Regulation of Expression of ob mRNA and Protein by Glucocorticoids and cAMP*

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Lawrence J. Slieker, Kyle W. Sloop, Peggy L. Surface, Aidas Kriauciunas, Frank LaQuier, Joseph Manetta, J ulie Bue-Valleskey, and Thomas W. Stephens

From the Endocrine Research and Technology Core Divisions, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

Regulation of obese gene (ob) expression in ob/ob and db/db mice and in cultured rat adipocytes was examined. It has been demonstrated that exogenous human OB protein (leptin) treatment reduces food intake and weight gain, as well as insulin, glucose, and corticosterone levels in ob/ob mice. In the present report we show that leptin treatment down-regulates endogenous adipose ob mRNA. However, treatment of isolated rat adipocytes with 100 ng/ml human or murine leptin had no direct effect on expression of endogenous ob mRNA, suggesting that leptin may be able to down-regulate its own expression by an indirect, non-autocrine mechanism. Glucocorticoids increased both ob mRNA levels and secreted leptin levels in vitro. Conversely, agents that increase intracellular cAMP, such as β-adrenergic agonists or Bt2CAMP itself, decreased ob mRNA expression and leptin secretion. Therefore, increased glucocorticoid levels and decreased sympathetic neural activity may contribute to the elevated ob mRNA expression observed in genetically obese, hyperglucocorticoid rodents. Furthermore, leptin might regulate its own expression through a feedback mechanism involving the hypothalamic pituitary axis.

Obesity is a major risk factor for a number of human diseases including cardiovascular disease, hypertension, and non-insulin-dependent diabetes. Although obesity in humans is apparently a polygenic disorder, numerous rodent models of obesity exist as single gene mutations. Several of these which have recently been identified include the agouti gene (1, 2), the fat gene (3), and the ob (obese) gene (4). The ob locus on chromosome 6 involves two mutations in the gene for a secretory protein, which result in either premature termination (sense mutation at Arg-105) in the original C57BL/6j ob/ob strain or complete absence of message in the SM/Ckc-1 mouse (5) in the original C57BL/6j ob/ob strain (4). The human homolog has been cloned (4, 5), and there appears to be no evidence of obesity-associated mutations in human OB comparable with the ob mouse (5) nor any linkage between OB mutations and susceptibility to non-insulin-dependent diabetes (6). The ob mutation was originally thought to involve a diminished satiety signal or factor based on parabiosis experiments in both ob/ob and db/db mice (7), which suggested that db/db mice did not express the satiety signal while db/db mice were defective in the signal transduction pathway. Recent experiments have supported this by demonstrating that exogenous recombinant mouse Ob protein and human OB protein decrease food intake and weight gain in the ob/ob, but not in the db/db, mouse (8–11). It has recently been suggested that the ob gene product be referred to as leptin (9).

Despite the evidence that leptin acts as a satiety factor, human obesity (5) and other forms of rodent obesity that involve different genetic loci, such as the AY mouse (12), or non-genetic lesions in the ventromedial hypothalamus (13, 14) are paradoxically associated with elevated levels of leptin expression. For this reason, we investigated the regulation of ob expression in ob/ob and db/db mice, and in 24 h cultures of mature rat adipocytes to determine what obesity associated factor(s) might regulate leptin expression.

**MATERIALS AND METHODS**

Animal Treatment—Five- to six-month-old obese (C57Bl/6j-ob/ob and C57Bk/Ks-db/db) mice were obtained from Jackson Laboratories, Bar Harbor, ME or Harlan, U.K. Mice were housed three to six per cage; water and Purina Formula Chow 5008 (Purina Mills, St. Louis, MO) were available ad libitum. Animals were maintained on a normal 12 h light/12 h dark cycle. Recombinant human leptin or saline was administered subcutaneously at 300, 200, and 100 μg/mouse/day for 9, 2, and 19 days, respectively, 1 h before the dark cycle as described previously (11). Blood samples for glucose, insulin, and corticosterone determinations were obtained between 8:00 and 10:00 a.m. at the end of the experimental period. Epididymal fat pads were processed as described below for Northern blot. Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). A full presentation of food consumption and body weight change data, as well as glucose, insulin, triglyceride, and glucocorticoid measurements, has been published elsewhere (11).

Isolation and Culture of Mature Rat Adipocytes—Adipocytes were obtained from rat epididymal fat pads from 250–300 g male rats by collagenase digestion (15). The cell suspension was filtered sequentially through 500-, 250-, and 100-μm mesh and washed 6 times with Dulbecco's modified Eagle's medium/Ham's F12 (3:1, Life Technologies, Inc.) supplemented with 20 μg/ml bovine serum albumin (RIA grade, Sigma), 20 μM Hepes, 0.1 μg/liter sodium selenite, and 4.88 mg/liter ethanolamine. Adipocytes were cultured in this medium for 24 h at approximately 5 × 10⁶ cells/ml in either Costar P6 wells or Corning 775 flasks. Medium was removed from beneath the adipocyte layer and stored at –20 °C until used for RIA and Western blot analysis. Adipocytes were used for RNA isolation as described below. Dibutyryl cAMP, isoprotroteren, dexamethasone, and hydrocortisone were obtained from Sigma. Recombinant human insulin was from Lilly.

Western Blot—10 ml of conditioned medium from 24-h cultures was treated with 100 μl of 20 mg/ml deoxycholate and 1.1 ml of 20% trichloroacetic acid (16). After centrifugation, the pellets were dissolved in 200 μl of Laemmi sample buffer, and 40-μl samples were run on 18% polyacrylamide gel electrophoresis gels (Novex, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes and blotted with affinity-purified antibody (1 μg/ml) that had been obtained in rabbits immunized by full-length recombinant murine leptin (11). Antisera were affinity-purified on agarose columns containing immobilized murine leptin (Aminolink immobilization kit, Pierce). Western blots were detected by the Supersignal chemiluminescent system (Pierce).

Northern Blot—Total RNA was isolated from rat adipocytes or from

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†To whom correspondence should be addressed: Endocrine Research Division, Drop Code 0540, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285.
Regulation of Expression of ob mRNA and Protein

We have previously reported that treatment of ob/ob and db/db mice with recombinant human leptin induced weight loss and reduced food consumption, and lowered glucose, insulin, and glucocorticoid levels in the ob/ob mice only (11). In the treated ob/ob mice, corticosterone levels were reduced 60% from 247 ± 9 to 95 ± 18 ng/ml (p < 0.05, n = 6) while insulin levels were reduced from 75 ± 28 ng/ml to <1.4 ng/ml, and glucose levels were reduced from 214 ± 16 mg/dl to 118 ± 7 mg/dl. Untreated lean Ob?/mice had glucose, insulin, and glucocorticoid values of 144 ± 12 mg/dl, <1.5 ng/ml, and 31 ± 6 ng/ml, respectively (11). In the db/db mice, neither glucose (676 ± 18 mg/dl), insulin (<1.5 ng/ml), nor corticosterone (318 ± 17 ng/ml) levels were altered significantly with treatment (11).

In the present study, Northern analysis of total RNA from epididymal fat pads of db/db mice that have a presumed central defect in its signal transduction, would suggest that leptin does not act directly at the adipocyte but instead through a centrally regulated mediator.

RESULTS AND DISCUSSION

Because of our initial observation that dexamethasone up-regulated ob mRNA expression in the ob/ob mouse, we examined a number of agents to investigate their role in regulating expression of leptin.

The development of a sensitive RIA allowed us to examine the number of agents to investigate their role in regulating expression of leptin. The limit of detection for this assay is approximately 0.5–1 ng/ml. Cross-reactivity between rat and mouse leptin was confirmed by showing that rat serum diluted in RIA buffer. Standards were prepared in RIA buffer and ranged from 0.25 to 500 ng/ml. After incubation at room temperature, tubes were centrifuged and decanted. Pellets were counted for 125I and data analyzed by RIA Aid™ (ICN Micromedic Systems, Huntsville, AL). In order to verify cross-reactivity of the anti-mouse leptin antibody with rat leptin, serum from obese Zucker fa/fa rats (100, 50, 25, 12.5, and 6.25 µl) was diluted into varying amounts of horse serum such that the total serum volume was 100 µl. Horse serum itself showed no detectable immunoreactivity with the anti-mouse antibody. The dilution curve generated from these samples was parallel over the entire range to a comparator standard curve generated from mouse leptin standards diluted in 100 µl of horse serum.

Fig. 1A, chronic effects of exogenous human leptin on endogenous murine ob mRNA expression in db/db and db/db mice. RNA was isolated and hybridized to 32P-labeled murine ob probe as described under “Materials and Methods.” RNA loading was evaluated by quantitation of 18 S RNA. B, graph quantitates db mRNA relative to 18 S RNA. Data are presented as relative ratios ± S.E. (n = 6). C, effect of 24-h incubation of rat adipocytes on endogenous ob mRNA levels: lane 1, control; lane 2, 25 nm dexamethasone; lane 3, 100 ng/ml human leptin; lane 4, 100 ng/ml mouse leptin. These data represent a single experiment.

However, these data do not rule out the possibility that a putative adipocyte leptin receptor is also defective in db/db mouse.

In order to examine direct (autocrine regulation at the adipocyte) versus indirect effects of leptin on mRNA regulation, we treated rat adipocytes in vitro with either 25 nm dexamethasone or 100 ng/ml human or mouse leptin for 24 h. Northern analysis (Fig. 1C) shows that leptin treatment did not alter endogenous ob mRNA levels but that 25 nm dexamethasone up-regulated ob expression dramatically. Therefore, the ability of exogenous human leptin to down-regulate endogenous expression of ob mRNA in the ob/ob mouse argues for an indirect mechanism, possibly secondary to reduced glucocorticoid levels. It should be noted, however, that our measurements are of steady-state mRNA levels, and in the absence of nuclear run-on experiments we cannot distinguish transcriptional regulation from effects on mRNA stability. The recent report that treatment of rats with dexamethasone and hydrocortisone increased adipocyte ob mRNA expression levels is consistent with these in vitro results (18).

The development of a sensitive RIA allowed us to examine a number of agents to investigate their role in regulating expression of leptin. The limit of detection for this assay is approximately 0.5–1 ng/ml. Cross-reactivity between rat and mouse leptin was confirmed by showing that rat serum diluted in parallel with the mouse leptin standard (data not shown). However, the absolute level of cross-reactivity has not been determined quantitatively with recombinant rat leptin standard. The anti-mouse leptin antibody also cross-reacted with secreted rat leptin in a Western blot. These results are reasonable based on the high degree of conservation of the protein sequence between rat and mouse leptin (96% at the protein level (13)) and suggest that this assay is suitable for measuring relative, if not necessarily absolute, changes in rat leptin levels.

Because of our initial in vitro observation that dexamethasone up-regulated ob mRNA expression, we examined a number of steroids, including glucocorticoids, mineralocorticoids, estrogens, and androgens, for their ability to regulate leptin expression and secretion (data not shown). Preliminary experiments indicated that glucocorticoids such as dexamethasone,
hydrocortisone, and corticosterone had a pronounced 3-4-fold stimulatory effect on leptin expression (as measured by RIA). Testosterone and aldosterone were relatively ineffective (data not shown), while 17β-estradiol increased leptin release approximately 2-fold. Fig. 2 shows a dose response for stimulation of leptin secretion by dexamethasone, hydrocortisone, and 17β-estradiol. Both glucocorticoids reach the same maximal response (3-3.5-fold increase over basal) with the synthetic glucocorticoid being more potent (EC50: dexamethasone, 3.3 nM; hydrocortisone, 24.7 nM). These values are consistent with other glucocorticoid-induced functions in adipocytes, such as inhibition of preadipocyte proliferation (19). Dexamethasone and hydrocortisone increased ob mRNA expression (Fig. 3A) as well as leptin secretion (Fig. 3, B and C).

Insulin treatment increased leptin secretion only 20-25% as measured by RIA (Fig. 3). Effects of insulin on ob mRNA expression have been variable, probably reflecting the difficulty in measuring small changes in RNA by Northern analysis. Therefore, the effect of insulin under these conditions appears modest. Incubation of adipocytes with 25 mM glucose had no effect on ob mRNA expression (data not shown), suggesting that ob expression may not be regulated directly by acute changes in glucose availability. A recent report by Saladin et al. (20) indicated that a single insulin injection or a hyperinsulinemic clamp of rats increased ob mRNA expression severalfold, independent of glucose concentration. In vitro treatment of adipocytes with insulin for 24 h also doubled ob mRNA. However, Murakami et al. (21) reported that in vitro insulin treatment of adipocytes increased ob mRNA only 10%. Insulin clearly increases leptin expression, but the degree would appear to be less than that of glucocorticoids.

Increases in intracellular cAMP result in decreased expression of ob mRNA and leptin secretion in rat adipocytes. This is demonstrated in Fig. 3, where it is shown that dibutyryl cAMP, the non-selective β-adrenergic receptor agonist isoproterenol, and the selective β3 agonist ICI 201,651 (22) all reduce ob mRNA and protein expression. This would suggest that when lipolysis is stimulated in adipocytes, leptin expression is reduced. This hypothesis is consistent with decreased expression of a satiety signal under conditions of starvation. A recent report has demonstrated that fasting of lean Ob/ob mice (but not Ob/Ob mice) is associated with a reduction of ob mRNA levels, which is reversed upon refeeding (23). Pertussis toxin (50 ng/ml) and adenosine deaminase treatment (0.5 unit/ml) reduced leptin secretion (as measured by RIA) 60 and 20%, respectively, suggesting a role for G, in regulating leptin secretion. The effect of adenosine deaminase was mimicked by the adenosine antagonist 8-phenyltheophylline (2 μM) and was blocked by the adenosine agonist phenylisopropyl adenosine (10 nM) (data not shown).

Interestingly, ICI 201,651 is less effective than isoproterenol at lowering ob expression, even at the relatively high concentration of 10 μM. This is similar to the effect of another β3 selective agonist BRL 37344 on cAMP production in rat adipocytes and may represent a weaker coupling of the β3 receptor to cAMP production (24). Since β3 agonists induce weight loss through increased thermogenesis in rats (25), it is interesting that they also down-regulate expression of leptin, which similarly increases the basal metabolic rate in rats (10).

We have previously demonstrated that leptin treatment of ob/ob mice reduces synthesis and release of hypothalamic NPY, and that this may represent its mechanism of action on food intake and basal metabolic rate (11). It has been suggested that glucocorticoids and insulin regulate energy balance by their central regulation of food intake through NPY and through their peripheral effects on energy storage (26). Central glucocorticoids increase NPY levels and food consumption (26), while insulin inhibits NPY release (27). The data presented here suggest that leptin may represent another arm of this regulatory pathway. Increased glucocorticoids directly stimulate leptin secretion from adipose tissue, which then negatively modulates NPY release in the hypothalamus (11). Increased NPY levels would also lead to decreased sympathetic activity (28) which, based on our results, would lead to further increases in leptin secretion. This would explain the observed increased leptin expression in genetic models of rodent obesity, such as the Zucker fa/fa rat (29), since these animals are characterized by hyperglucocorticoidism, elevated hypothalamic NPY, and reduced sympathetic activity (30). Increased NPY is also associated with increased insulin secretion, which
Adipocytes with leptin mRNA expression, while similar treatment of ob-resistant db/db mice has no effect. Treatment of isolated rat adipocytes with leptin in vitro has no effect on endogenous ob mRNA levels, while glucocorticoids up-regulate expression of ob mRNA and protein secretion. Agents that increase intracellular cAMP down-regulate ob expression. These results suggest a centrally mediated mechanism of feedback regulation of ob expression involving the hypothalamic-pituitary-adrenal axis.

In summary, we have demonstrated that treatment of ob/ob mice with exogenous human leptin down-regulates endogenous murine ob mRNA expression, while similar treatment of ob-resistant db/db mice has no effect. Treatment of isolated rat adipocytes with leptin in vitro has no effect on endogenous ob mRNA levels, while glucocorticoids up-regulate expression of ob mRNA and protein secretion. Agents that increase intracellular cAMP down-regulate ob expression. These results suggest a centrally mediated mechanism of feedback regulation of ob expression involving the hypothalamic-pituitary-adrenal axis.

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