Hepatic Responses to Dietary Stress in Zinc- and Metallothionein-Deficient Mice

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Metallothionein (MT) and zinc are both reported to be protective against oxidative and inflammatory stress and may also influence energy metabolism. The role of MT in regulating intracellular labile zinc, thus influencing zinc (Zn)-modulated protein activity, may be a key factor in the response to stress and other metabolic challenges. The objective of this study was to investigate the influence of dietary zinc intake and MT on hepatic responses to a pro-oxidant stress and energy challenge in the form of a high dietary intake of linoleic acid, an omega-6 polyunsaturated fatty acid. Male MT-null (KO) and wild-type (WT) mice, aged 16 weeks, were given semisynthetic diets containing 16% fat and either 5 (marginally zinc-deficient [ZD]) or 35 (zinc-adequate [ZA]) mg Zn/kg. For comparison, separate groups of KO and WT mice were given a rodent chow diet containing 3.36% fat and 86.6 mg Zn/kg. After 4 months on these diets, the body weights of all mice were equal, but liver size, weight, and lipid content were much greater in the animals that consumed semisynthetic diets compared to the chow diet. The increase in liver size was significantly lower in ZA but not ZD KO mice, compared with WT mice. Principally, MT appears to affect the diet-induced increase in liver tissue but it also influences the concentration of hepatic lipid. Plasma levels of C-reactive protein (CRP), a marker of inflammation, were increased by zinc deficiency in WT mice, suggesting that marginal zinc deficiency is proinflammatory. CRP was unaffected by zinc deficiency in KO mice, indicating a role for MT in modulating the influence of zinc. Neither zinc nor MT deficiency affects the level of soluble liver proteins, as determined using two-dimensional (2D) gel proteomics. This study highlights the close association between zinc and MT in the manifestation of stress responses.
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Introduction

Diet-induced inflammatory stress is thought to be a major factor in promoting the initiation and pathogenesis of chronic disease (1, 2). Physiological defense mechanisms and dietary antioxidants may retard this process, and zinc has the potential to protect against oxidative stress–induced cellular damage (3, 4). Metallothionein (MT) is a stress-response protein that appears to function in protection against oxidative (5, 6) and other forms (7–9) of stress. It may do this as a consequence of its primary or secondary protein structure characteristics or as a consequence of its capacity to sequester and donate metals, principally zinc. Because MT binds zinc and is thought to regulate the availability of the cellular labile zinc that is responsible for triggering signaling pathways, the relationship between zinc status and MT has to be taken into account when evaluating their protection efficacy against stress. A useful model for this purpose is the MT-1 and MT-2–deficient mouse, which has targeted disruptions of the MT-1 and MT-2 genes (10, 11). This and other models have been used extensively to demonstrate the role of MT in protecting against metal toxicity (12–14), oxidative stress (5, 15), carcinogenic compounds (16, 17), ionizing radiation (18,19), and toxic drugs (20, 21).

We have utilized the MT-1 and MT-2 null mouse model in order to evaluate the role of zinc and MT, and their interdependency, in moderating the response to diet-related stress. Linoleic acid (C18:2, n-6), a polyunsaturated fatty acid found in many edible oils, has been shown to induce oxidative stress in vascular endothelial cells (22), where it promotes an inflammatory response and atherogenesis (23, 24). For the purposes of the present study, a semisynthetic diet known to be proatherogenic even in rodents was identified (25) and prepared to contain 16% fat as safflower oil (65% linoleic acid). The objective of this study was to

Table 1. Nutritional Composition of the Semisynthetic Mouse Diets

Zinc status	Deficient	Adequate
No. of mice	20	20
Major nutrients (g/kg)		
Egg white solids, spray-dried	244.72[a]	
Sucrose	100	
Corn starch	249.9929	250.0889
Maltodextrin	150	
Safflower oil	158	
Cholesterol	12.5	
Sodium cholate	5	
Cellulose	50	
Mineral mix		
Corn starch (bulking agent)	25.6902	
Zinc sulfate ($ZnSO_4 \cdot H_2O$)	0.0124	0.1084
Biotin	0.0044	
Vitamin B_{12} (0.1% trituration)	0.025	
Calcium pantothenate	0.066	
Choline dihydrogen citrate	3.497	
Folic acid	0.002	
Menadione	0.05	
Niacin	0.099	
Pyridoxine HCl	0.007	
Riboflavin	0.022	
Thiamin HCl	0.022	
Vitamin A palmitate (500,000 U/g)	0.0397	
Vitamin D_3 (500,000 U/g)	0.0044	
Vitamin E acetate (500 U/g)	0.15	

[a] For those nutrients that were present in identical amounts in both diets, the values are placed between the columns.

evaluate zinc and MT deficiency–related changes in inflammation, liver lipids, and hepatic protein expression in the presence of a pro-oxidant stress and energy challenge. Liver was investigated because it is the primary site for diet-derived fatty acid metabolism and because of the known effect of zinc deficiency on hepatic lipid metabolism (26, 27).

Materials and Methods

Animals, Diets, and Tissues. Mice with targeted disruptions in the MT-1 and MT-2 genes (10) and appropriate wild-type (WT) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were maintained as breeding colonies at the Rowett Research Institute. Fifty adult male MT-null (KO) and 50 adult male WT mice were randomly divided into three groups of 20, 20, and 10 animals. The animals were group-housed in plastic cages and were given deionized, trace element–free water. Semi-synthetic diets were prepared by Harlan Teklad (Madison, WI) according to a formula that has previously been shown to be proatherogenic (25). Zinc-deficient (ZD) and zinc-adequate (ZA) diets nominally contained 5 and 35 mg Zn/kg, respectively, which were analytically confirmed by Harlan Teklad and in our own laboratory. The two groups of 20 mice for each genotype were given the ZA and ZD diets, and the remaining groups of 10 mice were given commercial mouse chow (CRM[P] diet; Special Diet Services, Witham, UK). The composition of the semi-synthetic diets is shown in Table 1, and the chow diet contained 3.36% fat and 86.6 mg Zn/kg. The diets were fed to the mice for 4 months and the animals were then killed by a lethal intraperitoneal injection of pentabarbitone. Blood was removed from the posterior vena cava using heparinized syringes and centrifuged to obtain plasma. Livers were removed, weighed, and perfused with PBS. Plasma and liver samples were frozen in liquid nitrogen and stored at −80°C.

Plasma Zinc and Markers of Inflammation. Plasma zinc was analyzed by atomic absorption spectrophotometry (Unicam Solaar 969) after 1:10 dilution with 0.1 *M* hydrochloric acid (HCl) and centrifugation at 2500 *g*. Standards and a standard reference material were also prepared in 0.1 *M* HCl.

C-reactive protein (CRP) was measured in plasma samples using a high sensitivity CRP ELISA kit (Kamiya Biomedical Company, Seattle, WA). Tumor necrosis factor-α (TNF-α) and γ-interferon (IFN-γ) were analyzed using a cytometric bead array assay (Mouse Th1/Th2 Cytokine CBA; BD Biosciences, San Diego, CA) and a FACSCalibur flow cytometer (BD Biosciences).

Hepatic MT. The total MT level in liver was estimated using the silver binding assay (28). Approximately 200 mg of liver was homogenized in 1 ml deionized water and 0.5 ml was used for the assay. Standards were prepared by adding various amounts of purified rabbit liver MT (Sigma-Aldrich Co. Ltd., Gillingham, UK) to liver homogenates from a KO mouse. Sheep red blood cell lysate was used for scavenging excess silver. Atomic absorption spectrophotometry was used for analysis of silver binding to MT.

Diet and Liver Lipid Content and Composition. Total lipid was extracted from each sample using the method of Folch (29) and weighed. A sample of total lipid was then converted to methyl esters using acid-catalyzed transesterification. Methyl esters were purified on small silicic acid columns prior to analysis by gas chromatography, which was performed on an Agilent 6890 GC system (Agilent Technologies UK Ltd., Stockport, UK) fitted with a flame ionization detector. A 30m DB23 column (Jones Chromatography, Hengoed, UK) was used and the carrier gas was helium.

Liver Soluble Protein Proteomics. Liver samples from 6 ZD and 6 ZA WT and KO mice were randomly selected for individual proteomics analysis. Liver cytosol preparation and 2D gel proteomics were as previously described in detail (30). Briefly, liver samples (125 mg) were homogenized in 50 m*M* Tris-HCl buffer, pH 7.1 (50 m*M* Tris, 100 m*M* potassium chloride [KCl], 20% glycerol, 1.4 μ*M* pepstatin A, 1.0 m*M* phenylmethylsulfonyl fluoride [PMSF], and the protease inhibitor cocktail Complete according to the manufacturer's instructions [Roche Diagnostics Ltd., Lewes, UK]). Cytosol samples obtained by

Table 2. Plasma Zinc and Hepatic MT Levels

Genotype/ Treatment[a]	Plasma Zn (μM)	Liver MT (μg/g)
WT-Chow	14.7 ± 1.2	<5.0
KO-Chow	13.8 ± 1.6	<5.0
WT-ZA	17.0 ± 1.2	14.4 ± 0.5
WT-ZD	16.6 ± 1.0	13.1 ± 0.2*
KO-ZA	14.7 ± 0.5	<5.0
KO-ZD	17.3 ± 0.7	<5.0

[a] WT and KO mice were fed either rodent chow (Chow) or semisynthetic diets adequate (ZA) or deficient (ZD) in zinc. The asterisk indicates a significant difference ($P < 0.05$) when comparing ZA and ZD diets for the same genotype.

centrifugation at 108,000 *g* for 30 min at 4°C in a Beckman TL-100 ultracentrifuge (Beckman Coulter U.K., Ltd., High Wycombe, U.K.) were applied to isoelectric point (pI) 3–10 immobilized pH gradient (IPG) strips. Isoelectric focusing was carried out as described (30), and after blocking sulfhydryl groups with iodoacetamide, the IPG strips were applied to 18 × 18-cm 8%–16% gradient polyacrylamide gels. Proteins were separated by electrophoresis using a 24 m*M* Tris, 0.2 *M* glycine, and 0.1% sodium dodecyl sulfate (SDS), pH 8.6 buffer at 200V for about 9.5 hrs. Up to six gels were separated concurrently and then stained with Coomassie blue (30). Destained gels were scanned to obtain grayscale images in PDQuest software (BioRad Laboratories Ltd., Hemel Hempstead, UK).

Statistical Analysis. Data were analyzed using a one-way ANOVA followed by Fisher's multiple comparisons. For the proteomics work, spot densities were normalized and different treatments were statistically compared using *t* tests. Matched spot densities were also analyzed by principal component analysis using SIMCA-P software (Umetrics Ltd., Winkfield, UK).

Results

Zinc Status and Hepatic MT. Diets, treatment combinations, and genotype had no significant effect on plasma zinc levels (Table 2). Mice that consumed the chow diet tended to have lower zinc levels than mice that consumed the semisynthetic diet.

Hepatic MT levels of all KO mice were below the detection limit, as was the MT level in WT mice on the chow diet. However, livers of WT mice that consumed the semisynthetic diets contained MT levels that were at least 3-fold higher than MT in livers of WT animals on the chow diet (Table 2). Hepatic levels of MT in ZD mice were slightly but significantly ($P < 0.05$) lower than in ZA mice.

Liver Weight and Hepatic Lipid Content and Composition. Livers from animals that ate the semisynthetic diets were much paler in color and considerably larger than the livers from mice on the chow diet. Both genotypes were similar in this respect. In WT mice, the increase in liver weight as a consequence of eating the semisynthetic diet was the same regardless of the zinc status of the diet (Fig. 1). However, the liver weight of KO mice did not increase as much in the ZA group (Fig. 1). Analysis of hepatic lipids showed that the livers of mice fed the semisynthetic diets contained substantially more lipid and had a higher lipid concentration than the livers of mice that ate the chow diet. KO-ZA mouse livers contained substantially less total lipid and a slightly lower concentration of lipid than those of the WT-ZA mice (Fig. 1). The composition of hepatic lipids was unaffected by dietary zinc intake or MT genotype, with the predominant fatty acids identified as linoleic acid and oleic acid (Table 3). Hepatic lipids of mice fed the rodent chow diet contained a higher proportion of palmitic acid (Table 3).

Markers of Inflammation. Mice fed ZA semisynthetic diets had very similar levels of plasma CRP compared to mice fed the chow diet (Fig. 2). However, WT mice fed a ZD diet had significantly higher levels of plasma CRP. In contrast, plasma CRP levels in KO mice that received the ZD diet were the same as those found in ZA mice and the animals that ate the chow diets. Control levels of TNF-α and IFN-γ were at the lower limit of detection for the method, and no treatment or genotype-related changes in these cytokines were observed (data not shown).

Proteomic Analysis of Soluble Liver Proteins. A total of 1001 spots were detected on 2D gels, of which 283 were successfully matched in all gels. Statistical tests indicate that only 6–13 spots were significantly affected by the treatments, which, at a significance level of 5%, might be expected by chance. The lack of significant treatment effects was confirmed by principal component analysis of the 283 matched spots. There was no clear treatment-related structure in the data for the first and second or subsequent components (data not shown).

Discussion

The high-fat semisynthetic diets used in this study had a considerable impact on liver appearance, size, weight, and lipid content. The principal fatty acid present in these livers was found by gas chromatography to be linoleic acid, reflecting the predominance of this lipid in the diet. In spite of the increased liver weight in mice on the semisynthetic diet compared with the chow diet, body weight was unaffected by dietary composition, zinc intake, and genotype. We showed previously that KO mice in a mixed C57BL-129Ola genetic background are obese (31), and mild obesity has been confirmed in KO mice that have been backcrossed sufficiently into a C57BL genetic background to be considered congenic with that strain (20). A metabolic mechanism based on gene array studies, which could explain this phenotype, has been proposed (32). However, KO mice in a 129Sv genetic background do not show an obese phenotype (33), and the body weights of KO and WT mice in the present study were not significantly different (*P*

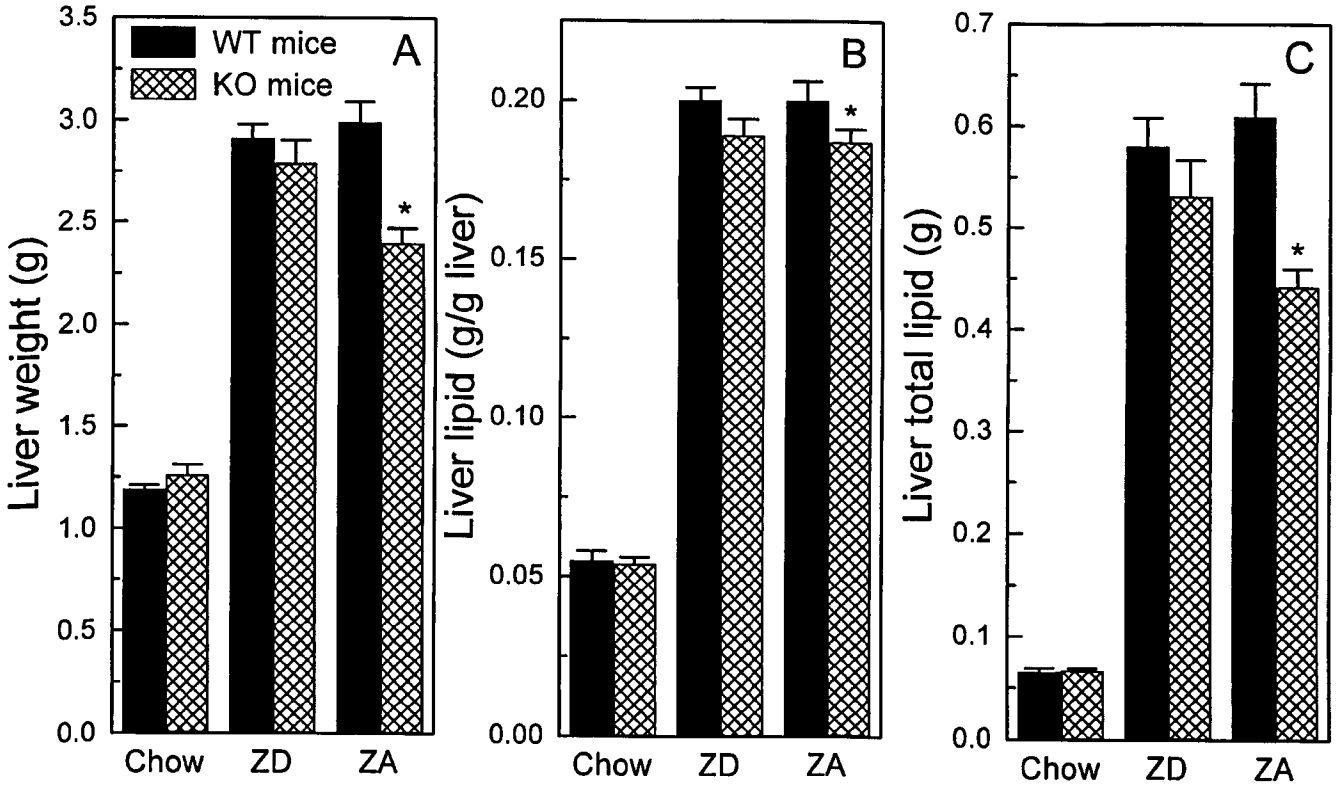

Figure 1. (A) Liver weight, (B) hepatic lipid concentration, and (C) total liver lipid content of KO and WT mice fed either rodent chow or a ZA or ZD semisynthetic diet. Error bars indicate the standard error of the mean and asterisks indicate statistically significant differences ($P < 0.05$) when comparing genotypes. Liver weights for all mice fed semisynthetic diets were significantly greater than those of chow-fed mice ($P < 0.001$).

< 0.05). Nevertheless, the hepatic response to high dietary fat intake was significantly affected by genotype, and less fat accumulated in the livers of KO-ZA mice. However, there was also less tissue in KO-ZA mouse livers, and the concentration of lipid was only slightly decreased by MT deficiency. This indicates that the difference in liver weight was not only due to accumulation of less fat, but that the liver also did not increase in size as much in the KO mice as in WT animals. This effect was not observed upon comparison of ZD genotypes, which indicates that an interaction between MT and zinc is necessary to elicit this response. Recent evidence suggests that cell proliferation during liver regeneration after partial hepatectomy is lower in KO mice than in WT mice (34), and it is possible that the retarded increase in liver tissue due to high dietary lipid intake observed in the present study is related to suppressed cell proliferation in the absence of MT.

Since a clear effect of genotype on liver weight was detected, which was also influenced by zinc status, we investigated the influence of zinc and MT on the expression

Table 3. Fatty Acid Composition of the Chow and Semisynthetic (S-S) Diets and also of the WT and KO Livers from Mice Fed ZA and ZD Diets

	Palmitic	Stearic	Oleic	Linoleic	Arachidonic	Saturated	Others
	% composition (mean ± SE)						
Chow diet	NA[a]	NA	19.2	55.1	NA	17.4	8.3
S-S diet	NA	NA	13.7	74.8	NA	9.8	1.8
Liver:							
WT-Chow	28.7 ± 1.1	11.7 ± 0.6	12.0 ± 0.6	18.2 ± 0.6	14.2 ± 0.8	NA	15.1 ± 1.1
KO-Chow	29.5 ± 0.8	13.5 ± 1.1	11.2 ± 3.1	17.1 ± 0.8	14.4 ± 1.8	NA	14.2 ± 1.3
WT-ZA	8.1 ± 0.2	2.4 ± 0.1	27.2 ± 0.3	45.7 ± 0.3	3.4 ± 0.3	NA	13.4 ± 0.3
WT-ZD	7.5 ± 0.2	2.4 ± 0.2	26.3 ± 0.5	48.4 ± 0.9	3.0 ± 0.1	NA	12.7 ± 0.7
KO-ZA	7.7 ± 0.2	2.5 ± 0.1	26.4 ± 1.0	47.7 ± 1.0	3.4 ± 0.1	NA	12.2 ± 0.2
KO-ZD	7.8 ± 0.3	2.2 ± 0.2	27.6 ± 0.6	46.4 ± 0.2	2.9 ± 0.3	NA	13.2 ± 0.3

[a] Some fats were not analyzed (NA) in some samples.

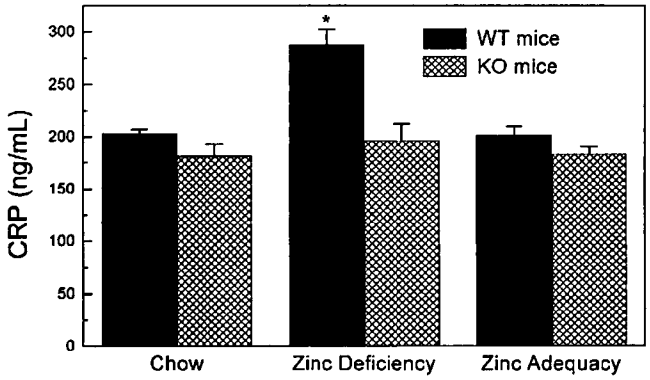

Figure 2. Plasma CRP levels in KO and WT mice given a rodent chow diet or a ZA or ZD semisynthetic diet. Error bars indicate the standard error of the mean and asterisks indicate statistically significant differences ($P < 0.05$) when comparing zinc adequacy with zinc deficiency for the semisynthetic diets and for the same genotype, and when comparing different genotypes for the same zinc intake.

of hepatic soluble proteins. Proteomic analysis using 2D gels is a powerful technique to display not only genotype-related changes in protein levels, but also post-translational modifications and protein-protein interactions. The centralized proteomics facility at the Rowett Institute routinely produces gels of high quality with clear protein resolution and good reproducibility. It was therefore surprising that we could not find an effect of zinc deficiency, MT deficiency, or a combination of the two factors on hepatic protein levels. Principal component analysis confirmed a lack of treatment or genotype effect, although highly significant differences of some hepatic protein levels in mice that consumed a high-fat semisynthetic diet, compared with those that ate a chow diet, were found for both mouse genotypes (results not shown). The proteomic analysis was repeated twice, and the same conclusions were obtained each time. These results contrast with the many genotype-related changes in gene expression in KO mouse livers compared with WT animals observed in a gene array study (32). The mice were of the same genetic background as those used in the present study, but one possible explanation for the different results is the age of the animals. Mice for the gene array study were 4-weeks old, whereas the animals in the present study were 32-weeks old by the end of the study. Another possible reason for the difference in results is that we only studied soluble liver proteins, whereas the gene array may have included genes that express soluble and insoluble proteins. It is also possible that changes in the expression of certain genes do not affect the corresponding protein levels, due to homeostatic regulation.

Since linoleic acid is considered pro-oxidant and proinflammatory, exposure to a high level of this fatty acid should have stimulated an anti-inflammatory response and raised levels of inflammation markers (22). A general marker of inflammation is CRP, which is produced in the liver and secreted into plasma (35). Having noted the marked effect of the semisynthetic diet on mouse livers, we measured the level of plasma CRP. With adequate zinc intake, the CRP level in WT mice was unaffected by the semisynthetic diet, but a marginal zinc intake resulted in a significantly raised level of CRP. The effect of marginal zinc deficiency on the level of CRP was not found in KO mice, indicating that MT modulates the metabolism of CRP when zinc is limiting. This result appears to contradict the hypothesis that MT protects against stress; on the other hand, if an adequate stress response is lacking in the absence of MT, mice deficient in MT might be more susceptible to the adverse effects of stress, which seems to be the case in practice. The elevated hepatic MT level observed in WT mice on the semisynthetic diets compared to the chow diet indicates that the high dietary-lipid challenge had indeed elicited a stress response. A treatment or genotype-related increase in the inflammatory cytokines TNF-α and IFN-γ was not observed and so the effect was specific to CRP. In general, cytokine assays can barely detect control levels of hormones in rodent plasma, but are designed to detect levels that are attained during inflammation.

In conclusion, the livers of mice that consumed a semisynthetic diet with 16% fat content, mostly as linoleic acid, were considerably larger and contained much more lipid than livers from mice that consumed a chow diet with 3.36% fat. The increase in liver size was not only due to a higher tissue lipid content but also to an increase in liver tissue *per se*. The increase in liver tissue and to a lesser extent the tissue lipid concentration were retarded in MT deficiency combined with zinc adequacy. This effect was not observed in zinc deficiency, indicating the importance of an interaction between MT and zinc. Plasma CRP levels were significantly increased in WT-ZD mice but not in KO-ZD animals, which again indicates an interaction between MT and zinc in CRP expression or secretion, since the half-life, and therefore degradation/excretion, of CRP is reported to be constant under all conditions of health and disease (36). Given that liver size and the liver-derived protein CRP were affected by MT and zinc status, it is surprising that no treatment- or genotype-related changes in the expression of soluble hepatic proteins was detected by 2D gel proteomics. Since the matched proteins detected by this technique constituted <1% of the total proteome and were the most highly expressed soluble liver proteins, it is possible that there are changes in lesser-expressed proteins that we were unable to detect. Further proteomic analysis is underway to investigate proteins from other tissues.

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