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Effects of dietary zinc deficiency on homocysteine and folate metabolism in rats

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In rats, zinc deficiency has been reported to result in elevated hepatic methionine synthase activity and alterations in folate metabolism. We investigated the effect of zinc deficiency on plasma homocysteine concentrations and the distribution of hepatic folates. Weanling male rats were fed ad libitum a zinc-sufficient control diet (382.0 nmol zinc/g diet), a low-zinc diet (7.5 nmol zinc/g diet), or a control diet pair-fed to the intake of the zinc-deficient rats. After 6 weeks, the body weights of the zinc-deficient and pair-fed control groups were lower than those of controls, and plasma zinc concentrations were lowest in the zinc-deficient group. Plasma homocysteine concentrations in the zinc-deficient group (2.3 ± 0.2 μmol/L) were significantly lower than those in the ad libitum-fed and pair-fed control groups (6.7 ± 0.5 and 3.2 ± 0.4 μmol/L, respectively). Hepatic methionine synthase activity in the zinc-deficient group was higher than in the other two groups. Low mean percentage of 5-methyltetrahydrofolate in total hepatic folates and low plasma folate concentration were observed in the zinc-deficient group compared with the ad libitum-fed and pair-fed control groups. The reduced plasma homocysteine and folate concentrations and reduced percentage of hepatic 5-methyltetrahydrofolate are probably secondary to the increased activity of hepatic methionine synthase in zinc deficiency. (J. Nutr. Biochem. 11:165–169, 2000) © *Elsevier Science Inc. 2000. All rights reserved.*

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Introduction

In the last several years, there has been increasing interest in homocysteine metabolism, as a number of investigators have reported an association between elevated plasma homocysteine concentrations (hyperhomocysteinemia) and an increased risk for occlusive-vascular diseases.[1,2] Although several mechanisms have been proposed to explain the effects of hyperhomocysteinemia on vascular integrity, a clear understanding of the role of hyperhomocysteinemia in vascular damage is still lacking.[1–3]

Homocysteine is metabolized through the transsulfuration and transmethylation pathways, which require 5-methyltetrahydrofolate (5-CH_3-H_4folate), methylcobalamin, and pyridoxal-5′-phosphate.[4] Consequently, changes in the nutritional status of these vitamins may alter homocysteine concentrations in the circulation. In the transmethylation pathway, methylation of homocysteine to methionine is catalyzed by methionine synthase, which requires methylcobalamin as a cofactor and 5-CH_3-H_4folate as a substrate.

In 1987, Tamura et al.[5] reported increased hepatic methionine synthase activity and decreased plasma folate concentrations in zinc-deficient rats compared with zinc-sufficient controls. We undertook the current study to evaluate the effects of zinc deficiency in rats on plasma homocysteine concentrations and the distribution of hepatic folates using a combination of high performance liquid chromatography (HPLC) and microbiological assay methodologies.

Materials and methods

Animals

Male Sprague-Dawley rats (n = 33) weighing 80 to 100 g were obtained from Charles River (Gilroy, CA USA) and housed in

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Table 1 Various parameters in zinc-deficient, pair-fed, and *ad libitum*-fed control rats

Parameters	Zinc deficient	Pair-fed control	*Ad libitum*-fed control
Body weight (g)	137 ± 5[a]	171 ± 5[b]	401 ± 10[c]
Dietary intake (g/day)	10.5 ± 0.2[a]	10.5 ± 0.2[a]	24.0 ± 0.6[b]
Methionine intake (mg/day)	88 ± 2[a]	88 ± 2[a]	202 ± 5[b]
Plasma zinc (μmol/L)	7.3 ± 0.7[a]	21.0 ± 0.5[b]	22.0 ± 0.5[b]
Hepatic zinc (μmol/g)	0.36 ± 0.01[a]	0.38 ± 0.01[a]	0.40 ± 0.01[b]
Plasma folate (nmol/L)	68 ± 5[a]	109 ± 8[b]	154 ± 10[c]
Red-cell folate (μmol/L)	2.44 ± 0.26	2.32 ± 0.14	2.24 ± 0.17
Total hepatic folate (nmol/g)	27 ± 2	26 ± 1	23 ± 2
Hepatic methionine synthase (μmol of [^{14}C]methionine formed/h/g wet weight)	2.35 ± 0.19[a]	1.80 ± 0.10[b]	1.43 ± 0.11[b]
Plasma homocysteine (μmol/L)	2.3 ± 0.2[a]	3.2 ± 0.4[b]	6.7 ± 0.5[c]

Values are mean ± SEM of 11 rats in each group.
Values in a row not sharing the same superscript are significantly different ($P < 0.05$) by ANOVA with Fisher's exact post hoc test.

stainless-steel, wire-bottom cages in a room with a 12-hour light-dark cycle at constant temperature (23°C) and humidity (50%).[6] Rats were fed a commercial rat diet (5001, Purina, St. Louis, MO USA) for several days upon arrival and were assigned to one of three treatment groups. The zinc-deficient group received an egg-white protein-based diet containing 7.5 nmol zinc/g diet, and the pair-fed zinc-sufficient (pair-fed control) and ad libitum-fed zinc-sufficient (ad libitum-fed control) groups received the same diet except that it contained 382.0 nmol zinc/g diet. Diets were fed for 6 weeks, during which time food intake and body weight were monitored on a daily basis. The pair-fed control group was given the same amount of diet as that consumed by the zinc-deficient group on the previous day. These diets were prepared based on the recommendation by National Research Council[7] and contained 145 nmol of copper and 1.4 nmol of folate per gram of diet by analysis in our laboratory.[6] At the end of the 6-week period, rats were sacrificed by exsanguination using cardiac puncture after exposure to carbon dioxide (CO_2), and tissues were rapidly removed. Heparinized blood was immediately placed on ice, and plasma was separated after an aliquot of whole blood was separated for hematocrit and red-cell folate determinations.[6]

Determinations of zinc, folate, and homocysteine

Plasma and hepatic zinc concentrations were determined by inductively-coupled argon plasma-atomic emission spectrophotometry after wet ashing (Trace Scan ICP, Thermo-Jarrel Ash Corp., Franklin, MA USA).[8] Plasma, red-cell, and total hepatic folate contents were measured by microbiological assay using *Lactobacillus casei* as previously described.[9] Red-cell and hepatic folate contents were measured after treatment with rat serum folate conjugase. Plasma total homocysteine concentrations were measured using an HPLC method with a fluorescent detector.[10] The coefficients of variation for folate and homocysteine analyses using pooled human plasma were approximately 10% and 6%, respectively.

Determinations of methionine synthase activity and hepatic folate distribution

The activity of hepatic methionine synthase was determined using a method described by Koblin et al.[11] with slight modifications. Briefly, liver tissue was homogenized in 10 volumes of 0.01 M potassium phosphate buffer (pH 7.3) containing 1% mercaptoethanol using a Teflon-glass tight-fitting homogenizer on ice. The homogenates were centrifuged at 25,000 × g for 10 minutes at 4°C, and the supernatant fractions were collected after carefully removing the fat layer on the top. The supernatant (100 μL) was added to a mixture (90 μL) containing 0.5 mM S-adenosylmethionine, 15 mM homocysteine, 58 mM dithiothreitol, and 20 μM cyanocobalamin. After the mixture was incubated for 5 minutes at 37°C, 10 μL of 5-[^{14}C]H_3-H_4folate (93 MBq/100 μL, barium salt, 2.15 GBq/mmol; Amersham, Arlington Heights, IL USA) was added and incubated for an additional 10 minutes at 37°C. The reaction was terminated by heating at 100°C for 3 minutes, and the heated mixture was centrifuged at 5,000 × g for 5 minutes after cooling on ice. The supernatant of the mixture (100 μL) was applied to a column (total volume of 1.0 mL of AG1-X8, Bio Rad, Richmond, CA USA). The product ([^{14}C]methionine) was eluted by washing with a total of 1.5 mL of 0.02 M sodium acetate buffer (pH 4.5). The whole eluent was collected and radioactivity was counted (Wallac 1410, Wallac Inc., Gaithersburg, MD USA). The activity is expressed as nmol of [^{14}C]methionine formed per gram of wet weight liver per hour.

The distribution of hepatic folates after folate conjugase treatment was measured using an HPLC method with a combination of *L. casei* microbiological assay as originally described by Horne and colleagues[12,13] with slight modifications.[6] To calibrate the column, the following authentic folates were used: 5-[^{14}C]H_3-H_4folate (Amersham); [^{3}H]-5-formyltetrahydrofolate ([^{3}H]-5-HCO-H_4folate, 0.94 TBq/mmol; Moravek, Brea, CA USA); [^{3}H]-10-formyltetrahydrofolate ([^{3}H]-10-HCO-H_4folate prepared from [^{3}H]-5-HCO-H_4folate[14]); and tetrahydrofolate (H_4folate, prepared from nonradioactive pteroylglutamic acid[15]).

Statistical analyses

Comparisons in various parameters between the three groups were performed by analysis of variance using Fisher's exact test as a post hoc test. The differences in the distribution of folate coenzymes were evaluated by Wilcoxon rank-sum test between the zinc-deficient and pair-fed control groups. The correlations between hepatic or plasma zinc concentrations and plasma homocysteine concentrations or hepatic methionine synthase activities were evaluated using linear regression. A *P*-value less than 0.05 was considered significant.

Results

After 6 weeks of feeding, the rats in the zinc-deficient group showed reduced food intake compared with the control group (*Table 1*), and a cyclic pattern of food intake, which is characteristic of zinc deficiency in rats, was observed.[16] Plasma zinc concentrations were significantly lower in the

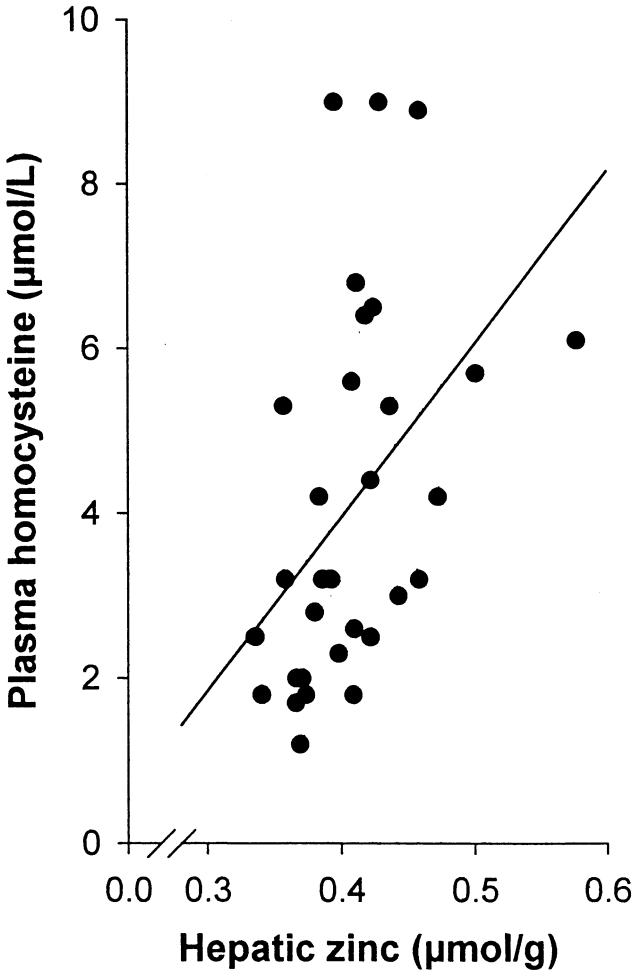

Figure 1 Relationship between plasma homocysteine concentrations and hepatic zinc concentrations in all three groups of rats. Correlation coefficient between plasma homocysteine and hepatic zinc concentrations was 0.46 ($P < 0.01$, n = 31).

zinc-deficient group than in the pair-fed and ad libitum-fed control groups. Similarly, hepatic zinc concentrations were significantly lower in the zinc-deficient group than in the ad libitum-fed control group; however, hepatic zinc concentrations in the zinc-deficient and pair-fed control groups were similar (*Table 1*).

Plasma folate concentrations were significantly lower in the zinc-deficient group than in the pair-fed and ad libitum-fed control groups; red-cell and hepatic folate concentrations were similar in the three groups (*Table 1*). As shown in *Table 1*, plasma homocysteine concentrations in the zinc-deficient group were significantly lower than in the two control groups. Furthermore, plasma homocysteine concentrations in the pair-fed control group were significantly lower than those in the control group. As shown in *Figure 1*, plasma homocysteine concentrations were positively correlated with hepatic zinc concentrations ($r = 0.46$, $P < 0.01$). The correlation between plasma homocysteine and zinc concentrations was also significant ($r = 0.56$, $P < 0.001$).

Hepatic methionine synthase activities were significantly higher in the zinc-deficient group than in the pair-fed or ad libitum-fed control groups, and hepatic methionine synthase activities were similar in the pair-fed and ad libitum-fed control groups (*Table 1*). The correlation between hepatic methionine synthase activities and zinc concentrations was significant ($r = 0.42$, $P < 0.0001$).

Table 2 Distribution (%) of hepatic folate derivatives in zinc-deficient and pair-fed control rats

Folates	Zinc-deficient	Pair-fed control	*P*
10-HCO-H_4folate	29 ± 10	16 ± 4	0.025
H_4folate	14 ± 6	14 ± 6	NS
5-HCO-H_4folate	16 ± 2	9 ± 2	0.01
5-CH_3-H_4folate	41 ± 7	61 ± 3	0.01

Each group consisted of five animals.
Difference between the two groups were estimated using Wilcoxon-rank-sum test.
NS, not significantly different.

The distribution of hepatic folate derivatives in the zinc-deficient and pair-fed control groups is shown in *Table 2*. The percentage of 5-CH_3-H_4folate in the zinc-deficient group was significantly lower than in the pair-fed control group ($P = 0.01$). In contrast, the percentages of both 10-HCO-H_4folate and 5-HCO-H_4folate in the zinc-deficient group were significantly higher than those in the pair-fed control group ($P < 0.03$), whereas the percentage of H_4folate was similar in the two groups.

Discussion

In the study presented here, we found that dietary zinc deficiency in rats induced an increase in hepatic methionine synthase activity, which in turn resulted in decreased plasma homocysteine and folate concentrations. A decreased proportion of hepatic 5-CH_3-H_4folate in the zinc-deficient rats compared with pair-fed controls was identified using an HPLC method. The observation of increased hepatic methionine synthase activity in zinc-deficient rats is consistent with the data reported by Tamura et al.,[5] who also showed decreased plasma folate concentrations and hepatic 5-CH_3-H_4folate by differential microbiological assay using *L. casei* and *Streptococcus faecium*. In the study presented here, there were significant correlations between hepatic zinc concentrations and plasma homocysteine concentrations (*Figure 1*) or hepatic methionine synthase activities. The relationship between plasma homocysteine and plasma zinc concentrations was also significant.

In 1972, Käferstein and Jaenicke[17] were the first to report the possible nutrient interaction between zinc and folate. They demonstrated that chicken pancreas folate conjugase is a zinc metalloenzyme. Since then, several groups of investigators have shown that folate conjugase in various mammalian tissues, including the intestine of humans, liver of cows, and pancreas of pigs and rats, are zinc dependent.[18] Furthermore, Hsu et al.[19] investigated methionine metabolism in zinc-deficient rats. They found a higher production of $^{14}CO_2$ after the administration of [^{14}C]H_3-methionine in zinc-deficient rats compared with that in zinc-supplemented controls. However, the produc-

tion of $^{14}CO_2$ was similar after the injection of 2-[^{14}C]-methionine and 1-[^{14}C]-methionine in the two groups of rats. Those data suggest that transmethylation reactions are more efficient in zinc-deficient rats than in controls. Thus, our present findings, and a previous report by Tamura et al.,[5] are in agreement with the report by Hsu et al.[19] To our knowledge, methionine concentrations in tissues of zinc-deficient animals are not available at the present time; thus, it is important to obtain the information to understand the exact metabolism of homocysteine and methionine in zinc-deficient animals. However, Duerre and Wallwork[20] reported that methionine concentrations in zinc-deficient rats were similar to those in the control groups using the isolated liver after perfusion with two levels of methionine.

We are at present unable to explain why plasma homocysteine concentrations are lower in the pair-fed control group than in the ad libitum-fed control group. As shown in *Table 1*, the mean dietary intakes of methionine in these groups were 88 and 202 mg/day, respectively. When we divided these intake values by mean body weights, the means of methionine intake were 0.51 and 0.50 mg/g body weight for the pair-fed and ad libitum-fed control groups, respectively. Thus, the difference in plasma homocysteine concentrations between these two groups does not appear to be secondary to dietary intakes of methionine, although homocysteine in vivo is exclusively derived from methionine. Dietary restriction is known to induce a decline in protein turnover.[21] However, to our knowledge, no information is available as to whether tissue methionine concentrations decline under the conditions of starvation; therefore, it is unclear if the decline in plasma homocysteine concentrations is due to reduced availability of methionine. Considering that zinc deficiency can also result in decreased protein turnover,[22] it is likely that the low plasma homocysteine observed in the zinc-deficient group is due to a combination of increased hepatic methionine synthase activity and a decreased protein turnover. However, it should be noted that the methionine intake in the zinc-deficient group was 0.64 mg/g body weight. Thus, if the decline in protein turnover affects certain biochemical parameters, the pair-fed control group may not be an ideal control for the zinc-deficient rats. Careful consideration may be needed for certain indices as to what is the best control for experimental zinc deficiency in the rat model. Further studies are warranted for the clarification of this point.

Recently, we reported that hepatic methionine synthase activity is decreased in copper-deficient rats.[6] In these rats, increased plasma homocysteine and plasma folate concentrations and an increased proportion of hepatic 5-CH_3-H_4folate in copper-deficient rats compared with controls were observed. Thus, the findings in the zinc-deficient rats are opposite to those in copper-deficient rats (*Table 3*). One interpretation of these findings in rats is that "zinc-copper interaction" may in part regulate the activity of hepatic methionine synthase. It is important to note that the group of Matthews[23,24] reported that both cobalamin-dependent and cobalamin-independent methionine synthases of *Escherichia coli* contain zinc, which is essential for the catalytic activity. However, we do not know why the requirement for specific metals for the catalytic activity or the synthesis of methionine synthase is different between bacteria and mammalian cells.

Table 3 Comparison of various parameters of folate metabolism between zinc deficiency and copper deficiency in rats

	Zinc deficiency	Copper deficiency
Plasma folate	decreased	increased
Red-cell folate	unchanged	unchanged
Total hepatic folate	unchanged	unchanged
5-Methyltetrahydrofolate	decreased	increased
Hepatic methionine synthase	increased	decreased
Plasma homocysteine	decreased	increased

Furthermore, one may question why the activities of hepatic methionine synthase were higher in the zinc-deficient groups than in the two control groups despite similar hepatic zinc concentrations among all three groups (*Table 1*). This is likely due to different subcellular zinc distributions between zinc-deficient and zinc-sufficient control rats. Although it is impossible to precisely determine the subcellular distribution of zinc in animal tissues using the currently available experimental techniques, it has been long assumed that the redistribution of zinc occurs when animals are given a low-zinc diet. For example, zinc concentrations in the soluble fraction of brain homogenate obtained from zinc-deficient rats were significantly lower than those from the control groups.[25] Considering that methionine synthase is located in the cytoplasmic fraction of mammalian cells,[26] it may be reasonable to find altered activity of this enzyme in zinc deficiency.

Our experimental models are the extreme cases of either zinc or copper deficiency, and it is unknown what extent of alterations a mild deficiency of either of these minerals would cause in methionine synthase activity. The investigation of the alteration of methionine synthase activity in rats fed varying levels of dietary zinc or copper is needed. Furthermore, it is unclear whether our findings of altered plasma homocysteine concentrations, presumably due to the changes in hepatic methionine synthase activity in zinc-deficient or copper-deficient rats can be extrapolated into human conditions. Further studies are warranted to investigate whether altered metabolism of folate and homocysteine occurs in zinc or copper deficiency in humans.

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