Effects of leptin replacement on hypothalamic-pituitary growth hormone axis function and circulating ghrelin levels in ob/ob mice

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Luque RM, Huang ZH, Shah B, Mazzone T, Kineman RD. Effects of leptin replacement on hypothalamic-pituitary growth hormone axis function and circulating ghrelin levels in ob/ob mice. Am J Physiol Endocrinol Metab 292: E891–E899, 2007. First published November 22, 2006; doi:10.1152/ajpendo.00258.2006.—Leptin-deficient obese mice (ob/ob) have decreased circulating growth hormone (GH) and pituitary GH and ghrelin receptor (GHS-R) mRNA levels, whereas hypothalamic GH-releasing hormone (GHRH) and somatostatin (SST) expression do not differ from lean controls. Given the fact that GH is suppressed in diet-induced obesity (a state of hyperleptinemia), it remains to be determined whether the absence of leptin contributes to changes in the GH axis of ob/ob mice. Therefore, to study the impact of leptin replacement on the hypothalamic-pituitary GH axis of ob/ob mice, leptin was infused for 7 days (sc), resulting in circulating leptin levels that were similar to wild-type controls (~1 ng/ml). Leptin treatment reduced food intake, body weight, and circulating insulin while elevating circulating n-octanoyl ghrelin concentrations. Leptin treatment did not alter hypothalamic GHRH, SST, or GHS-R mRNA levels compared with vehicle-treated controls. However, leptin significantly increased pituitary GH and GHRH-R expression and tended to enhance circulating GH levels, but this latter effect did not reach statistical significance. In vitro, leptin (1 ng/ml, 24 h) did not affect pituitary GH, GHRH-R, or GHS-R mRNA but did enhance GH release. The in vivo effects of leptin on circulating hormone and pituitary mRNA levels were not replicated by pair feeding ob/ob mice to match the food intake of leptin-treated mice. However, leptin did prevent the fall in hypothalamic GHRH mRNA and circulating IGF-I levels observed in pair-fed mice. These results demonstrate that leptin replacement has positive effects on multiple levels of GH axis function in ob/ob mice.

growth hormone-releasing hormone; GHRH receptor; ghrelin receptor; neuropeptide Y

LEPTIN IS PRODUCED primarily in adipocytes, and circulating leptin levels are elevated in obese states and decreased in response to food restriction and fasting (18). Experimental data in rat models suggest that leptin supports hypothalamic-pituitary growth hormone (GH) axis function. Central infusion of leptin antiserum to GH-releasing hormone (GHRH), coupled with the stimulatory effect of leptin on GH output can be blocked by the fact that central delivery of leptin increases GHRH mRNA levels in both intact and hypophysectomized fasted rats, has led to the conclusion that leptin mediates its positive effects on GH release by enhancing GHRH production (8). A positive link between leptin, GHRH, and GH is further supported by the observation that GH and GHRH mRNA levels are reduced in leptin receptor (ObR) mutant fa/fa rats (2, 10, 54, 55).

Consistent with the observations made in fa/fa rats, ob/ob mice that lack a functional leptin gene maintain hypothalamic expression of GHRH, whereas circulating GH and pituitary GH mRNA levels are decreased compared with lean controls (29, 52). Also, in contrast to the rat, fasting increases circulating GH levels in mice (30, 39, 47), as well as all other mammalian species studied to date (16, 17, 19, 34, 44, 56), despite the fall in circulating leptin. These divergent results bring into question the importance of leptin in maintaining hypothalamic-pituitary GH axis function across diverse species. To help clarify this issue, the following study examined the effects of leptin replacement on the hypothalamic-pituitary GH axis of the ob/ob mouse. This study included a group of ob/ob mice pair fed to match the food intake of leptin-treated mice, in order to differentiate between the direct effects of leptin and those mediated indirectly by leptin-induced reduction in food intake and weight loss.

RESEARCH DESIGN AND METHODS

All experimental procedures were approved by the animal care and use committees of the University of Illinois at Chicago and the Jesse Brown Veteran’s Administration Medical Center. Male ob/ob mice of the C57BL/6d background were purchased at 7 wk of age from Jackson Laboratories (Bar Harbor, ME). Lean mice homozygote or heterozygote for the wild-type allele, designated collectively as +/+ , were used as controls. Mice were housed under standard conditions of light (12:12-h light-dark cycle, lights on at 0700) and temperature (22–24°C), with free access to standard rodent chow (LabDiet, cat. no. 5008; fat 17% kcal, carbohydrate 56% kcal, protein 27% kcal) and tap water. Mice were allowed to acclimate to the facility, personnel, and daily handling for 3 wk. At 10 wk of age, ob/ob mice were anesthetized with ketamine/xylazine and implanted subcutaneously with osmotic minipumps (model 1007D; Alzet, Palo Alto, CA) containing either recombinant mouse leptin (1.3 mg/ml saline; R&D Systems, Minneapolis, MN) or vehicle (n = 5 animals/treatment group). The pumps released at a rate of 0.5 µl/h, delivering a total of 15.6 µg of leptin each day. One week later, an additional group of ob/ob mice (n = 5) were also anesthetized and sham operated and then provided food to match the mean food intake of mice receiving leptin. Mice were maintained on heating pads (37°C) until they fully recovered from anesthesia. Mice were weighed prior to anesthesia and each day following pump placement and killed between 0700 and 0900 by decapitation without anesthesia.

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blood was collected for hormone (insulin, IGF-I, corticosterone, and ghrelin) and metabolite (glucose and free fatty acids) determinations, whereas hypothalami, pituitaries, and stomachs were collected for analysis of mRNA by quantitative real-time RT-PCR (qRT-PCR; see below for details). It should be noted that tissues and blood samples from all three groups (vehicle treated, leptin treated, and pair fed) were processed and end points measured at the same time. In addition, plasma and tissue samples taken from nonanesthetized, ad libitum-fed ob/ob and lean (+/-) male mice (10 wk of age) used in a previous study (29) to assess the impact of genotype (i.e., lack of leptin) on the GH axis were further evaluated to explore the effects of genotype on ghrelin synthesis and release.

Primary pituitary cell cultures. To determine whether leptin could directly regulate pituitary expression of genes important in GH synthesis and release, pituitaries of 10-wk-old male mice (C57BL/6J x FVB/N mixed background, n = 7-10 pooled/experiment, 4 separate experiments) were dispersed into single cells and plated at 2 x 10^5/well in 500 μl of 5% horse serum (Sigma, St. Louis, MO), 0.15% BSA (Sigma), and penicillin-streptomycin (Invitrogen) as previously reported (27-30). After a 48-h incubation, cultures were rinsed in serum-free medium and incubated for 2 h, and medium was replaced with serum-free medium containing 0 (control group), 1, 10, or 100 ng/ml of recombinant mouse leptin (3–4 wells/treatment group). Cultures were incubated for an additional 24 h, and cells were extracted for total RNA expression of GHRH, GHS-R, ghrelin, and cyclophilin A mRNA levels did not significantly vary between experimental groups within tissue type.

Table 1. Circulating hormone and metabolite levels in ob/ob male mice infused (7 days) with either vehicle or leptin or pair fed to match food intake of leptin-treated mice

<table>
<thead>
<tr>
<th></th>
<th>ob/ob, Vehicle</th>
<th>ob/ob, Leptin Treated</th>
<th>ob/ob, Pair Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting body wt, g</td>
<td>44.6±3</td>
<td>43.6±0.7</td>
<td>48.5±0.5</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>46.2±3.1</td>
<td>36.5±1.1</td>
<td>41.3±0.5</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>375±58.6</td>
<td>113±6.3</td>
<td>135±10.6</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>16.9±9.2</td>
<td>4.3±1.5</td>
<td>31.6±6.6</td>
</tr>
<tr>
<td>FFA, nmol/μl</td>
<td>1.6±0.3</td>
<td>0.92±0.05</td>
<td>0.98±0.006</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>406±19</td>
<td>408±38</td>
<td>269±45</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 5 mice/treatment group). FFA, free fatty acid. Hormone and metabolite values are from samples run in the same assay. Values that do not share a common letter (a, b, or c) differ statistically. P < 0.05 is considered statistically different.

RNA isolation and reverse transcription. Tissues and cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep kit (Stratagene, La Jolla, CA), with DNase treatment as previously described (29, 27). The amount of RNA recovered was determined using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1 μg of whole tissue extracts and 0.25 μg of pituitary cell culture extracts) was reverse transcribed (RT) in a 20-μl volume using random hexamer primers, with enzyme and buffers supplied in the cDNA First Strand Synthesis kit (Molecular Probes, Eugene, OR). cDNA was treated with RNaseH, and duplicate aliquots (1 μl) were amplified by qRT-PCR, where samples were run against synthetic standards to estimate mRNA copy number (see below).

qRT-PCR. Details regarding the development, validation, and application of a qRT-PCR to measure expression levels of mouse GHRH, somatostatin (SST), neuropeptide Y (NPY), GH, GHRH receptor (GHRH-R), ghrelin receptor (GHS-R), ghrelin, and cyclophilin A have been reported previously (27, 29). For real-time PCR reactions, Brilliant SYBR Green QPCR Master Mix (Stratagene) was used, where thermocycling and fluorescence detection was performed using a Stratagene Mx3000P real-time PCR machine. The final volume of the PCR reaction was 25 μl: 1 μl of RT sample, 12.5 μl of the QPCR Master Mix, 0.375 μl of each primer (10 μM stock solution), 0.375 μl of the reference dye, and 10.375 μl of H2O. Thermal cycling profile consisted of a preincubation step at 95°C for 10 min, followed by 40 cycles of denaturation (95°C, 30 s), annealing (61°C, 1 min), and extension (72°C, 30 s). Final PCR products were subjected to graded temperature dependent dissociation to verify that only one product was amplified. To determine the starting copy number of cDNA, RT samples were PCR amplified and the signal compared with that of a standard curve run on the same plate. Standard curves consisted of 10^1, 10^2, 10^3, 10^4, 10^5 copies of synthetic cDNA template for each of the transcripts of interest. Standard curves were generated by the Stratagene Mx3000P software, and the slopes were ~1, indicating that the efficiency of amplification was 100%, meaning that within the detectable range all templates in each cycle were copied. In addition, total RNA samples that were not RT and a no-cDNA control were run on each plate to control for genomic DNA contamination and to monitor potential exogenous contamination, respectively. Also, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (a peptidyl isomerase), where cyclophilin A mRNA levels did not significantly vary between experimental groups within tissue type.

Assessments of hormones and metabolites. Glucose levels were determined in fresh whole blood samples using the SureStep Glucose meter (Johnson & Johnson, Milpitas, CA). The remaining trunk blood...
(200–500 μl) was immediately mixed with 15 μl of MiniProtease inhibitor (Roche, Nutley, NJ) and placed on ice until centrifugation. Plasma was collected and stored at −80°C until analysis of insulin (Linco rat/mouse ELISA; Linco Research, St. Charles, MO), corticosterone (IDS Octeia rat/mouse ELISA; IDS, Fountain Hills, AZ), free fatty acids (Wako, Richmond, VA), leptin (Linco mouse ELISA), total and n-octanoyl ghrelin (Linco rat/mouse ELISA, cat. nos. EZG-DAC-87K and EZGAC-86K, respectively), and IGF-I (DSL IGF-I mouse/rat ELISA, Webster, TX). Plasma and media samples from in vitro experiments were analyzed for GH concentrations using the DSL mouse/rat GH ELISA (DSL, Webster, TX).

**RESULTS**

As previously reported (6, 15, 20, 48), treating ob/ob mice with leptin significantly reduced food intake and body weight.
compared with vehicle-treated controls (Fig. 1, A and B, respectively), and pair feeding ob/ob mice to match the food intake of leptin-treated mice resulted in a proportional weight loss (Fig. 1B and Table 1). Both leptin replacement and pair feeding significantly reduced circulating levels of insulin, glucose, and free fatty acid (Table 1). However, the impact of pair feeding on circulating insulin was less than that observed following leptin replacement. Corticosterone levels did not differ between vehicle-treated and pair-fed ob/ob mice, whereas leptin replacement significantly suppressed corticosterone to less than that observed in the pair-fed group (Table 1). Also consistent with an earlier report (48), leptin replacement reduced hypothalamic NPY mRNA levels to less than that observed in pair-fed controls (Fig. 2C).

Hypothalamic GHRH and SST mRNA levels in leptin-treated ob/ob mice did not differ from vehicle-treated controls (Fig. 2, A and B, respectively). However, leptin did prevent the fall in hypothalamic GHRH mRNA (Fig. 2A) observed in pair-fed mice. At the pituitary level, leptin replacement, but not pair feeding, enhanced GH and GHRH-R mRNA levels compared with vehicle-treated controls (Fig. 2, D and E, respectively). Leptin also increased pituitary GHS-R mRNA levels; however, these values did not differ from pair-fed controls, indicating that this change is due in part to secondary effects of decreased food intake and weight loss (Fig. 2F). This overall stimulatory effect of leptin on somatotrope function was reflected by an increase in circulating GH levels (Fig. 2G); however, this effect did not reach statistical significance due to the small sample size and the pulsatile nature of GH release (31, 37).

Our laboratory has reported previously (29) that circulating levels of total ghrelin and pituitary GHS-R expression are reduced in ob/ob mice compared with lean controls. Since ghrelin acts through the GHS-R at both the hypothalamic and pituitary level to stimulate GH release (23), it is possible that the changes in GH axis function observed in the ob/ob mouse may be related to changes in ghrelin synthesis, release, or sensitivity. To explore this possibility, we evaluated in detail the effect of genotype, leptin replacement, and pair feeding on...
circulating ghrelin levels and tissue-specific ghrelin expression, and the results are presented in Figs. 4 and 5. Consistent with the decrease in total ghrelin output previously observed by our laboratory and others (3, 29), n-octanoyl ghrelin and stomach ghrelin mRNA levels were also suppressed in ob/ob mice compared with lean controls (Fig. 4, A and B, respectively). Leptin replacement, but not pair feeding, increased n-octanoyl ghrelin levels (Fig. 4D) and prevented the fall in total circulating ghrelin levels observed in pair-fed ob/ob mice (Fig. 4C). Ghrelin mRNA levels were also suppressed in the hypothalamus and pituitary of ob/ob mice compared with lean controls (Fig. 5). The positive effects of leptin replacement on circulating ghrelin concentrations were not matched with respect to hypothalamic and pituitary regulation of ghrelin expression (Fig. 5). It should also be noted that hypothalamic GHS-R mRNA levels were not altered by genotype, leptin treatment, or food restriction (data not shown).

**DISCUSSION**

Our laboratory (29) has reported previously that hypothalamic GHRH mRNA levels do not differ between ob/ob mice and lean controls, indicating that leptin is not required for basal GHRH expression in the mouse. These results are in contrast to observations made in the fa/fa rat (2, 10, 54, 55), where defects in leptin signaling result in a decrease in hypothalamic GHRH production. Therefore, under basal conditions, the impact of leptin on GHRH expression is model (species) dependent. However, the results of the present study reveal that leptin replacement can prevent the fall in GHRH expression observed in food-restricted ob/ob mice, supporting a stimulatory role for leptin in central regulation of GH axis function in response to nutrient deprivation similar to that observed in the fasted rat (8, 9, 59). Data have accumulated suggesting that a positive effect of leptin on GHRH expression in nutrient-deficient states may be mediated indirectly by suppression of NPY. A functional link between leptin, NPY, and GHRH on fasting-mediated changes in GH release is based on the observations that NPY neurons located in the arcuate nucleus express the ObR (14, 35, 49), central infusion of leptin can block the fasting-induced rise in NPY as well as the fall in GHRH mRNA (8, 59), central infusion of NPY decreases GHRH mRNA levels in the fed state in both rats and mice (42, 43), and fasting does not suppress GHRH mRNA levels in the NPY knockout mouse despite the fall in circulating leptin levels (12, 39).
present study, leptin replacement did suppress NPY mRNA levels compared with pair-fed controls. Taken together, we might speculate that leptin-mediated suppression of NPY neuronal activity allows for sustained GHRH expression. However, it should be noted that hypothalamic GHRH mRNA levels are not altered in fed ob/ob mice, compared with lean controls, despite an elevation in hypothalamic NPY expression (29). Therefore, the role of NPY in mediating GHRH expression is dependent on nutrient availability. This conclusion is supported by our previous findings (39) demonstrating that GHRH mRNA levels do not differ between NPY-intact and NPY knockout mice in the fed state, whereas the absence of endogenous NPY protects against the fasting-induced decline in GHRH expression.

Leptin-specific effects on corticosterone may also contribute to the maintenance of GHRH neuronal function in the ob/ob mouse. It has been shown previously (15, 20) that corticosterone levels are elevated in the ob/ob mouse and that leptin replacement, but not pair feeding, significantly reduces glucocorticoid output. A similar pattern was observed in the present study; however, in our report, the inhibitory effect of leptin on corticosterone levels, compared with vehicle-treated controls, did not achieve statistical significance. Leptin is thought to suppress adrenal-axis function by suppressing ACTH release (1) and by directly inhibiting basal and ACTH-stimulated steroidogenesis (5, 41). It is well recognized (11) that hypercortisolemia, associated with Cushing syndrome or long-term immunosuppressive therapy, reduces circulating GH levels in humans. Also, prolonged treatment with high doses of dexamethasone decreases basal GH release and hypothalamic GHRH mRNA levels in rats (24, 50, 58). Taken together, we might speculate that the ability of leptin to reduce circulating glucocorticoids and hypothalamic NPY mRNA levels, relative to pair-fed mice, might contribute to the maintenance of GHRH neuronal activity in the nutrient-deprived state.

It is well established (25) that GHRH enhances somatotrope function by stimulating the expression and release of GH as well as enhancing expression of its own receptor. Therefore, the leptin-mediated upregulation of pituitary GH and GHRH-R expression observed in the present study would be consistent with enhanced GHRH input. In addition, the leptin-mediated fall in circulating insulin levels may also contribute to enhanced somatotrope function, since insulin has been shown to inhibit GH, GHRH-R, and GHS-R gene expression and GH release in primary pituitary cell cultures (28, 29, 60). It should be noted that both leptin replacement and pair feeding significantly reduced circulating insulin levels; however, leptin was more effective in this regard, lowering insulin to less than one-half that observed in pair-fed mice. It is also possible that leptin directly mediates somatotrope function, since pituitaries have been shown to express the ObR (21, 22). However, the direct effects of leptin on GH synthesis and release are con-

Fig. 5. Effect of genotype [lean (+/?) vs. ob/ob; A and B] and leptin replacement and pair feeding of ob/ob mice (C and D) on hypothalamic and pituitary ghrelin mRNA levels. Ghrelin mRNA copy number was determined by qRT-PCR and the values adjusted by cyclophilin A copy number as an internal control and expressed as either %lean (+/?; A and B) or vehicle-treated ob/ob mice (C and D) set at 100%. Values represent means ± SE. Group means that do not share a common letter (a or b) differ statistically. P < 0.05 was considered significant.
troversial in that leptin has been shown (4, 13, 36, 45, 46, 61) to either increase, decrease, or have no effect on basal and stimulated GH release and GH mRNA levels, depending on the dose used, duration of treatment, and animal model tested. In the present study, in vitro treatment of primary mouse pituitary cell cultures with a dose of leptin that mimicked levels achieved in the leptin-treated ob/ob mouse failed to alter GH, GHRH-R, or GHS-R expression. However, this same dose of leptin did enhance GH release. These data indicate that in vivo leptin might contribute directly to enhance GH secretory vesicle release, whereas leptin effects on somatotrope expression of GH and GHRH-R may be due to indirect effects on central and systemic inputs. However, we cannot exclude the possibility that the in vitro conditions used in the present study were not optimum to observe leptin effects on transcriptional regulation.

Another factor that may influence leptin-mediated regulation of GH axis function is ghrelin. The primary source of circulating ghrelin is the gastrointestinal tract, and under normal circumstances ghrelin levels rise just prior to a meal and in response to food deprivation (23). Ghrelin circulates in two forms, desacylated and acylated (n-octanoyl), but only the n-octanoyl form of ghrelin binds and activates the GHS-R1a and is responsible for positive effects of ghrelin on GH release both at the hypothalamic and pituitary levels (23). Centrally, ghrelin has been shown to increase GHRH neuronal activity (53), whereas a positive effect of ghrelin on GHRH expression is supported by the recent findings (32) that transgenic rats with attenuated expression of hypothalamic GHS-R1a levels have reduced GHRH expression compared with wild-type controls. This effect may be direct in that the GHS-R1a is colocolated to GHRH neurons in the rat arcuate nucleus (33). At the level of the pituitary, ghrelin directly stimulates basal GH release and augments GHRH-stimulated cAMP production (23). Therefore, the leptin-induced rise in circulating n-octanoyl ghrelin and pituitary GHS-R expression observed in the present study could serve to enhance GH axis function at both the hypothalamic and pituitary level.

Although circulating levels of total and n-octanoyl ghrelin are suppressed in ob/ob mice compared with lean controls, as previously reported (3, 29) and confirmed in the present study, ghrelin levels are also suppressed in diet-induced obesity (40, 51), a state of hyperleptinemia. These divergent observations at first suggest that the low levels of ghrelin in the ob/ob mouse are due to the obese state independently of leptin. However, in the present study, we observed that leptin replacement increased circulating levels of n-octanoyl ghrelin, whereas pair feeding ob/ob mice did not. These results demonstrate that leptin has specific and positive effects on n-octanoyl ghrelin production independently of food intake. This stimulatory effect may be centrally regulated because adenoviral expression of a leptin transgene within the hypothalamus of normal rats was reported (38) to elevate circulating ghrelin levels independently of food intake and weight loss. Central expression of a leptin transgene in ob/ob mice was also shown (57) to be associated with elevated levels of ghrelin. In contrast, leptin has been shown (26) to directly inhibit ghrelin release in isolated perfused stomachs.

In summary, our present findings demonstrate that leptin replacement enhances multiple aspects of the hypothalamic-pituitary GH axis function and circulating ghrelin levels in ob/ob mice. At first glance, these observations appear counter-intuitive to the negative relationship between GH/ghrelin and leptin levels observed when normal mice are subjected to the metabolic extremes of acute fasting and diet-induced obesity. However, it should be emphasized that the ob/ob mouse is a model of complete leptin deficiency, resulting in severe metabolic disturbances that are unique to those observed in response to fasting (hypoileptinemia) and diet-induced obesity (hyper-leptinemia). Although we observed that leptin does have a direct stimulatory effect on pituitary GH release, we provide evidence that suggests some of the effects of leptin on GH axis endpoints are indirect and related to its ability to rapidly “normalize” metabolic function.

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