

Leptin Inhibition of the Hypothalamic-Pituitary-Adrenal Axis in Response to Stress*

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ABSTRACT

Leptin is a newly identified protein hormone that is synthesized and secreted by adipose tissue. Absence of the mature hormone is responsible for the obese phenotype of *ob/ob* mice. The hypothalamic-pituitary-adrenal axis (HPAA) is activated in *ob/ob* mice, and chronic administration of leptin to *ob/ob* mice decreases plasma corticosterone levels, suggesting that the adipose hormone is capable of inhibiting the HPAA. The aim of this study was to determine whether leptin feeds back acutely to inhibit the HPAA of normal mice and rats. Male C57BL mice were injected ip with 100 μ l saline and 2 or 4 μ g/g BW mouse leptin in saline vehicle, and 4 h later they were subjected to 2 h of restraint stress by taping the hind limbs together or no stress. Hind leg restraint stimulated the HPAA as measured by significant ($P < 0.05$) elevation of both ACTH and corticosterone. Pretreatment with recombinant mouse leptin blocked the stress-mediated stimulation of both plasma hormones. To determine whether this inhibition was exerted at the hypothalamic level through inhibition of CRH, we studied leptin action on isolated rat hypothalamus perfused with

Krebs-Ringer buffer containing glucose (5.5 mM). CRH secretion was stimulated by decreasing the glucose concentration of the buffer to 1.1 mM. A surge of CRH was released over a 2-h period (basal integrated release was 14.4 ± 1.6 pg/2 h, $n = 5$ and increased to 34.7 ± 3.1 pg/2 h, $n = 14$). This response was blocked by mouse leptin in a dose-dependent manner (integrated stimulated CRH secretion was 30.6 ± 2.5 pg/2 h, $n = 5$; 20.5 ± 3.6 pg/2 h, $n = 7$; 15.3 ± 4.3 pg/2 h, $n = 3$ for 1 nM, 3 nM and 30 nM, respectively). Leptin did not alter secretion of ACTH from rat primary cultured pituitary cells. These data demonstrate that leptin can inhibit hypothalamic CRH release, either directly or indirectly through another hypothalamic neuropeptide such as neuropeptide-Y. Dysfunctional leptin, insufficient leptin levels, or leptin resistance should each result in a partial open loop, thereby accounting for elevated glucocorticoid levels that accompany and contribute to many obese phenotypes. Leptin's ability to inhibit CRH release is the likely explanation for its ability to inhibit activation of the HPAA in response to stress. (*Endocrinology* **138**: 3859–3863, 1997)

A NEW gene that is thought to function as an integral component in the physiological system for regulation of body fat stores was identified late in 1994 (1). Leptin, the protein hormone encoded by this *obese* gene, is synthesized in adipose tissue and is secreted into the blood (2). Synthesis (3–5) and secretion (5) of leptin are stimulated by glucocorticoids, and receptors for leptin have been reported in the hypothalamus (6–8), where they transduce the signal to neuropeptide-Y (NPY) neurons of the arcuate nucleus (9, 10). Leptin is capable of both rapid and more prolonged actions, because it inhibits both release (9) and synthesis of NPY message (9, 10). Recently, Glaum *et al.* (11) reported that the rapid actions of leptin are likely a result of inhibition of excitatory postsynaptic membrane potentials of NPY neurons.

The mutation in the *obese* gene (*ob/ob* mice) results in profound obesity, hyperinsulinemia, and hypercorticosteronemia (12). A similar phenotype is observed in *db/db* mice (6) or *fa/fa* rats (6,8) suffering from mutations in the leptin receptor. Chronic leptin replacement in *ob/ob* mice but not *db/db* mice corrects the hypercorticosteronemia (9), and injections

of leptin in wild-type mice blunt the fasting-mediated surge in plasma ACTH and corticosterone (13). Whether leptin is capable of providing important feedback to the hypothalamic-pituitary-adrenal axis (HPAA) during stress is unknown. In this report we demonstrate that leptin can blunt the stress-induced activation of the HPAA, and it is capable of exerting this effect at the hypothalamic level through inhibition of CRH release. Such inhibition could complete a HPAA feedback loop that extends the axis to include adipose tissue and leptin.

Materials and Methods

Leptin inhibition of HPAA response to restraint stress

Male C57BL mice (Jackson Labs., Bar Harbor, ME), age 8 weeks were housed in groups of 4 under 12 h light (0600–1800 h) and 12 h dark (1800–0600 h) and were allowed free access to chow and water for 2 weeks until use. The animal facility where these studies were performed is a fully accredited, institutional member of the American Association for the Accreditation of Laboratory Animal Care and provides a committee that approved the protocol used. Mice were divided into four groups ($n = 8$ /group), and handling was minimized to cage cleaning. One group was injected ip with 100 μ l saline between 0700–0800 h of the light cycle. The others were injected with saline and 2 or 4 μ g/g BW mouse leptin [biosynthetically prepared in *Escherichia coli* (see Ref. 9)] in 100 μ l saline vehicle (0700–0800 h). Two hours later they were subjected to 2 h of restraint stress by taping the hind limbs together. They were then killed by decapitation 4 h after injection, and plasma was obtained for measurement of ACTH, corticosterone, and leptin with RIA kits from Diagnostic Products Corp. (Los Angeles, CA), ICN Biomedicals (Costa

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Mesa, CA), and Linco Research (St. Charles, MO) as described previously (13).

Hypothalamic perfusion

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing from 250–300 g, were acclimated for at least 2 weeks in an identical environment to that described above. The animal facility in which this study was performed is a fully accredited, institutional member of the American Association for the Accreditation of Laboratory Animal Care and provides a committee that approved the animal use protocol used in this part of the study. Five rats were housed in each cage that had water and food (Ralston-Purina, St. Louis, MO) continuously available. Rats were killed by decapitation, and the brain was quickly removed.

A region bordered dorsally by the thalamus, rostrally by the optic chiasm, and caudally by the mamillary bodies was excised and bisected sagittally through the third ventricle. Hemihypothalamic sections were randomly assigned to one of four wells containing 3 ml Krebs-Ringer bicarbonate buffer (KRB) with 5.5 mM glucose (KRBhG) that was placed on ice. A total of 20 hemisections (10 hypothalami) per well were washed twice with KRBhG at room temperature and transferred to 1.5 ml Acusyst-S micro chambers (Endotronics, Minneapolis, MN) containing 0.8 ml KRBhG. This buffer was pumped to each of four chambers simultaneously at 100 μ l/min under an atmosphere of O₂/CO₂ 95:5% at 37 C. The time lag for buffer to reach chambers is 10 min. We stimulated CRH release by decreasing the glucose concentration (KRB containing either 2.8 mM or 1.1 mM glucose, KRBLoG) after 180 min. Mouse leptin was added during this 60-min challenge period. Treatments were then washed out by changing back to KRBhG for a final 30 min. Perifusate was collected at 30-min intervals into tubes containing 750 μ l 1 M trifluoroacetic acid (TFA; Aldrich, Milwaukee, WI) and rapidly frozen.

Fractions were thawed, and CRH was extracted and concentrated using Isolute solid phase C-18 columns (International Sorbent Technology, Mid-Glamorgan, UK). After conditioning the columns with 7 ml H₂O, 7 ml MeOH, and 7 ml 0.1% TFA, the perifusates were applied. The columns were then washed with 5 ml 0.1% TFA, and CRH was eluted with 4 ml 60% acetonitrile (Mallinckrodt, Paris, KY) in 0.1% TFA. Eluant was evaporated with a Speed Vac concentrator (Savant Instruments, Farmingdale, NY). Neuropeptide was reconstituted with 250 μ l (16-fold concentration) RIA buffer [0.05 M PBS containing 0.01% BSA (Sigma, St. Louis, MO), 0.01% sodium azide (Sigma), and 0.001% Triton X-100 (Sigma)]. At least 95% of CRH could be recovered by such extraction.

Duplicate 100- μ l determinations were made for each fraction by standard RIA. [¹²⁵I]-CRH was purchased from Dupont NEN (Boston, MA). CRH primary antisera, normal rabbit serum, and goat antirabbit IgG were purchased from Peninsula Laboratories (Belmont, CA) and diluted as instructed by the manufacturer. Coefficients of variation calculated for a set of standards was less than 12% for both inter- and intraassay measurements.

Primary pituitary cell culture

Rat anterior pituitary cells were dispersed and cultured as described previously (14) except for the following modifications. Pituitary sections were collected in Spinner's MEM (S-MEM; GIBCO-BRL, Grand Island, NY) and washed. Tissue was sectioned into eights and then placed into 15-ml conical centrifuge tubes containing 10 ml S-MEM (20 hypophyseal equivalents per tube). Fragments were allowed to settle and were washed once. Medium was discarded and 10 ml S-MEM (pH = 7.8)

containing BSA (1 mg/ml), NaHCO₃ (12 mM), HEPES 13 mM, DNase (15 μ g/ml; Sigma), and trypsin 1:250 (3 mg/ml) (DIFCO, Detroit, MI) was added. Tubes containing tissue were slowly inverted (10 rpm) at 37 C for 20 min and then centrifuged for 5 min at 200 \times g. Medium was aspirated, discarded, and pellets were resuspended in 4 ml S-MEM containing lima bean trypsin inhibitor (1 mg/ml) (Worthington, Freehold, NJ) and DNase (0.1 mg/ml). Cells were dispersed by pipetting with a Pasteur pipette, and pituitary fragments were allowed to settle before transferring the cell suspension to a 50-ml conical centrifuge tube. This cell dispersion was repeated two more times. Pooled dispersed cells were filtered through a series of three Swinnex (Millipore, Bedford, MA) filter units containing Nylon mesh of 74, 20, and 15 μ m (Tetko, Elmsford, NY). Each filter unit was rinsed with 1 ml S-MEM, and the cells were centrifuged for 5 min at 1000 \times g. Supernatant was aspirated, discarded, and the collected cells were resuspended with 8 ml S-MEM containing DNase and lima bean trypsin inhibitor. Cells were cultured and washed as described previously (14), except that fresh rat serum was omitted. Cultured cells were washed with α -MEM and challenged with the same medium containing mouse leptin at concentrations ranging from 0–1 μ M. After 3 h at 37 C, medium was removed and stored at –20 C until assayed for ACTH by RIA (Diagnostic Products Corp.).

Data and statistical analyses

CRH released was integrated using the trapezoidal rule (SigmaPlot; Jandel Scientific, San Rafael, CA). Integrated release is presented as mean \pm SEM, and treatment groups were compared by ANOVA followed by Scheffé's F test (StatView; BrainPower, Calabasas, CA). Plasma leptin, ACTH, and corticosterone levels were compared by ANOVA and Fisher's protected least significant differences test (PSLD). Significance was accepted at $P < 0.05$.

Results

Leptin inhibition of HPAA response to stress

Restraint of the hind legs for 2 h significantly stimulated the HPAA as evidenced by a dramatic increase in both ACTH and corticosterone ($P < 0.05$; Table 1). Pretreatment with 2 μ g/g BW mouse leptin significantly attenuated the plasma corticosterone response to stress, but plasma ACTH and leptin values were not different than stressed controls. The higher dose (4 μ g/g BW), however, was capable of blocking stress-mediated stimulation of both plasma ACTH and corticosterone. Plasma leptin levels at the time HPAA hormones were measured (4 h after injection) were significantly elevated by only the 4- μ g/g dose.

Leptin inhibition of CRH release

Decreasing the glucose concentration in perfusion medium from levels typical of normoglycemia (5.5 mM) to those characteristic of severe hypoglycemia (1.1 mM) was a potent and concentration-dependent stimulus for CRH release (Fig. 1). The hypoglycemic stimulus also released GHRH and GH-release-inhibiting factor (SRIF) but does not stimulate

TABLE 1. Leptin inhibition of HPAA response to restraint stress

Treatment	Plasma		
	Corticosterone (ng/ml)	ACTH (pg/ml)	Leptin (ng/ml)
Control	101 \pm 15.9 ^a	78.5 \pm 9.7 ^a	4.6 \pm 0.7
Stress + saline	343 \pm 28.1	365 \pm 63.9	7.1 \pm 2.1
Stress + 2 μ g/g BW leptin	266 \pm 23.6 ^a	273 \pm 383.8	7.1 \pm 1.0
Stress + 4 μ g/g BW leptin	196 \pm 33.6 ^a	174 \pm 28.5 ^a	15.1 \pm 1.8 ^a

Male C57BL mice (8/group) were injected with saline or leptin 2 h before restraint stress. Plasma levels of circulating hormone were measured by RIA 2 h after initiation of the stress. Values represent mean \pm SEM.

^a $P < 0.05$ compared with restraint stress + saline.

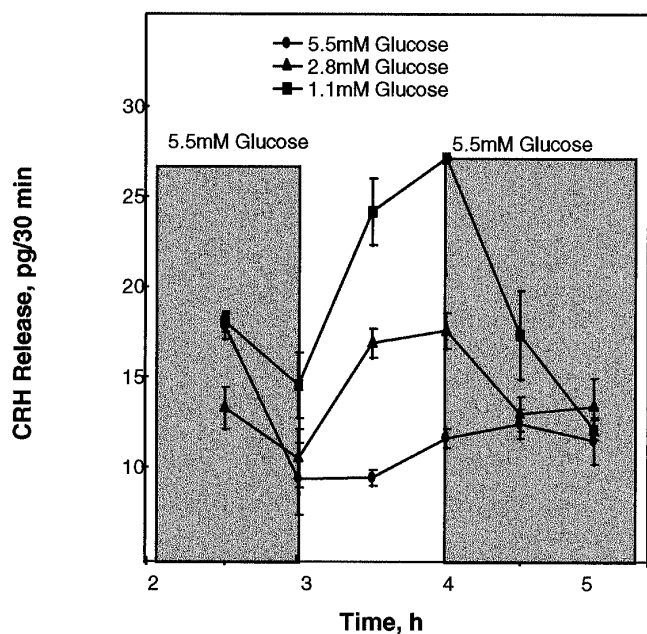


FIG. 1. Hypoglycemia-mediated CRH secretion from rat hypothalami in perfusion. Values represent mean CRH secreted \pm SEM during a 30-min period from 20 hypothalamic bisections (equivalent to 10 hypothalami). After 3 h perfusion with buffer containing 5.5 mM glucose, hypothalami were challenged by decreasing glucose concentration to 2.8 mM or 1.1 mM. A chamber of hypothalami remained at 5.5 mM glucose throughout the experiment. Initial perfusion buffer was restored after 60 min. These data are representative of four experiments.

secretion of NPY nor GnRH (data not shown). Stimulated integrated release during this challenge period was 34.7 ± 3.1 pg/2 h ($n = 14$) and was significantly ($P < 0.05$) greater than basal integrated release during the same period [14.4 ± 1.6 pg/2 h ($n = 5$)]. Such stimulated release was inhibited by leptin in a dose-dependent manner (Fig. 2 and Table 2). Integrated CRH secretion when 30 nM leptin was added in the presence of 1.1 mM glucose was not different than that measured under nonstimulated (5.5 mM glucose) conditions ($P > 0.05$) and was near the limit of detection (8 pg CRH/2 h).

Failure of leptin to directly alter ACTH secretion

To examine whether leptin inhibits pituitary release of ACTH directly, we added the protein hormone to rat primary pituitary cells. These cells responded to CRH (0.5 nM) stimulation with more than an 8-fold increase in medium ACTH (Table 3). However, neither basal nor 0.5 nM CRH-mediated ACTH secretion were altered by presence of 1 μ M leptin.

Discussion

Obesity is associated with increased HPA activity and resulting hypercorticism in some humans (15, 16) and in many rodent models including *fa/fa* Zucker rats (17, 18) and *ob/ob* mice (12). The etiology of obesity observed in *ob/ob* mice involves the failure to produce mature leptin (1), and replacement of leptin by exogenous administration to *ob/ob* mice corrects the hypercorticism (9). Obesity in both *db/db* mice and *fa/fa* rats is a consequence of mutations in the leptin

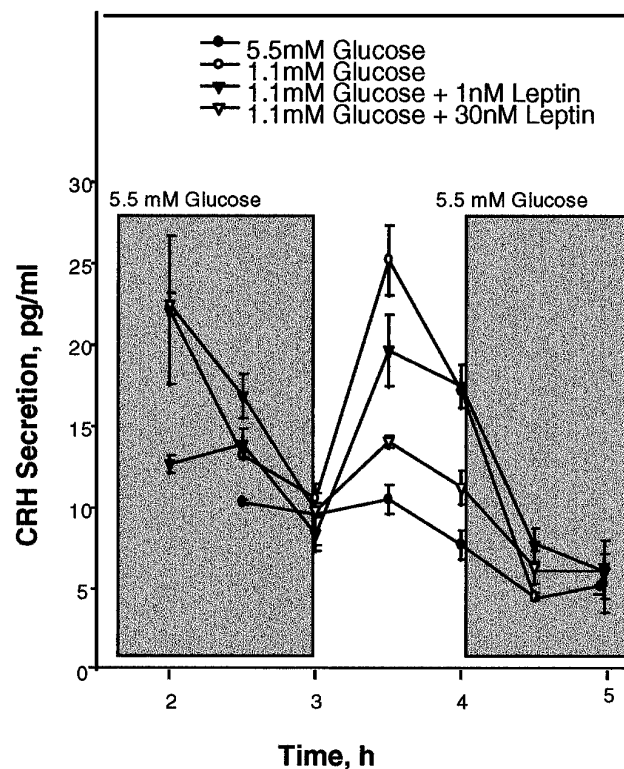


FIG. 2. Leptin inhibition of hypoglycemia-mediated CRH secretion from rat hypothalami in perfusion. Values represent mean CRH secreted during a 30-min period from 20 hypothalamic bisections (equivalent to 10 hypothalami). After 3 h perfusion with buffer containing 5.5 mM glucose, hypothalami were challenged by decreasing glucose concentration to 1.1 mM with and without addition of mouse leptin. Initial perfusion buffer was restored after 60 min. A chamber of hypothalami remained at 5.5 mM glucose throughout the experiment. These data are representative of 3–14 similar experiments.

TABLE 2. Leptin inhibition of hypoglycemia-stimulated CRH release

Leptin dose (nM)	Integrated CRH release (pg/2 h)	n	P
0	34.7 ± 3.1	14	
1	30.6 ± 2.5	5	>0.05
3	20.5 ± 3.6	7	<0.05
30	15.3 ± 4.3	3	<0.05

CRH secretion from hypothalamic hemisections was integrated during period of exposure to both hypoglycemia and leptin (challenge period indicated in Fig. 1) ranging from 0–30 nM. Values represent mean \pm SEM of 3–14 separate experiments (n). Leptin concentrations of 3 nM and 30 nM significantly inhibited CRH release.

receptor gene (6–8). Human obesity does not appear to be caused by mutations in the *ob* gene or insufficient *ob* expression (19) but could involve leptin resistance (20). Our data are the first to directly demonstrate that leptin feeds back to the HPA to inhibit release of CRH. Resistance to leptin or failure to secrete mature leptin would be expected to result in hypercorticism by compensatory increases in CRH secretion.

Stress is the classic stimulus to the HPA (for review see Ref. 21). One type of stress, starvation, markedly stimulates this endocrine axis (22). Administration of leptin to fasting wild-type mice attenuates stimulation of this axis, and both

TABLE 3. Leptin does not inhibit ACTH secretion from rat primary pituitary cells

Leptin dose (nM)	CRH dose (nM)	Medium ACTH (pg · ml · 3 h)	n	P
0	0	1496.4 ± 34.9	3	
10	0	1648.5 ± 230.7	3	>0.05
100	0	2090.3 ± 74.9	3	>0.05
1000	0	1920.7 ± 155.1	3	>0.05
0	0.5	12840.8 ± 628.6	3	<0.01
1000	0.5	13241.8 ± 470.2	3	<0.01

ACTH secretion from rat primary pituitary cultured cells was measured after 3 h exposure to CRH, leptin, both, and neither. Values represent mean ± SEM of three separate experiments (n). Only CRH significantly altered ACTH secretion.

ACTH and corticosterone levels are almost normalized to that of the fed state (13). Stress induced by immobilization is another means to activate the HPA (23, 24). Our data demonstrate that as in starvation, leptin attenuates restraint stress-induced increases in plasma ACTH and corticosterone. We also demonstrate that such inhibition may be exerted at the hypothalamic level, because the adipose hormone directly inhibited CRH release in response to hypoglycemia, and because it did not directly alter ACTH secretion. That hypothalamic CRH secretion is a counter-regulatory response to prevent hypoglycemia has been demonstrated *in vivo* (25–27) and *in vitro* (28). We confirm those *in vitro* data, and demonstrate that leptin is capable of impeding such CRH release. Leptin may inhibit CRH release as opposed to changing messenger RNA (mRNA) levels, because daily leptin administration to *ob/ob* mice for 5 days did not alter expression of CRH mRNA in the paraventricular nucleus (PVN) (10). Unfortunately, neither plasma corticosterone nor ACTH were measured in that study, however, treatment of *ob/ob* mice with leptin for 30 days did significantly decrease corticosterone levels in plasma (9). In addition, acute intracerebroventricular injections of leptin to normal rats fasted for 40 h increased CRH mRNA levels in PVN without increasing plasma corticosterone (29). To reconcile these divergent findings, it is possible that the rapid action of leptin to decrease the readily releasable storage pool of hypophysiotropic CRH is independent of actions of leptin to influence hypothalamic CRH mRNA in the PVN. Further, the PVN is functionally and morphologically divided into several divisions (30). Neurons that project to the median eminence are concentrated in medial regions of the parvocellular subdivision (31, 32). Neurons of the dorsal, ventral, and lateral parvocellular subdivision give rise to descending inputs of autonomic centers (33). These autonomic subdivisions contain neurons of several phenotypes, including those that express arginine vasopressin, oxytocin, and CRH (33). Interestingly, induction of Fos protein after acute *iv* leptin administration was detected in only these latter autonomic regions of the PVN (34). Fos immunoactivity was not noticeably altered in the medial subneurons, which contain CRH and project to the median eminence. Conversely, Van Dijk and colleagues (35) found a prominent induction of Fos protein in the PVN following intracerebroventricular infusion of leptin. However, they did not specifically describe the subnuclear distribution of Fos. Moreover, intracerebroventricular administration creates a fundamentally different model than *iv* injection, because it is still unclear how and to what extent leptin gains access to different brain regions.

These data demonstrating a rapid action of leptin to inhibit release of CRH independent of changes in gene expression are consistent with the recent demonstration that leptin produced a robust inhibition of excitatory postsynaptic membrane potentials in NPY neurons of the arcuate nucleus (11). They are also consistent with a recent demonstration in human that leptin levels in blood are pulsatile and are temporally related in an inverse fashion to levels of ACTH and cortisol (36).

The HPA is an endocrine regulatory system that responds quickly to stress (for review see Ref. 21). Further, the system adapts to chronic stress, so that further responsiveness of the axis is maintained. Negative feedback control of the HPA by glucocorticoids even during stress is well documented (21). Our data indicate that leptin could provide a further source of negative feedback inhibition to this axis. It has been proposed that adipose tissue participates in the adaptation to starvation by both supplying stored calories and by decreasing secretion of leptin (13). We now extend the concept of leptin as a regulator of the HPA to include another classical paradigm of stress, and have demonstrated that the site of inhibition may be hypothalamic secretion of CRH. Further study of leptin interactions with the HPA is necessary to clarify this concept.

In summary, we demonstrated that leptin inhibits CRH release from the hypothalamus *in vitro*, as well as blunted the plasma ACTH and corticosterone responses to restraint stress *in vivo*. We speculate that this feedback is an important component in the HPA, and we propose that this axis now be extended to include adipose tissue and leptin. In addition to the classical endocrinology of the HPA, glucocorticoids are capable of stimulating leptin synthesis and secretion (3–5). Circulating leptin could then limit activity of the HPA by inhibiting CRH release. During acute and chronic stress, leptin secretion may decrease, and thus facilitate the responsiveness of the HPA, which appears to be important for survival. Future studies are needed to test this speculation of a hypothalamic-pituitary-adrenal-adipose axis. If confirmed, dysfunctional leptin, insufficient leptin levels, and leptin resistance could all contribute to increased hypothalamic-pituitary-adrenal-adipose axis activity and hypercorticism.

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