Attenuation of Diabetic Hyperphagia in Neuropeptide Y–Deficient Mice

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The combined effects of increased hypothalamic signaling by neuropeptide Y (NPY) and decreased signaling by melanocortins are hypothesized to stimulate food intake when body fat stores are depleted. To investigate NPY’s role in the hyperphagic response to uncontrolled diabetes, streptozotocin (STZ) (200 mg/kg intraperitoneally) or saline vehicle was given to NPY-deficient (Npy−/−) and wild-type (Npy+/+) mice. In Npy+/+ mice, STZ-induced diabetes increased mean daily food intake to plateau values 50% above baseline intake (+2.0 ± 0.6 g/day; P ≤ 0.05), an effect that was not seen in STZ-treated Npy−/− mice (+0.8 ± 0.1 g/day; NS), despite comparably elevated levels of plasma glucose and comparably decreased levels of body weight, fat content, and plasma leptin. Unlike the impaired feeding response to uncontrolled diabetes, Npy−/− mice exhibit intact hyperphagic responses to fasting (Erickson et al. [1], Nature 381:415–418, 1996). To investigate whether differences in hypothalamic melanocortin signaling can explain this discrepancy, we used in situ hybridization to compare the effects of STZ-diabetes and fasting on pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP) mRNA levels in the hypothalamic arcuate nucleus (ARC) of Npy−/− and Npy+/+ mice. AgRP mRNA levels were increased by both fasting and STZ-diabetes, but the increase in STZ-diabetes was small (50–80%) compared with the effect of fasting (~20-fold increase of AgRP mRNA). STZ-diabetes also lowered POMC mRNA levels by 65% in the ARC of Npy+/+ mice (P ≤ 0.05) but by only 11% in Npy−/− mice (NS); fasting significantly lowered POMC mRNA levels in both genotypes. We conclude that NPY is required for both the increase of food intake and the decrease of hypothalamic POMC gene expression induced by uncontrolled diabetes. In contrast, NPY is not required for either of these responses when the stimulus is food deprivation. Moreover, fasting is a more potent stimulus to hypothalamic AgRP gene expression than is STZ-diabetes. Therefore, central nervous system melanocortin signaling appears to be suppressed more effectively by fasting than by uncontrolled diabetes, which provides a plausible explanation for differences in the feeding response to these two stimuli in mice lacking NPY. Diabetes 51: 778–783, 2002

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daptive increases of food intake induced by depleted body energy stores are important for survival and appear to involve the coordinate regulation of multiple hypothalamic pathways that can influence feeding behavior (2). For example, hypothalamic neurons that contain neuropeptide Y (NPY), a potent stimulator of food intake, are activated when body fat content is reduced by energy restriction (3). Conversely, the hypothalamic production of melanocortins (peptides with anorectic properties that are cleaved from pro-opiomelanocortin [POMC]) is reduced in this setting (4). Acute or chronic energy deficits also increase expression of the gene encoding agouti-related peptide (AgRP), an endogenous antagonist of central nervous system (CNS) melanocortin receptors (5) that is co-expressed with NPY in neurons of the hypothalamic arcuate nucleus (ARC) (6). Fasting is an especially potent stimulus to AgRP gene expression in mouse ARC, increasing AgRP mRNA by ~20-fold (6). The combined effects of decreased melanocortin signaling (due to both increased AgRP and decreased POMC biosynthesis) and increased NPY signaling, therefore, comprise an integrated mechanism to mediate hyperphagia in response to a sustained energy deficit.

The original description of mice with NPY deficiency (Npy−/−) due to targeted NPY gene disruption revealed them to have normal levels of daily food intake and body weight and to exhibit normal increases of food intake after a fast (1). One possible explanation for this finding is that neural control over feeding under these circumstances is sufficiently redundant that the loss of NPY is compensated for by other responses. Of potential importance in this context is the marked reduction of melanocortin signaling induced by fasting, which may allow Npy−/− mice to increase food intake appropriately during refeeding. One approach to test this hypothesis is to identify and study a model in which hyperphagia depends more on increased NPY signaling than on reduced melanocortin signaling. In
such a model, the ability to increase food intake should be compromised in NPY-deficient mice.

Uncontrolled insulin-deficient diabetes induced by the β-cell toxin streptozotocin (STZ) is an established model of sustained hyperphagia in rodents. Like the response to fasting, diabetic hyperphagia appears to involve increased hypothalamic signaling by NPY (7–10) and is also accompanied by increased AgRP mRNA and reduced POMC mRNA expression in ARC neurons (11–13). However, the increase of AgRP mRNA levels induced by STZ-induced diabetes in mice appears to be much smaller (~2-fold) (13) than that induced by fasting (~20-fold) (5). Diabetic hyperphagia may therefore depend to a greater extent on NPY than on AgRP when compared to the hyperphagic response to fasting.

Based on this reasoning, we hypothesized that mice lacking NPY would manifest impaired feeding responses to uncontrolled diabetes, despite intact responses to fasting (which induces a much larger increase of AgRP expression). Evidence that the orexigenic response to centrally administered AgRP is heightened in Npy+/+ mice (14) provides additional support for this hypothesis. We therefore measured food intake and hypothalamic levels of AgRP and POMC mRNA in Npy−/− and Npy+/+ mice made diabetic with STZ and compared these responses to those induced by a 48-h fast in separate groups of each genotype.

RESEARCH DESIGN AND METHODS

Animals. Adult mice (C57Bl/6x129Sv/Ev mixed genetic background or pure 129Sv/Ev background) with targeted knockout of the Npy gene (Npy−/−) and wild-type littermate controls (Npy+/+) were housed in individual cages in standard vivarium conditions (12 h:12 h light:dark cycle). Standard rodent chow (Harlan Teklad Rodent Diet, Purina) was provided ad libitum, except standard vivarium conditions (12 h:12 h light:dark cycle). Standard rodent (8:00 a.m.), food was removed from a subset of these mice (n ~ 10) and the remaining mice were maintained on ad libitum feeding. At the time of food removal, mice were binned into groups of equal weight.

Experiment 1: STZ-induced diabetes. Baseline measurements of 24-h food intake, weight, and body weight were obtained for 2 days before administration of STZ (200 mg/kg i.p. in 0.5 mmol/l citrate buffer, pH 4.5) to mice of each genotype (C57Bl/6x129Sv/Ev mixed genetic background) to induce diabetes. Mice were randomized into four groups to receive injection of the citrate buffer only and remained nondiabetic (Npy+/+), STZ-treated Npy+/+ mice, STZ-treated Npy−/− mice fed ad libitum for the fasting study) was considered to represent 100% of the control value of relative body weight, and individual AUC values for the diabetic groups were expressed as a percent of this value.

Plasma assays. Blood samples were collected on heparin and placed immediately on ice in heparinized tubes. Plasma was separated by centrifugation and stored at −20 °C until determination of glucose and leptin concentrations. Plasma glucose was determined by the glucose oxidase method (Beckman Instruments, Brea, CA). Plasma leptin levels were determined by radioimmunoassay (Linco Research, St. Louis, MO).

Statistics. All values are reported as group means ± SE. The level of significance was taken as P ≤ 0.05, two-tailed. Data were analyzed for differences from baseline values using one-way ANOVA with repeated measures, and Fisher's protected least significance difference test was used for multiple comparisons when significant F ratios were obtained. Interactions between treatment and genotype were analyzed by group mean comparisons using two-way ANOVA. Statistical comparisons were made using Statview (Calabasa, CA) software.

RESULTS

Experiment 1: STZ-induced diabetes. Plasma glucose values (Fig. 1A) obtained at the end of the study (day 18) were significantly and comparably elevated in both genotypes of STZ-treated mice (Npy+/+ 332 ± 25; Npy−/− 285 ± 20 mg/dl; P ≤ 0.05 vs. respective vehicle-treated controls; NS between the two diabetic groups) relative to nondiabetic controls (Npy+/+ 105 ± 6; Npy−/− 107 ± 6 mg/dl). Plasma leptin levels did not differ according to genotype among vehicle-treated animals (Npy+/+ 2.9 ± 0.4; Npy−/− 4.1 ± 0.8 ng/ml; NS) and were significantly and comparably decreased in the two diabetic groups (Npy+/+ 2.0 ± 0.2; Npy−/− 2.0 ± 0.2 ng/ml; P ≤ 0.05 vs. respective vehicle groups) (Fig. 1B).

Baseline (preinjection) 24-h food intake (Fig. 2) in vehicle-treated Npy+/+ mice was 4.1 ± 0.3 g/day, and this value did not change over the course of the study, averaging 4.0 ± 0.2 g/day for the last 7 days of the experiment. Baseline 24-h food intake was 3.5 ± 0.2 g/day in vehicle-treated Npy−/− mice and increased to 4.3 ± 0.2 g/day (P ≤ 0.05 vs. baseline food intake) for the last 7 days of the experiment. After STZ injection, food intake declined transiently in Npy+/+ mice before returning to baseline levels on day 4 (4.1 ± 0.2 g/day) and increased subsequently to ~50% over baseline (to 6.2 ± 0.5 g; P ≤ 0.05) for the last 7 days of the study. NPY-deficient mice also experienced a transient decline in food intake from baseline values (3.8 ± 0.5 g) following STZ injection, but unlike wild-type mice, food intake of Npy−/− mice did not increase significantly above baseline levels over the remainder of the experiment (4.8 ± 0.3 g/day, days 11–18; NS
By two-way ANOVA, the change of food intake induced by diabetes differed significantly according to genotype ($P < 0.05$) and was significantly increased only in Npy$^{+/+}$ mice (Figs. 2 and 3). Diabetes was also associated with a marked increase of daily water intake in Npy$^{+/+}$ mice, from a baseline of 7.1 ± 0.5 ml/day to mean values of 30.9 ± 1.6 ml/day ($P < 0.05$) over the last 7 days of the study. In STZ-treated Npy$^{-/-}$ mice, daily water intake also increased, but to a lesser degree (to 14.4 ± 0.9 ml; $P < 0.05$ vs. all other groups).

Among vehicle-treated nondiabetic animals, body weight increased in both genotypes by 0.2–0.9 g over the course of the experiment, whereas it decreased gradually in both diabetic groups, and by day 18, there was no significant effect of genotype on weight loss in STZ-treated mice (Fig. 3). A similar pattern was revealed by MRS-derived measures of relative body fat content. Whereas body fat mass was comparable among vehicle-treated Npy$^{+/+}$ and Npy$^{-/-}$ mice, fat content of diabetic Npy$^{+/+}$ mice was 44 ± 6% ($P \leq 0.05$) of vehicle-treated Npy$^{+/+}$ values. Similarly, body fat mass of STZ-treated Npy$^{-/-}$ mice was 60 ± 8% ($P \leq 0.05$) of vehicle-treated Npy$^{+/+}$ mice. Like the decreases of body weight, the effect of diabetes on relative fat content did not differ significantly by genotype (Fig. 3).

By in situ hybridization, NPY mRNA levels measured in the ARC of Npy$^{+/+}$ mice receiving STZ were 230 ± 50% higher than in vehicle-treated controls ($P < 0.05$) (Fig. 4A). As expected, NPY mRNA was not detected in Npy$^{-/-}$ mice.

**FIG. 1.** Plasma glucose and leptin levels in Npy$^{+/+}$ (KO) and Npy$^{-/-}$ (WT) mice. Plasma levels of glucose (A) and leptin (B) from vehicle-treated (WT-V and KO-V; $n = 7$ per genotype) or STZ-diabetic (WT-D and KO-D; $n = 6$ per genotype) mice obtained on day 18 of experiment 1. *$P \leq 0.05$ vs. vehicle-treated controls.

**FIG. 2.** Change in daily food intake in Npy$^{+/+}$ (KO) and Npy$^{-/-}$ (WT) mice. Change of daily food intake during baseline (days 0–1) and after injection (days 1–18) of either vehicle or STZ in Npy$^{+/+}$ and Npy$^{-/-}$ mice of experiment 1. Baseline food intake: Npy$^{+/+}$ mice, 4.1 ± 0.3 g/day (WT-V); 4.1 ± 0.2 g/day (WT-D); Npy$^{-/-}$ mice, 3.5 ± 0.2 g/day (KO-V); 3.8 ± 0.5 g/day (KO-D). Data points are means ± SE. *$P \leq 0.05$ vs. baseline.

**FIG. 3.** Effect of STZ-diabetes on food intake, body weight, and body fat content by genotype. Data (means ± SE) were obtained from Npy$^{+/+}$ and Npy$^{-/-}$ mice on day 18 of experiment 1 and are expressed as a percent of nondiabetic controls of the same genotype.
AgRP mRNA expression (Fig. 4B) was also increased in the ARC of diabetic Npy+/+ mice (by 54 ± 29% relative to nondiabetic wild-type mice; *P < 0.05) and was similar to the response detected in Npy−/− mice, with AgRP mRNA levels being increased by 83 ± 43% over values obtained in vehicle-treated Npy−/− mice. There was no significant effect of genotype on AgRP mRNA levels in either diabetic or vehicle-treated groups. By comparison, POMC mRNA levels (Fig. 4C) were reduced in the rostral ARC of diabetic Npy+/+ mice by 65 ± 7% (P ≤ 0.05 vs. vehicle-treated controls) but fell by only 13 ± 1.6% in the ARC of diabetic Npy−/− mice (NS vs. vehicle-treated Npy−/− mice; *P ≤ 0.05 vs. diabetic Npy+/+ mice). Among vehicle-treated animals, POMC mRNA levels were not significantly altered by NPY deficiency.

**Experiment 2: responses to fasting.** Consistent with a previous report in male mice (6), food intake increased significantly and similarly in response to a 48-h fast in female mice of both genotypes (Table 1). Four hours after the return of food, intake was increased by 80–120% in both Npy+/+ and Npy−/− mice compared with controls fed ad libitum, and at 24 h of refeeding, intake was increased by ~60% in both genotypes. No difference by genotype was detected in the effect of fasting on food intake (Table 1). In a separate group of Npy+/+ mice, fasting reduced POMC mRNA levels in the rostral ARC by 52% (P < 0.05 vs. Npy+/+ mice fed ad libitum) (Table 1). Hypothalamic POMC mRNA levels were also significantly reduced by fasting in Npy−/− mice, and although the magnitude of this effect (~33%) was below that detected in wild-type mice, mean levels of POMC mRNA in fasted Npy+/+ and Npy−/− mice were not significantly different from one another.

**DISCUSSION**

To investigate the specific contribution of NPY to the pathogenesis of diabetic hyperphagia, we compared food intake of NPY-deficient and wild-type mice after inducing diabetes with STZ. Whereas food intake increased by 50% within 2 weeks of the onset of diabetes in Npy+/+ mice, hyperphagia was not detected in diabetic mice that lack NPY. Reduced feeding in response to uncontrolled diabetes in Npy−/− mice cannot be attributed to differences in the severity of diabetes, since levels of plasma glucose, plasma leptin, body weight, and relative fat content were comparable in the two diabetic groups. Combined with our finding that STZ-diabetes induces increased NPY mRNA levels in the ARC of Npy+/+ mice, consistent with several earlier reports in both rats and mice (7–13), these results support the conclusion that increased hypothalamic NPY signaling is required for the development of diabetic hyperphagia.

The attenuation of diabetic hyperphagia in Npy−/− mice

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**TABLE 1**

Food intake and hypothalamic POMC and AgRP mRNA levels in response to fasting in Npy+/+ and Npy−/− mice

<table>
<thead>
<tr>
<th>Genotype and condition</th>
<th>Food intake (g)</th>
<th>Hypothalamic mRNA level</th>
<th>mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
<td>POMC</td>
</tr>
<tr>
<td><strong>Npy+/+</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>0.8 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td>100 ± 11% (n= 8)</td>
</tr>
<tr>
<td>Fasted</td>
<td>1.4 ± 0.1†</td>
<td>5.1 ± 0.9†</td>
<td>48 ± 6%† (n=12)</td>
</tr>
<tr>
<td><strong>Npy−/−</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>0.6 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>102 ± 12% (n= 7)</td>
</tr>
<tr>
<td>Fasted</td>
<td>1.4 ± 0.1†</td>
<td>5.6 ± 0.3†</td>
<td>67 ± 8%† (n=12)</td>
</tr>
</tbody>
</table>

Date are means ± SE.*Previously published (14). †p<0.05 vs. fed.
contrasts sharply with the hyperphagic response to fasting, which is completely intact in mice lacking NPY. Our current findings of comparable refeeding hyperphagia in Npy \(^{-/-}\) and Npy \(^{+/+}\) mice replicate those published previously (1) and support the conclusion that NPY is not required for this response. This finding is at odds with a recent report showing impaired refeeding responses after fasting in Npy \(^{-/-}\) mice (18). The basis for this discrepancy is unclear, but our efforts to document abnormal levels of intake in Npy \(^{-/-}\) mice 24 h after fasting have been consistently unsuccessful. Differences in housing conditions of mice before study is a potential factor, since mice in our studies were housed individually before and during all experimental interventions, whereas mice from the study by Bannon et al. (18) were group-housed before study. Because Npy \(^{-/-}\) mice can have heightened stress responses to novel stimuli (1), this difference in housing may have influenced intake during refeeding. Another possibility is that differences in background strain of mice used in the different laboratories contributed to differences in their feeding response.

Our observation of intact refeeding after a 48-h fast in Npy \(^{+/+}\) mice, therefore, suggests that factors that stimulate food intake after fasting must differ from those involved in diabetic hyperphagia. That plasma glucose levels are increased in diabetes and reduced by fasting raises the possibility that differences in CNS glucose delivery contributed to differences in feeding in response to the two conditions in NPY-deficient mice. By comparison, both fasting and uncontrolled diabetes lower circulating insulin and leptin levels and induce comparable increases of hypothalamic NPY gene expression. Because reduced melanocortin signaling is implicated in the hyperphagic response to both fasting and diabetes, and because melanocortin-producing neurons are sensitive to input from NPY, we determined the effect of these two conditions on hypothalamic expression of POMC and AgRP mRNA in wild-type and Npy \(^{+/+}\) mice. Although neuropeptide mRNA levels are an indirect measure of the activity of hypothalamic neurons, their use in assessing the response to hormonal and metabolic stimuli is supported by a large literature (5,7–13,15,16).

The hypothalamic melanocortin system is made up of neurons that synthesize melanocortin receptor (Mcr) agonists, such as α-melanocyte-stimulating hormone (α-MSH), a product of POMC, and those that contain the Mcr antagonist, AgRP. Both POMC- and AgRP-containing cell bodies are located in the ARC, with AgRP being co-localized with NPY in this brain area. Since Mc4r antagonists stimulate feeding (19,20), and since genetic deletion of either POMC or Mc4r causes hyperphagia and obesity in mice (21,22), defective CNS melanocortin signaling is a potent stimulus to increase food intake. Evidence that STZ-diabetes increases AgRP and decreases POMC mRNA in rodent hypothalamus suggests that reduced Mcr signaling might, along with increased NPY signaling, contribute to diabetic hyperphagia (12,13). Our current finding that POMC mRNA levels were markedly reduced by uncontrolled diabetes in the ARC of Npy \(^{+/+}\) mice is consistent with this hypothesis and is in agreement with data obtained in diabetic rats (12).

By comparison, POMC mRNA levels were not signifi-

cently reduced in NPY-deficient mice with STZ-induced diabetes. Failure of NPY-deficient mice to suppress melanocortin signaling may therefore have contributed to the attenuation of their hyperphagic response. Moreover, this finding suggests that NPY is required for the effect of uncontrolled diabetes to reduce hypothalamic POMC gene expression. Together with evidence that POMC expression in ARC neurons is, in general, reduced in conditions that activate ARC NPY neurons (e.g., fasting, uncontrolled diabetes, and genetic leptin deficiency), our results suggest that activation of NPY neurons contributes to the inhibition of ARC POMC neurons in these conditions. This possibility is supported by several findings. Both NPY Y1 and Y5 receptor subtypes are present in the ARC (23,24), chronic central NPY administration reduces hypothalamic POMC mRNA levels in vivo (25), and NPY neurons exert inhibitory effects on the firing of ARC POMC neurons in vitro (26).

In contrast to the effect of NPY, leptin administration increases ARC POMC gene expression in vivo (16) and increases electrical activity of these neurons in vitro (26). These effects are likely mediated, at least in part, via direct stimulatory effects of leptin, since a majority of these neurons express the signaling form of the leptin receptor (OB-Rb) (27,28). Thus, conditions of negative energy balance such as fasting can inhibit POMC neurons in the ARC via at least two mechanisms: decreased input from leptin and activation of adjacent NPY neurons (a response also triggered in part by leptin deficiency). In the current studies, we found that although the effect of fasting to lower ARC POMC mRNA levels was less pronounced in Npy \(^{-/-}\) than in Npy \(^{+/+}\) mice, these differences did not achieve statistical significance. Thus, unlike the situation with uncontrolled diabetes, NPY is not required for the effect of a 48-h fast to lower hypothalamic POMC gene expression, although it may contribute to this response. NPY-independent mechanisms may therefore play a more important role to inhibit POMC neurons during fasting than they do in STZ-deficient diabetes, and additional studies are required to identify these factors. Fasting-induced decreases of leptin signaling are unlikely to explain reduced expression of POMC mRNA in this setting, since fasting and STZ-diabetes lower leptin levels comparably.

In data from a previously published study, fasting induced marked (~20-fold) increases of AgRP mRNA in the ARC of both Npy \(^{-/-}\) and Npy \(^{+/+}\) mice (Table 1 and reference 14). Thus, NPY deficiency did not significantly alter the effect of either diabetes or fasting on hypothalamic AgRP mRNA levels, but the increase of hypothalamic AgRP gene expression induced by fasting is much greater than that induced by STZ-diabetes in both genotypes. Although AgRP is co-localized with NPY in ARC neurons (5,14,29), our results suggest that NPY is not required for the effect of fasting or diabetes to induce AgRP gene expression. However, the relatively modest effect of diabetes to increase of hypothalamic AgRP expression (50–80%) was evidently inadequate to increase food intake in NPY-deficient mice. Because the AgRP response to uncontrolled diabetes was small in comparison to that induced by fasting, it can be concluded that NPY-deficient mice respond to fasting with a much greater
increase of AgRP and a greater decrease of POMC gene expression in the ARC. These observations suggest that fasting inhibited CNS melanocortin signaling to a greater extent than did STZ diabetes in Npy−/− mice, which provides a plausible explanation for the intact hyperphagic response to fasting in these animals. This hypothesis is strengthened by evidence that the orexigenic response to AgRP is heightened in Npy−/− mice (14).

In conclusion, our results support a major role for NPY in the stimulation of food intake triggered by uncontrolled diabetes. Unlike wild-type mice, diabetic mice lacking NPY also fail to suppress hypothalamic expression of POMC mRNA. Combined with evidence that diabetes-associated increases of AgRP mRNA in the ARC are much smaller than those induced by fasting, our results support a model in which failure to suppress hypothalamic melanocortin signaling contributes to the attenuation of diabetic hyperphagia in mice lacking NPY. By comparison, food deprivation appears to inhibit hypothalamic melanocortin signaling more effectively than STZ-diabetes in Npy−/− mice, a mechanism that may help explain why these animals exhibit intact hyperphagia during refeeding but not in response to uncontrolled diabetes.

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