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 corticotropin-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 growth1,2. 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 corticotropin-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 levels5.

Zn may be a mediator of leptin production in human subjects6,7 and rodents8–9. Circulating leptin levels and white adipose tissue leptin mRNA levels are decreased in Zn deficiency7,8. 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 deficiency12. 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 cycling.
cycling behaviour characteristic of acute Zn deficiency\textsuperscript{3}. 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±32 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.\textsuperscript{3}. 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±0.5°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 determination and eWAT and the brain hypothalamic (Sigma, St Louis, MO, USA). Pancreas was collected for Zn analysis of a standard block was removed for RT–PCR.

Food and protein assay

Tissue and cell samples were analysed for Zn and protein levels as previously described\textsuperscript{9}. Analysis of a standard reference material (NIST SRM 1577b, bovine liver) confirmed the accuracy of analysis (120 (SEM 7)μg/g, n=3, compared to the reference value of 127 (SEM 16)μg/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'-TTC TTT GCT ATC TGC AGC AC-3'; CRH (355 bp): forward 5'-CCG CAG CCG TGG AAT TTC TTG C-3'; reverse 5'-AGG TGA GAT CCA GAG AGA TGG GCG-3'. NPY (543 bp): forward 5'-ATC CCT GCT GTG TTT GGG C-3'; reverse 5'-GGG TCT TCA AGC CTT GGT GG-3'; β-Actin (543 bp): forward 5'-ATC GTG GGG CGC CCC AGG CAC-3'; reverse 5'-CTT AAT GTC ACG CAT 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)\textsuperscript{a}, 4.45 (SEM 0.68)\textsuperscript{ab}, 3.40 (SEM 1.32)\textsuperscript{b} and 4.40 (SEM 0.66)\textsuperscript{ab} μg/mg protein (blood mononuclear cells) and 14.2 (SEM 3.2)\textsuperscript{c}, 14.4 (SEM 1.7)\textsuperscript{c}, 12.3 (SEM 2.1)\textsuperscript{b} and 16.3 (SEM 2.6)\textsuperscript{c} μg/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.
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 rats and one possible explanation is that Zn directly or indirectly regulates leptin gene expression. Ott and Shay 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 depletion. 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 intake\textsuperscript{15}. 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 anorexia\textsuperscript{8}. 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 considered\textsuperscript{9}.

The lack of a response of eWAT leptin mRNA to reduced food intake due to pair-feeding in an acute Zn-deficiency study\textsuperscript{8} is surprising given that starvation of rats decreases eWAT leptin gene expression within 24 h\textsuperscript{16}. Overall food intake in rat acute Zn deficiency is actually only reduced by about 40\%\textsuperscript{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 about 40\% over 6 d while leptin mRNA levels in abdominal subcutaneous adipose tissue were unchanged\textsuperscript{17}. 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 leptin\textsuperscript{18}, and it is of interest that Zn has insulinomimetic effects in cells due to its role in potentiating insulin receptor signalling\textsuperscript{19}. Thus Zn deficiency might be expected to diminish insulin-stimulated secretion of leptin in adipocytes, but the exact opposite result has been demonstrated \textit{ex vivo}\textsuperscript{9}. 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 previously\textsuperscript{12}, 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 hNPY\textsuperscript{20}, 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 reported\textsuperscript{15}, but this has not been confirmed elsewhere\textsuperscript{21}. 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


