Lead Does Not Affect Transcription of Intestinal Zinc-Binding Proteins in Growing Rats

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Environmental lead exposure remains a serious concern for the growth and development of children. Micronutrient status may affect the absorption and tissue accumulation of lead, but the mechanisms of gastrointestinal uptake and transport remain unknown. Thus, our objective was to investigate the effects of lead on the mRNA levels of intestinal zinc transporter 4 (ZIP4), metallothionein (MT), cysteine-rich intestinal protein (CRIP), and divalent metal transporter 1 (DMT1) in growing rats fed marginal, adequate, and supplemental zinc diets. Weanling Sprague Dawley rats were assigned to marginal zinc (MZ; 8 mg Zn/kg diet), zinc-adequate control (CT; 30 mg Zn/kg), zinc-adequate diet-restricted (DR; 30 mg Zn/kg), or supplemental zinc (SZ; 300 mg Zn/kg) groups, with and without lead acetate–containing drinking water (200 mg Pb/l) for 3 weeks. Duodenum was analyzed for ZIP4, MT, CRIP, and DMT1 mRNA levels by real-time reverse transcription–polymerase chain reaction and MT immunolocalization. Tissues were analyzed for zinc, lead, and iron by inductively coupled plasma spectrometry. MZ rats had higher duodenal ZIP4 mRNA levels, lower MT mRNA levels, lower MT immunostaining intensity, and lower zinc concentrations than DR, CT, and SZ. Duodenal DMT1 mRNA levels were lower in DR and SZ compared with MZ. Tissue lead concentrations responded to dietary zinc with SZ < CT < DR ≤ MZ. The greater accumulation of hepatic lead in MZ rats was associated with zinc deficiency as well as diet restriction. Lead treatment resulted in higher hepatic iron concentrations but had no effect on duodenal ZIP4, MT, CRIP, or DMT1 mRNA levels. Thus, tissue lead accumulation was not directly mediated by the transcriptional induction of zinc and iron binding or transport proteins. The mechanisms of lead absorption during nutritional deficiency and supplementation require further investigation. Exp Biol Med 232:744–753, 2007

Key words: lead; zinc; ZIP4; DMT1; metallothionein

Introduction

Chronic exposure to environmental lead remains a significant public health issue among low-income populations (1). Lead toxicity targets the nervous, renal, hematopoietic, skeletal, and reproductive systems, with the effects being most insidious during growth and development (1, 2). Poor nutritional status may increase lead absorption and potentiate toxic effects (3); thus, supplementation with essential minerals may compete for lead during intestinal uptake and transport, thereby decreasing absorption and/or toxicity. This interaction between zinc nutrition and lead exposure is a potentially significant health concern, as marginal or subclinical zinc intakes in human populations may be more common than previously thought (4).

There is experimental evidence that lead-exposed rats maintained on zinc-deficient diets have higher blood and tissue lead levels than rats fed zinc-replete or zinc-supplemented diets (5–8). However, these responses cannot be attributed to zinc deficiency alone, as weight loss associated with zinc deficiency was not accounted for in these earlier studies. In addition, zinc recycling and contamination may not have been well controlled for in the past, as suggested by low-zinc diets that failed to demonstrate a reduction in rate of weight gain (5). Zinc contamination can occur through contact with glassware that is not properly acid washed, use of metal equipment not made of stainless steel (e.g., galvanized coatings contain zinc), and cross-contamination of zinc-replete and zinc-deficient diets, for example. Nonetheless, the protective effect of zinc against tissue lead accumulation has been attributed to an interaction at the gastrointestinal level, as intraperitoneal zinc administration does not have the same protective effect (5).

Toxic metals often have characteristics similar to those

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of essential metals, and therefore may take advantage of the transport and binding proteins for essential metals in order to gain access to enterocytes and other target cells (9). Lead is thought to target proteins that naturally bind zinc and has shown strong affinity for the cysteine-rich zinc sites of these proteins (10). While various studies have investigated the impact of mineral nutrition on measures of lead absorption and toxicity, the molecular mechanisms of intestinal uptake and transport of lead remain unknown. Thus, it is hypothesized that zinc supplementation may decrease lead absorption at the gastrointestinal tract through competition for binding to an intestinal, zinc transport protein, such as zinc transporter ZIP4. In addition, the zinc-binding proteins metallothionein (MT) and cysteine-rich intestinal protein (CRIP) may function in lead detoxification through sequestration of lead within the enterocyte or transport to intestinal Paneth cells for storage.

Paneth cells are widely distributed in the base of the crypt of Lieberkuhn (11) and have high concentrations of zinc (11, 12). This property may be related to zinc storage for functional use or possibly as a route for elimination of heavy metals, although these hypotheses are lacking in critical evidence (11, 13). CRIP is a 77–amino acid, 8.6-kDa protein specific to the intestine, monocytes, and macrophages (11). Intestinal CRIP is predominantly localized within Paneth cells within the intestinal crypt (11).

MT proteins are ubiquitous and are characterized by their highly conserved cysteine content, low molecular weight (6–7 kDa), high metal content, and lack of aromatic amino acids (14, 15). MT is thought to function in the regulation of zinc metabolism (16) and is inducible by essential trace elements, such as zinc and copper, several toxic metal ions, hormones, cytotoxic and inflammatory agents, and cellular stress (15). Strong MT immunostaining has been reported in the intestinal epithelia, which diminished in response to zinc deficiency (17). The localization of MT within the intestinal crypt and proliferative region of the villi was thought to support a role for MT in heavy metal detoxification (17). Furthermore, studies in MT knockout mice indicate that MT is the source of zinc for the zinc finger motifs of CRIP, suggesting a role for CRIP in zinc nutritional status (14).

Several zinc transporters have recently been cloned from a variety of organisms. In the enterocyte, transporter ZIP4 is the primary importer of dietary zinc (19). It is a member of the ZIP family transporters (SLC39A gene family) found on the apical membrane of the enterocyte, mainly lining the intestinal villi (19). Preliminary studies indicate that ZIP4 expression in the enterocyte is regulated by extracellular zinc concentration (19); however, the transcriptional response to lead has not been previously reported.

It has also been suggested that lead uptake may accompany iron absorption through a common gastrointestinal pathway (20). Cell culture studies have reported that the major intestinal ferrous iron transport protein, divalent metal transporter (DMT1), transports both iron and lead with similar affinity in yeast cells (20). Lead transport by DMT1 may also explain the correlation between low dietary iron intake and increased lead absorption in animal models, as DMT1 mRNA is sharply upregulated when an iron-deficient diet is provided (21).

While remediation is always the top priority in cases of childhood lead intoxication, there may be circumstances in which this is not possible. The roles of intestinal transport and binding protein(s) for mitigating lead exposure have not been extensively investigated to date. Thus, further research in this area may lead to additional methods to reduce lead exposure. Therefore, the objective of this study was to investigate the *in vivo* effects of lead on the transcription of intestinal MT, CRIP, ZIP4, and DMT1 and the immunolocalization of MT with conditions of low, adequate, and excess dietary zinc supply in growing rats.

Materials and Methods

Weanling male Sprague Dawley (SD) rats ($n = 64$) weighing approximately 55–65 g were obtained from Charles River Laboratories (St. Constant, PQ, Canada). Animals were housed in a temperature-controlled (21°C–23°C) and humidity-controlled (55%) room with a 14:10-hr light:dark cycle. Following a 5-day acclimatization period to a nutritionally complete control diet, rats were randomly assigned to treatment groups ($n = 8$ per group) for 3 weeks. Treatment groups (mg Zn/kg diet and mg Pb/l drinking water) included marginal zinc intake (MZ^- group: 8 mg Zn/kg, 0 mg Pb/l), lead-exposed and marginal zinc intake (MZ^+: 8 mg Zn/kg, 200 mg Pb/l), supplemental zinc intake (SZ^-: 300 mg Zn/kg, 0 mg Pb/l), lead-exposed and supplemental zinc intake (SZ^+: 300 mg Zn/kg, 200 mg Pb/l), a control diet group (CT^-: 30 mg Zn/kg, 0 mg Pb/l), a lead-exposed group fed control diet (CT^+: 30 mg Zn/kg, 200 mg Pb/l), a diet-restricted group, which was to maintain a weight comparable to the MZ^- group (DR^-: 30 mg Zn/kg, 0 mg Pb/l), and a lead-exposed, diet-restricted group, so as to maintain a weight comparable to the MZ^+ group (DR^+: 30 mg Zn/kg, 200 mg Pb/l). Of note, when dietary groups (MZ, DR, CT, and SZ) are expressed without the +/– designation, the Pb and non-Pb data have been combined as a main effect of dietary zinc ($n = 16$), as per the statistical analysis. Similarly, treatment groups designated Pb^- and Pb^+ represent the pooled data of all non–lead- and lead-treated groups ($n = 32$), respectively. This was done in order to analyze the main effects in the absence of a significant interaction effect between lead and zinc. DR groups were included in the study to control for the lower rate of weight gain seen in zinc-deficient animals. MZ and DR rats were weighed daily and were weight matched by restricting the feed of the DR rats.

Lead (as acetate) was administered in the drinking water at 200 mg Pb/l and provided *ad libitum* to produce a subclinical toxicity, as this dosage given for 10 weeks with a

standard laboratory rat chow has been shown as not producing significant alterations in hematopoiesis, renal size, histology, and function (22). Water was chosen as the administration route to ensure a more uniform dosage, as well as to avoid airborne exposure during diet preparation. Non–lead-treated animals received double deionized water *ad libitum*. All drinking water was provided in plastic bottles with stainless steel sipper tubes. A marginal rather than a severe (<1 mg Zn/kg) zinc deficiency diet was chosen in order to reflect the mild states of zinc deficiency most commonly seen in human populations (23). Zinc supplementation at 300 mg Zn/kg was selected as a high but nontoxic dose in growing rats. All dietary treatments except DR^- and DR^+ were provided *ad libitum*. Experimental diets were modified AIN-93G diets containing egg white, additional biotin (2 mg/kg), and potassium phosphate (5.4 g/kg diet for the growth formulation), as previously described (24) and as necessary to deliver the MZ diet. Zinc was added to each diet as $ZnCO_3$ according to the desired concentration for each dietary group. All diets were powdered and made in stainless steel bowls that had been rinsed previously with distilled water. Rats were housed individually in stainless steel hanging cages, and special precautions were taken to avoid zinc and lead recycling and contamination throughout the experimental period. Lead-treated animals were housed in a separate cage rack, and low-zinc groups were housed above the groups with higher zinc diets.

Feed intake and water intake were recorded throughout the study, and diet spillage was measured. Body weight was recorded weekly for all groups, except after Day 10, when it was recorded daily in order to restrict the feed intake of the DR^- and DR^+ rats to match the weight of the MZ^- and MZ^+ treatments, respectively. The protocol for animal care procedures was approved by the University of Manitoba Protocol Management and Review Committee.

Tissue Collection. All animals were euthanized by CO_2 asphyxiation and exsanguination in accordance with the guidelines of the Canadian Council on Animal Care (1993). Body weights were recorded, and trunk blood was collected. Blood samples were stored on ice until centrifuged (Beckman TJ-6R centrifuge; Beckman Coulter, Inc., Fullerton, CA) at 1290 *g* for 15 mins to obtain serum. The small intestines and livers were removed. Sections of the duodenum (1 cm distal to the pylorus) and jejunum (midsection) were excised and briefly rinsed with phosphate-buffered saline to remove superficial blood and intestinal contents. Intestinal samples then were fixed in 10% phosphate-buffered formalin for 24 to 48 hrs in preparation for immunohistochemical studies. The remaining intestinal tissue and liver were immediately frozen in liquid nitrogen and later stored at –80°C, along with the serum samples. Other tissues were collected and analyzed from these animals as previously described (25, 26).

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Total RNA extraction was carried out on 200 mg intestinal tissue using TRIzol (Invitrogen Corp., Carlsbad, CA). Isolated RNA was treated for 15 mins at room temperature with DNAse I (Invitrogen Corp.) to remove DNA. Custom primers were designed using the Internet-based National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov.proxy2.lib.umanitoba.ca/entrez/query.fcgi?db=PubMed) for MT (accession no. BC 058442), CRIP (accession no. AA925488), DMT1(accession no. NM 013173), and ZIP4 (accession no. XM 216970). Oligonucleotide sequences were: MT sense, 5′-GCC TTC TTG TCG CTT ACA CC-3′, MT antisense, 5′-CTT CTT GCA GGA GGT GCA TT-3′; CRIP sense, 5′-CCC TGC TGT CTA GGG ACA AG-3′, CRIP antisense, 5′-ACT GCA ACC ATC CCT GCT AC-3′; DMT1 sense, 5′-ACT ATG ACC GGC ACC TAT GC-3′, DMT1 antisense, 5′-CCT CAG GTC TCG GAA GAC AG-3′; and ZIP4 sense, 5′-CTT GGC TCT AGG CAA ACC TG-3′, ZIP4 antisense, 5′-GCA TGT TTT CTC TGG GTC GT-3′. The housekeeping gene L32 (accession no. X06483) was included in the analysis as a control, with an oligonucleotide sequence of: L32 sense, 5′-AAG ATT CAA GGG CCA GAT CC-3′, and L32 antisense, 5′-GTT GCA CAT CAG CAG CAC TT-3′. Real-time RT-PCR reactions were performed on 500 ng template RNA using the QuantiTect SYBR Green kit (Qiagen, Mississauga, ON, Canada) and on a Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA) sequence detection system. The following protocol was employed: 30 mins for RT at 50°C, 15 mins of incubation at 95°C for PCR activation, followed by 15 secs at 94°C, 30 secs at 55°C, 30 secs at 72°C, and 40 cycles of amplification. Melt-curve analyses were performed on each set of primers to confirm product size. Relative amounts of mRNA were determined by comparing cycle threshold (CyT) values for equal amounts of amplified RNA. The difference between control and treatment mRNA expressions was calculated from the difference in CyT values using the formula $2^{\Delta CyT}$. Of note, the housekeeping gene L32 was not affected by dietary treatment when analyzed by 2-way ANOVA. Additionally, the incorporation of L32 mRNA levels as a statistical covariate did not alter the MT, CRIP, DMT1, and ZIP4 mRNA results.

Immunohistochemical Localization of MT. Standard procedures for indirect immunoperoxidase staining were used to determine MT localization in the small intestine. Briefly, endogenous peroxidase activity was inactivated by treatment with 3% hydrogen peroxide, and slides were incubated for 1 hr with monoclonal mouse anti-MT antibody (clone E9 [DAKO, Carpinteria, CA] diluted 1:25 in phosphate-buffered saline) at room temperature. Sections were then layered with goat anti-mouse/anti-rabbit peroxidase-labeled polymer (DAKO Envision System; DAKO; diluted 1:10 in phosphate-buffered saline) and incubated at room temperature for 30 mins. The reaction product was visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride (DAB·4HCl; Polysciences Inc., Warrington, PA). Tissues were counterstained with Harris

hematoxylin-eosin. The specificity of the reaction was confirmed by omission of the anti-MT antibody from the procedure. Using a light microscope, the intensity of MT staining was estimated subjectively at four levels: nil, defined as the absence of staining; weak, defined as the staining just visible above the background at lower magnification (×10 objective); moderate, defined as the staining easily visible at lower magnification; and strong, defined as the staining easily visible at lowest magnification (×2.5 objective). The evaluator was blinded to the animal number and treatment group during the evaluation process. Computer images of immunostained sections were obtained with Northern Eclipse software version 6.0 (Empix Imaging Inc., Toronto, ON, Canada).

Mineral Analysis. After obtaining wet and dry weights, organ and diet samples were wet ashed using trace-element–grade nitric acid, as previously described (27). Acid digests were diluted appropriately with double deionized water before analysis of lead and zinc (all tissues and diets) and iron (liver) by ICP-AE analysis (Varian Liberty 200; Varian, Georgetown, Ontario, Canada). Bovine liver standard reference 1577b (National Institute of Standards and Technology, Gaithersburg, MD) was processed in triplicate as a quality control. The detection limits for lead, zinc, and iron were 0.5 μg/ml, 0.1 μg/ml, and 0.1 μg/ml, respectively.

Statistical Analysis. Data were analyzed for both main effects of lead and zinc and interactions of lead and zinc by two-way ANOVA using SAS software version 9.1 (SAS Institute, Cary, NC). However, when no lead was detectable in the tissues of non–lead-treated animals, a one-way ANOVA was used to analyze the MZ^+, DR^+, CT^+, and SZ^+ groups only. Repeated measures analysis was performed for main effects and interactions on weekly feed efficiency. Data were checked for normality and homogeneity of variance and transformed when necessary, although nontransformed means are reported. In the instance in which log transformation did not normalize the data but the data set had homogeneity of variance and sample sizes were equal, ANOVA was considered robust. For main effects, significant differences among treatment group means were determined with Duncan multiple range test. Differences were considered significant at $P < 0.05$. All data are reported as means ± SEM. As there was no interaction between lead and zinc, table and figures report means of main effects of lead ($n = 32$) and zinc ($n = 16$) only.

Results

Growth. There was no interactive effect of lead and zinc on weight gain (Table 1). Since there was a main effect of lead and a main effect of zinc, treatment groups were pooled as described in Materials and Methods. As a main effect, Pb^+ rats had 10% less total weight gain than Pb^- rats. As a main effect of zinc, MZ and DR rats gained 17%–19% less total weight than CT rats, whereas SZ rats gained 7% more weight than CT rats. When analyzed by week, MZ rats had less weight gain (17%–23%) than DR, CT, and SZ rats during Week 1. However, during Week 3, DR rats had less weight gain (36%–43%) than MZ, CT, and SZ rats.

Feed efficiency was calculated as weekly weight gain divided by weekly feed intake (Table 1). There was no main effect of lead on feed efficiency over time. As a main effect of zinc, the feed efficiency of MZ rats was 9% lower than CT rats over the first week of the study, and it was 13% less than SZ rats over the second week. During the third and final week of the study, the feed efficiency of MZ rats recovered to the same level as CT and SZ rats, whereas DR rats dropped to a level 34% lower than CT rats. There was no interaction of lead and zinc on feed efficiency over time.

There was a main effect of zinc on intestinal weight, with MZ rats having a 12%–17% lower intestinal weight than CT and SZ rats, but this was not significant when corrected for body weight (Table 1).

Trace Mineral and Lead Assessment. Serum zinc (Fig. 1a), duodenal zinc, (Fig. 1b), and jejunal zinc (data not shown) concentrations were reflective of dietary zinc intake ($MZ < DR = CT < SZ$) as a main effect of zinc, but they were not altered by lead treatment. Hepatic zinc concentration was not affected by lead and was not different among MZ, CT, and SZ groups (data not shown). DR groups had 10% higher hepatic zinc concentrations than MZ rats (1.75 ± 0.04 vs. 1.58 ± 0.03 μmol Zn/g dry weight), but they were not different from CT or SZ rats.

Total lead consumed during the 3-week study was not affected by dietary zinc intake or diet restriction (Table 1). Tissue lead concentrations were only detectable in the four lead-treated groups. Hepatic lead concentration was 52% higher in MZ^+ rats than in CT^+ rats, and was 63% lower in SZ^+ rats than it was in CT^+ rats (Fig. 1c). There was no difference between DR^+ and MZ^+ rats or DR^+ and CT^+ rats in terms of hepatic lead concentration. Lead concentration was not affected by dietary treatment in the duodenum ($P = 0.3485$) or jejunum ($P = 0.8594$; data not shown).

There was a main effect of lead on hepatic iron concentration (Fig. 1d), resulting in Pb^+ rats having a 16% higher iron concentration than Pb^- rats, but there was no effect of dietary zinc on hepatic iron.

Duodenal mRNA Levels. There was a main effect of zinc on duodenal ZIP4 (Fig. 2a) and duodenal MT (Fig. 2b) mRNA levels. ZIP4 mRNA was 10-fold higher in MZ compared with CT rats, whereas there was no difference between CT and DR rats or CT and SZ rats. Conversely, MT mRNA levels reflected dietary zinc intake, as MZ rats' levels were 67% lower than those in CT and 82% lower than those in SZ rats. MT mRNA levels also were lower in MZ rats than in DR rats. There was no main effect of lead on ZIP4 or MT mRNA levels and no interaction between lead treatment and dietary zinc. Similar results for ZIP4 and MT also were found in the jejunum (data not shown). DMT1 (Fig. 2c) mRNA levels as a main effect of zinc were approximately 3- to 4-fold higher in MZ than in DR and SZ

Table 1. Effects of Lead, Dietary Zinc, and Diet Restriction on Growth and Lead Dose[a]

	Lead treatment[b]			Dietary group[c]				
Measurement	Pb^-	Pb^+	*P*	MZ	DR	CT	SZ	*P*
Weight gain[d]								
Week 1 (g/week)[e]	60 ± 1	54* ± 2	<0.05	48^B ± 2	59^A ± 1	58^A ± 2	63^A ± 1	<0.05
Week 2 (g/week)	50 ± 2	44* ± 2	<0.05	39^B ± 2	42^B ± 3	51^A ± 2	56^A ± 1	<0.05
Week 3 (g/week)[e]	48 ± 2	45 ± 2	NS	48^B ± 2	31^C ± 3	53^{AB} ± 2	55^A ± 1	<0.05
Total (g/3 weeks)	159 ± 3	143* ± 5	<0.05	135^C ± 4	132^C ± 3	162^B ± 4	174^A ± 3	<0.05
Feed efficiency (gain to feed)								
Week 1 (g/g)	0.52 ± 0.01	0.50 ± 0.01	NS	0.47^B ± 0.01	0.52^A ± 0.01	0.52^A ± 0.01	0.53^A ± 0.01	<0.05
Week 2 (g/g)	0.38 ± 0.01	0.37 ± 0.01	NS	0.35^B ± 0.01	0.36^{AB} ± 0.02	0.39^{AB} ± 0.01	0.40^A ± 0.01	<0.05
Week 3 (g/g)	0.31 ± 0.01	0.32 ± 0.01	NS	0.36^A ± 0.01	0.23^B ± 0.02	0.34^A ± 0.01	0.34^A ± 0.01	<0.05
Intestine								
Wet weight (g)	6.15 ± 0.17	5.82 ± 0.16	NS	5.45 ± 0.16^C	5.72 ± 0.26^{BC}	6.19 ± 0.24^{AB}	6.59 ± 0.16^A	<0.05
Relative wet weight	0.023 ± 0.001	0.023 ± 0.001	NS	0.023 ± 0.001	0.024 ± 0.001	0.023 ± 0.001	0.024 ± 0.001	NS
Lead dose (mg/3 weeks)	—	—	—	97.3 ± 5.5	92.8 ± 6.5	90.1 ± 6.8	79.1 ± 10.0	NS

[a] As there was no interaction between lead and zinc but there was a main effect of lead and a main effect of zinc, data were pooled to show means of main effects only. Pb^- represents MZ^-, DR^-, CT^-, and SZ^- groups. Pb^+ represents MZ^+, DR^+, CT^+, and SZ^+ groups. MZ represents MZ^- and MZ^+. DR represents DR^- and DR^+. CT represents CT^- and CT^+. SZ represents SZ^- and SZ^+. Values are means ± standard error for $n = 32$ (lead effect) and $n = 16$ (zinc effect), as determined by 2-way ANOVA, except for total lead dose and feed efficiency. Values for lead dose are means ± standard error ($n = 8$) for MZ^+, DR^+, CT^+, and SZ^+, as determined by 1-way ANOVA. Feed efficiency, reported as means ± standard error for $n = 32$ (lead effect) and $n = 16$ (zinc effect), was determined by repeated measures analysis.

[b] Statistical differences are indicated by an asterisk (lead effect).

[c] Statistical differences are indicated by capital letters (zinc effect).

[d] Initial body weights at the onset of the study were not different, ranging from 104 ± 3 g to 108 ± 2 g among all 8 treatment groups ($n = 8$).

[e] Data are not normal but have homogeneity of variance and equal sample size.

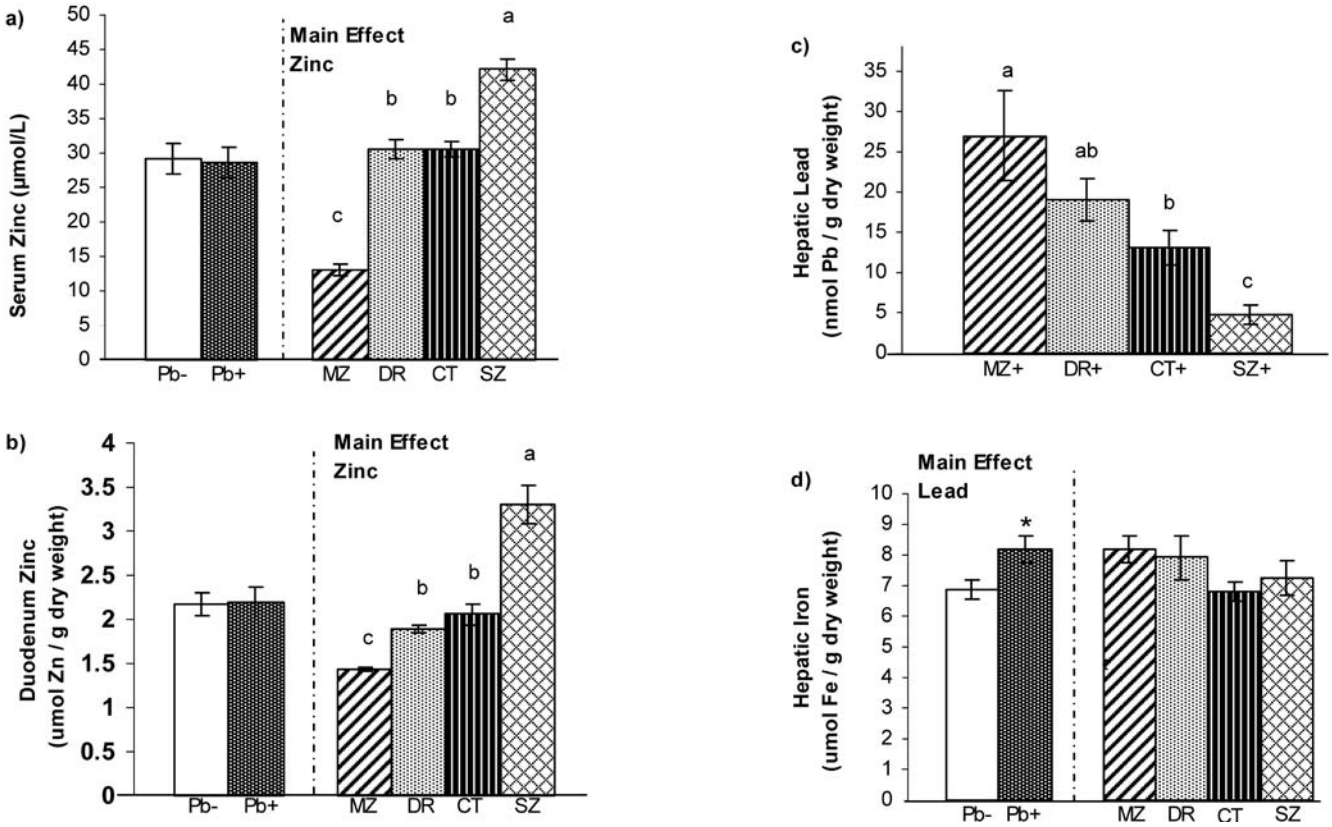

Figure 1. Effects of lead, dietary zinc, and diet restriction on serum zinc (a), duodenal zinc (b), hepatic lead (c), and hepatic iron (d) concentrations. Values are means ± standard error for $n = 31$ to 32 (lead effect) and $n = 14$ to 16 (zinc effect), as determined by 2-way ANOVA (a, b, d) and means ± standard error for $n = 8$, as determined by 1-way ANOVA (c). Statistical differences among means ($P < 0.05$) are indicated by an asterisk (lead effect) and lowercase letters (zinc effect). As there were no interactions between lead and zinc but there were main effects of lead and zinc, data were pooled to show means of the main effects only. Pb^- represents MZ^-, DR^-, CT^-, and SZ^- groups. Pb^+ represents MZ^+, DR^+, CT^+, and SZ^+ groups. MZ represents MZ^- and MZ^+. DR represents DR^- and DR^+. CT represents CT^- and CT^+. SZ represents SZ^- and SZ^+.

rats, although none of the dietary treatment groups were different than CT rats. There was no main effect of lead on DMT1 mRNA and no interaction between lead treatment and dietary zinc. CRIP mRNA levels were not affected by lead exposure or dietary zinc intake in the duodenum (Fig. 2d) or jejunum (data not shown).

Duodenal MT Immunolocalization. All negative control sections were absent of MT staining (data not shown), indicating that the staining was specific for MT. There were no apparent differences in staining intensity or distribution between the lead and non-lead treatments for intestinal MT. Thus, these results will only be discussed in terms of the response to dietary zinc. In addition, duodenal and jejunal sections were similar, so only the duodenum is reported here. There was strong nuclear and cytoplasmic MT staining in Paneth cells of intestinal crypts in all treatments except the MZ groups (Fig. 3a and b). MZ treatment resulted in weak staining that was limited to very few cells in each intestinal section. MT staining of the nuclei and cytoplasm of villi surface epithelial cells, with the exception of goblet cells, was weak to nil in all treatment groups, except in the duodenal sections of SZ groups, which stained strongly. In general, MT staining was present mainly in the proliferative region of the villi, rather than the apical tip. There was no MT staining detectable in the lamina propria, submucosa, muscularis, or vasculature in intestinal sections of any treatment group.

Discussion

This is the first study to investigate the mRNA levels of ZIP4, MT, CRIP, and DMT1 in response to lead exposure at varying intakes of dietary zinc. Zinc supplementation was highly protective against tissue lead accumulation, as SZ rats had 60%–87% lower lead concentrations than MZ rats in the liver (Fig. 1c), kidney (26), and femur (25). Despite this strong response, lead does not appear to induce the synthesis of intestinal zinc-binding and transport proteins or the iron transporter DMT1 (Fig. 2). Although the dose used in the present study resulted in growth inhibition, this dose may have been insufficient to induce a transcriptional response of intestinal proteins over the 3-week treatment. Alternatively, lead may also take advantage of other available metal-binding proteins in the enterocyte or may affect protein expression through posttranslational mechanisms. ZIP4 mRNA levels were 10-fold higher in MZ than

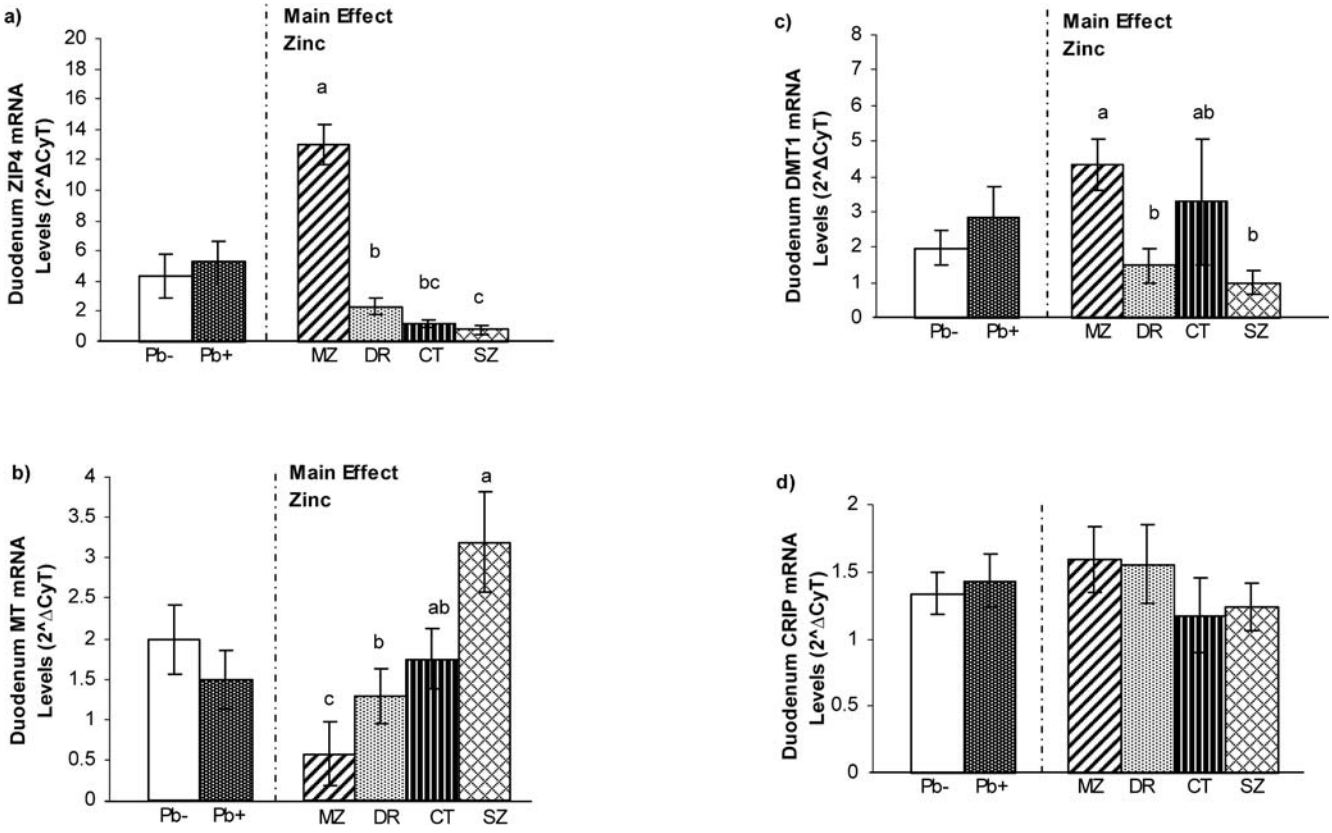

Figure 2. Effects of lead, dietary zinc, and diet restriction on duodenal ZIP4 (a), MT (b), DMT1 (c), and CRIP (d) mRNA levels. Values are means ± standard error for $n = 16$ to 19 (lead effect) and $n = 8$ to 10 (zinc effect), as determined by 2-way ANOVA. Data are expressed as the difference between control and treatment mRNA levels, calculated using the formula $2^{\Delta CyT}$. Statistical differences among means ($P < 0.05$) are indicated by lowercase letters (zinc effect). As there were no interactions between lead and zinc but there were main effects of zinc, data were pooled to show means of the main effects only. Pb^- represents MZ^-, DR^-, CT^-, and SZ^- groups. Pb^+ represents MZ^+, DR^+, CT^+, and SZ^+ groups. MZ represents MZ^- and MZ^+; DR represents DR^- and DR^+. CT represents CT^- and CT^+. SZ represents SZ^- and SZ^+.

in CT rats (Fig. 2a). Thus, ZIP4 upregulation may have contributed to the increased tissue lead deposition seen in this dietary group without being directly induced by lead, as toxic metals are thought to be inadvertently absorbed through the transport pathways for essential nutrients such as zinc or iron (28).

It was also hypothesized that MT induced in the intestine of DR, CT, and SZ rats may sequester lead within the enterocyte, contributing to the lower tissue lead levels seen in these rats. However, lead did not affect the cellular localization of MT in the duodenum or jejunum, and lead concentration in the intestine did not vary with low- or high-zinc diets. Thus, these results do not support a role for MT in lead sequestration in the gut. Based on previous studies, the primary role of MT in lead detoxification may be in the formation of nuclear lead inclusion bodies in target tissues (29) rather than a direct role in cellular uptake. It is also possible that lead may compete for binding with intracellular proteins; however, the specifics regarding intracellular metal competition with respect to lead are poorly defined at this time.

This study was unique in its use of diet-restricted animals to determine effects of zinc deficiency versus its associated malnutrition on lead uptake, as well as in the comparison of outcome measures in the proximal duodenum to the jejunum. Previously, MT, CRIP, and zinc levels have only been assessed in the small intestine as a whole (17, 30). In the present study, ZIP4, MT, and CRIP levels (Fig. 2) and zinc concentrations (Fig. 1b) were similar in the duodenum and jejunum, strengthening the confidence in these results. While previous studies have reported increased tissue lead levels in zinc-deficient animals (5, 6, 8), this is the first study to control for zinc deficiency–induced anorexia by including diet-restricted groups while investigating lead uptake and toxicity. Gastrointestinal lead absorption is thought to be influenced by meal size and total feed intake, as well as nutrient composition (31). However, reduced feed intake did not seem to strongly contribute to higher lead levels in the soft tissues (liver [Fig. 1c] and kidney [26]), as DR^+ was not different from MZ^+ or CT^+. Furthermore, lead deposited in the femur, reflecting whole-body accumulation, was 2- to 2.5-fold higher in MZ^+ than in DR^+ or CT^+ (25). Therefore, while reduced feed intake may play a small role in soft tissue lead accumulation, this may not significantly contribute to whole-body lead retention in the skeleton.

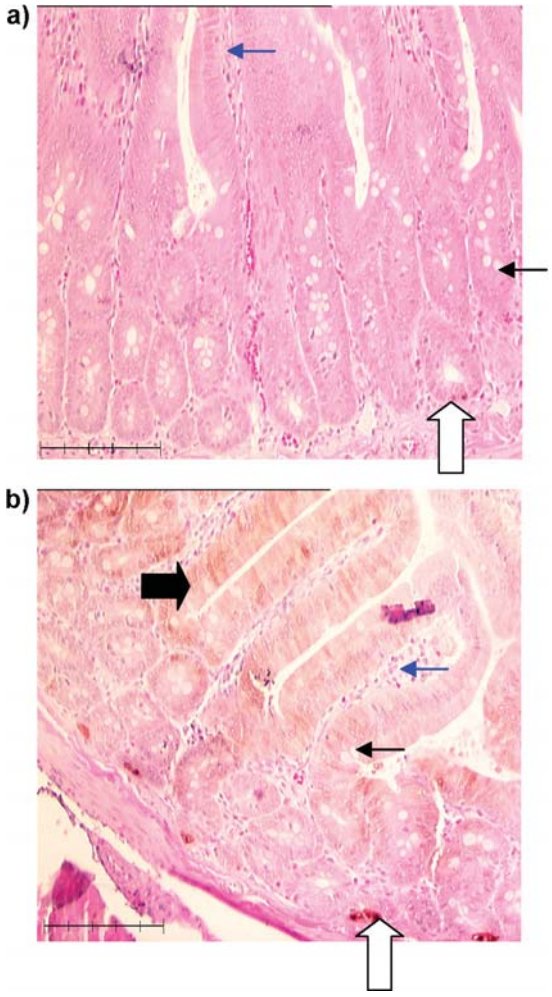

Figure 3. Immunohistological staining for MT in rat duodenum. All negative control sections were absent of MT staining (data not shown). As there was no effect of lead on MT staining and results were similar between DR, CT, and SZ groups, only images to compare MZ^- (a) and SZ^- (b) are shown. There was strong nuclear and cytoplasmic MT staining in Paneth cells (white block arrow) in all treatments except MZ. MT staining in villi epithelial cells (black block arrow) was strong in SZ and nil in MZ rats. No detectable MT staining was present in goblet cells (black arrow) and lamina propria (blue arrow) in any treatment. Scale bars shown at the bottom left corner equal 100 μm.

Also of note, the weanling rats fed 8 mg Zn/kg diet in the present study demonstrated a modest reduction in rate of weight gain, as expected (Table 1). When Cerklewski and Forbes (5) fed weanling rats the same level of dietary zinc for 3 and 7 weeks, there was no difference in weight gain compared with control rats, suggesting possible zinc contamination. In the present study, low zinc status of MZ rats was confirmed by lower serum (Fig. 1a) and femoral (25) zinc concentrations, downregulated intestinal MT levels (Figs. 2b and 3), and upregulated ZIP4 transcription (Fig. 2a). Zinc-supplemented rats had higher serum and duodenum zinc concentrations (Fig. 1a and b), as well as weight gain (Table 1), than CT rats. Interestingly, the growth pattern of MZ rats was distinct from DR rats and Pb^+ rats. While the feed efficiency and weight gain of MZ rats (Table 1) was initially impaired, both measures were able to recover by the third week of the study to levels equivalent with CT rats, suggesting compensatory mechanisms to the limited zinc supply. In contrast, DR rats grew well during the first week but fell behind MZ, CT, and SZ rats by Week 3 with respect to feed efficiency and weight gain (Table 1). This is likely due to the technique of pair-weighing, as DR feeding was adjusted only after MZ intake and growth could be assessed. With respect to weight gain, growth in Pb+ rats was impaired during the first 2 weeks of the study, but not the third (Table 1). Thus, there may also be compensatory responses to a low-dose, chronic lead exposure that will allow growth to recover over time.

The upregulation of ZIP4 transcription seen in MZ rats (Fig. 2a) confirms previous observations in severely zinc-deficient mice (32–35). The induction of ZIP4 expression by dietary zinc depletion also has been shown to result in the localization of the protein to the enterocyte apical membrane, although the stimulus of this upregulation and cellular translocation is unclear (35). ZIP4 regulation by dietary zinc is thought to occur through both transcriptional and posttranslational mechanisms and is a key homeostatic response to increased luminal zinc uptake (32, 36).

DMT1 mRNA is regulated by dietary iron supply (21); however, there is *in vitro* evidence that DMT1 may also act as a transporter for lead (28). For example, lead uptake is elevated in human fibroblasts and yeast cells when DMT1 is overexpressed, but it is inhibited by the presence of iron (21). In the present experiment there was no effect of lead exposure on duodenal DMT1 mRNA levels at any level of dietary zinc (Fig. 2c). Thus, oral lead exposure does not appear to have the capacity to induce intestinal DMT1 transcription under these conditions. However, there was a trend toward upregulation of intestinal DMT1 transcription with MZ versus DR, CT, and SZ treatments, although this effect failed to reach statistical significance with respect to CT values. Increased DMT1 transcription is likely a compensatory response to zinc deficiency, as DMT1 has been shown to transport zinc in a frog oocyte model (21). DMT1 does not appear to be related to tissue lead accumulation in the present study.

The lead-induced hepatic iron accumulation (Fig. 1d) in the present study was not explained by lead-induced DMT1 mRNA levels in the intestine. Intraperitoneal lead administration has been shown to increase hepatic iron by as much as 24% (20). This response was attributed to an increased hepatic iron uptake and/or iron retention in the liver upon exposure to lead and was supported by a 26% reduction in total iron in the blood (20). Furthermore, based on the results of this study, intestinal DMT1, at least at the mRNA level, does not appear to be responsible for hepatic iron accumulation. Thus, the redistribution of body iron stores may be primarily responsible for this effect. However, an increased rate of iron transfer in the enterocyte or increased

IREG-1 activity (the intestinal transporter responsible for iron efflux) could also be involved.

There was no effect of lead treatment on intestinal MT mRNA levels (Fig. 2b) or the intensity or distribution of MT immunostaining (Fig. 3) in the intestine. This is in contradiction to the ability of cadmium, silver, and other toxic metals that are strong inducers of the zinc-binding protein MT (15). Previous studies have reported lead induction of MT and low affinity binding of lead to MT in hepatic but not renal tissues (37); however, the intestinal response was not measured or reported in those studies. This is an important consideration, as the most critical interaction between lead and zinc may occur at the gastrointestinal level. Furthermore, hepatic MT induction by lead has been relatively well characterized with intraperitoneal and subcutaneous injection (37), but not with oral lead exposure. The lack of a response in intestinal MT mRNA to lead was reflected in MT immunostaining, as lead treatment did not obviously affect the intensity or distribution of MT.

Intestinal CRIP mRNA levels were not affected by lead treatment, suggesting that it is not involved in the absorption or detoxification of lead. Dietary zinc also had no affect on intestinal CRIP mRNA, confirming previous work (30). There is considerable evidence that CRIP appears to be involved in the immune response (18); however, further roles in the uptake of other heavy metals such as cadmium have not been investigated.

In summary, the protective effects of dietary zinc supplementation against tissue lead accumulation were not associated with changes in mRNA levels of duodenal zinc and iron binding or transport proteins, or with increased lead accumulation in the intestine of rats. Of note, dietary restriction had no effect on the intestinal mRNA levels of ZIP4 and MT, serum and duodenal zinc concentrations (Figs. 1–2), or bone lead concentrations (25), implying that the effects of MZ on these parameters are a direct consequence of zinc deficiency rather than the effects of reduced feed intake and the associated energy malnutrition. While DR may have contributed to lead concentrations in soft tissues, this effect did not translate into bone lead concentrations. This is critical, as skeletal lead deposition is the primary storage reservoir for lead in the body (38). Although there was no evidence of lead-induced transcription of intestinal MT, ZIP4, CRIP, or DMT1 in the present study, these results should be confirmed with high acute doses of lead, as well as chronic exposures over several months to 1 year. In addition, further studies to elucidate the mechanisms of intestinal lead absorption are required. The significance of these mechanisms must then be studied in the context of human health, as mineral absorption pathways are known to vary between humans and rodents, especially with respect to paracellular and transcellular involvement.

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