Dietary Deficiency of Calcium and/or Iron, an Age-Related Risk Factor for Renal Accumulation of Cadmium in Mice

Kyong-Son Min, a Erika Sano, a Hidenori Ueda, a Fumitoshi Sakazaki, a Keita Yamada, a Masaoki Takano, b and Keiichi Tanaka a

a Faculty of Pharmacy, Osaka Ohtani University; 3–11–1 Nishikiori-Kita, Tondabayashi, Osaka 584–8540, Japan; and
b Faculty of Pharmaceutical Sciences, Kobe Gakuin University; 1–1–3 Minatojima, Chuo-ku, Kobe 650–8586, Japan.
Received April 17, 2015; accepted July 16, 2015; advance publication released online July 29, 2015

The major route of cadmium (Cd) intake by non-smokers is through food ingestion. Cd is a non-essential metal absorbed through one or more transporters of essential metal ions. Expression of these transporters is affected by nutritional status. To investigate the risk factors for Cd toxicity, the effects of deficiency of essential metals on hepatic and renal accumulation of Cd were studied in mice of different ages. Mice were administered a control diet or one of the essential metal-deficient diets, administered Cd by gavage for 6 weeks, and killed; then, Cd accumulation was evaluated. Iron deficiency (FeDF) or calcium deficiency (CaDF) resulted in remarkable increases in hepatic and renal Cd accumulation compared with control-diet mice and other essential metal-deficient mice. Cd accumulation in hepatic and renal tissue was increased significantly at all ages tested in FeDF and CaDF mice. Renal Cd concentrations were higher in 4-week-old mice than in 8- and 25-week-old mice. Increase in intestinal mRNA expression of calcium transporter (CaT)1, divalent metal ion transporter-1, and metallothionein (MT)1 was also higher in 4-week-old mice than in other mice. Renal accumulation of Cd showed strong correlation with intestinal mRNA expression of CaT1 and MT1. These data suggest that CaDF and FeDF at younger ages can be a risk factor for Cd toxicity.

Key words cadmium; age-related risk factor; essential metal-deficiency; intestinal metal transporter
MATERIALS AND METHODS

Animals All animal experiments were conducted under the guidance of the Animal Research Committee of Osaka Ohtani University (Osaka, Japan) and in accordance with the Guidelines on Animal Experiments set by Osaka Ohtani University and Japanese Government Animal Protection and Management Law (number 105). These guidelines comply with the guidelines on animal experimentation published by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Male mice (ddY strain; 5 weeks) were purchased from Nihon SLC (Shizuoka, Japan). Mice were housed in plastic cages and maintained under a 12-h light–dark cycle and had access to water ad libitum.

Control and EMDF Diets The diet used as a control was AIN-93M (Ca, 5.0 g/kg; Cu, 6.3 mg/kg; Fe, 38.8 mg/kg; Mg, 0.5 g/kg; Zn, 33.7 mg/kg) purchased from Oriental Yeast (Osaka, Japan). EMDF diets (Ca-deficient diet (CaDF), Cu-deficient diet (CuDF), Fe-deficient diet (FeDF), Mg-deficient diet (MgDF), and Zn-deficient diet (ZnDF)), were prepared to remove calcium carbonate, copper carbonate, ferric citrate, magnesium oxide, or zinc carbonate from the AIN-93M mineral mixture, respectively (Oriental Yeast). Amounts of EMs in EMDF diets were determined by wet-ashing digestion with nitric acid at 80°C for 5 d and by atomic absorption spectrophotometry (AAS; Z2300 Polarized Zeeman Atomic Absorption Spectrophotometer; Hitachi, Tokyo, Japan). Metal contents relative to the control diet were: Ca of CaDF, 1.4%±0.3%; Cu of CuDF, 9.5%±0.9%; Fe of FeDF, 21.0%±6.5%; Mg of MgDF, 3.4%±0.9%; Zn of ZnDF, 3.4%±0.4%. Control diet and EMDF diets were fed to five mice each for 3 d before repeat administration of Cd via the oral route.

Hepatic and Renal Accumulation of Cd after Repeated Long-Term Administrations of CdCl₂ to EMDF Mice CdCl₂ solution was administered by gavage to control-diet mice and EMDF mice (n=18) at 0.2 mg/kg/d for 1–6 weeks. Diets were removed before Cd administration and reinstated 2h later. All mice were killed by exsanguination under anesthesia, and

![Graph](image-url)
tissues collected 24 h after the final Cd administration. Tissue sections were digested with nitric acid and the Cd concentration in each tissue determined by atomic absorption spectrophotometry (AAS). To evaluate MT protein, ~0.2 g of the small intestine was homogenized with 1 mL of 10 mM Tris–HCl buffer (pH 7.6). After centrifugation of the sample ($10,000 \times g$ for 60 min at 4°C), the concentration of the Zn–MT complex in the resulting supernatant fraction was determined by a Cd-hemoglobin binding assay, as described previously.10

**CdCl$_2$ Administration to Mice of Different Ages** CdCl$_2$ solution was administered per os (p.o.) to control-diet, FeDF, and CaDF groups of 4-, 8-, and 25-week-old mice ($n$=5 or 6 in each group; fasted for 2 h) at 2.5 mg Cd/kg/d for 4 weeks. Diets were reinstated 2 h later. All mice were exsanguinated under anesthesia with ether 24 h after Cd administration. Livers and kidneys were collected and used for determination of the concentrations of Cd, Ca, and Fe in tissues by AAS after digestion of sections with nitric acid.

**RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses of Samples of Small Intestine in Control, CaDF, and FeDF Mice of Different Ages** Samples of dissected small intestine were homogenized in TRIzol (Invitrogen Japan K.K., Osaka, Japan), and total RNA isolated as specified in the manufacturer’s instructions. Isolated RNA was reverse transcribed to cDNA with a ReverTra Ace qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan). Quantification of mRNAs was undertaken by real-time PCR with RT-PCR Master Mix (Toyobo Co., Ltd.) on a DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories, K.K., Tokyo, Japan). Intestinal mRNA levels of DMT1 (GenBank: AF029758), CaT1 (GenBank: AB037373), MT1 (GenBank: NM_013602.3), and $\beta$-actin (GenBank: M12481.1) were assessed by real-time RT-PCR with the primers reported previously6,11–13 from Gene Design Inc. (Osaka, Japan).

**Statistical Analyses** Results are the mean±standard deviation (S.D.) Statistical significance between groups was determined using one-way ANOVA. Post hoc tests were executed by Dunnett’s test for comparisons against control values. Bonferroni’s modified t-test was employed for comparisons among groups. Correlations were analyzed by Pearson’s correlation factor. Statistical analyses were conducted using SPSS v20 (IBM, Armonk, NY, U.S.A.). $p<0.05$ was considered significant.

**RESULTS**

**Hepatic and Renal Accumulation of Cd after Repeated Oral Administration of CdCl$_2$ to EMDF Mice for 6 Weeks** Mice were fed the control diet or EMDF, administered Cd for 6 weeks, and Cd contents in the liver and kidney were evaluated (Fig. 1). Hepatic and renal accumulation of Cd was increased significantly after repeated long-term administration of Cd to FeDF and CaDF mice compared with control-diet mice (Figs. 1a, b). Compared with control-diet mice, hepatic Cd contents were increased 8-fold in FeDF mice and 1.8-fold in CaDF mice after 6-week administration (Fig. 1A). Renal Cd contents were increased 6-fold in FeDF mice and 8-fold in CaDF mice after 6-week administration (Fig. 1B).

**Intestinal MT Concentrations in Mice Fed EMDF Diets** Intestinal concentrations of MT were evaluated in EMDF mice after being fed an EMDF diet for 4 weeks (Fig. 2). Intestinal MT concentrations were increased in CaDF and FeDF mice. Compared with control mice, MT concentrations were 8-fold higher in CaDF mice and 1.5-fold higher in FeDF mice.

**Accumulation of Cd in Tissues after Oral Administration of Cd to Mice of Different Ages** Mice aged 4, 8, and 25 weeks were fed the control diet, CaDF, or FeDF, administered Cd for 4 weeks, and hepatic and renal Cd accumulation were evaluated (Fig. 3). FeDF and CaDF resulted in significant increases in Cd accumulation compared with control-diet mice of each age group (Figs. 3A, B). In the control-diet group, hepatic and renal Cd accumulation in 4-, 8-, and 25-week-old mice were similar to each other (Figs. 3A, B). However, in FeDF mice, Cd accumulations in 4- and 8-week-old mice were greater than that of 25-week-old mice (Figs. 3A, B). In CaDF mice, Cd accumulation in 4-week-old mice was greater than those of 8- and 25-week-old mice (Figs. 3A, B).

**Hepatic Concentrations of Fe and Ca in Mice of Different Ages Fed Control and EMDF Diets** Hepatic concentrations of Fe and Ca in mice of different ages fed the control diet, FeDF, or CaDF without oral administration of Cd are shown in Fig. 4. FeDF resulted in a significant decrease in hepatic Fe concentrations (Fig. 4A), but CaDF did not affect hepatic Fe concentrations (Fig. 4B). Plasma concentrations of Fe were decreased in FeDF and plasma concentrations of Ca were decreased in CaDF mice (plasma Fe concentration in FeDF mice: 33.7±22.3%; plasma Ca concentration in CaDF mice: 59.3±6.3% of control).

**Intestinal mRNA Expression of Metal Transporters and MT1 in Mice of Different Ages Fed Control and EMDF Diets** Intestinal mRNA expressions of DMT1, CaT1, and MT1 in mice of different ages fed the control diet, FeDF, or CaDF are shown in Fig. 5. Intestinal mRNA expression of DMT1 was increased markedly in 4-week-old mice, but not in 8-week-old mice, in the FeDF group (Fig. 5A). The enhancement of DMT1 mRNA expression in 8-week-old and 25-week-old mice were significantly weaker than that in 4-week-old in the FeDF group (Fig. 5A). CaT1 mRNA expression was increased significantly in mice of 4-week-old, but not of 8- and 25-week-

---

**Fig. 2. Intestinal Concentrations of MT in Mice Fed Control or EMDF Diets for 4 Weeks**

A part of the small intestine was homogenized with 1 mL of 10 mM Tris–HCl buffer (pH 7.6). After centrifugation of the sample ($10,000 \times g$ for 60 min at 4°C), intestinal concentrations of MT were determined by the Cd-hemoglobin binding assay. Data points represent the mean±S.D. of five mice. Post hoc tests were executed by Dunnett’s test for comparisons against control values. *$p<0.05$, **$p<0.01$
old mice in the CaDF group (Fig. 5B). The enhancements of CaT1 mRNA expression in 8- and 25-week-old were significantly weaker than that on 4-week-old mice (Fig. 5B). Intestinal mRNA expression of MT1 was similar with intestinal mRNA expression of CaT1 (Fig. 5C).

**Correlation between Intestinal mRNA Expression of Metal Transporters, MT1 and Tissue Accumulation of Cd Given via the Oral Route** The correlation between intestinal mRNA expression of transporters or MT1 and tissue accumulation of Cd is shown in Fig. 6. Hepatic accumulation of Cd had a significant correlation with DMT1 mRNA expression (Fig. 6A) and renal accumulation of Cd had a strong correlation with CaT1 and MT1 mRNA expression (Figs. 6E, F). Moreover, intestinal mRNA expression of CaT1 had a strong correlation with intestinal mRNA expression of MT1 (Fig. 6I).

**DISCUSSION**

Reports from our research team have demonstrated that transporters for EMs may be involved in intestinal absorption of Cd because total Cd accumulation was increased when mice were fed other EMDFs, such as those lacking Ca, Fe, Cu, Mg, and Zn.8) In the present study, FeDF or CaDF resulted in remarkable increases in hepatic and renal accumulation of Cd compared with the control after long-term oral administration of Cd at a lower dose (Figs. 1A, B). This finding suggests that FeDF and CaDF among EMDFs are risk factors for tissue accumulation of Cd administered via the oral route.

*National Health and Nutrition Survey in Japan, 2012* reported that dietary intake of Fe and Ca among teenagers is not sufficient.14) The recommended dietary allowance for Fe and Ca is higher in teenagers than for other age groups. It has been suggested that the critical level of Fe and Ca for teenagers may be lower than for other age groups. We found that
FeDF and CaDF during a growth period (4 weeks of age) resulted in greater renal accumulation of Cd compared with that at other ages (Fig. 3B). FeDF and CaDF might be risk factors in human teenagers even if they consume similar amounts of Cd compared with other age groups.

Little is known about the mechanisms of gastrointestinal absorption and tissue distribution of Cd. Gunshin et al. demonstrated that DMT1 has an unusually broad substrate range that includes Fe\(^{2+}\), Zn\(^{2+}\), and Cd\(^{2+}\). CaT1-mediated Ca\(^{2+}\) uptake is inhibited by divalent metal ions such as the lead ion (Pb\(^{2+}\)) and Cd\(^{2+}\). Suzuki et al. showed that intestinal Cd accumulation in DMT1-dysfunctional MK/Rej-(mk)/(mk) mice was the same as in wild-type mice even though the loss of DMT1 function led to decreased Fe concentrations in the intestine. Previously, we have suggested that Cd may be transported as a “hitchhiker” through highly expressed EM transporters. Among tested EMDF groups, FeDF and CaDF caused a significant increase in hepatic and renal Cd accumulation compared with the control-diet group (Figs. 1A, B). FeDF induced expression of DMT1 mRNA (Fig. 5A), and CaDF induced expression of CaT1 mRNA (Fig. 5B). FeDF did not increase expression of CaT1 (Fig. 5B), and CaDF did not increase expression of DMT1 (Fig. 5A). These data suggest that the apical transmembrane transporters DMT1 and CaT1 are strong candidates for intestinal transport of Cd in FeDF and CaDF mice, respectively.

Contribution of DMT1 and CaT1 to Cd absorption was confirmed by the correlation between mRNA expression of those transporters and Cd accumulation (Fig. 6). Hepatic and accumulation of Cd had moderate correlation with expression of DMT1 mRNA (Fig. 6A), and renal accumulation of Cd had strong correlation with expression of CaT1 mRNA (Fig. 6E). These data suggest that DMT1 and CaT1 may participate in Cd transport from the lumen. When focusing on only renal Cd accumulation, it had a strong correlation with intestinal mRNA expression of CaT1 and mRNA expression of MT1 (Figs. 6E, F).

MTs are defensive factors that can bind with Cd and transfer Cd to the kidney. Some reports have concluded that the impact of MTs on intestinal Cd absorption is of minor importance, but Cd bound to MTs has been found to be distributed preferentially in rodent kidneys. Pre-administration of Zn has been shown to significantly induce intestinal levels of MTs and renal accumulation of Cd, but such effects of Zn pretreatment have not been observed in MT-null mice. Renal accumulation is increased significantly after oral administration of Cd but not after parenteral administration in FeDF and CaDF mice, suggesting that intestinal MTs have a role in Cd distribution in the kidney. Additionally, FeDF and CaDF induced expression of MT protein and mRNA (Figs. 2, 4). These results suggest that absorbed Cd may be bound to intestinal MTs that may protect cells from toxicity, and that the Cd–MT complex that leaks into the circulation transfers preferentially to the kidney in CaDF mice.

Cd accumulation was higher in FeDF and CaDF 4-week-old mice than in 8- and 25-week-old mice, and mRNA expression of DMT1 and CaT1 were also higher in 4-week-old mice than in 8- and 25-week-old mice (Figs. 3, 4). These observations suggest that FeDF or CaDF may induce intestinal expression of DMT1 and CaT1 to stimulate uptake of Ca or Fe from the gut, respectively, and that FeDF and CaDF result in severe Cd toxicity, particularly during periods of growth in 4-week-old mice. Neonates are more sensitive to Cd toxicity than adults because of higher gastrointestinal absorption.

---

**Fig. 5. Intestinal mRNA Expression of Transporters and MT1 in Control, FeDF, and CaDF Mice**

Total RNA was isolated from the small intestine, and intestinal mRNA expression assessed by RT-PCR. Data points represent the mean±S.D. of five mice. A, mRNA expression of DMT1. B, mRNA expression of CaT1. C, mRNA expression of MT1. Post hoc tests were executed by Bonferroni’s modified t-test for comparisons among groups. *p<0.05, **p<0.01 versus control-diet mice at each age were considered significant. †p<0.05, ‡p<0.01 versus 4-week-old mice at each diet group were considered significant.
et al. demonstrated that Cd transport in a model of neonatal intestinal cells correlated with expression of multidrug resistance-associated protein-1 but not with non-heme iron transporters such as DMT1. Susceptibility of the intake and accumulation of Cd could vary depending on age.

Strong correlation between the hepatic accumulation of Cd and DMT1, and correlation among the renal accumulation of Cd, CaT1 and MT1 were observed (Fig. 6). Those correlation might suggest that the hepatic accumulation is due to DMT1, and the renal accumulation of Cd is due to CaT1 and MT1. The strong correlation between CaT1 and MT1 could suggest that the transcription of both genes share the same mechanism (Fig. 6I). Further investigation would be needed.

In 2006, the Codex Alimentarius Commission set a new international standard for maximum permitted levels of Cd. However, this standard does not deal with age- or malnutrition-

---

**Fig. 6.** Correlation between Tissue Accumulation of Cd and Intestinal mRNA Expression of Transporters and MT1 at Various Ages in Control-Diet and EMDF Mice

A, Correlation between hepatic accumulation of Cd and DMT1. B, Correlation between hepatic accumulation of Cd and CaT1. C, Correlation between hepatic accumulation of Cd and MT1. D, Correlation between renal accumulation of Cd and DMT1. E, Correlation between renal accumulation of Cd and CaT1. F, Correlation between renal accumulation of Cd and MT1. G, Correlation between DMT1 and CaT1. H, Correlation between DMT1 and MT1. I, Correlation between CaT1 and MT1. Hepatic and renal accumulation of Cd were calculated from the time periods in Fig. 3. Intestinal mRNA expression of transporters and MT1 were calculated from the data in Fig. 5. Pearson’s correlation factor, p value, and regression line for each combination were shown.
related susceptible groups. Our results suggest that hepatic and renal accumulation of Cd given via the oral route was 5–10 times higher in CaDF and FeDF mice than in control mice (Fig. 3). Renal Cd accumulation was 4-fold higher in 4-week-old mice fed CaDF than in older mice, which can lead to chronic Cd toxicity. Especially, in the condition of present experiments CaDF was mild and did not cause the decrement of Ca in liver while the FeDF cause decrement of Fe in liver although the plasma content of Ca or Fe was decreased (Fig. 2). This means that a light deficiency of Ca can enhance the accumulation of Cd. These observations suggest that teenagers whose dietary intake of Fe and Ca is not sufficient might be more sensitive to Cd toxicity than adults. An imbalance of Fe and/or Ca in the diet may be an age-related risk factor for Cd toxicity.

CONCLUSION

Among EMDFs, FeDF and CaDF resulted in marked increases in hepatic and renal accumulation of Cd compared with control-diet mice. Renal Cd concentrations, intestinal induction of DMT1 in FeDF mice, and intestinal induction of CaT1 in CaDF mice were higher in 4-week-old mice than in older mice. Renal Cd concentrations were strongly correlated with intestinal mRNA expression of CaT1 and MT1. These data suggest that dietary FeDF and CaDF may increase renal Cd accumulation after an increase in intestinal expression of DMT1, CaT1, and MT1. We propose that an imbalance in nutritional Fe and Ca (especially at younger ages) is an age-related risk factor for Cd toxicity.

Acknowledgment  The present study was supported by a Grant-in-Aid for Scientific Research (No. C-22510077) from the Japan Society for the Promotion of Science.

Conflict of Interest  The authors declare no conflict of interest.

REFERENCES