Hypothalamic NPY, AGRP, and POMC mRNA responses to leptin and refeeding in mice

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Food deprivation (FD) increases hypothalamic neuropeptide Y (NPY) and agouti-related protein (AGRP) mRNA levels and decreases proopiomelanocortin (POMC) mRNA levels; refeeding restores these levels. We determined the time course of changes in hypothalamic NPY, AGRP, and POMC mRNA levels on refeeding after 24 h FD in C57BL mice by in situ hybridization. After 24 h deprivation, mice were refed with either chow or a palatable mash containing no calories or were injected with murine leptin (100 μg) without food. Mice were perfused 2 or 6 h after treatment. Food deprivation increased hypothalamic NPY mRNA (108 ± 6%) and AGRP mRNA (78 ± 7%) and decreased hypothalamic POMC mRNA (15 ± 1%). Refeeding for 6 h, but not 2 h, was sufficient to reduce (but not restore) NPY mRNA, did not affect AGRP mRNA, and restored POMC mRNA levels to ad libitum control levels. Intake of the noncaloric mash had no effect on mRNA levels, and leptin administration after deprivation (at a dose sufficient to reduce refeeding in FD mice) was not sufficient to affect mRNA levels. These results suggest that gradual postabsorptive events subsequent to refeeding are required for the restoration of peptide mRNA to baseline levels after food deprivation in mice.

Palatable; preabsorptive; postabsorptive; time course; arcuate nucleus; in situ hybridization; neuropeptide Y; agouti-related protein; proopiomelanocortin

Caloric deprivation invokes a complex array of hormonal, neural, and metabolic responses to reduce energy expenditure to defend body weight. Associated with reduced caloric availability is an increased drive to eat. Both the reduced energy expenditure and increased appetite associated with reduced energy availability may be regulated by a network of hypothalamic neuropeptides (30, 52, 61). Agouti-related protein (AGRP), a melanocortin receptor antagonist, is coexpressed with neuropeptide Y (NPY) in most hypothalamic NPYergic arcuate nucleus neurons (8), and hypothalamic peptide content and mRNA levels of both NPY (7, 21, 31) and AGRP (33, 42) are increased by food deprivation. Proopiomelanocortin (POMC) is expressed in a separate population of arcuate nucleus neurons and via α-melanocortin stimulating hormone (α-MSH), a posttranslational product of POMC and a melanocortin receptor agonist, tonically inhibits food intake (5, 34), whereas food deprivation results in decreased POMC mRNA levels in the arcuate nucleus (42). Therefore, during conditions of altered energy balance, NPY and AGRP signaling via NPY-Y1/Y5 and melanocortin MC-4 receptors, respectively, and POMC/α-MSH signaling also via MC-4 receptors are coordinately regulated to defend body weight and stimulate appetite, thus maintaining energy homeostasis.

Little is known about the time course of the restoration of food deprivation-induced changes in hypothalamic peptide mRNA levels on refeeding, i.e., how soon after refeeding are signals of caloric compensation or sufficiency transduced into changes in peptide mRNA levels. The first purpose of this study was to determine the time course of changes in hypothalamic NPY, AGRP, and POMC mRNA levels on refeeding after 24 h food deprivation in mice by in situ hybridization. Specifically, we attempted to bracket the earliest time point on refeeding at which signals of caloric compensation are transduced into changes in NPY, AGRP, and POMC mRNA levels.

Several lines of evidence suggest that these three peptides are downstream targets of leptin action: arcuate nucleus NPY/AGRPergic neurons as well as POMC-containing neurons express the long-form leptin receptor critical in leptin signaling (17, 36, 61). NPY and AGRP mRNA levels are increased, whereas POMC mRNA levels are decreased, in leptin-deficient ob/ob mice. Exogenous leptin administration not only reverses these changes in ob/ob mice (16, 41, 43, 55), but also blunts the increases in NPY and AGRP mRNA levels and the decrease in POMC mRNA levels induced by food deprivation (42, 51, 59, 62). However, the relative contribution of increased leptin levels in reversing deprivation-induced changes in these three peptide mRNA levels during refeeding is less clear. Furthermore, the relative contribution and mechanism of other

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afferent feeding-related signals in correcting deprivation-induced changes in peptide mRNA levels are unknown. These include short-term, preabsorptive signals such as taste and gut distension (27, 60), as well as other long-term postabsorptive signals that reflect caloric sufficiency, such as insulin (4).

Therefore, the second purpose of this study was to examine the contribution of short-term preabsorptive signals (e.g., taste, gut distension), as well as long-term postabsorptive signals of adiposity or caloric sufficiency (e.g., leptin), in restoring food deprivation-induced changes in hypothalamic NPY, AGRP, and POMC mRNA levels. To evaluate the contribution of preabsorptive signals, we measured mRNA levels after deprivation in mice refead a palatable mash that contained no calories. To determine the contribution of postabsorptive signals of caloric sufficiency, we measured mRNA levels in deprived mice receiving a dose of leptin sufficient to reduce compensatory hyperphagia.

METHODS

Animals

Eight- to twelve-week-old male C57BL mice (Charles River) were individually housed in polycarbonate cages with wood chip bedding at 22–24°C under 12:12-h light-dark cycle with lights on 7:00 AM-7:00 PM. Animals were maintained on standard pelleted rodent chow and had ad libitum access to water throughout all experimental procedures. All protocols and procedures were approved by the Florida State University Institutional Animal Care and Use Committee.

Experimental Protocols

Protocol 1: Leptin treatment and refeeding. Two groups of mice (n = 6/group) were food deprived for 24 h (food removed at 7:00 AM). After deprivation, at 7:00 AM, one group received an intraperitoneal injection of 100 µg (0.2 ml) murine leptin (Peprotech) in 5 mM sodium citrate vehicle (pH 4.0); the other group received vehicle. Both groups were given access to chow, and food intake and body weight were measured at 2 and 6 h.

Protocol 2: Measurement of neuropeptide mRNA levels. Control mice (n = 12) were allowed ad libitum access to food throughout the experiment. To control for circadian effects in the 2-h treatment groups, control mice (C-al) were perfused at 9:00 AM.

FOOD DEPRIVATION. Two groups of mice (n = 6/group) were food deprived for 24 h. Food was removed at 7:00 AM. To control for circadian effects in the 2- and 6-h treatment groups, one group was perfused after 26 h deprivation at 9:00 AM (FD + 2 h) and one group after 30 h deprivation at 1:00 PM (FD + 6 h).

FOOD DEPRIVATION AND REFEEDING. Food was removed from three groups of mice (n = 6/group) at 7:00 AM. After 24 h deprivation, at 7:00 AM, mice were allowed ad libitum access to pelleted chow for either 2 h (RF + 2 h), 6 h (RF + 6 h), or 26 h (RF + 26 h). We selected the 2-h time point to evaluate potential rapid changes after refeeding, the 6-h time point to assess hypothalamic mRNA at a time well after postabsorptive signals have been released into plasma, and 24 h as a check for complete restoration after fasting. The RF + 2 h group received an intraperitoneal injection (0.2 ml) of 5 mM sodium citrate buffer (pH 4.0) at 7:00 AM for control purposes. The RF + 2 h group was perfused at 9:00 AM, RF + 6 h group at 1:00 PM, and the RF + 26 h group at 9:00 AM.

FOOD DEPRIVATION AND NONCALORIC REFEEDING. Food was removed from two groups of mice (n = 6/group) at 7:00 AM. After 24 h deprivation, at 7:00 AM, mice were allowed ad libitum access to a palatable mash containing no nutrients for either 2 h (M + 2 h) or 6 h (M + 6 h). The mash contained 2.5 parts by weight α-cellulose (Sigma), 1.0 part mineral oil, and 10.0 parts of a deionized water solution of 0.1% sodium saccharin (Fisher) and 0.2% artificial vanilla extract (generic, nonalcoholic) (38). The M + 2 h group was perfused at 9:00 AM and the M + 6 h group at 1:00 PM.

FOOD DEPRIVATION AND LEPTIN TREATMENT. Two groups of mice (n = 6/group) were food deprived for 24 h (food removed at 7:00 AM). After deprivation, at 7:00 AM, all mice received an intraperitoneal injection of 100 µg (0.2 ml) murine leptin (Peprotech) in 5 mM sodium citrate buffer (pH 4.0). One group was then perfused after 2 h at 9:00 AM (L + 2 h) and the other after 6 h at 1:00 PM (L + 6 h).

Tissue collection and in situ hybridization. All mice were weighed before and after food deprivation and again before being perfused. Mice were overseed with a cocktail containing ketamine-HCl (100 mg/ml), acepromazine maleate (10 mg/ml), and xylazine (20 mg/ml), and then transcardially perfused first with heparinized saline containing 0.5% NaNO2 and then with 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were rapidly dissected, blocked, postfixed for 24 h, and transferred into 30% sucrose for cryoprotection.

Coronal 40-µm sections were cut on a sliding freezing microtome through the rostrocaudal extent of the hypothalamus. Alternate sections were placed into ice-cold 2× SSC. Free-floating tissue sections were prehybridized in glass vials in 1 ml of 60% formamide, 0.02 M Tris pH 7.4, 1 mM EDTA, 10% dextran sulfate, 0.8% Ficoll, 0.8% polyvinylpyrrolidone, 0.8% BSA, 2× SSC, 0.1 M dithiothreitol, and 1.6 mg/ml herring sperm DNA for 2 h at 37°C. After 2 h prehybridization, radiolabeled probe was added (1.0× 107 cpm/vial) and incubated for 16–20 h at 37°C. Hybridization was performed with either tail-labeled prepro-NPY antisense oligonucleotide (bases 59–88), AGRP antisense oligonucleotide (bases 1–45), or POMC antisense oligonucleotide (bases 482–517). Sections were then sequentially rinsed in 2× SSC, 1× SSC, and 0.5× SSC for 10 min at 37°C. The tissue sections were mounted from 0.05 M sodium phosphate onto gelatin-coated slides, air-dried, and exposed to autoradiographic film (β-max, Amersham) for 2–3 days. Tissue sections from different groups were hybridized in parallel and exposed to film together to ensure that in situ hybridization was carried out on representative members of each experimental group at the same time under identical conditions, allowing direct comparison of mRNA expression.

The 30 h-FD, 24 h-RF and 6 h treatment groups (RF + 6 h, M + 6 h, L + 6 h) with a subset of C-al mice (n = 6) were processed in a separate experiment from the C-al, 26 h-FD and 2 h treatment groups (RF + 2 h, M + 2 h, L + 2 h). The 6-h data were normalized to the 2-h data by scaling the mRNA levels from the 6-h data by the ratio of the mRNA levels in the C-al groups from the two experimental runs.

Quantification of hybridization. mRNA levels and area of tissue hybridization were quantified by densitometry (Zeiss Stemi-2000 stereoscope attached to a Dage-MTI CCD 72 camera and Macintosh image analysis system). The relative gray scale values (relative optical density) of hybridization signal and the area of hybridization in the arcuate nucleus (NPY, AGRP, and POMC) were determined for each section. The mean background pixel density (measured as a gray scale value from 1 to 256) and variance were measured in a
single section for each mouse in a region of adjacent hypothalamus. A threshold was then calculated for each mouse as two standard deviations above the mean background. All densities above this threshold value were considered to represent total hybridization. The mean gray scale value of total hybridization was then measured for all sections, and the relative gray scale value of specific hybridization (mRNA level) for each section was calculated by subtracting the background value. The mean level of mRNA expression was calculated across the three sections with the greatest area of specific hybridization in each mouse.

Statistical Analysis

Data were analyzed using one-way ANOVA followed by Tukey's post hoc test for all pairwise comparisons. All results are presented as means ± SE. Differences were considered to be statistically significant at \( P < 0.05 \).

RESULTS

Effects of Leptin Administration on Food Intake After Deprivation

One-way ANOVA revealed significant treatment effects on leptin on food intake and body weight after 6 h (Table 1). The refed group consumed 50% of total 6-h food intake during the first 2 h of refeeding. Leptin administration did not affect 2-h food intake, but significantly reduced food intake between 2 and 6 h of refeeding and thus total 6-h food intake compared with refed chow + vehicle group. The mean body weight of mice was 24.3 ± 0.3 g before food deprivation and body weight loss after deprivation was on average 4.6 ± 0.1 g. At the 2- and 6-h time points, body weight gain in the refed chow + leptin group was significantly less than the refed chow control group.

Effects of Refeeding and Leptin Administration After Deprivation on Hypothalamic NPY, AGRP, and POMC mRNA Levels

The mean body weight of mice was 26.1 ± 0.5 g before food deprivation and body weight loss after deprivation was on average 5.2 ± 0.4 g. Total food intake in the RF + 2 h and RF + 6 h groups was the same and this was reflected in body weight gain (Table 2). Total mash intake in the M + 6 h group was 50% higher than in the M + 2 h group; however, body weight gain was similar in both groups (Table 2). The RF + 24 h group consumed 8.1 ± 0.7 g of chow and restored body weight to predeprivation levels (Table 2). Leptin treatment did not affect body weight (Table 2).

One-way ANOVA revealed significant treatment effects on NPY (\( F_{9,55} = 29.3, P < 0.001 \)), AGRP (\( F_{9,55} = 33.8, P < 0.001 \)), and POMC (\( F_{9,55} = 7.9, P < 0.001 \)) mRNA levels.

Food deprivation for 26 h significantly increased NPY (Fig. 1A) and AGRP (Fig. 1B) mRNA and decreased POMC (Fig. 1C) mRNA levels. In general, these responses were not rapidly corrected. Thus neither refeeding with nutrient-containing chow, refeeding with a palatable, but nonnutritive, mash, or intra-peritoneal leptin administration altered NPY, AGRP, or POMC mRNA levels.

Food deprivation for 30 h was associated with similar increases in NPY and AGRP mRNA and reductions in POMC mRNA levels compared with those observed after 26 h of food deprivation. These responses were differentially influenced by refeeding of chow for 6 h. NPY mRNA levels were reduced, but remained elevated compared with ad libitum conditions. Interestingly, AGRP mRNA levels remained elevated at levels observed after deprivation even 6 h after refeeding. In contrast, POMC mRNA levels were restored to control levels 6 h after refeeding. In contrast to refeeding with standard rat chow, we observed no effect of either feeding a palatable, noncaloric mash or of leptin administration on hypothalamic neuropeptide levels 6 h after treatment.

After a full day of ad libitum refeeding, we observed that NPY mRNA levels remained significantly elevated above ad libitum conditions, whereas AGRP and POMC mRNA levels were normalized.

DISCUSSION

In this study we examined the time course and potential mechanisms of changes in hypothalamic NPY, AGRP, and POMC mRNA levels on refeeding after 24 h food deprivation in C57 mice. The main finding of this study is that 6 h, but not 2 h, of refeeding after food deprivation was sufficient to reduce (but not restore) NPY mRNA levels, did not affect AGRP mRNA levels, and restored POMC mRNA to ad libitum control levels. Because intake of the noncaloric mash had no effect on these mRNA levels, the act of feeding per se and preabsorptive signals including taste and gut dis-
tension do not appear to be sufficient to modulate mRNA levels. Furthermore, leptin administration after deprivation, at a dose that reduces refeeding in food-deprived mice, was not sufficient alone to affect the deprivation-induced changes in NPY, AGRP, or POMC mRNA levels. Taken together, these findings suggest that gradual postabsorptive events subsequent to refeeding are required for the restoration of NPY, AGRP, and POMC mRNA after a period of food deprivation.

The first experiment in this study was designed to determine whether leptin administration after food deprivation would affect food intake on refeeding. Leptin is a key afferent signal of caloric status, affecting short- and long-term regulation of food intake (9, 10, 18, 19). Exogenous leptin administration decreases food intake in ad libitum-fed rats (29, 58, 59) and mice (39) and normalizes body weight in genetically obese leptin-deficient ob/ob mice (25, 46). We therefore hypothesized that leptin administration in mice after 24 h food deprivation would reduce ad libitum food intake on refeeding.

Leptin administration [at a dose previously reported to induce SOCS-3 mRNA within 2 h of administration (6)] significantly reduced postdeprivation food intake between 2 and 6 h of refeeding (Table 1). Correlated with the decrease in food intake was a significant reduction in body weight gain during the refeeding period compared with the refed chow. These results are consistent with previously reported rapid effects of leptin administration to reduce food intake in ad libitum-fed animals (39, 58, 59). Thus we used this dose in subsequent studies designed to examine the role of leptin in the restoration of hypothalamic neuropeptides after refeeding.

Few reports have examined the restoration of food deprivation-induced changes in hypothalamic peptide mRNA levels within 24 h of refeeding: NPY mRNA levels in rats have been reported to be normalized after 5 h (20), after 24 h (49), or after 3 days (15) of ad libitum feeding after food deprivation, whereas NPY peptide concentration in the paraventricular nucleus was reportedly restored within 1 h of refeeding in wild-type lean, but not ob/ob, mice (28). Fasting-induced decreases in POMC mRNA were not normalized after 5 h of refeeding in rats (20). The details of the time course of reversing deprivation-induced changes in peptide mRNA levels during refeeding can, however, provide important information as to the underlying signals and mechanisms regulating these changes. Multiple short-term, meal-related signals (e.g., taste, gut distension) and long-term peripheral hormonal (e.g., leptin) and nutritive (e.g., glucose) signals are likely to contribute to the modulation of hypothalamic mRNA levels on refeeding. The relative contributions and temporal dynamics of these afferent signals in restoring deprivation-induced changes in peptide mRNA levels are not well understood.

For example, during normal ad libitum feeding, both ob (leptin) mRNA and plasma leptin levels are increased within the first 4 h of dark-phase feeding (30,
Caloric deprivation results in rapid decreases of both ob mRNA and plasma leptin levels (3, 13, 30, 35, 40), and these changes are significantly and rapidly reversed within the first 1–6 h of refeeding (22, 45, 50, 56). As discussed, NPY, AGRP, and POMC are downstream targets of leptin action; therefore, if an increase in circulating leptin during refeeding contributes to reversing the deprivation-induced changes in expression of these genes, the time course of reversal on refeeding should be subsequent to changes in circulating leptin levels.

Our work adds to a growing body of evidence indicating differential regulation of hypothalamic neuropeptides involved in the regulation of energy balance. Indeed, NPY and AGRP, which are coexpressed within the same neurons, appear to be differentially regulated under various circumstances such as treatment with fatty acid synthase inhibitors (53) or changes in photoperiod (37). Chronic infusion of leptin was shown to reduce NPY mRNA but had no effect on POMC or cocaine- and amphetamine-regulated transcript mRNA (1). In our study, we observed that AGRP mRNA was restored more slowly than NPY after refeeding in the 24-h fasted C57 mouse. The specific mechanisms involved in the differential regulation of NPY, AGRP, and POMC mRNAs remain poorly understood and require further study.

Although we did not measure plasma leptin in our study, the time course of refeeding effects on NPY and POMC mRNA levels supports potential regulation by circulating leptin on refeeding. Our results show that 6 h, but not 2 h, of refeeding after 24 h food deprivation was sufficient to significantly decrease NPY mRNA levels compared with food-deprived mice and restore POMC mRNA to ad libitum control levels. Thus, between 2 and 6 h of refeeding, afferent signals of caloric compensation were transduced into changes in mRNA levels. Interestingly, AGRP mRNA levels were not affected by 6 h of refeeding.

Given that the time course of restoration of NPY and POMC mRNA levels are consistent with a regulatory role for circulating signals such as leptin, we were surprised that exogenous leptin administration in food-deprived mice had no effect on NPY, AGRP, or POMC mRNA levels, in contrast to the relatively rapid effects of refeeding. We selected a high dose of leptin (100 µg or 4 mg/kg) to maximize the possibility of observing a role for leptin in regulation of hypothalamic gene expression. Unfortunately, we did not measure leptin levels in our study, but the dose of leptin produces superphysiological levels for at least 3 h postinjection (2) and thus should have been adequate to test the hypothesized role for leptin in the regulation of NPY, AGRP, and POMC gene expression. The effects of leptin-receptor signaling are mediated via the JAK-STAT signal transduction pathway (24). Specifically, peripheral leptin administration induces dose-dependent activation of STAT-3 within 15 min (57), whereas intracerebroventricular leptin induces nuclear translocation of STAT-3 in hypothalamic nuclei (26). STAT-3 has been localized to both NPY and POMC arcuate nucleus neurons (24). In addition, leptin administration induces SOCS-3 (suppressor of cytokine signaling) expression in hypothalamic nuclei that express the long-form leptin receptor (6). In our study, however, a dose of leptin sufficient to reduce compensatory hyperphagia (experiment 1) and induce SOCS-3 mRNA (6) was not sufficient to effect any change in NPY, AGRP, or POMC mRNA levels. It is reasonable to speculate that in our study, the reduction in food intake after leptin administration was mediated via rapid mechanisms independent of changes in neuropeptide gene expression or modulation of protein synthesis. This is consistent with several studies indicating that leptin induces alterations in membrane potential and ion channels that may mediate rapid changes in neurotransmitter secretion (12, 23, 43, 48, 54).

The lack of effect of leptin administration on the deprivation-induced changes in mRNA levels further supports the concept that afferent input from multiple signals is required to regulate mRNA expression. In further support of this concept, previous studies have reported food deprivation-induced changes in NPY, AGRP, and POMC mRNA levels in the db/db mouse (41, 43) and obese Zucker rat (NPY) (47), which, due to mutations in the gene coding for the long form of the leptin receptor, are unable to detect changes in circulating leptin levels. We speculated that postingestion signals related to taste and gut distension could contribute to the regulation of gene expression in the arcuate hypothalamus. To examine this possibility, mice were refed a novel palatable mash diet containing no calories. One potential limitation of this approach was that additional groups consuming the mash diet with calories were not studied and thus we do not know that refeeding with a palatable mash diet produces a similar pattern of peptide gene expression compared with refeeding with chow. Nonetheless, the results from our study are consistent with the concept that nonnutritive refeeding is insufficient to restore food deprivation-induced changes in NPY, AGRP, and POMC gene expression.

Further potential modulators of mRNA levels include reversal of the deprivation-induced decrease in circulating glucose, insulin, and increase in glucocorticoid levels (14). Hypothalamic circuitry is activated by physiological changes in plasma glucose (64), and conditions of altered energy balance such as obesity and diabetes are associated with alterations in central glucose sensing (32). Insulin receptors are expressed in the arcuate nucleus, and exogenous insulin administration decreased food intake as well as suppressed NPY mRNA levels (4, 63). Glucocorticoids stimulate feeding (14) and dexamethasone treatment (synthetic glucocorticoid) induces rapid NPY release from arcuate nucleus neurons (11) as well as NPY expression in pancreatic islet cells (44).

Thus multiple factors presumably coordinate the restoration of deprivation-induced changes in hypothalamic peptide mRNA levels on refeeding. We showed that 2–6 h of caloric refeeding decreased the depriva-
tion-induced increase in NPY mRNA and reversed the decrease in POMC mRNA to ad libitum control levels. Leptin administration alone was not sufficient to mediate these changes. The time course of restorative changes in mRNA levels on refeeding described here provides a template from which to further understand the complex interplay required between multiple afferent signals and upstream mechanisms mediating these changes, as well as subsequent downstream physiological and behavioral responses.

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