Zinc Deficiency Reduces Leptin Gene Expression and Leptin Secretion in Rat Adipocytes

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The present study was conducted to measure ob mRNA abundance in the zinc-deficient (ZD) rats and the secretion of leptin from adipose tissue obtained from ZD, zinc-adequate (ZA), and pair-fed (PF) rats. It was found that ob mRNA abundance was greatest ($P < 0.05$) in adipose tissue obtained from ZA and PF rats. Ob mRNA abundance was similar in PF and ZD rats. To study leptin secretion from adipose tissue in a cell culture model, a method was developed to use excised epididymal adipose tissue from ZD, ZA, and PF rats. Tissue was incubated in Opti-modified Eagle's medium (MEM) cell culture medium in which concentrations of zinc and insulin were manipulated. It was observed that leptin secretion was higher ($P < 0.05$) in adipose tissue obtained from ZA than ZD and PF rats. Secretion of leptin was higher in adipose tissue of PF than ZD rats ($P < 0.05$). Surprisingly, media zinc content in this *ex vivo* model tended to suppress secretion of leptin. This suppression seems to be zinc specific and might be caused by the sequestration of insulin in the culture medium. Our results indicate that the reduction in serum leptin observed in ZD rats is likely caused by not only a reduction in body fat, but also by a decrease in leptin synthesis and secretion per gram of adipose tissue. Taking these results into account along with a prior study (1), it is possible that even a marginal zinc deficiency could affect leptin secretion and serum leptin concentrations. Impaired leptin secretion caused by zinc deficiency might be one factor contributing to hypogonadism observed in zinc deficiency.

[Exp Biol Med Vol. 226(9):841–846, 2001]

Key words: zinc deficiency; leptin; ob mRNA; adipocyte

This work was supported by the National Institutes of Health Grant AG13586.

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Received December 19, 2000.
Accepted May 7, 2001.

1535-3702/01/2269-0841$15.00

Zinc is essential for plants, animals, and humans, and is involved in numerous biological processes. Humans and animals experiencing zinc deficiency exhibit a wide variety of symptoms, including impaired growth, anorexia, impaired sexual development, geophagia, dwarfism, anemia, and dermatitis (2).

The mechanisms by which zinc deficiency induces anorexia are unclear. Changes in amino acid metabolism during zinc deficiency might play a role. To provide further insight into possible mechanisms behind zinc deficiency-induced anorexia, macronutrient preferences of zinc-deficient (ZD) and zinc-adequate (ZA) rats were studied (3). Within the group of ZD rats, the decrease in food intake was mainly the result of a reduction in carbohydrate consumption. It was then speculated that neuropeptide Y (NPY), a potent appetite stimulant, was involved in anorexia of ZD rats because NPY is known to specifically increase intake of carbohydrate (4). It was found that hypothalamic NPY was increased during zinc deficiency-induced anorexia (5, 6). Elevated NPY would normally trigger an increase in food intake, specifically carbohydrate intake, but the opposite, decreased food intake, occurs during zinc deficiency. This situation has been described as "NPY resistance," in which NPY is high, yet food intake remains low.

The recent discovery of the hormone leptin provides another tool for elucidating the physiology of zinc deficiency-induced anorexia. Serum concentrations of leptin reflect the nutritional status and body fat mass of an individual. Increased fat mass, food intake, and elevated concentrations of insulin and glucocorticoids produce increases in *ob* mRNA and circulating leptin. Conversely, lower body fat, fasting, and decreased serum insulin concentrations are correlated with lower concentrations of leptin (7–10). Leptin was reduced in the serum of ZD rats (11), which was not surprising, since anorexic rats have less body fat compared with controls. Since leptin is known to inhibit the synthesis and release of NPY (12), the reduction in serum leptin in ZD rats is consistent with the finding that hypothalamic NPY is

elevated during zinc deficiency. However, the NPY resistance observed during zinc deficiency remains unexplained.

A better understanding of NPY, leptin, and insulin action during zinc deficiency may help explain the NPY resistance observed during zinc deficiency. We wished to determine if reduced leptin synthesis and secretion was responsible for the reduced concentrations of serum leptin previously reported (11). Alternatively, increased turnover of serum leptin could result in decreased concentrations, despite normal synthesis in adipocytes. To investigate these hypotheses, the present study was undertaken. We quantitated ob mRNA in rat adipose tissue during zinc deficiency. Leptin secretion from adipose tissue obtained from control and ZD rats was measured. Finally, we investigated the effect of zinc and insulin on rat adipose tissue in an *ex vivo* cell culture system. Understanding the paradox between high NPY and low food intake will be one key to better understanding the physiology of anorexia. Understanding the regulation of leptin during zinc deficiency may also shed light on the development of reproductive dysfunction associated with this nutritional deficiency.

Materials and Methods

Animals: General Methods. Male Sprague-Dawley outbred rats (Harlan, Indianapolis, IN) were used. Rats were typically acclimated to our facility and the light/dark cycle for 1 week. They were then weighed and separated into three groups (ZD, ZA, and pair fed [PF]) so that the average weights of the rats in each group were similar. Powdered diets were described previously (5). ZA diets contained 30 mg Zn/kg, and ZD diets were formulated to contain 1 mg Zn/kg. Zinc content of all diets was confirmed using atomic absorption spectrophotometry as described previously (5). Measured values of zinc content in diets were deemed acceptable at ± 3 mg Zn/kg for the ZA diet and ± 0.3 mg Zn/kg for the ZD diet. Rats were housed individually in stainless steel, wire-bottom cages and they were maintained on a 12:12-hr light:dark cycle, with the dark cycle beginning at 15:00 hr and ending at 03:00 hr. All rats were allowed *ad libitum* access to deionized, distilled water in glass bottles with zinc-free, silicone stoppers and stainless steel sipping tubes. Food intake was recorded daily and body weights were recorded every 2 to 3 days. PF rats were provided the average amount of diet consumed on the previous day by the ZD rats. Rats were sacrificed in the middle of the light cycle and this was done by decapitation after CO_2 inhalation. Bone zinc values were determined as described previously (3).

Animals: Study 1. Starting body weights ranged from 125 to 150 g. Rats were maintained on ZD, ZA, and PF diets for a period of 21 days. Adipose tissue was collected from epididymal fat pads from 32 rats for RNA isolation and Northern analysis. The epididymal fat depots were used because they were the only depots from which appreciable amounts of fat from ZD rats could be obtained consistently. After eliminating samples that were judged to contain degraded total RNA, the total number in each group used for Northern analysis (containing intact RNA) was 10 ZD, 13 ZA, and 9 PF.

Northern Analysis. Our protocol follows that previously described (5). To measure ob mRNA expression, 15 μg of total RNA was analyzed by electrophoresis, blotting, hybridization, and phosphorimaging. Any individual mRNA samples showing any indication of degradation as indicated by a reduction in the expected ~2:1 ratio of ethidium bromide staining of the 28s/18S rRNA was not included in any densitometric evaluation of leptin mRNA quantitation. A cDNA probe corresponding to the mouse *ob* mRNA sequence was prepared in our laboratory by using the polymerase chain reaction corresponding to nucleotides 500 to 599 of the leptin mRNA sequence. The Rediprime DNA Labeling System (Amersham Life Sciences, Arlington Heights, IL) was used to produce radiolabeled cDNA by following the manufacturer's suggested protocol. Approximately 100 ng of cDNA were labeled with 10 μCi of ^{32}P-dCTP (3000 Ci/mmol). Total activity of the radiolabeled probes ranged from 1.25 to 1.5 × 10^8 cpm and the specific activity ranged from 8 × 10^7 to 1 × 10^8 cpm/μg DNA. The probe was added to 20 ml of RapidHyb (Amersham Life Science) hybridization solution, which was then added to autoblot tubes containing the blots, and placed in a hybridization oven at 55°C. Hybridization proceeded for 2 to 4 hr. After the blots were washed, they were exposed to a phosphorimaging screen for 24 to 48 hr. A similar protocol was used to detect 28S rRNA expression. Bound cDNA probe was quantified by using phosphorimaging software and was analyzed by ANOVA. *ob* mRNA content is expressed as the ratio of *ob* mRNA to28S rRNA expression.

Animals: Study 2 and 3. After finding zinc deficiency to significantly reduce body fat, we chose to use larger rats for subsequent studies. Starting weights for these rats ranged from 190 to 250 g. Housing, diets, and feeding regimes for these rats were similar to that of the rats in Study 1.

***Ex Vivo* Secretion Studies.** Pilot testing was performed to establish a reliable model for cell culture studies to measure secretion of leptin from adipocytes. We tested both isolated primary adipocytes (13) and excised pieces of adipose tissue (14) maintained in cell culture conditions. We chose to use excised adipose tissue (14) after finding that the shorter preparation time without protease treatment resulted in more consistent results. Both methods resulted in good cell viability: >95%, based on trypan blue dye exclusion. After dietary treatment, rats were sacrificed and epididymal fat pads were extracted, divided into proximal and distal portions, and placed into 1% bovine serum albumin (BSA) in PBS. Tissues were divided, weighed, and placed in sterile, 24-well dishes so that each well contained one proximal and one distal tissue fragment, each ~50 mg, from the same rat. Tissue fragments were incubated at 37°C in Opti-modified Eagle's medium (MEM) (Gibco-Life Technologies, Grand Island, NY). Zinc concentrations in Opti-

MEM medium after 10% serum was added were 5 to 6 μM, therefore a concentration of the zinc chelator diethylenetriaminepentaacetic acid (DTPA) was initially set at ~100-fold excess, or 600 μM. Thus, a zinc-free culture condition was simulated with 10% serum + 600 μM DTPA, whereas treatment containing zinc included zinc and did not include DTPA. Specific details are included with each study. After appropriate culture conditions, media were collected and frozen at −20°C for subsequent leptin measurement. Media were analyzed for leptin by radioimmunoassay (RIA).

Leptin Secretion: Time Course. Rat epididymal fat was incubated for 0, 2, 6, 12, and 24 hr in Opti-MEM containing 10% fetal bovine serum (FBS), 50 nM insulin, and 25 μM $ZnSO_4$. Three independent incubations were maintained for each time. After the appropriate length of incubation, media were collected and frozen at −20°C for later leptin measurement by RIA.

Study 2: Effect of Zinc Deficiency, Insulin, and $ZnSO_4$ on Leptin Secretion. Study 2 utilized a 2 × 2 × 3 factorial design, evaluating the effect of insulin, zinc, and diet treatment, respectively. Two rats each received ZD, ZA, and PF diet treatments. After 21 days of feeding, rats were sacrificed and adipose tissue was collected as described for *ex vivo* secretion tests. Tissues were incubated at 37°C for 6 hr in Opti-MEM containing 10% FBS, 25 mM glucose, and varying concentrations of insulin (700 vs. 0 nM) and $ZnSO_4$ (50 vs. 0 μM + 600 μM DTPA). After 6 hr of incubation, media were collected and frozen as before. Media were analyzed for leptin by RIA. Analysis utilized a three-way analysis of variance (ANOVA) evaluating the effect of diet treatment, media zinc, and media insulin on leptin secretion. Tukey's test was used as a *post hoc* test when significant differences were observed. Study 2 was repeated two other times; all three trials produced similar results.

Study 3: Effect of Zinc and DTPA on Leptin Secretion. Six rats were used in this experiment. Two rats each received ZD, ZA, and PF diet treatments. Tissue and incubation procedures were performed as described above. Tissues were incubated at 37°C for 6 hr in Opti-MEM containing 10% FBS, 25 mM glucose, 700 nM insulin, and varying concentrations of DTPA (600, 100, 25, or 0 μM) or $ZnSO_4$ (0, 25, 100, or 500 μM). After the appropriate incubation period, media were collected and frozen as before. Media were analyzed for leptin by RIA. Data was analyzed using two-way ANOVA assessing effect of diet versus the presence or absence of zinc in the culture medium. After significant differences were observed, *post hoc* testing used Tukey's test.

Leptin RIA. RIA for leptin was conducted on the media samples for Studies 2 and 3 by using a Rat Leptin RIA kit (Linco Research, Inc., St. Charles, MO). The manufacturer's suggested protocol was followed. Data were analyzed by using ANOVA. The intra- and interassay coefficients of variation were 5% and 8%, respectively.

Results

Study 1: Ob mRNA and Leptin Secretion. Rats that consumed the ZD diet had a significantly ($P < 0.05$) lower food intake than that of ZA controls, indicating an anorexia had been induced. Body weights of ZD, ZA, and PF rats in this study were all different from one another ($P < 0.05$). Mean increases in body weight for ZD, ZA, and PF rats were 20, 125, and 40 g, respectively. Bone (femur) zinc concentrations were determined from selected rats within each dietary treatment, and the levels were similar to those previously reported from our laboratory (3, 5). We considered the considerable anorexia exhibited by every ZD rat and selected bone zinc measurements to indicate that zinc deficiency had been established. Ob mRNA content (Fig. 1), expressed as a ratio to 28S rRNA abundance, was higher in both ZA and PF rats compared with ZD rats ($P < 0.05$). Serum leptin concentrations were higher ($P < 0.05$) in the ZA rats compared with both the ZD and PF groups. Leptin concentrations were: ZD, 2.2 ± 0.3; ZA, 4.8 ± 0.3; and PF, 2.5 ± 0.3 ng leptin/ml serum. Although numerically higher in the PF compared with ZD rats, ANOVA indicated that concentrations in the ZD and PF rats were equivalent.

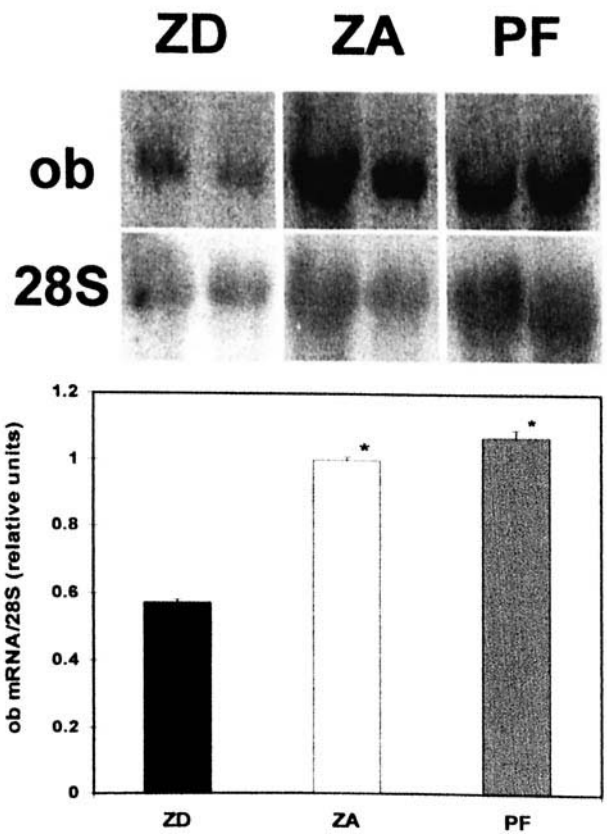

Figure 1. (Top) Northern analysis of *ob* mRNA versus 28S rRNA in ZD, ZA, and PF rats. Figure shows two different samples from each condition. (Bottom) *ob* mRNA expression in epididymal adipose tissue isolated from ZD, ZA, and PF rats. Audioradiographs were analyzed by densitometry and ob mRNA levels are expressed as a ratio of ob mRNA to 28S rRNA content. Values represent means ± SEM and significant differences were calculated by ANOVA. Asterisk indicates significantly greater ($P < 0.05$) than ZD. $n = 9$ to 13 per group.

Leptin Secretion: Time Course. The *ex vivo* secretion model (14) uses pieces of adipose tissue incubated in a cell culture medium that allows conditions to be carefully controlled. Our first trials included several time course studies. Although leptin concentrations in the medium increased throughout the first 24 hr of culture, the rate of secretion was most robust for the first 6 hr (data not shown). Therefore, the remainder of our secretion studies uses a 6-hr incubation as an appropriate time.

Study 2: Effect of Zinc Deficiency, Insulin, and $ZnSO_4$ on Leptin Secretion. Study 2 (Figs. 2 and 3) tested the effect of medium zinc and insulin on the secretion of leptin from adipose tissue after 6 hr of incubation. There was a significant interaction between insulin and the presence of zinc in the culture media: insulin increased leptin secretion ($P < 0.05$) when zinc was not present in the incubation media. This may be observed in bars 2 vs. 4, 6 vs. 8, and 10 vs. 12 in Figure 2. Leptin secretion was different ($P < 0.05$) between all three dietary treatments (Fig. 3), being least from adipose tissue obtained from ZD rats. Leptin secretion from adipose tissue obtained from PF rats was nearly double ($P < 0.05$) that measured from ZD tissue samples. Leptin secretion from adipose tissue obtained from ZA rats was more than double that of the ZD condition and nearly 25% greater than the PF condition ($P < 0.05$). As a main effect in this experiment and others (not shown), the secretion of leptin from insulin-stimulated adipose tissue was greater ($P < 0.05$) than from insulin-free conditions. Curiously, there was also an unanticipated effect of media zinc concentration on leptin secretion. As a main effect, zinc-free culture conditions paradoxically resulted in greater leptin secretion ($P < 0.05$). In five of the six combinations of diet and insulin, secretion of leptin tended to be higher in the zinc-free condition. Other tests (data not shown) included evaluating the effect of supplemental magnesium and calcium at 2 and 10 mM, respectively. Neither mineral proved to have a significant effect on leptin secretion.

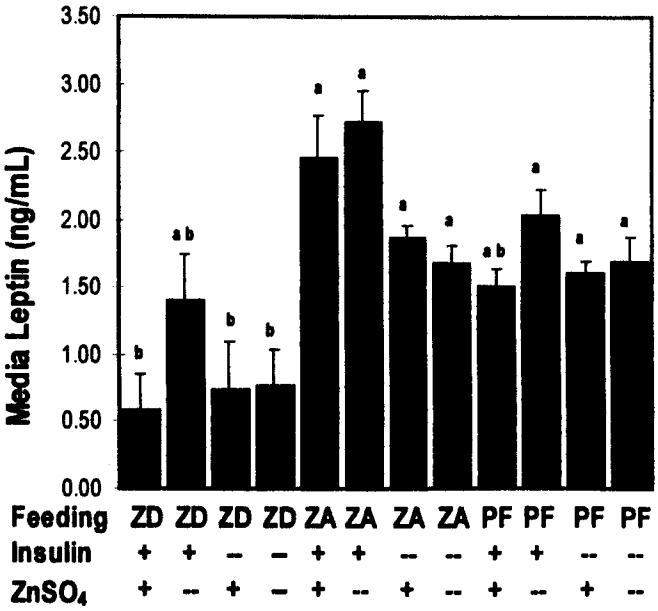

Figure 2. Media leptin concentrations after a 6-h incubation of 100 mg of epididymal adipose tissue obtained from ZD, ZA, and PF rats. Media included varying insulin (700 nM [+] vs. 0 nM [-]) and $ZnSO_4$ (50 μM [+] vs. 0 μM + 600 μM DTPA [-]) concentrations. Bars represent mean values ± SEM. Values not sharing a common letter indicate significant differences ($P < 0.05$) as determined by 3-way ANOVA. $n = 4$ per group.

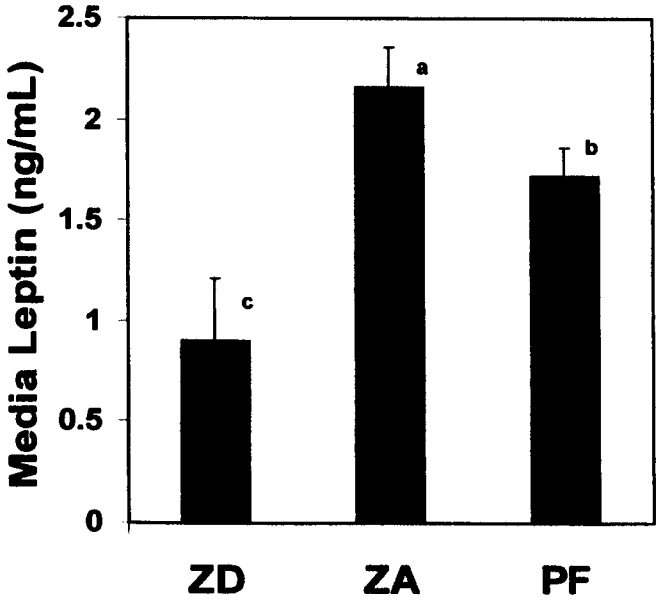

Figure 3. Media leptin concentrations after a 6-h incubation of 100 mg of adipose tissue from ZD, ZA, and PF rats. Bars represent mean values ± SEM. Values not sharing a common letter indicate significant differences ($P < 0.05$) as determined by ANOVA. $n = 16$ per group.

Study 3: Effect of Zinc and DTPA on Leptin Secretion. Study 3 (Fig. 4) was designed largely to explore the paradoxical result from Study 2 in which zinc content in media seemed to have a negative effect on leptin secretion. As in Study 2, adipose tissue obtained from ZA rats secreted more leptin ($P < 0.05$) than tissue from ZD rats, while incubation with zinc (versus zinc-free) in the medium reduced leptin secretion ($P < 0.05$). In this study, secretion of leptin was 3-fold greater ($P < 0.05$) in the absence of $ZnSO_4$. After observing the results of Study 2, one concern was that including DTPA in experiments as a zinc chelator was introducing artifactual results. Thus, zinc-free incubations were performed with DTPA included at concentrations of 600, 100, 25, and 0 μM. In the absence of DTPA, zinc-containing incubations included zinc at 0, 25, 50, 100, and 500 μM. Nearly every combination of medium DTPA and zinc showed that secretion of leptin from adipose tissue obtained from ZD rats was reduced in comparison with tissues obtained from ZA or PF rats. As a main effect of all treatments in Study 3, secretion of leptin was equivalent in ZA and PF rats and was reduced ($P < 0.05$) in ZD rats. However, when examining the secretion of leptin from adipose tissue obtained from ZD, ZA, and PF rats and incubated in 25, 50, or 100 μM zinc, the pattern of secretion was

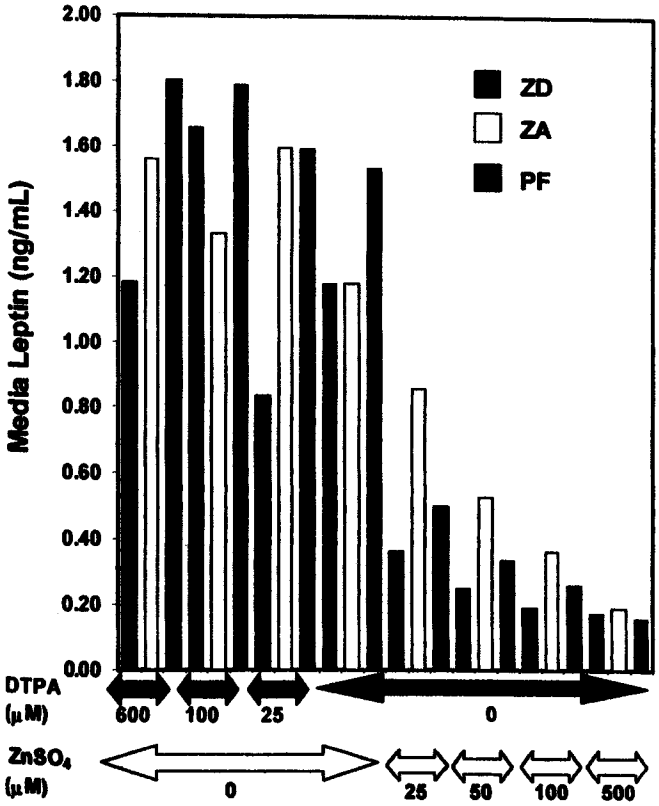

Figure 4. Media leptin concentrations after a 6-h incubation of 100 mg epidydimal adipose tissue from rats fed ZD, ZA, and PF diets. Incubation medium contained 10% fetal bovine serum, 25 mM glucose, 700 nM insulin, and varying DTPA (600, 100, 25, or 0 μM ; black arrows) or $ZnSO_4$ (0, 25, 50, 100, or 500 μM; white arrows) concentrations. Each bar represents the mean of two measurements. Analysis of main effects indicated leptin secretion was greater ($P < 0.05$) in zinc-free conditions and that secretion was greater ($P < 0.05$) in both ZA and PF conditions compared with ZD. The experiment shown in this figure is representative of four identical experiments conducted independently, and each experiment produced similar results.

similar to that found in Study 2 (Fig. 3). Secretion again followed the ZA>PF>ZD pattern.

Discussion

Ob mRNA levels and Leptin Secretion. Northern analysis indicated that adipose tissue from ZD rats had reduced expression of the *ob* gene when compared with tissue obtained from both ZA and PF rats (Fig. 1). Considering that leptin decreases the synthesis and release of NPY (12), these findings are consistent with the result that ZD rats have higher hypothalamic NPY content than control animals. These results also indicate that lower serum leptin of ZD rats are likely caused by both decreased leptin production and reduced body fat (11). It has not been determined if the decrease in leptin gene expression is caused by a decrease in transcription, reduced mRNA stability, or a combination of both factors. Although zinc is involved in the structure and function of RNA polymerases (15) and transcription factors containing zinc fingers (16), we consider it most likely that ob gene expression is reduced indirectly as a result of reduced insulin concentration or effect during zinc deficiency (20). Zinc is essential for insulin synthesis and release (17), and zinc deficiency seems to impair release of insulin (18). Additionally, leptin secretion and gene expression is induced by insulin (14, 7, 13, 10, 19). Therefore, reduced insulin action in ZD rats may be partially responsible for decreased expression of the *ob* gene. Recently, we investigated the effect of zinc on the insulin signaling pathway and showed that zinc enhances the activity in this pathway (20). *In vivo*, zinc deficiency may be contributing to both a reduction in insulin secretion and cellular responsiveness to insulin, thus reducing insulin-stimulated *ob* gene expression.

***Ex Vivo* Secretion Studies.** Our *ex vivo* model showed similarities to intact adipose tissue as determined previously by *in vivo* studies. Adipose tissue obtained from ZA and PF rats secreted more leptin than similar tissue obtained from ZD rats. This observation is entirely consistent with a prior report (11). It seems that the reduction in serum leptin observed in ZD rats depends on the amount of adipose tissue and also less leptin secretion per gram than comparable tissue from ZA rats. Our model also showed that insulin stimulates the release of leptin from adipocytes as others have shown before (14). However, the *ex vivo* model showed unusual results in that the absence of zinc in the incubation media increased the secretion of leptin. Without zinc in the culture medium, incubation with 600, 100, 25, or 0 μM DTPA produced largely similar results. This effect may be caused in part by zinc-insulin interactions in culture since zinc can stabilize insulin into a hexameric form in solution (21). This stabilization could make insulin less available for receptor binding, and thus, in our model, insulin might be sequestered from cell surface binding. Our data suggest that this might be true, because even the lowest concentration of zinc (25 μM) dramatically suppressed leptin secretion from adipose tissue (Fig. 4, bars 10 through 12 vs. 13 through 15). The depression of leptin secretion caused by adding zinc to culture medium was observed in at least eight additional independent trials conducted in our laboratory (data not shown). We also tested other divalent cations for their effect on leptin secretion, but is seems that zinc is specific (data not shown). Testing included magnesium and calcium at 2 and 10 mM, respectively. We expect that future studies investigating insulin-stimulated parameters in this tissue culture system, such as insulin-stimulated genes or insulin-stimulated phosphorylation, will help us understand the paradoxical results between zinc-free culture conditions and *in vivo* zinc deficiency.

Epididymal fat was used mainly as a matter of practicality, as it was the only depot of body fat in the rat that would remain in ZD rats at the end of a feeding study. We have observed in other longer-term studies that ZD rats can become essentially free of body fat reserves, as determined by visual inspection of the body cavity after a study is completed. It is entirely possible that leptin expression and secretion may be regulated differently in the different adipose depots of the body.

The "NPY resistance" observed in anorexia caused by zinc deficiency may be a part of anorexia in general. We suggest that human anorexia, characterized by reduced intake and very low body fat content, may be similar to this rat model in which serum leptin levels are low and hypothalamic NPY levels are high. Many other brain peptides are involved in the regulation of food intake, including galanin, proopiolmelanocortin, cocaine- and amphetamine-related transcript peptide, and agouti-related peptide. For example, it has already been shown that galanin content in the hypothalamus of ZD rats is reduced. (23). Galanin and NPY are two appetite-stimulating peptides, yet the expression of NPY is increased during zinc deficiency, while galanin is decreased. It is possible that zinc deficiency more directly impacts anorexia by affecting a hypothalamic peptide other than NPY, or that another factor, perhaps galanin, is dominant to NPY during anorexia, thus reducing NPY effect. We have previously shown that ZD rats are responsive to administration of exogenous NPY delivered to the hypothalamus (5). However, there were differences noted in the sensitivity of ZD and ZA rats to NPY administration. ZD rats did not tolerate doses of NPY normally tolerated by ZA rats. Our results are consistent with a model suggesting that zinc deficiency elevates NPY mRNA and intracellular content of NPY, but perhaps secretion of active NPY during zinc deficiency is blocked, perhaps by the action of another appetite-regulating peptide.

The present results may have implications for individuals with a marginal zinc deficiency. Recent studies have indicated that a marginal zinc deficiency may regulate serum leptin concentration in humans (1, 22). Although we suggest that insulin action is a likely target affected by zinc deficiency, others have suggested that changes in circulating IL-2 and TNF-alpha are an important part of the physiology of zinc deficiency (22). Decreased leptin levels resulting from a marginal zinc deficiency could have long-term effects on tissues containing leptin receptors, for example, reproductive tissues. As it is already recognized that zinc affects levels of hypothalamic NPY, it will remain to be determined if reproductive changes associated with zinc deficiency are caused directly, or through the hypothalamic-pituitary-gonadal axis. The present data may help provide an explanation behind the hypogonadism that is seen during severe zinc deficiency (2).

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