

Short Communication

Marginal zinc deficiency in rats decreases leptin expression independently of food intake and corticotrophin-releasing hormone in relation to food intake

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Zn deficiency reduces food intake and growth rate in rodents. To determine the relationship between Zn deficiency and the regulation of food intake, we evaluated leptin gene expression in epididymal white adipose tissue (eWAT), and hypothalamic corticotrophin-releasing hormone (hCRH) and hypothalamic neuropeptide Y (hNPY) of rats Zn-deficient only to show reduced food intake and growth rate but not food intake cycling. Growing male Sprague-Dawley rats (240 g) were randomly assigned to one of four dietary groups: Zn-adequate (ZA; 30 mg/kg diet), Zn-deficient (ZD; 3 mg/kg diet), pair-fed with ZD (PF; 30 mg/kg diet) and Zn-sufficient (ZS; 50 mg/kg diet) (*n* 8), and were fed for 3 weeks. Food intake and body weight were measured, as were blood mononuclear cells and pancreas Zn levels. eWAT leptin, hCRH and hNPY mRNA levels were determined. Food intake was decreased by about 10% in ZD and PF rats compared to ZA and ZS rats. Growth and eWAT leptin mRNA levels were unaffected in PF rats but were significantly ($P < 0.05$) decreased in ZD rats. However, hNPY showed a tendency to increase, and hCRH significantly ($P < 0.05$) decreased, in both ZD and PF rats. These results suggest that while leptin gene expression may be directly affected by Zn, hNPY and hCRH are likely responding to reduced food intake caused by Zn deficiency.

Zinc deficiency: Corticotrophin-releasing hormone: Leptin: Food intake: Growth

Zn is an essential component of numerous enzymes and transcription factors and influences many biological processes including the control of food intake and growth^{1,2}. Zn deficiency in experimental animals and in man causes anorexia, poor appetite, weight loss and growth retardation. Food intake is controlled by appetite and satiety centres in the central nervous system involving systemic hormonal and nervous feedback of fat reserves and food ingestion³. Leptin, a 16 kDa protein secreted primarily by white adipocytes, targets receptors in neurons of hypothalamic nuclei, informing the central nervous system about fat reserves and regulating the expression and signalling of orexigenic and anorexigenic neuropeptides³. Leptin negatively and positively regulates the release of neuropeptide Y (NPY) and corticotrophin-releasing hormone (CRH), respectively, from the hypothalamic paraventricular nucleus⁴. NPY is a potent stimulator of appetite whereas CRH inhibits food intake, and so in starved animals, NPY expression is increased and CRH decreased due principally to decreased circulating leptin levels⁴.

Zn may be a mediator of leptin production in human subjects^{5,6} and rodents^{7–9}. Circulating leptin levels and white adipose tissue leptin mRNA levels are decreased in Zn deficiency^{7,8}. Also, Zn supplementation of obese mice¹⁰ and diabetic mice¹¹ increased circulating leptin levels. As would be expected, in acute Zn deficiency where food intake is reduced, body adipose tissue diminishes resulting in reduced leptin synthesis and secretion. Hypothalamic NPY mRNA and protein levels increase in Zn deficiency¹², although the release of NPY by the paraventricular nucleus is reported to be reduced by Zn deficiency¹³. However, intracerebroventricular administration of NPY does not normalize food intake¹⁴ and there is therefore no clear consensus about the relationship between Zn status and NPY signalling.

We proposed to examine the response of epididymal white adipose tissue (eWAT) leptin and hypothalamic NPY (hNPY) and hypothalamic CRH (hCRH) mRNA to marginal Zn deficiency. We used a Zn level of 3 mg/kg diet because it reduces food intake in rats but does not induce food intake

Abbreviations: eWAT, epididymal white adipose tissue; CRH, corticotrophin-releasing hormone; hCRH, hypothalamic corticotrophin-releasing hormone; hNPY, hypothalamic neuropeptide Y; NPY, neuropeptide Y; PF, pair-fed with Zn-deficient; ZA, Zn-adequate; ZD, Zn-deficient; ZS, Zn-sufficient.

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cycling behaviour characteristic of acute Zn deficiency¹. Our objective was to try to identify Zn-specific effects on leptin, hNPY and hCRH mRNA.

Materials and methods

Animal care and experimental diets

After 1 week of adaptation, thirty-two growing Sprague-Dawley male rats (SLC, Shizuoka, Japan), weighing 240.0 ± 3.2 g and 6–7 weeks old, were randomly assigned to one of four dietary Zn groups (n 8): Zn-adequate (ZA; 30 mg/kg diet), Zn-deficient (ZD; 3 mg/kg diet), pair-fed with the ZD group (PF; 30 mg/kg diet) and Zn-sufficient (ZS; 50 mg/kg diet). The composition of the experimental diets and mineral mixes were used as described by Lee *et al.*⁹. The prepared purified diet used 20% casein as the protein source. The nominal Zn levels in the ZD, ZA and ZS diets were 3, 30 and 50 mg/kg diet, respectively, and the analysed Zn levels were 2.6, 32.9 and 52.8 mg/kg diet, respectively. The experimental diets were made by modifying the rodent AIN-76 mineral mix (30 mg Zn/kg diet) and were fed for 3 weeks.

Individual PF rats were matched to ZD rats and, at 18.00 hours each day, each was given the same quantity of ZA diet as consumed by its ZD pair the previous day. Rats were individually housed in stainless steel wire-bottom cages in an environmentally temperature-controlled room ($22 \pm 0.5^\circ\text{C}$) with alternate 12 h light and dark cycles. All rats had free access to distilled and deionized water from plastic bottles with silicon stoppers. The diet was stored at 4°C in plastic containers and handled with plastic gloves and appropriate utensils to avoid contamination.

Food intake was recorded daily and body weights were measured every 3 d. Diet spillage, common in ZD animals, was measured and accounted for in determining food consumption. The study was approved and performed in accordance with the guidelines for the care and use of laboratory animals and with the Animal Care and Use Committee at Andong National University.

Tissue and blood sample collection

On the last day of dietary Zn treatment for ZS, ZA and ZD groups, and 1 d later for the PF group, the assigned rats were anaesthetized with ketamin-HCl (10 mg/kg body weight). Blood was collected from the abdominal aorta and mononuclear cells were separated using Histopaque-1077 (Sigma, St Louis, MO, USA). Pancreas was collected for Zn level determination and eWAT and the brain hypothalamic block was removed for RT-PCR.

Zn and protein assay

Tissue and cell samples were analysed for Zn and protein levels as previously described⁹. Analysis of a standard reference material (NIST SRM 1577b, bovine liver) confirmed the accuracy of analysis (120 (SEM 7) $\mu\text{g/g}$, n 3, compared to the reference value of 127 (SEM 16) $\mu\text{g/g}$). The measured Zn value was within the reference range.

RT-PCR for epididymal white adipose tissue leptin, hypothalamic corticotropin-releasing hormone and hypothalamic neuropeptide Y mRNA

The guanidinium-thiocyanate method using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction from eWAT and hypothalamus according to the manufacturer's instructions. For adipose tissue, the method was modified by including an additional low-speed spin and by increasing the volume of TRIzol Reagent by 1.5-fold. RNA samples were reverse transcribed and PCR amplified using standard protocols. The primers used for amplification of leptin, CRH, NPY and β -actin were as follows. Leptin (320 bp): forward 5'-AAG AAG ATC CCA GGG AGG AA-3'; reverse 5'-TCA TTG GCT ATC TGC AGC AC-3'. CRH (355 bp): forward 5'-CCG CAG CCG TTG AAT TTC TTG C-3'; reverse 5'-AGG TGA GAT CCA GAG AGA TGG GCG-3'. NPY (543 bp): forward: 5'-ATC CCT GCT CGT GTG TTT GGG C-3'; reverse: 5'-GGG TCT TCA AGC CTT GTT CTG GG-3'. β -Actin (543 bp): forward 5'-ATC GTG GGG CGC CCC AGG CAC-3'; reverse 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3'.

The PCR products were separated on 1.2% agarose gels, stained with ethidium bromide and photographed under UV light using a digital camera for band intensity quantification.

Statistical analysis

Data were analysed with a one-way ANOVA and Tukey's multiple comparisons using Minitab statistical software (Minitab Ltd, Coventry, UK).

Results

Daily food intake and body weight gain

Daily food intake was significantly lower in the ZD group compared to the ZA or ZS groups ($P < 0.05$; Fig. 1(C)) and, by design, food intake in the PF group was the same as in the ZD group. There was no cyclical pattern of food intake, a characteristic of acute Zn deficiency in rats. Consistent with a reduced intake of food, body weight gain for ZD rats was significantly lower than that for ZA and ZS rats ($P < 0.05$; Fig. 1(A)). However, despite consuming the same amount of food as the ZD rats, the PF animals showed a similar body weight gain to the ZA and ZS rats.

Zn concentration in tissues

The mean tissue Zn concentrations for ZA, PF, ZD and ZS groups were 5.10 (SEM 0.90)^a, 4.45 (SEM 0.68)^{ab}, 3.40 (SEM 1.32)^b and 4.40 (SEM 0.66)^{ab} $\mu\text{g/mg}$ protein (blood mononuclear cells) and 14.2 (SEM 3.2)^c, 14.4 (SEM 1.7)^c, 12.3 (SEM 2.1)^d and 16.3 (SEM 2.6)^c $\mu\text{g/g}$ wet weight (pancreas), respectively (mean values with unlike superscript letters were significantly different ($P < 0.05$)). Both mononuclear cells and pancreatic Zn levels were significantly lower in the ZD group as compared to the ZA group, indicating the Zn-deficient status of ZD rats.

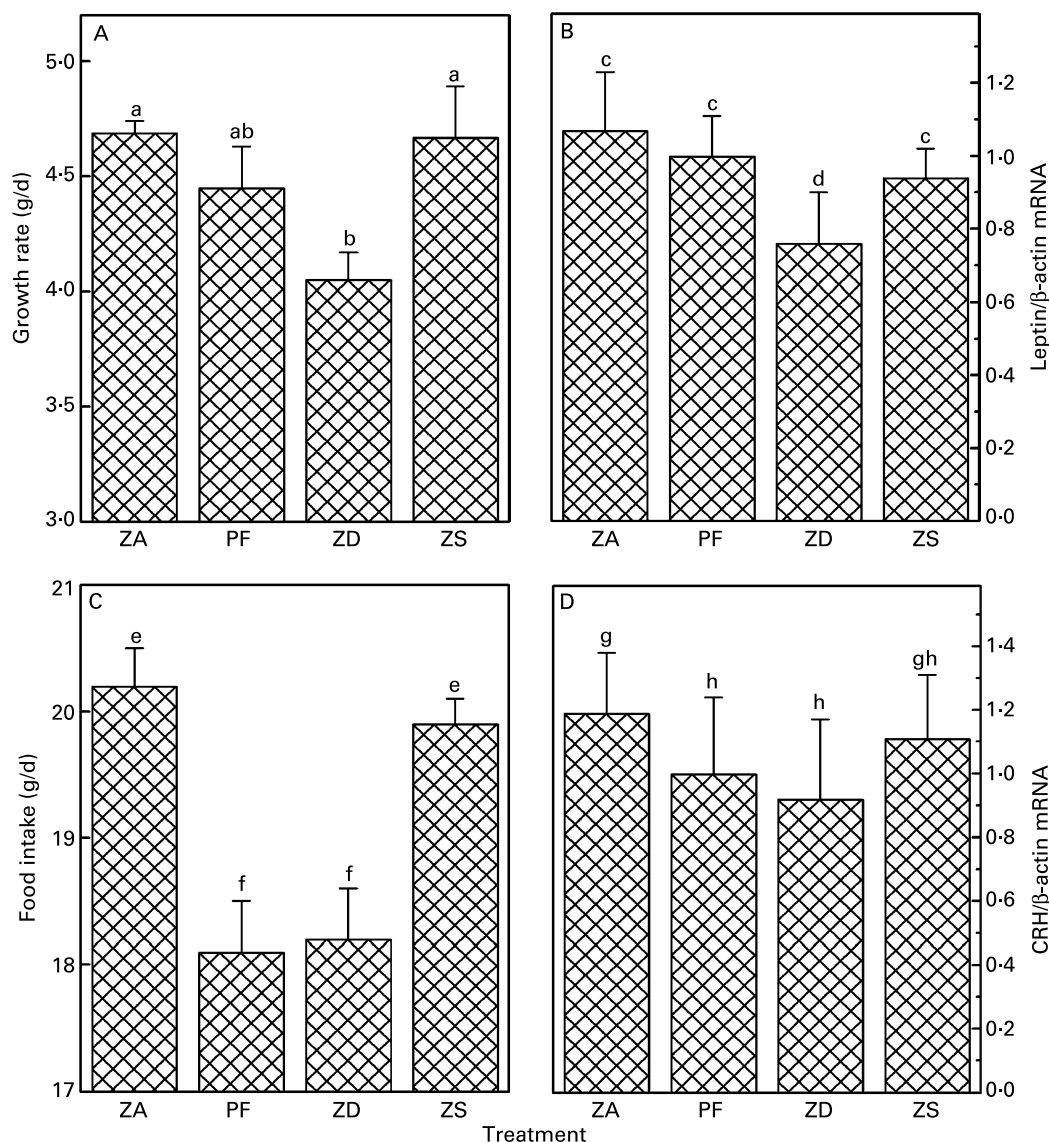


Fig. 1. Zinc deficiency (ZD) and pair-feeding (PF) effects on rat growth (A), epididymal white adipose tissue leptin mRNA levels (B), food intake (C) and hypothalamic corticotrophin-releasing hormone (CRH) mRNA levels (D) compared to zinc adequacy (ZA) and zinc sufficiency (ZS). Values are means with their standard errors depicted by vertical bars (n 8 for growth and food intake, n 5 for leptin, n 6 for CRH). Levels of target mRNA are normalized for β -actin mRNA and expressed relative to the PF mean values. ^{a-h}Mean values with unlike superscript letters were significantly different ($P < 0.05$).

Epididymal white adipose tissue leptin, hypothalamic corticotrophin-releasing hormone and hypothalamic neuropeptide Y mRNA levels

Relative mRNA levels of leptin in eWAT and hCRH are shown in Fig. 1(B, D). eWAT leptin mRNA levels in ZD rats were significantly lower than in ZA or ZS rats ($P < 0.05$). They were also significantly lower than the PF leptin mRNA levels ($P < 0.05$), which were unaffected by the reduced food intake. hCRH mRNA levels in the ZD and PF rats were significantly lower than those in the ZA but not the ZS rats ($P < 0.05$). hNPY mRNA levels in ZD rats showed a tendency to increase, compared to those of the ZA or PF rats, although without statistical significance (data not shown).

Discussion

Using marginal deficiency conditions, we have demonstrated that Zn deficiency decreases growth and eWAT leptin mRNA levels independently of food intake. A similar observation has been made in eWAT of acutely Zn-deficient rats⁸ and one possible explanation is that Zn directly or indirectly regulates leptin gene expression. Ott and Shay⁸ have hypothesized that Zn-regulated synthesis and secretion of insulin by the endocrine pancreas is a likely mechanism, since insulin stimulates leptin gene expression in adipocytes. The influence of Zn on leptin gene expression in isolated adipocytes is unknown but the insulin-stimulated secretion of leptin protein from *ex vivo* culture of rat eWAT was actually increased in response to Zn depletion⁸. The authors suggested that an

interaction between Zn and insulin in the medium might have been responsible for this unexpected result.

Circulating levels of plasma leptin decrease in acute Zn deficiency, but not independently of food intake¹⁵. Plasma leptin may therefore respond to the effect of Zn deficiency on food intake rather than being regulated directly by Zn. Indeed, a Zn deficiency-related reduction in circulating leptin may largely be caused by loss of adipose tissue due to anorexia⁸. Previously published discrepancies between leptin mRNA levels, leptin secretion and circulating leptin levels may therefore have logical explanations, and effects on mRNA stability and clearance of circulating protein also cannot be discounted. Also, differences in Zn-regulated expression of leptin in different adipose tissue depots should be considered⁹.

The lack of a response of eWAT leptin mRNA to reduced food intake due to pair-feeding in an acute Zn-deficiency study⁸ is surprising given that starvation of rats decreases eWAT leptin gene expression within 24 h¹⁶. Overall food intake in rat acute Zn deficiency is actually only reduced by about 40%¹, but the cycling pattern of intake may influence leptin gene expression. In the present study of marginal Zn deficiency, where no food cycling was observed, food intake was reduced by about 10% in pair-fed rats, but this also had no effect on eWAT leptin mRNA levels. It also had no effect on growth, indicating an improvement in energy efficiency that was not matched in the Zn-deficient rats. In a human study of starvation, circulating leptin levels decreased by 40% over 6 d while leptin mRNA levels in abdominal subcutaneous adipose tissue were unchanged¹⁷. There appears, therefore, to be a capacity for the dislocation of transcriptional and translational regulation of leptin production or of cellular leptin levels with rates of secretion. Insulin rapidly stimulates secretion of leptin from adipose tissue resulting in decreased adipocyte levels of leptin¹⁸, and it is of interest that Zn has insulinomimetic effects in cells due to its role in potentiating insulin receptor signalling¹⁹. Thus Zn deficiency might be expected to diminish insulin-stimulated secretion of leptin in adipocytes, but the exact opposite result has been demonstrated *ex vivo*⁸. Clearly, adipose tissue or adipocytes in culture are not subjected to sympathetic nerve and other hormonal influences which may modulate responses to Zn and insulin.

An increase in hNPY mRNA levels might be expected in response to decreased leptin expression, as has been observed previously¹², but the increase observed in the present study was not statistically significant. However, in concordance with the levels of eWAT leptin mRNA, hCRH mRNA levels were significantly decreased in the ZD group. In contrast to eWAT leptin expression, hCRH mRNA also decreased in PF rats. This suggests that hCRH is responding to plasma leptin, which is well known to decrease in response to Zn deficiency and reduced food intake. Thus it would appear that, like hNPY²⁰, hCRH expression is not directly affected by Zn, but is instead responding to a Zn deficiency-induced reduction in food intake.

In conclusion, we have demonstrated that both rat growth rate and leptin mRNA levels in eWAT are decreased by Zn deficiency independently of food intake, suggesting that Zn targets one or more factors affecting energy utilization efficiency and leptin gene expression. A food

intake-independent, suppressive effect of Zn deficiency on metabolic rate has been reported¹⁵, but this has not been confirmed elsewhere²¹. Our observation that hCRH mRNA levels are decreased in Zn deficiency has not previously been reported. hCRH mRNA probably responds to reduced food intake caused by Zn deficiency and may not be specifically targeted by Zn. This work highlights the need for a more comprehensive analysis of Zn-regulated mechanisms of leptin expression and secretion in adipocytes.

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References

1. Chesters JK (1997) Zinc. In *Handbook of Nutritionally Essential Mineral Elements*, pp. 185–230 [BL O'Dell and RA Sunde, editors]. New York: Marcel Dekker.
2. Tapiero H & Tew KD (2003) Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomed Pharmacother* **57**, 399–411.
3. Morton GJ, Cummings DE, Baskin DG, Barsh GS & Schwartz MW (2006) Central nervous system control of food intake and body weight. *Nature* **443**, 289–295.
4. Sahu A (2004) Hypothalamic role in energy balance with special emphasis on leptin. *Endocrinology* **145**, 2613–2620.
5. Chen MD, Song YM & Lin PY (2000) Zinc may be a mediator of leptin production in humans. *Life Sci* **66**, 2143–2149.
6. Mantzoros CS, Prasad AS, Beck FWJ, Grabowski S, Kaplan J, Adair C & Brewer J (1998) Zinc may regulate serum leptin concentrations in humans. *J Am Coll Nutr* **17**, 270–275.
7. Mangian HF, Lee RG, Paul GL, Emmert JL & Shay NF (1998) Zinc deficiency suppresses plasma leptin concentrations in rats. *J Nutr Biochem* **9**, 47–51.
8. Ott ES & Shay NF (2001) Zinc deficiency reduces leptin gene expression and leptin secretion in rat adipocytes. *Exp Biol Med* **226**, 841–846.
9. Lee SL, Kwak EH, Kim YH, Choi JY, Kwon ST, Beattie JH & Kwun IS (2003) Leptin gene expression and serum leptin levels in zinc deficiency: implications for appetite regulation in rats. *J Med Food* **6**, 281–289.
10. Chen MD & Lin PY (2000) Zinc-induced hyperleptinemia relates to the amelioration of sucrose-induced obesity with zinc repletion. *Obes Res* **8**, 525–529.
11. Chen MD, Song YM & Lin PY (2000) Zinc effects on hyperglycemia and hypoalbuminemia in Streptozotocin-induced diabetic mice. *Horm Metab Res* **32**, 107–109.
12. Lee RG, Rains TM, Tovar-Palacio C, Beverly JL & Shay NF (1998) Zinc deficiency increases hypothalamic neuropeptide Y and neuropeptide Y mRNA levels and does not block neuropeptide Y-induced feeding in rats. *J Nutr* **128**, 1218–1223.
13. Levenson CW (2003) Zinc regulation of food intake: new insights on the role of neuropeptide Y. *Nutr Rev* **61**, 247–249.
14. Williamson PS, Browning JD, Sullivan MJ, O'Dell BL & Macdonald RS (2002) Neuropeptide Y fails to normalize food intake in zinc-deficient rats. *Nutr Neurosci* **5**, 19–25.

15. Gaetke LM, Frederich RC, Ox HS & McClain CJ (2002) Decreased food intake rather than zinc deficiency is associated with changes in plasma leptin, metabolic rate, and activity levels in zinc deficient rats. *J Nutr Biochem* **13**, 237–244.
16. Becker DJ, Ongemba LN, Brichard V, Henquin JC & Brichard SM (1995) Diet- and diabetes-induced changes of ob gene expression in rat adipose tissue. *FEBS Lett* **371**, 324–328.
17. Andersen PH, Kristensen K, Pedersen SB, Højllund E, Schmitz O & Richelsen B (1997) Effects of long-term total fasting and insulin on ob gene expression in obese patients. *Eur J Endocrinol* **137**, 229–233.
18. Barr VA, Malide D, Zarnowski MJ, Taylor SI & Cushman SW (1997) Insulin stimulates both leptin secretion and production by rat white adipose tissue. *Endocrinology* **138**, 4463–4472.
19. Haase H & Maret W (2003) Intracellular zinc fluctuations modulate protein tyrosine phosphates activity in insulin/insulin-like growth factor-1 signaling. *Exp Cell Res* **291**, 289–298.
20. Shay NF & Mangian HF (2000) Neurobiology of zinc-influenced eating behavior. *J Nutr* **130**, 1493S–1499S.
21. Evans SA, Overton JM, Alshingiti A & Levenson CW (2004) Regulation of metabolic rate and substrate utilization by zinc deficiency. *Metabolism* **53**, 727–732.