporters such as DMT1 and FPN can transport Cd from the intestinal lumen to the blood (Park et al., 2002; Ryu et al., 2004). We examined hepatic Fe concentrations in control
and individual EMDF mice (Fig. 3). Hepatic Fe concentration was decreased in FeDF mice and increased in FeSP mice (Fig. 1). Moreover, hepatic Fe concentration was significantly \((p < 0.01)\) greater in CaDF, CuDF, MgDF and ZnDF mice than in control mice. We then examined the intestinal expression of the mRNAs of Fe-related transporters (DMT1 and FPN) and of CaT1 in control, EMDF, and FeSP mice (Fig. 4). Intestinal expression of both DMT1 and FPN mRNAs was significantly higher in FeDF mice than in controls \((p < 0.01)\). In contrast, intestinal expression of DMT1 mRNA was significantly lower in FeSP mice than in controls. Interestingly, the intestinal level of DMT1 mRNA was also significantly \((p < 0.01)\) lower in the other EMDF mice than in the controls. Intestinal expression of FPN mRNA was significantly increased in FeDF mice and decreased in CaDF mice \((p < 0.01)\), but not affected in FeSP or other EMDF mice. Although hepatic Cd accumulation was increased in all groups of EMDF mice (Fig. 2A), levels of intestinal expression of DMT1 mRNA were high in FeDF mice but low in the other EMDF groups. These data indicate that intestinal DMT1 expression was not involved in the increase in hepatic Cd accumulation in the EMDF groups other than the FeDF group.

CaT1, like DMT1, can transport Cd from the gastrointestinal lumen to the blood (Min et al., 2008a; Peng et al., 1999). We compared the intestinal expression of Cat1 mRNAs in control, EMDF, and FeSP mice (Fig. 4C). As was the case for intestinal DMT1 expression in FeDF mice, intestinal expression of Cat1 mRNA in CaDF mice was significantly higher than in the controls \((p < 0.01)\). However, it was decreased in the other EMDF groups. These data indicate that neither DMT1 nor CaT1 transports oral Cd in EMDF mice apart from FeDF and CaDF mice, respectively. Although individual EM deficiencies affected the metabolism of not only the target EM but also other EMs (Fig. 3), different transporters may have been involved in increased body Cd accumulation in each of the EMDF groups.

**DISCUSSION**

Several previous reports have demonstrated that EM transporters may be involved in the intestinal absorption of Cd (Bressler et al., 2004; Gunshin et al., 1997; Leazer et al., 2002; Min et al., 2008a; Park et al., 2002) The apical transmembrane Fe transporter DMT1 is a strong candidate for this role, because it transports various divalent metals (such as Cd) in addition to Fe \(\text{in vitro}\) (Gunshin et al., 1997). Upregulation of DMT1 in the small intestines of rats fed a FeDF diet significantly increased the uptake of Fe and Cd from the gastrointestinal lumen and their transfer to other tissues (Park et al., 2002). The correlation between Cd absorption and DMT1 expression in pregnant rats (Leazer et al., 2002) suggests a role for DMT1 in

![FIG. 3](https://academic.oup.com/toxsci/article-abstract/106/1/284/1705725)

**FIG. 3.** Hepatic concentration of Fe in control and EMDF mice. Tissues were obtained from mice fed control or EMDF diet for 4 weeks. All samples were digested with concentrated nitric acid, and then metal concentrations were determined by AAS. Data points represent means ± SD of five mice. **\(p < 0.01\) versus values for control mice.

![FIG. 4](https://academic.oup.com/toxsci/article-abstract/106/1/284/1705725)

**FIG. 4.** Intestinal expression of DMT1 mRNA (A), FPN mRNA (B), and CaT1 mRNA (C) in control, EMDF, and FeSP mice. Total RNA was isolated from the small intestine, and intestinal expression of DMT1, FPN, and CaT1 mRNAs was assessed by RT-PCR. Data points represent means ± SD of five mice. *\(p < 0.05\), **\(p < 0.01\) versus values for control mice.
the increased absorption of Cd during pregnancy. Ryu et al. (2004) demonstrated that expression of the basolateral Fe exporter FPN mRNA is also correlated with the Cd body burden in rats. In our recent study (Min et al., 2008b), hemolytic anemia caused tissue Cd accumulation and a significant increase in intestinal expression of DMT1 mRNA but not FPN mRNA, despite the fact that the anemic mice were fed a normal Fe diet. These results suggest that marked increases in body Cd accumulation are dependent on the upregulation of DMT1 expression by the body’s Fe requirement.

In our present study, increases in either intestinal expression of DMT1 or FPN mRNA and hepatic Cd accumulation occurred in our FeDF mice, in which the hepatic Fe concentration was significantly less than normal (p < 0.01). Conversely, a significant increase in hepatic Cd accumulation was not seen in FeSP mice, which showed decreased intestinal DMT1 mRNA expression and increased hepatic Fe concentration (Fig. 1C). These data correspond with those of previous reports (Bressler et al., 2004; Kim et al., 2007; Park et al., 2002; Ryu et al., 2004). However, the hepatic Fe concentrations of mice fed diets deficient in other EMs (i.e., Ca, Mg, Cu, and Zn) for 4 weeks were significantly higher than in the controls, despite their receiving the same concentration of Fe as in the control diet. Intestinal expression of DMT1 mRNA in these other EMDF mice was significantly lower than in control mice. These data indicate that intestinal DMT1 expression is regulated by body Fe status (Gunshin et al., 2001; Tchernitchko et al., 2002). Interestingly, although intestinal DMT1 mRNA expression was decreased in these other EMDF mice, hepatic Cd accumulation was significantly increased after oral administration of Cd to all EMDF mice. However, neither oral administration of Cd to EMSP mice nor i.p. injection of Cd in EMDF mice gave significant increases in hepatic Cd accumulation. Individual EMSPs did not result in an increase in the hepatic concentration of each target EM, except in the case of Fe. EMSP therefore presumably resulted in a decrease in intestinal absorption of EM and/or an increase in renal excretion and thus tissue-safe accumulation of EMs for individual EM homeostasis. For example, FeSP resulted in a decrease in the intestinal expression of DMT1 mRNA and an increase in hepatic Fe accumulation, because hepatocytes respond by producing ferritin, which binds to the excess iron in hepatocytes, limiting its ability to exert a toxic effect (Drake et al., 2007). CaSP resulted in a reduction in the renal Ca concentration to 50% of that in the control group at same experiment as Figure 1C (data not shown). It suggests that renal Ca excretion may accelerate by the regulatory effects of several hormones (Honda et al., 2007). In contrast to EMSPs, individual EM deficiencies caused increases in intestinal expression of metal transporters for either target EM or non-EMs such as Cd. Consequently, the host’s nutritional EM status may affect the rate of body accumulation of ingested Cd.

Furthermore, the ratio of the Cd content in the liver to that in kidney was significantly increased in our CuDF and MgDF mice and decreased in the CaDF mice (data not shown), suggesting that Cd is absorbed and accumulated through multiple pathways that maintain EM homeostasis in the body and that DMT1 is not the sole transporter of Cd. Levels of intestinal accumulation of Cd are the same in DMT1-dysfunctional MK/Rej-(mk)/(mk) mice as in WT mice, even though the loss of DMT1 function leads to decreased intestinal Fe concentration (Suzuki et al., 2008). Our recent study showed that CaT1 might be a Cd transporter, because intestinal expression of CaT1 mRNA and tissue Cd accumulation were significantly increased in CaDF mice (Min et al., 2008a). Because, like Mn, Cd has higher transport affinity for DMT1 than does Fe (Garrick et al., 2006), Cd may be transferred instead of Fe during overexpression of DMT1. However, when the production of other EM transporters is upregulated in response to deficient EM nutritional status, some of these transporters, including CaT1, may also shuttles Cd. Therefore, an imbalance in the EM nutritional status is a risk factor for increased body Cd accumulation.

In 2006, the Codex Alimentarius Commission set new international standards for the maximum allowable levels of contaminants, including cadmium. Dietary Cd intake is usually considered to be the main determinant of body Cd retention. Our findings indicate that hepatic Cd accumulation was at least 1.5 times higher in EMDF mice than in control mice after only a single oral dose. Because insufficient dietary intake of EMs is a common human health concern, these host-associated risk factors cannot be neglected.

In summary, hepatic Fe concentration was significantly decreased in FeDF mice but increased in other EMDF mice fed each EMDF diet for 4 weeks. Intestinal expression of DMT1 mRNA was negatively correlated with hepatic Fe concentration in EMDF mice (Figs. 3 and 4). Interestingly, although intestinal DMT1 mRNA expression was decreased in EMDF mice (other than FeDF ones), hepatic Cd accumulation was significantly increased after oral Cd administration to all the groups of EMDF mice. Hepatic Cd accumulation was not increased after oral administration of Cd in either EMSP mice or control mice simultaneously supplemented with each of the EMs evaluated. These results suggest that DMT1 is not the sole transporter of Cd, and that an imbalance in EM nutritional status is a risk factor for increased body Cd accumulation.

FUNDING

Scientific Research Grant (C-17510050) from the Japanese Ministry of Education, Science and Culture.

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