Reduction of liver manganese concentration in response to the ingestion of excess zinc: identification using metallomic analyses

Tomoya Fujimura, Tomohiro Terachi, Masayuki Funaba* and Tohru Matsui

Division of Applied Biosciences, Kyoto University Graduate School of Agriculture, Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan

Corresponding author: Masayuki Funaba, Ph.D.

Division of Applied Biosciences

Kyoto University Graduate School of Agriculture

Kitashirakawa Oiwakecho, Kyoto 606-8502, Japan

Tel.: +81-75-753-6055

Fax: +81-75-753-6344

E-mail: mfunaba@kais.kyoto-u.ac.jp

Abstract

To date, minerals of interest have been analyzed individually to understand mineral dynamics and metabolism. Our recent development of metallomic analyses enabled us to evaluate minerals in an unbiased and global manner. Here, we evaluated effects of the ingestion of excess zinc on the plasma and tissue concentrations of minerals in growing rats. A total of 26 minerals were simultaneously evaluated by the metallomic analyses using an inductively-coupled plasma-mass spectrometry in semi-quantification mode; the concentrations of several minerals exhibited consistent changes in response to the concentration of dietary zinc. The manganese concentrations in plasma and femur were increased, whereas those in the liver and pancreas were decreased with increasing dietary zinc concentrations. Because the interaction between zinc and manganese has not been known, we further focused our analysis on liver manganese. Quantitative analyses also indicated that the hepatic concentration of manganese decreased in response to the ingestion of diets containing excess zinc, a result that was partly explained by the decreased expression of hepatic *Zip8*, a manganese transporter. The present study reveals a mineral interaction using metallomic analyses and proposes a possible mechanism underlying the novel interaction.

Introduction

Many minerals are essential for a variety of biological processes because of the ability of these minerals to serve as enzyme co-factors, oxygen transporters, second messengers to elicit cell responses, and body supports. Therefore, excesses and deficiencies of minerals cause serious metabolic disorders.[1,2] In addition, inappropriate mineral intake induce secondary disturbances in metabolism of other minerals through agonistic or antagonistic interactions between minerals.[1,3] Furthermore, there are interactions between mineral metabolism and the metabolism of macronutrients such as sugars, fats and proteins.[4-6] However, because minerals of interest have been analyzed individually to date, the information is limited. Recently, we developed the "metallomic analyses" using a semi-quantitative procedure based on inductively-coupled plasma-mass spectrometry (ICP-MS).[7] This method enables the unbiased and comprehensive evaluation of mineral dynamics through global measurement of the tissue mineral concentrations. The present study uses this method to explore the effects of excess levels of dietary zinc (Zn) on the mineral concentrations in tissues and plasma in growing rats.

Materials and methods

Samples

The samples collected in the previous study[8] were used; a total of 28 4-wk-old male specific-pathogen-free Sprague-Dawley rats were used. They were housed individually in stainless-steel cages under constant conditions (24°C, 50% humidity) with a fixed light-dark cycle (lights on from 0500 to 1900) and were randomly assigned to receive diets containing differing Zn concentrations, i.e., 24, 1016, 2008 and 3000 mg Zn/kg (n = 7). Weaning piglets are frequently given a high Zn diet, because the diets containing this level of Zn improved body weight gain (3000 mg Zn/kg diet).[9-11] Therefore, diets with Zn levels as high as 3000 mg Zn/kg diet were included and given to growing rats.[8] Ingredients and composition of diets were previously described.[8] Briefly, egg-white was used as a protein source, because this is a part of the study examining responses to dietary Zn status, i.e., excess intake as well as deficiency. D-biotin was added to the diet due to the high avidin content of egg-white to prevent biotin deficiency. Zn was added as ZnO at the expense of glucose. The measured concentrations of dietary manganese (Mn) were 7.55, 7.80, 7.47 and 8.47 mg/kg in the diets containing 24, 1016, 2008 and 3000 mg Zn/kg, respectively. All rats showed no signs of Zn toxicity- namely, they did not vomit or show any gastrointestinal dysfunction throughout the study, although two

rats fed a diet with 3000 mg Zn/kg excreted soft stools for the last 3 d of the experimental period. No significant differences between the dietary groups were detected on body weight and daily feed intake.[8] These results are consistent with the previous results that a diet with 2500 mg Zn/kg did not interfere with growth, reproduction and normal function of rats through three generations.[12] After 10 d of feeding the experimental diet, tissues and plasma were collected. The experiments were approved by the Kyoto University Animal Experiment Committee (20-19).

Metallomic analyses and quantification of Mn

The metallomic analysis was performed for screening to detect mineral interactions. Plasma (1 ml), liver (ca. 0.5 g), pancreas (ca. 0.5 g), kidney (one kidney) and femur (one femur) from each rat were wet-ashed with trace-element grade nitric acid and hydrogen peroxide, and the minerals in the sample were dissolved in 2% nitric acid to 50 ml. The tissue samples were accurately weighed. The femur samples were further diluted 10-fold with 2% nitric acid, whereas the other samples were diluted 5-fold. To obtain the representative tissue concentration of the minerals, samples from each group were pooled. Comprehensive analyses of the minerals were performed using an ICP-MS (Elan6000, Perkin Elmer, Norwalk, CT) in semi-quantification mode (TotalQuant III).[7] A total of 26 minerals was evaluated by this metallomic analysis. The results were visualized by a two-dimensional graphical representation of the mineral contents in red and green using Treeview.[13] As in transcriptomic analyses, the graphical representation makes the analyses of metallomic data much easier.[14]

The Mn concentrations in the liver and the diets were individually quantified by ICP-MS in quantification mode. The analytical accuracy for the Mn quantitation (mass number: 55) was confirmed by analysis of a certified reference material derived from bovine liver (standard reference material 1577b, National Institute of Standards and Technology, Gaithersburg, MD, USA); intrassay CV and interassay CV were 0.5% and 3.1%, respectively, and recovery was 98.6%.

RNA isolation and qRT-PCR

RNA isolation from the liver and qRT-PCR were performed as described previously.[8] The following primers were used: 5’-ttttggtgggcaacaacttt-3’ and 5’-gcatgtcgttcatctctgga-3’ for *Zip8* (NCBI accession number: NM_001011952) and 5’-ttcctcagtgtctcactgattaa-3’ and 5’-ggaaaagtgcgttagagagc-3’ for *Zip14* (NM_001107275). *Hprt1* was used as a reference gene as described previously.[8] The

relative level of each gene transcripts was expressed as a ratio with respect to *Hprt1* mRNA, and the level in rats fed the basal diet were set to 1.

Statistical analyses

Data are expressed as the least square mean ± SEM. All analyses were performed with SAS[15] using the supercomputer of ACCMS, Kyoto University. The effects of dietary Zn on the Mn concentration and gene expression were analyzed with the GLM procedure. When the effect of diet was significant, the differences among the groups were examined by Tukey's multiple comparison test. Differences were considered significant at $P < 0.05$.

Results and discussion

Metallomic analyses indicated that feeding diets containing various Zn contents for 10 d expectedly resulted in changes in plasma Zn concentrations that paralleled the dietary Zn concentrations, except for rats fed the diet containing 3000 mg Zn/kg (Fig. 1). This result is consistent with the quantified concentrations of the plasma Zn as shown in the previous report[8]; the Zn concentrations in plasma linearly increased with increasing concentrations of dietary Zn up to 2008 mg Zn/kg, after which the plasma concentrations plateaued.

Several minerals other than Zn exhibited consistent changes in their concentrations in response to changing dietary Zn concentrations (Fig. 1). Among them, we paid attention to Mn concentrations; Mn concentrations in the plasma and femur were increased, whereas those in the liver and pancreas were decreased with increasing dietary Zn concentrations.

Because Mn is an essential mineral in animals[2,16,17] and because the interaction between Zn and Mn has not been known,[3,17] we further focused our analysis on the liver Mn concentration. We performed quantitative analyses of the liver Mn concentration; as consistent with the semi-quantitative analysis, it was lower in rats fed diets containing more than 1016 mg/kg Zn than in rats fed the diet containing 24 mg/kg Zn (Fig. 2A). Gajula et al.[18] reported that liver concentrations of Mn were comparable among chicks fed diets containing 40, 80 and 120 mg Zn/kg for 27 d. Peixoto et al.[19] also reported that the subcutaneous administration of $ZnCl_2$ at a dose of 27 mg/kg body weight/d for 5 d did not affect the Mn concentrations in liver and kidney of 13-d-old rats. Differences

between the species, in the dietary Zn concentrations or in the duration may be responsible for the discrepant results. Additionally, the diet used by Gajula et al.[18] was mainly (< 90%) composed of corn and soybeans, which contain phytic acid, a compound that inhibits Zn absorption.[20] Thus, it is possible that the presence of phytic acid affected the interactions between Zn and Mn.

The present results suggested that the plasma and tissue concentrations of Mn are differentially affected by the levels of ingested Zn in a tissue-dependent manner. This result reflects the altered body distribution of Mn in response to the ingestion of excess Zn, suggesting that the expression and activity of Mn transporters are modulated in response to dietary Zn levels to regulate Mn delivery. Zip8 is a transporter responsible for Mn uptake.[21] The transcript level in the liver was lower in rats fed the diets containing 1016 mg Zn/kg and 2008 mg Zn/kg than in those fed the diet containing 24 mg Zn/kg, although the expression level in rats fed the diet containing 3000 mg Zn/kg was not significantly different from that in control rats (Fig. 2B). By contrast, the expression of *Zip14*, which is another Zn transporter[22] and is also involved in Mn uptake,[23] was unchanged, irrespective of dietary Zn levels (Fig. 2C). The down-regulation of *Zip8* but not *Zip14* in rats fed the diet containing 1016-2008 mg Zn/kg partly explains the decrease in the hepatic accumulation of Mn. The molecular bases how excess Zn intake affects *Zip8* expression are not clear yet. It should also be noted that factors other than the hepatic expression of *Zip8* and *Zip14* are involved in the decreased Mn concentrations in the liver of rats fed the diet containing 3000 mg Zn/kg. In view of the transcriptional and post-transcriptional regulation of the activities of the Zip family members,[24] the lower Mn concentration may be the result of additional modifications. Alternatively, it is also possible that Mn export from the liver is accelerated in rats fed the diet containing 3000 mg Zn/kg. Either way, further studies are needed for full clarification on modulation of Mn transporters.

The present study identified a novel interaction between minerals. There was a decrease in the hepatic concentration of Mn in response to the ingestion of diets containing excess Zn. The nutritional and pathological significance of the modulated Mn concentration should be clarified in future; because Mn is a co-factor of various enzymes including oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases, the changes in Mn content in the tissue may affect the activities of some enzymes. Thompson et al.[25] showed that hepatic Mn concentration was reduced from 2.88 μg/g to 1.22 μg/g by ingestion of Mn deficient diet in rats, and suggested the

increase in oxidative stress in the liver of rats fed the Mn deficient diet. In view of the similar extent of the reduction on hepatic Mn concentrations (Fig. 2A), it is possible that the excess Zn induces hepatic Mn deficiency and oxidative stress. In the present study, regulation of the expression of Mn transporters was further investigated based on knowledge obtained from the metallomic analyses. In conclusion, we demonstrate that the metallomic analysis using ICP-MS in semi-quantification mode is a powerful tool with which to comprehensively and deeply explore mineral dynamics in animals.

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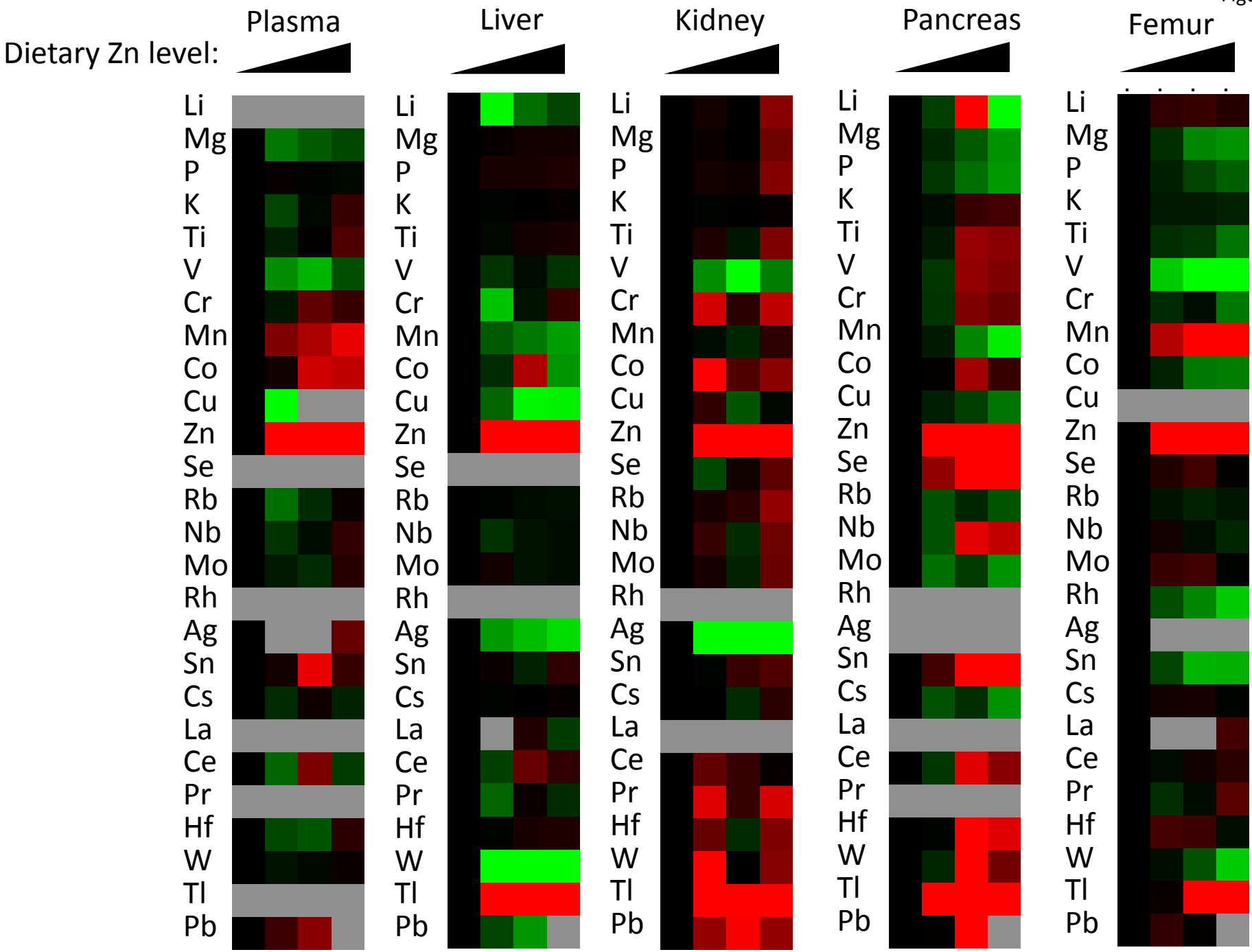
Figure legends

Fig. 1. Metallomic analyses of plasma and tissues of rats fed diets containing various levels of Zn

The plasma and tissues of rats fed diets containing 24, 1016, 2008 or 3000 mg Zn/kg were analyzed. The plasma and tissues concentrations of the rats fed the basal diet (24 mg Zn/kg) were set at 1, and concentrations relative to the concentration in rats fed the basal diet are shown in red (higher concentration) or green (lower concentration). Gray indicates concentrations below the detection limit.

Fig. 2. Quantitative measurement of Mn, and gene expression of Zip8 and Zip14 in the livers of rats fed diets containing various levels of Zn

The liver concentrations of Mn (A) and the expression levels of hepatic *Zip8* (B) and *Zip14* (C) were investigated in rats fed diets containing 24, 1016, 2008 or 3000 mg Zn/kg. The gene transcript levels were expressed as ratios relative to *Hprt1* with the level in rats fed the basal diet set to 1. Values are the means + SEM (n = 7). a, b: Mean values with different letters were significantly different ($P < 0.05$).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Sn
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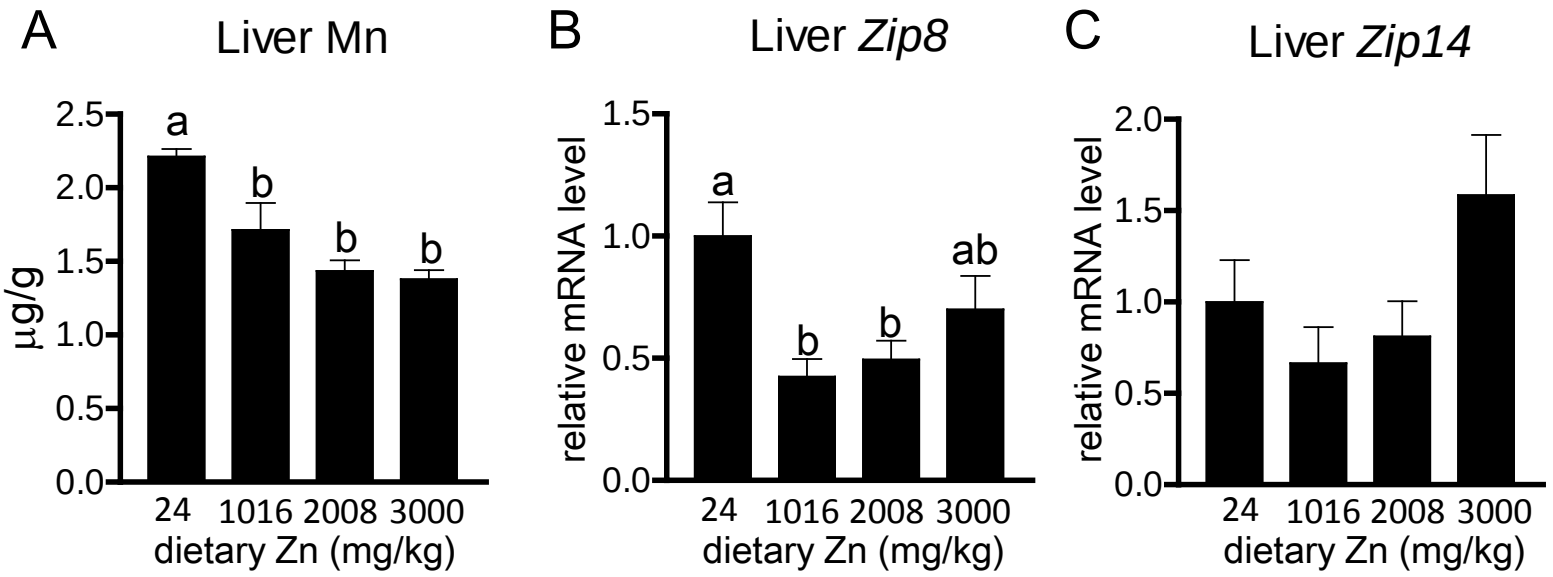
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