Zinc Deficiency Increases Hypothalamic Neuropeptide Y and Neuropeptide Y mRNA Levels and Does Not Block Neuropeptide Y–Induced Feeding in Rats

Rita G. Lee, Tia M. Rains, Claudia Tovar-Palacio, J. Lee Beverly* and Neil F. Shay†

Division of Nutritional Sciences, Department of Food Science and Human Nutrition and *Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801

ABSTRACT  Zinc deficiency reduces intake and produces an unusual ~3.5-d cycle of intake in rats. The mechanism underlying the anorexia and cycling has not yet been defined; current hypotheses suggest that alterations in amino acid metabolism and neurotransmitter concentrations may be a part of this anorexia. Recent reports indicate that appetite-stimulating neuropeptide Y (NPY) may be elevated during zinc deficiency. This suggests that a resistance to NPY may exist during zinc deficiency because NPY levels are high, yet appetite is low. The purpose of this study was to measure NPY peptide and mRNA concentrations during zinc deficiency in specific nuclei of the hypothalamus in which peptide and mRNA for NPY are known to be associated with appetite, and also to determine whether zinc-deficient rats are responsive to central infusions of NPY. Both NPY peptide levels in the paraventricular nucleus and NPY mRNA levels in the arcuate nucleus were higher (P < 0.05) in zinc-deficient rats than in zinc-adequate rats. When rats were administered exogenous NPY to the paraventricular nucleus, both zinc-deficient and zinc-adequate rats responded similarly by increasing food intake. These results suggest that NPY is elevated during zinc deficiency in an attempt to restore normal food intake levels, rather than being reduced and thereby contributing to the anorexia associated with zinc deficiency. During zinc deficiency, NPY receptors are able to bind NPY and initiate an orexigenic response. J. Nutr. 128: 1218–1223, 1998.

KEY WORDS: • rats • zinc deficiency • neuropeptide Y • food intake regulation • hypothalamus

Zinc deficiency causes a number of physiologic problems including anorexia, reduced growth and taste acuity, and hypogonadism (Prasad 1985). Zinc deficiency may be a secondary or sustaining feature of some human anorexias (Bakan 1984). The anorexia associated with the development of zinc deficiency has been studied for a number of years (Chesters and Quarterman 1970) and has several defining features. In growing rats, a reduction in intake begins to be observed ~3–5 d after a Zinc-deficient (Zn−) diet is provided. After 5 or more days, total intake may be reduced to ~50% of the diet consumed by zinc-adequate (Zn+) rats. Furthermore, individual Zn− rats are observed to eat with a recurring ~3.5-d cyclic pattern, in which intake varies from ~20 to 80% of control intake levels every 3–4 d (Tamaki et al. 1995). This pattern continues while rats remain zinc deficient. Upon zinc repletion with either zinc-containing diet or water, rapid restoration of normal intake is observed. In some individual zinc-repleted rats, levels of intake equivalent to control animals are observed within a few hours after repletion begins, and nearly 100% of all formerly Zn− rats will consume diet comparable to control levels within 24 h of zinc repletion. The cyclic intake associated with zinc deficiency disappears upon repletion.

Until recently, investigations of neural changes in the brain of zinc-deficient animals focused mainly on amino acid and catecholamine effects (O’Dell and Reeves 1989). Some investigators found that low protein, zinc-deficient diets affect food intake levels and reduce the magnitude of cycling observed in deficient rats (Griffith and Alexander 1972). It has been suggested that plasma amino acid concentrations, subsequent transport of amino acids through the large neutral amino acid transport system of the blood-brain barrier, and therefore the brain content of some amino acids may be rate-limiting for the synthesis of certain neurotransmitters, causing changes in selection of dietary protein and carbohydrate (Ashley and Anderson 1975). If zinc deficiency alters amino acid metabolism significantly, these factors could be affecting appetite. The catecholamine neurotransmitter, norepinephrine, has a role in food intake regulation. Reports quantitating norepinephrine content in excised brain tissue from Zn− rats have been mixed, with some investigators finding increases (Wallwork et al. 1982), whereas others have not (Reeves and O’Dell 1981). One aspect of appetite regulation during zinc deficiency...
that has been largely ignored until recently is the influence of the appetite-regulating neuropeptides. Neuropeptide Y (NPY) is the most potent appetite stimulant yet discovered (Clark et al. 1984). Although present in most neural tissues, NPY that is most closely associated with food intake is synthesized in cell bodies in the arcuate nucleus (ARC) and released primarily in the paraventricular nucleus (PVN). Generally, NPY mRNA levels in the ARC and NPY concentrations in the PVN are regulated coordinately during conditions that affect appetite, such as food restriction. Endogenous NPY concentrations in the PVN have been found to correlate with appetite; they are higher in food-restricted rats and reduced in fed rats (Sahu et al. 1988). Infusions of exogenous NPY into the PVN initiate immediate hyperphagia that results in obesity if rats are recurrently infused with NPY (Stanley et al. 1986). Infusion of NPY to the PVN has been shown to increase carbohydrate intake (Stanley et al. 1985) or intake of a preferred macronutrient when rats are allowed to select from three separate macronutrient-containing diets (Welch et al. 1994).

We have previously reported that the reduction in appetite in populations of Zn− rats was represented essentially by a reduction in carbohydrate intake (Rains and Shay 1995). The potent effect of NPY on appetite and a possible relationship with carbohydrate intake motivated this study. Our goals were twofold: to determine whether NPY content in the PVN and NPY gene expression in the ARC is changed by zinc deficiency (Studies 1 and 2) and whether central infusion of NPY affects either total intake or macronutrient preference in Zn− rats (Study 3).

MATERIALS AND METHODS

Format of studies. There were three separate studies. All studies utilized male Sprague-Dawley outbred rats (Harlan, Indianapolis, IN). Studies 1 and 2 involved diet treatments followed by analysis of NPY mRNA in the ARC and NPY in the PVN. Studies 1 and 3 utilized three-choice macronutrient diets that were Zn+/Zn−; study 2 utilized AIN-93G-based diets (Reeves et al. 1993) that were Zn+ and Zn− and included a pair-fed group. In Study 3, rats were fitted with cannulae to the PVN to allow exogenous NPY to be infused. All rats were acclimated to our facility and to control diets for ≥1 wk before beginning a study.

Animals and diets. All animal protocols were approved by the University of Illinois Laboratory Animal Care Advisory Committee. Rats were housed individually in stainless steel hanging cages under controlled conditions (21°C, 12-h light:dark cycle) with free access to distilled water in zinc-free plastic bottles. Atomic absorption spectrophotometry was used to measure femur zinc and diet zinc concentrations as described previously (Rains and Shay 1995). The three-choice diet system consisted of three separate carbohydrate-, protein- and fat-containing diets that were provided simultaneously to rats. These diets are based on a cornstarch/sucrose mixture (carbohydrate), spray-dried egg-white (protein), and soybean oil/celullose (fat). These diets were formulated to be isocaloric on the basis of Atwater energy values; the formulations, preparation and handling of these diets have been described previously (Rains and Shay 1995). The Zn− and Zn+ diets were designed to contain 1 or 30 mg Zn/kg diet, and atomic absorption tests on all batches of diet confirmed the nominal values ±2 mg/kg. Food intake for each rat was recorded every day before dark onset; after weighing, diet jars were returned to cages in a randomized fashion to avoid development of positional preferences. The use of three-choice diets allowed us to follow-up our earlier study (Rains and Shay 1995), which used the same diets to investigate macronutrient preferences during zinc deficiency and depletion. Use of the three-choice system in Study 3 enabled us to determine whether NPY infusions altered macronutrient preference along with total intake in Zn− and Zn+, NPY-infused rats. A single AIN-based diet was used in Study 2 to allow a pair-fed group to be included with Zn− and Zn+ rats from which NPY and NPY mRNA measurements were made. It is difficult to pair-feed rats provided three-choice diets. The AIN-93G-based diet (Reeves et al. 1993) for Study 2 used spray-dried egg white as the protein source, extra biotin added at 2 mg/kg diet, and minerals were reformulated to accommodate the changes in sodium and phosphorus as suggested by Reeves et al. (1993). Diets were made adequate or deficient in zinc (30 or 1 mg Zn/kg diet), and zinc concentration was confirmed via atomic absorption spectrophotometry.

Study 1. Male Sprague-Dawley outbred rats (n = 26) weighing 100–110 g were randomly divided into Zn+/Zn− and Zn− groups, n = 13 per group. All rats had free access to three macronutrient diets for 6 wk. After 6 wk of feeding, rats were killed at the onset of the dark phase. To obtain Zn− rats in a homogeneous state, rats were killed on days on which they had low intake with respect to the 3- to 4-d intake cycle associated with zinc deficiency. The brain was removed immediately and microdissected to isolate ARC for RNA isolation and PVN for peptide concentration measurements. Tissues were microdissected using a fine scalpel blade from slices with the use of a rat brain atlas (Paxinos and Watson 1986) to identify brain coordinates. We prepared 1-mm-thick slices centered about −1.80 mm for PVN and 2-mm-thick slices centered about −3.30 mm for ARC (both coordinates posterior to bregma). Both PVN and ARC tissues were dissected as 2 mm (lateral) × 1 mm (dorsoventral) rectangles from their respective slices.

Study 2. Male Sprague-Dawley rats (n = 24) weighing an average of 110 g were provided modified AIN-93G powdered diets. Rats (n = 8 per group) were randomly assigned to one of three groups, i.e., with unlimited access to their preferred macronutrient diet but at the amount consumed on average by the Zn− group of rats on the previous day (pair-fed, PF). Rats were fed on this schedule for 14 d, the cycling behavior of the Zn− rats assessed and for the next 6 d the pair-fed rats were provided Zn+ diet in a cyclical fashion as follows: 14.8 g (d 15 and 18), 10.1 g (d 16 and 19) and 5.4 g (d 17 and 20). The diet provided to pair-fed rats during these 6 d was adjusted to give each rat the amount of zinc contained in 14.8 g of Zn+/Zn− diet. In this way, the PF rats consumed constant and adequate zinc while being provided diet in a cyclical fashion similar to Zn− rats. After the 6 d of cyclical feeding, Zn+ and PF rats were killed at the onset of dark on d 21. The Zn− rats were killed starting on d 21, on a day with low intake with respect to the ~3.5-d intake cycle. Tissues were obtained for analysis as described in Study 1.

Study 3. Male Sprague-Dawley outbred rats (n = 24) weighing 100–110 g were housed individually in stainless steel hanging cages under controlled conditions (21°C, 12-h light:dark cycle) with free access to distilled water in zinc-free plastic bottles. Rats were randomly divided into Zn− and Zn+ groups, n = 12 per group. All rats had free access to three macronutrient diets and were provided free access to Zn+ or Zn− three-choice diets as in Study 1. The light cycle was maintained such that the onset of dark was at 1100 h. Zinc concentrations in diets were verified as in Study 1. Diets were provided in glass jars with a 2.5-cm opening in the stainless steel lid. Diets and spillage were weighed manually every day to determine intake, and 1 h after intakes. Differences in color and texture of the diets allowed identification of the source of the spilled macronutrients. Animals were fed Zn+ or Zn− diets for 3 wk; then surgeries were performed as described below at the beginning of wk 4. Rats were allowed to recover from surgery during wk 4 while continuing to receive the Zn+ or Zn− diets, and infusion tests were begun at the beginning of wk 5. Constant doses of NPY were administered to rats rather than dosing based on body weight. This decision was based on dosing a constant number of neurons within the paraventricular nucleus of the rat and follows typical central infusion studies such as that of Stanley et al. (1986) in which rats were administered a constant daily dose of NPY during growth. Study 3 was conducted as three separate trials. Results from these three trials were combined. After completion of infusion testing, selected femur samples were collected to further verify zinc status among groups. Femur zinc was measured by atomic absorption spectrophotometry as described previously (Rains and Shay 1995).

Quantitation of mRNA levels by Northern blot analysis. Total RNA was isolated, subjected to electrophoresis and blotted as described previously (Stanley and Cousins 1993). Radiolabeled cDNA probes were prepared by using a commercial kit (RadPrime DNA Labeling System, GIBCO BRL, Grand Island, NY), labeling with α-
PVN was produced by boiling tissue in 500 µL of 0.5 mol/L acetic acid for 15 min followed by centrifugation at 13,000 x g for 10 min at 4°C. The supernatant was collected and stored at −80°C until use. Protein concentration was determined by using a colorimetric assay (Sigma, St. Louis, MO) and I-125-labeled NPY (Amersham, Arlington Heights, IL) following the supplier’s recommended protocol. NPY content in Study 1 was determined by using an NPY Enzyme-linked immunos assay (ELISA) kit (Peninsula Laboratories, Belmont, CA) following the recommended protocol. The minimum level of detection of NPY in this assay was 0.10 µg/L NPY and the linear range for the assay was from 0.10 to 5 µg/L. Peptide samples required dilution to produce values within the linear range of this assay. Our intra-assay variation for the ELISA was <5%, the interassay variation was <14%. Results are given as a ratio of NPY/total protein content for each ARC sample individually. The MT-1 mRNA was also measured by using a RIA utilizing a rabbit anti-NPY antibody (Sigma, St. Louis, MO) and I-125-labeled NPY (Amersham, Arlington Heights, IL). The minimum level of detection for this assay was <0.10 µg/L, and the intra- and interassay coefficients of variation were <5 and <12%, respectively. Both the ELISA and RIA exhibited parallelism between dilutions of both samples and NPY standards.

RESULTS

In all three studies, zinc deficiency caused an anorexia, and in Studies 1 and 3, which used three-choice diets, macronutrient intake patterns resembled those we have reported previously (Rains and Shay 1995). In Study 1, the difference in intake first became significant between Zn− and Zn+ groups at d 7. After rats consumed the Zn− or Zn+ diets for 6 wk, body weights (BW) were 142 ± 6 versus 283 ± 8 g, Zn− versus Zn+ (P < 0.01). Average energy intake per rat during the final week of study 1 was 192 ± 23 versus 369 ± 7 kJ/d, Zn− versus Zn+, respectively (P < 0.01), or 135 ± 16 versus 130 ± 2.5 kJ/(d·100 g BW), Zn− versus Zn+. The reduced intake and growth, a reduced preference for carbohydrate within the three-choice system and the appearance of the ~3.5-d intake cycle indicated that zinc deficiency had been established. This was confirmed by bone zinc concentrations (85 vs. 288 mg Zn/kg bone, Zn− vs. Zn+, respectively; P < 0.01). We further confirmed differences in zinc status at the cellular level utilizing northern analysis to quantitate relative levels of MT-1 mRNA levels in both intestine and brain. Metallothionein gene expression is regulated by elevated dietary zinc and was highly regulated between the Zn− and Zn+ groups in the intestine (more than fivefold, Zn− vs. Zn+, P < 0.05, data not shown). The MT-1 mRNA was also measured in RNA isolated from the brain of Zn− and Zn− rats (Fig. 1). Differences in brain MT-1/28S ratios between Zn+ and Zn− rats were not significant. In Study 1, the NPY mRNA/28S rRNA ratio was 120% higher (P < 0.05) in ARC of Zn− versus Zn+ rats (Fig. 1). The concentration of NPY peptide was 60% higher (P < 0.01) in the PVN of Zn− than of Zn+ rats (Fig. 2).
were 92% higher ($P < 0.01$) in both Zn− and PF rats than in Zn+ rats.

In Study 3, Zn− rats again exhibited reduced intake and growth and a cyclical food intake pattern. At the beginning of infusion testing, rat weights averaged 197 ± 6 and 269 ± 8 g (Zn− vs. Zn+, $P < 0.01$). Mean daily energy intakes were 135 ± 22 versus 256 ± 23 kJ/d or 69 ± 9 versus 95 ± 8 kJ/(d·100 g BW), (both $P < 0.05$, Zn− vs. Zn+). Zinc concentrations of pooled femurs obtained at the end of infusion testing were 101 versus 224 mg Zn/kg bone (Zn− vs. Zn+). The combination of reduced intake and growth, cyclical intake and bone zinc concentrations indicated that zinc deficiency had been established.

The dose of NPY administered to rats significantly affected energy intake ($P < 0.001$), with a dose-dependent increase in NPY-induced eating observed in both groups of rats (Fig. 5). When considering intake either per rat (not shown), or per 100 g BW (Fig. 5), NPY-stimulated intake did not differ in Zn+ and Zn− rats at any given dose. At the 160 pmol dose, total energy intake per 100 g BW by Zn+ rats was 140% greater than after saline infusion. The same dose of NPY increased intake per 100 g BW of Zn− rats by 224%.

**DISCUSSION**

These results do not support the hypothesis that a defect in the NPY system is mediating the decreases in intake during zinc deficiency. Concentrations of NPY in the PVN and NPY mRNA in the ARC were higher in Zn− than in Zn+ rats. This difference appeared to be related more to decreased food intake than zinc deficiency because pair-fed rats exhibited similar increases in NPY concentrations. Sahu et al. (1988 and 1992) reported that both NPY peptide and mRNA concentrations were increased in food-restricted rats. The present data are consistent with the recent report of Selvais et al. (1997) with respect to NPY mRNA levels, but are not consistent with their report that NPY content is not different in Zn− vs. Zn+ rats. However, we utilized PVN microdissected from the hypothalamus, whereas Selvais et al. (1992) measured levels in the whole hypothalamus. NPY content in the PVN and NPY mRNA levels in the ARC generally change in parallel,
Neuropeptide Y concentration in the hypothalamic paraventricular nucleus of zinc-adequate (Zn+), pair-fed (PF) and zinc-deficient (Zn-) rats consuming AIN-93-based diets. A peptide fraction was produced by acid extraction and NPY content was determined by RIA. Values represent average ± SEM, n = 8. *Significantly different (P < 0.01) from Zn+.

Although there are a few examples of NPY content and gene expression not correlating (Wilding et al. 1991).

There was no impairment in the feeding response to exogenous NPY in Zn− rats. At the highest dose of NPY (160 pmol) administered in this study, energy intake per kilogram body weight did not differ in Zn+ and Zn− rats. This observation contrasts with two previous studies in which exogenous infusions of orexigenic agents were administered centrally to Zn− and Zn+ rats. In those studies, it was observed that Zn− rats were resistant to increases in intake when dynorphin (Essatara et al. 1984b) and norepinephrine, muscimol, and bromerogocryptine (Essatara et al. 1984a) were administered. Because the four compounds tested by Essatara and colleagues did not equalize intakes of the groups, one hypothesis they advanced was that receptor function or sensitivity was reduced by zinc deficiency. In this study, equivalent energy intake in Zn− and Zn+ groups when 160 pmol of NPY was infused suggests that...
NPY receptor function may not be compromised by zinc deficiency. It is not practical to include a pair-fed group of rats in short-term intake studies because pair-fed rats are voracious eaters and consume food almost continuously for an hour or more upon presentation of diet. Our preliminary tests demonstrated that intakes of PF rats were unaffected by treatment (data not shown). Essatara et al. (1984a and 1984b) did not use pair-fed rats for the same reason. A discussion of the range of problems encountered with pair-fed rats in zinc deficiency studies is presented by O’Dell and Reeves (1989).

It may be that during zinc deficiency, the NPY neurons extending to the PVN are less able to process NPY into its final, fully active form. Zinc deficiency impairs processing of the brain peptide, thyrotropin-releasing hormone (Pekary et al. 1991). Incompletely processed NPY has a lower affinity for NPY receptors, and the amidated C-terminal end of the NPY peptide interacts with NPY receptor binding sites (Schwartz et al. 1990). During zinc deficiency, the hypothalamus may be normally responsive or even hyperresponsive to infusions of NPY. We suggest that this could occur during zinc deficiency if less than normal amounts of NPY are being released from terminals within the PVN or if incompletely processed pro-NPY peptide is being released from cells. This hypothesis does not agree with the current results indicating higher, rather than lower concentrations of NPY during zinc deficiency, although the assay used in this study detects total NPY immunoreactivity and does not measure the various processed forms of NPY. Another study reporting increases in NPY in the NPY secretion during zinc deficiency (Tovar-Palacio et al. 1996) also used an assay that did not measure processed forms of NPY.

The hypothesis that zinc deficiency–induced anorexia is related to decreases in NPY now appears less likely. The increase in NPY during zinc deficiency appears to result from the reduced intake of Zn—rats. The parallel increase of NPY content and NPY gene expression associated with zinc deficiency may be a normal regulatory response made in an attempt to increase intake in Zn—rats. Yet, while consuming a Zn—diet, these rats continued to eat less, unlike food-restricted rats. Because Zn—rats increase food intake after infusion of exogenous NPY and are at least as responsive as Zn+ rats to similar infusions, the NPY system appears to be intact postsynthetically. If the processing of NPY is unaffected by zinc deficiency, then on the basis of these results, it appears likely that some other physiologic change is mediating this anorexia.

LITERATURE CITED