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Dietary zinc attenuates renal lead deposition but metallothionein is not directly involved

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Abstract Chronic lead exposure irreversibly damages the kidneys and may be associated with hypertension and renal insufficiency at sub-clinically toxic levels. Zinc supplementation reduces lead absorption and tissue retention in rodent models but the mechanisms are unknown. Metallothionein (MT) may function in lead detoxification. Our objective was to investigate the effects of marginal zinc (MZ) and supplemental zinc (SZ) intakes on renal lead and zinc accumulation, renal MT immunolocalization and levels. Weanling Sprague Dawley rats were assigned to MZ (8 mg Zn/kg diet), zinc-adequate control (CT; 30 mg Zn/kg), zinc-adequate diet-restricted (DR; 30 mg Zn/kg) or SZ (300 mg Zn/kg) groups, with and without lead acetate-containing drinking water (200 mg Pb/L) for 3 weeks. Kidneys were analyzed for lead and zinc by inductively coupled plasma spectroscopy and MT by immunolocalization and Western blotting. MZ had higher renal lead and lower renal zinc concentrations than CT. SZ was more protective than CT against renal lead accumulation. Renal MT levels reflected dietary intake (SZ ≥ DR ≥ CT ≥ MZ) but lead had no effect on MT staining intensity, distribution, or relative protein amounts. In summary, while SZ lowered renal lead concentration, MT did not appear to function in renal lead accumulation. Future studies should explore alternate mechanisms of renal lead detoxification.

Keywords Kidney · Lead · Zinc · Metallothionein · Rat

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Introduction

Chronic exposure to environmental lead remains a significant public health concern among low-income populations (Tong et al. 2000). While much attention has been paid to the effects of lead on neurocognitive function in children, new research on lead and renal toxicity has raised serious concerns with respect to long term health. Chronic lead exposure has been linked with hypertension (Hu et al. 1996), as well as renal insufficiency (Lin et al. 2003).

The kidneys are the primary site for the initial accumulation of lead following oral or inhalation

exposure (Nolan and Shaikh 1992). Although lead targets several organ systems, renal effects may be the most insidious (Nolan and Shaikh 1992). In general, lead-induced nephrotoxicity results in nephromegaly, cellular inclusion body formation, and renal tubular dysfunction (Qu et al. 2002). The kidney is vulnerable to the toxic effects of lead as urinary excretion is a main elimination route for lead from the body (Madden and Fowler 2000). In addition, the proximal convoluted tubules are especially susceptible as a result of their high reabsorptive activity (Madden and Fowler 2000).

Poor nutritional status is thought to increase the risk of adverse health effects from environmental lead exposure (Mahaffey 1981). Dietary deficiency of zinc has been shown to enhance lead absorption in rat models (Mahaffey 1981), while supplementation of dietary zinc reduces the absorption of lead (Cerklewski and Forbes 1976; Flora 2002) as well as reducing the susceptibility to lead toxicity in rats (Flora et al. 1991). However, studies examining the association between nutritional status and lead toxicity in humans have produced equivocal results, with some encouraging effects on blood lead levels (Hammad et al. 1996; Wright et al. 1999) but other reports with no effect (Lucas et al. 1996; Serwint et al. 1999). Clinical trials involving zinc supplementation in lead-exposed subjects are very limited.

The molecular mechanisms underlying renal lead toxicity are not well defined (Smith et al. 1998). However, the zinc binding protein, metallothionein (MT), may contribute to the cellular transport and toxicity of this metal. MT is a metal-binding protein characterized by a low molecular weight (6,000–7,000 Da), high cysteine content, and high affinity for heavy metals (Kagi 1991). MT is thought to play a key role in the homeostatic regulation of zinc metabolism at the cellular level (Davis and Cousins 2000), which is supported by the finding that MT immunolocalization and concentration responds to dietary zinc in the small intestine and liver (Szczurek et al. 2001). MT protein synthesis is especially sensitive to dietary zinc in organs involved in absorption, storage, secretion, and excretion, implicating functions in zinc absorption, transport, storage, and elimination (Aggett and Comerford 1995). In addition, MT-null mice have demonstrated a heightened sensitivity to lead toxicity as compared to wild-type controls, including dose-related nephromegaly and diminished renal function (Qu et al. 2002). Thus, MT may be involved in renal lead excretion and detoxification processes. In the presence of a low dietary zinc supply lead retention and toxicity may be exacerbated through reduced MT expression and conversely, zinc supplementation may offer a protective effect from lead exposure. While the ability of cadmium to induce MT synthesis and modulate the toxicity of this metal is well known, the transcriptional response of renal MT to lead is less clear. Additionally, the cellular localization of MT in response to lead treatment and interactions between lead and varying levels of dietary zinc have not been previously reported. Therefore, the objective of this study was to investigate the effects of marginal zinc (MZ) and supplemental zinc (SZ) intakes on the expression and cellular localization of renal MT.

Materials and methods

Sixty-four weanling male Sprague Dawley rats weighing approximately 55–65 g, were obtained from Charles River Laboratories (St Constant, PQ, Canada). Animals were housed in a temperature (21–23°C) and humidity (55%) controlled room with a 14:10-h 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. A factorial design was used to provide low, adequate, or high dietary zinc, with and without lead-treated drinking water as described in Table 1. Diet-restricted (DR) groups were included in order to maintain a weight comparable to the low zinc groups, as zinc deficient animals have reduced weight gain. Of note, when dietary groups 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.

Table 1 Diet and treatment groups

Lead	Zinc			
	MZ (8 mg Zn/kg diet; ad libitum)	DR[a] (30 mg Zn/kg diet; DR)	CT (30 mg Zn/kg diet; ad libitum)	SZ (300 mg Zn/kg diet; ad libitum)
Pb- (0 mg Pb/L)	MZ–	DR–	CT–	SZ–
Pb+ (200 mg Pb/L)	MZ+	DR+	CT+	SZ+

MZ marginal zinc diet, *DR* diet-restricted, *CT* control diet, *SZ* supplemental zinc diet

[a] Diet-restricted groups were included in order to maintain a weight comparable to the low zinc groups, as zinc deficient animals have reduced weight gain. DR groups received the nutritionally adequate control diet but the amount was restricted to match the weight of the MZ groups

Lead (as acetate) was administered in the drinking water at 200 mg Pb/L and provided ad libitum, to produce a sub-clinical toxicity, as this dosage given for 10 weeks with a standard laboratory rat chow has been shown not to produce significant alterations in hematopoiesis, renal size, histology, and function (Six and Goyer 1970). 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 MZ deficiency was chosen, rather than a severe deficiency (<1 mg/kg Zn), to reflect mild states of zinc deficiency most commonly seen in human populations (Aggett and Comerford 1995). Zinc supplementation at 300 mg/kg was selected as a high, but non-toxic 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 (Lepage et al. 1999) and 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 previously rinsed 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 on a separate rack and low zinc groups were housed above groups with higher zinc diets.

Feed intake and water intake were recorded regularly and diet spillage was recorded. 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 collected. Blood was immediately centrifuged in micro-hematocrit capillary tubes (75 mm; Fisher, Fair Lawn, NJ, USA) for 10 min to obtain hematocrit. Kidneys were excised and briefly rinsed with phosphate-buffered saline to remove superficial blood and sectioned longitudinally. Renal sections were then fixed in 10% phosphate-buffered formalin for 24–48 h in preparation for immunohistochemical studies. The remaining tissues were immediately frozen in liquid nitrogen and later stored at –80°C. Sections fixed in formalin were embedded in paraffin, cut in 5 μm sections and mounted on fixative-coated slides. Other tissues were collected and analyzed from these animals as previously described (Jamieson et al. 2006; in press).

Immunohistochemical localization of metallothionein

Standard procedures for indirect immunoperoxidase staining were used to determine MT localization in the kidney. Briefly, endogenous peroxidase activity was inactivated by treatment with 3% hydrogen peroxide and slides were incubated for 1 h with monoclonal mouse anti-MT antibody (clone E9; DAKO; 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 min. The reaction product was visualized by treatment with 3,3′-diaminobenzidine tetrahydrochloride (DAB·4HCl; Polysciences, Inc.). Tissues were counter-stained with Harris hematoxylin. 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.).

Western blot analysis

Frozen kidney tissue was ground with a mortar and pestle and suspended in 3 × SDS sample buffer. After 15 min the solution was clarified by centrifugation (2 cycles of 10 min at 12,000 rpm). Cellular proteins (5 μg total protein per lane) were separated by SDS-PAGE in a 15% gel and transferred to polyvinylidene difluoride membrane was conducted as previously described (Yau et al. 1999). The ECL chemiluminescent system (Amersham) was employed to detect horseradish peroxidase-conjugated secondary antibody and intensity was quantified by scanning densitometry. Antibodies used were monoclonal mouse anti-MT antibody (clone E9; DAKO) and p42/44 mitogen activated protein kinase (MAPK; Cell Signaling Technology) as the loading control. MT and MAPK were diluted 1:500 and 1:1,000, respectively. A CT sample was also run on each gel as an intensity control. MT and MAPK were corrected for their respective intensity controls on each gel and MT was adjusted with the loading control.

Renal tubule histology

Renal pelvic tubule area and perimeter were measured as an indicator of lead-induced morphological injury using Northern Eclipse software version 6.0 (Empix Imaging Inc.). Sections were stained with hematoxylin as described above. Five longitudinal tubule sections were arbitrarily selected (three medium, one large, and one small) from two sites in the renal pelvis of each kidney under 100 × magnification. Each site was captured by a frame freezer and measured for area and perimeter. The evaluator was blinded to the animal ID and treatment group during the analysis.

Mineral analysis

After obtaining wet and dry weights, renal and diet samples were wet-ashed using trace-element-grade nitric acid, as previously described (Clegg et al. 1981). Acid digests were diluted appropriately with double deionized water before analysis of lead and zinc by inductively coupled plasma spectroscopy analysis (Varian Liberty 200, Varian Canada). Bovine liver standard reference 1577b (National Institute of Standards and Technology) was processed in triplicate as a quality control. The detection limits for lead and zinc were 0.5 and 0.1 μg/mL, respectively.

Statistical analysis

Data were analyzed for main effects of lead and zinc, as well as interactions of lead and zinc, by two-way ANOVA using SAS software version 9.1 (SAS institute). 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. Data were checked for normality and homogeneity of variance, and transformed when necessary, although non-transformed means are reported. For main effects, significant differences among treatment group means were determined with Duncan's Multiple Range test. Differences were considered significant at $P < 0.05$. All data are reported as means ± SEM. As there were no interactions between lead and zinc, tables and figures report main effects of lead ($n = 32$) and zinc ($n = 16$) only.

Results

Nephrotoxicity

Lead exposure at 200 mg Pb/L for 3 weeks did not result in nephromegaly (Table 2) or histological alterations. Renal pelvic tubule area ranged from 1,712 ± 146 to 2,580 ± 252 μm and perimeter ranged from 300 ± 23 to 384 ± 28 μm, but was not different among treatment groups. Hematocrit levels were also not different among treatments, ranging from 35.5 ± 2.0 to 38.6 ± 2.4%.

Growth

There was no interactive effect of lead and zinc on final body weight (Fig. 1a). As there was a main effect of lead and a main effect of zinc, treatment groups were pooled as previously described under Sect. ''Materials and methods''. Pb+ rats had a 6% lower body weight than Pb- rats. MZ and DR body weights were not different, but MZ rats were 10% lower than CT rats. DR was not different from MZ, as expected. SZ treatment did not affect final body weight. These main effects were also mirrored by feed intake, analyzed as g feed/rat/day (Fig. 1b).

Lead and zinc status

Renal zinc concentration was 5% less in Pb+ than Pb- rats, as a main effect (Fig. 2a). There was also a main effect of dietary zinc on renal zinc concentration with MZ rats having a 16% lower zinc concentration than CT, and SZ rats having

Table 2 Effects of lead, dietary zinc, and diet restriction on kidney weight

Measurement	Lead treatment†			Dietary group‡				
	Pb-	Pb+	*P*-value	MZ	DR	CT	SZ	*P*-value
Kidney								
Wet weight (g)	3.14 ± 0.12	2.78 ± 0.13*	<0.05	2.43 ± 0.13^{c}	2.80 ± 0.15^{bc}	3.22 ± 0.18^{ab}	3.39 ± 0.15^{a}	<0.05
Wet weight (g/g body weight)	0.0111 ± 0.0004	0.0118 ± 0.0004	NS	0.0121 ± 0.0005^{a}	0.0119 ± 0.0005^{a}	0.0117 ± 0.0006^{a}	0.0101 ± 0.0005^{b}	<0.05

As there was no interaction between lead and zinc but there was a main effect of lead and a main effect of zinc, data was pooled to show 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 mean ± standard error for $n = 32$ (lead effect) and $n = 16$ (zinc effect), as determined by two-way ANOVA.

† Statistical differences are indicated by an asterisk (lead effect)

‡ Statistical differences are indicated with different lower case letters (zinc effect)

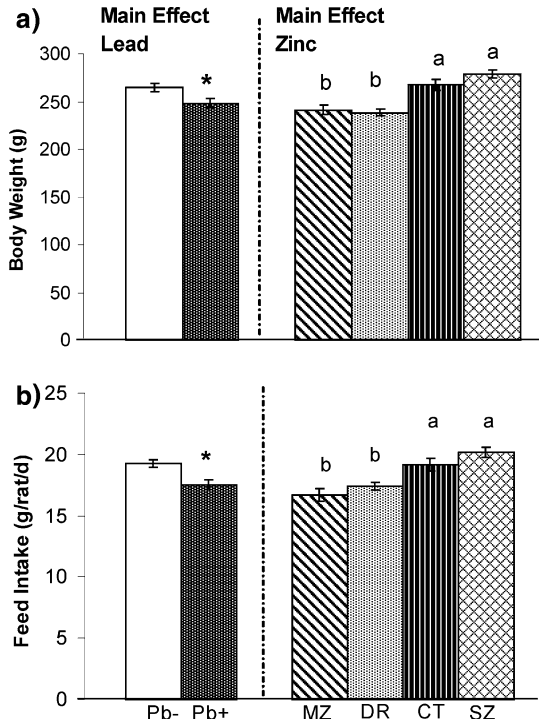

Fig. 1 Effects of lead, dietary zinc, and diet restriction on final body weight (**a**) and feed intake (**b**). Values are means ± standard error for $n = 32$ (lead effect) and $n = 16$ (zinc effect), as determined by two-way ANOVA. Statistical differences among groups ($P < 0.05$) are indicated by an *asterisk* (lead effect) and *lower case letters* (zinc effect; groups not sharing the same *letter* are significantly different). As there was no interaction between lead and zinc but there was a main effect of lead and a main effect of zinc, data was pooled to show 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+

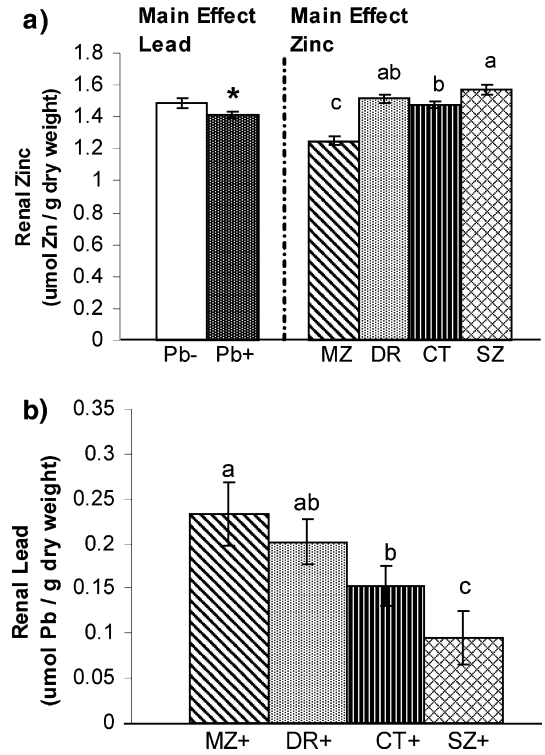

Fig. 2 Effects of lead, dietary zinc, and diet restriction on renal zinc concentration (**a**) and lead concentration (**b**). Values are means ± standard error for $n = 32$ (lead effect) and $n = 16$ (zinc effect), as determined by two-way ANOVA (**a**) and means ± standard error for $n = 8$, as determined by one-way ANOVA (**b**). Statistical differences among means ($P < 0.05$) are indicated by an *asterisk* (lead effect) or *lower case letters* (zinc effect; groups not sharing the same *letter* are significantly different). As there was no interaction between lead and zinc but there was a main effect of lead and a main effect of zinc, data was pooled to show main effects only (**a**). *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+

6% more than CT (Fig. 2a). Renal lead concentration was 35% higher in MZ+ rats and 33% lower in SZ+ than CT+ rats (Fig. 2b). Renal lead concentration of DR+ rats was not different than either MZ+ or CT+ rats.

Immunohistochemical localization of renal MT

All negative control sections were absent of MT staining (Fig. 3a), indicating that the staining was specific for MT. There were no apparent differences in staining intensity or distribution between the Pb+ and Pb- treatments, which is shown in Fig. 3c (DR-) vs Fig. 3d (DR+) as a representative result for all treatments. These results will mainly be discussed in terms of the response to dietary zinc.

There was moderate to strong MT immunostaining in the renal convoluted tubules of the DR, CT, and SZ groups (Fig. 3c, e, f), and weak to moderate staining in MZ groups (Fig. 3b). The staining was mainly localized within the epithelia of proximal convoluted tubules, rather than distal convoluted tubules, and was present in both the cytoplasm and nuclei of epithelial cells, when

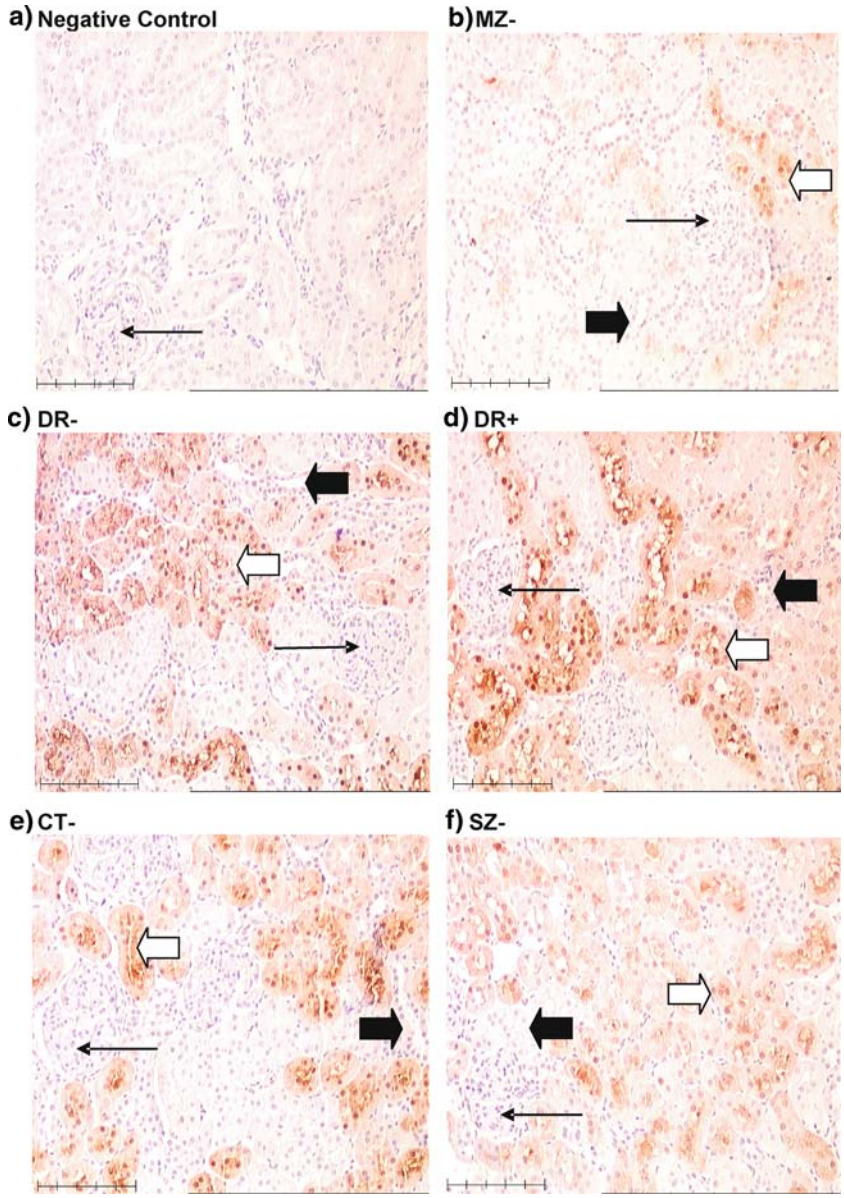

Fig. 3 Immunohistochemical staining for metallothionein (MT) in the renal cortex (counter-stained with hematoxylin). All negative control sections were absent of MT staining (**a**). As there was no effect of lead on MT staining results from all treatment groups are not shown. Representative images of MZ- (**b**), DR- (**c**), DR+ (**d**), CT- (**e**), and SZ- (**f**) are presented. MT immunostaining was primarily localized within proximal convoluted tubules (*white block arrow*) and weak or absent from distal convoluted tubules (*black block arrow*). No detectable MT immunostaining was present in glomeruli (*arrow*). Strong MT immunostaining was present in DR-, DR+, CT-, CT+, whereas SZ- and SZ+ treatments had greater variability in staining intensity (moderate to strong staining) and MZ- and MZ+ treatments had only weak to moderate staining. *Scale bars* at the bottom left corner equal 100 μm

examined at 400 × magnification (Fig. 4). All groups also had some staining in the lumina of renal tubules and no staining in glomeruli. The epithelia of large collecting ducts in the base of the renal pelvis also responded to dietary treatment in terms of MT intensity. DR rats had strong MT staining, whereas all other treatments had moderate to strong staining (images not shown). While

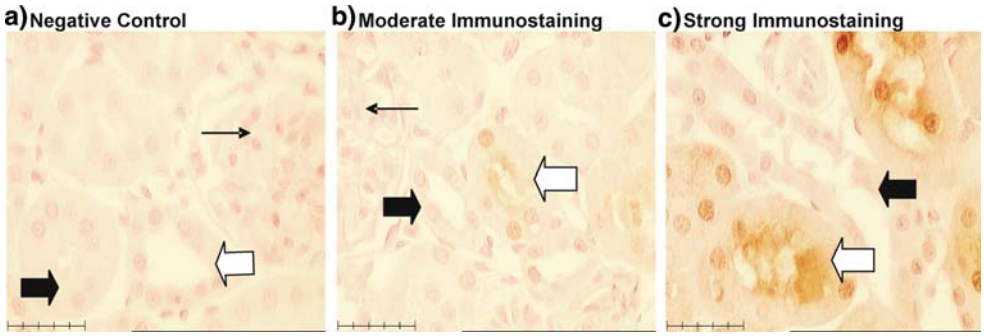

Fig. 4 High resolution immunohistochemical staining for metallothionein (MT) in the renal cortex (counter-stained with hematoxylin). All negative control sections were absent of MT staining (**a**). MT staining was localized within proximal convoluted tubules (*white block arrow*), with weak or absent staining in distal convoluted tubules (*black block arrow*). Proximal convoluted tubules are distinguishable by their distinct brush border, basally placed nuclei, and occluded lumen; whereas distal convoluted tubules lack a brush border, have apically located nuclei, and wide, open lumens. Representative images of moderate (**b**) and strong (**c**) immunostaining are presented. Immunostaining was absent from glomeruli (*arrow*). *Scale bars* in bottom left corners equal 40 μm

there were obvious differences in staining intensity, there were no apparent differences in MT distribution among the various levels of dietary zinc. Staining was generally present throughout the entire cortex and into the medulla.

Western blot analysis of renal MT

Western blotting analysis confirmed that MT levels were responsive to dietary zinc, but not to lead exposure (Fig. 5). The SZ rats had 86% higher MT levels than CT rats. MZ rats had 85 and 94% lower MT levels than DR and SZ rats, respectively, however the difference between MZ and CT was not significant. Statistically, there was no main effect of lead on renal MT levels and no interaction between lead and dietary zinc. However, upon visual inspection of the blots lead appeared to inhibit the induction of MT, especially in the CT and SZ groups (Fig. 5a). This difference likely did not reach significance due to the variability within groups and may require a larger sample size or higher dose to demonstrate this effect. The loading control, MAPK (Fig. 5a), did not change with dietary or lead treatment ($P < 0.8324$).

Discussion

Previous studies have independently investigated the effects of lead exposure and the effects of dietary zinc on MT (Waalkes et al. 1984; Maitani et al. 1986; Szczurek et al. 2001) in rodent and in vitro models, but this is the first study to examine the in vivo interaction of lead with low and high levels of dietary zinc on MT immunolocalization and protein levels. This study shows that renal MT immunostaining intensity and protein levels are responsive to dietary zinc, but not to chronic, low-level lead exposure in rats (Fig. 3). In addition, there is no interactive effect between lead and low or high dietary zinc on renal MT protein. Thus MT does not appear to be directly involved in renal lead accumulation. However, there was a trend toward lead-inhibited renal MT synthesis in CT and SZ groups. Although this effect could be seen visually on the Western blots, it did not reach statistical significance. This interaction should be further investigated at both the protein and transcriptional level. Furthermore, these results also do not exclude the possibility that MT may participate in other detoxification processes in the kidney or other organs, as previously suggested (Qu et al. 2002). There is some evidence that MT may participate in the formation of nuclear inclusion bodies, which may be a protective effect to mitigate lead toxicity (Qu et al. 2002). MT-null mice are more susceptible to cellular lead toxicity and incapable of producing the characteristic inclusion bodies seen with lead exposure (Qu et al. 2002). Although MT is not incorporated

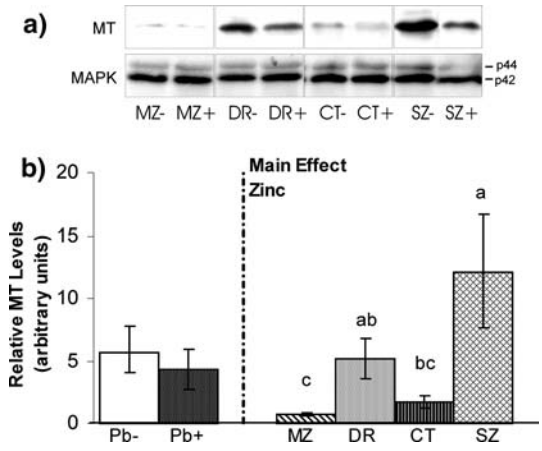

Fig. 5 Western blotting of MT in the kidney. Representative blots of all treatments for MT and MAPK (**a**) and arbitrary units for MT (**b**) for means ± standard error for $n = 24$ (lead effect) and $n = 12$ (zinc effect), as determined by two-way ANOVA. MT and MAPK were adjusted for an intensity control on each gel and MT was expressed as a ratio of the loading control MAPK. Data was analyzed by two-way ANOVA with statistical differences among groups ($P < 0.05$) indicated by *lower case letters* (zinc effect; groups not sharing the same *letter* are significantly different). As there was no interaction between lead and zinc but there was a main effect of zinc, data was pooled to show 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+

into the complex, it may play a facilitative or temporary transport role in inclusion body formation (Qu et al. 2002), thus sequestering the metal in a less toxic form. However, this study was done in mice, and therefore it cannot be ruled out that a protective effect of MT is specific to mice and not necessarily to rats.

Despite the lack of response to lead in renal MT, this study demonstrates a strong protective effect of adequate and supplemental zinc against lead accumulation in the kidney (Fig. 2). While this effect has been demonstrated previously (Cerklewski and Forbes 1976; Flora 2002), these studies are up to 30 years old and may have lacked the stringent controls currently followed in studies involving trace metals. For instance, Cerklewski and Forbes (1976) reported no difference in weight gain in growing rats fed low zinc (8 mg Zn/kg diet) diets compared to rats fed control or supplemented zinc diets, after 3 and 7 weeks. This finding is highly irregular and suggests zinc contamination in the low zinc group. Furthermore, nutritional studies of zinc deficiency require a weight-matched control in order to distinguish effects of zinc deficiency from effects of reduced feed intake or growth rate. This control was not present in previous studies.

The kidney accumulates the highest concentrations of lead present in soft tissues (Mahaffey 1981; Miller et al. 1983; Ashraf and Fosmire 1985), a finding also confirmed by the present study. The kidney had a higher lead concentration (approximately tenfold) and total content (approximately twofold) than the liver (Jamieson et al., in press). SZ feeding was more protective than the CT diet in terms of renal lead accumulation, and highly protective compared to the MZ rats. Thus, zinc nutrition may play a key role in reducing renal lead burden as previously hypothesized (Cerklewski and Forbes 1976; Cerklewski 1979; Mahaffey 1981; Flora 2002). Nevertheless, earlier studies cannot separate specific effects of zinc deficiency from indirect effects of reduced feed intake and/or growth rate, as the present study has done. Of note, renal lead concentrations of MZ and DR rats were not significantly different, suggesting that the lead accumulation in soft tissues may be at least partially due to undernutrition. However, femoral lead concentration, which is the best indicator of whole body lead burden (Peraza et al. 1998) was elevated in MZ compared to DR, and DR was not different from CT, as previously reported (Jamieson et al. 2006). Therefore, the mechanism of increased lead absorption and retention during zinc deficiency is likely a direct effect of zinc, per se, and not the result of reduced feed intake.

The low zinc status of MZ rats was confirmed by lower renal (Fig. 2), serum (Jamieson et al., in press) and femoral zinc concentrations (Jamieson et al. 2006), down-regulated renal MT immunostaining intensity (Fig. 3), and MT protein levels (Fig. 4). The diminished levels and distribution of renal MT immunostaining seen in MZ rats, is in accordance with previous results in the kidneys, liver and small intestine of severely zinc restricted rats (Szczurek et al. 2001; E.I. Szczurek, personal communication) and the intestine of marginally zinc deficient rats (Jamieson et al., in press). In

addition, this response in MT was reflective of lower tissue zinc concentrations. Conversely, SZ rats had higher renal zinc concentrations than CT rats, which was reflected in elevated renal MT levels. Of note, the immunostaining procedure was not sensitive enough to detect the more subtle differences among DR, CT, and SZ treatments, as seen by Western blotting.

Interestingly, DR rats had higher renal MT expression than MZ rats (Fig. 3, 5). Thus, lower MT levels in MZ rats can be attributed directly to zinc deficiency, per se, and not an indirect effect of reduced feed intake and/or growth rate. However, pair-weighing and pair-feeding techniques, which involve diet restriction, are stressful and have been shown to elevate MT synthesis (Szczurek et al. 2001). Although there was a trend for higher MT levels in DR compared to CT, this did not reach statistical significance.

The regulation of MT gene expression by zinc occurs through binding of the zinc-finger transcription factor MTF-1 to metal response elements on the MT gene promoter (Andrews 2000). The zinc-activated DNA-binding activity of MTF-1 is thought to be both specific and reversible, suggesting that this protein can act as an indicator of cellular zinc concentrations. In addition, transcriptional regulation of MT has been demonstrated by mercury (Zhang et al. 2003), cadmium, and oxidative stress (Andrews 2000). The cadmium response element has been identified on the MT-1 isoform promoter (Andrews 2000). However, transcriptional regulation of MTF-1 by other heavy metals such as lead has not been shown. Thus, if MT participates in lead detoxification processes, it does not appear to occur through transcriptional regulation via MTF-1.

The presence of MT in renal tubular epithelial cells and the lumina of renal tubules is indicative of a role in zinc excretion and/or reabsorption. These observations are consistent with studies on rats treated with dietary zinc (Szczurek et al. 2001), as well as $CdCl_2$ (Zhang et al. 2003). The localization of MT within the epithelia of collecting ducts was also previously reported (Szczurek et al. 2001), although the reason for this observation remains unclear. The primary function of the collecting duct is osmotic equilibration of the ultrafiltrate, thus a potential role in zinc homeostasis is not clear.

Urinary zinc excretion accounts for only 2–10% of total endogenous zinc excretion, as the major route of excretion is through the gastrointestinal tract (King and Keen 1999). In addition, urinary zinc losses tend to remain stable over a wide range of zinc intakes unless dietary zinc is severely restricted or given in great excess (King and Keen 1999). Urinary zinc mainly originates from the ultrafilterable portion of plasma zinc through proximal tubule secretion (Vallee and Falchuk 1993) and up to 95% of filtered zinc appears to be reabsorbed in the distal parts of the renal tubule (King and Keen 1999). Thus, the strong concentration of MT in the proximal convoluted tubules in CT-, CT+, DR-, DR+, SZ-, and SZ+ treatments may be contributing to zinc excretion, although this was not directly tested. The lack of urinary analysis, including an assessment of renal function, is a limitation to this study.

The lead dose given in the present study was uniform across the various treatment groups (Jamieson et al. 2006) and did not result in nephromegaly (Table 2), histological alterations, or differences in hematocrit, suggesting that a sub-clinical lead toxicity was produced. In fact, Pb+ rats had smaller kidneys than Pb- rats, although this effect can be attributed to the smaller body weight of Pb+ rats.

The effect of lead on renal zinc concentration (Fig. 2) confirms previous reports (Mahaffey 1981; Ashraf and Fosmire 1985), although the biochemical and physiological consequences of this are not known. This response may be due to a redistribution of zinc among the tissues or an increase in zinc excretion, as a 200 mg Pb/L treatment has been shown to increase urinary zinc excretion by more than twofold (Victery et al. 1987). While the mechanism for this remains unknown, subsequent studies have shown that it is not due to competition for renal reabsorption (Nolan and Shaikh 1992). This observation should be further investigated in the context of the trend for diminished MT synthesis in several of the lead-exposed groups (Fig. 5b).

In summary, renal MT expression was responsive to dietary zinc. Renal MT levels were highest in the zinc supplemented rats and lowest in the zinc deficient rats, although statistically there was

no difference between low zinc and control. However, MT does not appear to be involved in renal lead accumulation despite the fact that zinc supplemented rats had greatly reduced renal lead concentrations. Chronic, low-dose lead treatment did not affect MT levels or distribution in the kidney. Thus, the protective effect of zinc supplementation against tissue lead accumulation may be primarily mediated at the gastrointestinal level. However, there was an interesting trend toward inhibition of MT synthesis by lead which should be further investigated with a higher lead dose and at various time points. Zinc deficiency has been shown to enhance tissue lead accumulation and this appears to be a direct effect of zinc rather than a generalized effect of reduced feed intake. The DR groups were not as affected as low zinc groups when exposed to lead, suggesting that a specific mineral deficiency may be more detrimental during lead exposure than general malnutrition. Further studies on the role of zinc in lead toxicity should focus on molecular mechanisms of lead uptake and transport in the kidney as well as the enterocyte.

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