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Leptin is a Metabolic Signal to the Reproductive System

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ABSTRACT

Leptin, a newly-discovered hormonal product of the obese *(ob)* gene, is expressed by adipocytes and thought to play a role in the regulation of food intake and metabolism. We tested the hypothesis that leptin signals metabolic information to the reproductive system by examining its effects on the reproductive system of *ob/ob* mice, which have a congenital deficiency in leptin and are infertile. We treated pair-fed males and females with leptin (50 μg twice daily, ip) or vehicle (n=10/group) for 14 days, after which the animals were bled and killed. Leptin-treated females had significantly elevated serum levels of LH, increased ovarian and uterine weights, and stimulated aspects of ovarian and uterine histology compared to controls. Leptin-treated males had significantly elevated serum levels of FSH, increased testicular and seminal vesicle weights, greater seminal vesicle epithelial cell height, and elevated sperm counts compared to controls. These results demonstrate that leptin stimulates the reproductive endocrine system in both sexes of *ob/ob* mice and suggest that leptin may serve as a permissive signal to the reproductive system of normal animals.

Fertility in mammals requires adequate nutrition and reserves of metabolic fuel (1, 2). People experiencing severe dietary restriction (e.g., anorexia nervosa), wasting diseases (e.g., insulin-dependent diabetes), or who are high performance athletes (e.g., long distance runners and ballet dancers) have severely impaired reproductive systems (3-5), and even short-term changes in food intake can result in perturbations of reproductive hormone levels in rats and monkeys (6, 7). The effect of nutritional status on reproduction is postulated to reflect the action of metabolic signals that are recognized by the brain and serve as indices of metabolic state (8); however, the identity of these metabolic factors has remained elusive.

Recently, a hormonal product of the obese *(ob)* gene, leptin, was cloned and shown to serve as a satiety factor (9). Leptin is synthesized and secreted from fat cells in response to improved metabolic status, and it has the effect of increasing general metabolic rate and activity levels while decreasing appetite (10-13). Based on these relationships, it has been suggested that leptin serves as a metabolic signal to the areas of the brain governing appetite and metabolism (10, 13-16). The discovery of leptin receptor expression in the brain lends credence to this argument (17-20). Extending this logic, we postulated that leptin could inform the reproductive axis about the body's nutritional state, permitting reproduction to go forward if sufficient metabolic reserves are available and blocking it if reserves are low or the metabolic system is stressed. We tested this hypothesis on the reproductive system of genetically obese *ob/ob* mice, which have a mutation in the gene encoding leptin and are infertile because of what is thought to be a defect in the feedback regulation of the hypothalamic-pituitary axis (21-23).

Materials and Methods

Expression and Purification of Leptin

Recombinant full length human leptin was produced in *Saccharomyces cerevisiae*, purified to >95% homogeneity by analytical HPLC, and quantified by mass spectroscopy as described elsewhere (24).

Animals

C57Bl/6J *ob/ob* mice were obtained from Jackson Laboratories and maintained on standard rodent chow (from Teklad, Madison, WI) and water, *ad libitum*, until the time of the experiment. Males were between 11 and 17 weeks old at the beginning of the experiment and females were 10 weeks old.

Experimental Design

All experimental procedures were approved by ZymoGenetics Animal Care Committee. Body weight and food intake were recorded daily; control animals (10 male, 10 female) were pair-fed to experimental animals (10 male, 10 female) of the same sex and age ± 1 week. On day -1 of the experiment, blood was obtained from the orbital sinus under ether anesthesia at 1000 h. Control animals were given saline and experimental animals were given 50 μg leptin as 0.5 ml intraperitoneal injections twice daily (1000 and 1700 h) for 14 days. On day 14, animals were again bled under ether anesthesia at 1000 h, then killed by cervical dislocation. Seminal vesicles, testes, ovaries, and uteri were removed, weighed, and placed in either formalin or Bouin's solution.

Hormone Assays

Serum was collected from the blood samples and frozen at -20 C until assayed for LH and FSH by RIA with reagents from NIH. The standard used for the FSH assay was rFSH-RP2, and the antiserum was anti-rFSH-S11. For LH the standard was rLH-RP3, and the antiserum was anti-rLH-S11. The tracers for both assays were purchased from Corning Hazelton, Inc. (Vienna, VA). Final values are expressed as nanograms of rat LH or FSH per ml of serum. The intraassay coefficients of variation were 16% and 7% for LH and FSH, respectively.

Histology

The uteri, seminal vesicles, ovaries, and testes were dehydrated with a graded series of ethanol, cleared with xylene, and infiltrated and embedded in Paraplast X-TRA. Cross sections of uteri and testes, and longitudinal sections of seminal vesicles and ovaries, were stained for light microscopy and histomorphometry using a camera-lucida attached to a light microscope (Olympus, BH-2) with a BioQuant System IV image analysis system (B & M Biometrics, Inc.).

Statistical Analysis

Unless otherwise noted, all data are presented as mean ± SEM. Body weight profiles were compared by ANOVA. All other statistical comparisons were performed by Mann-Whitney U tests, with $p<0.05$ considered significant.

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Table 1. Ovarian histology: number of different follicular types in one cross section of each ovary from each animal.

	Primordial Follicles	Primary Follicles	Early Primary Follicles	Secondary Follicles	Graafian Follicles	Total
Saline	0.9±0.43	3.0±0.79	5.7±0.83	2.1±0.43	0	11.7±1.7
Leptin	2.3±0.50	5.5±0.79[a]	7.8±1.2	3.6±0.58	1.6±0.31[b]	20.8±2.3[c]

Data are presented as mean ± SEM.

[a] $p<0.05$ versus saline-treated animals

[b] $p<0.005$ versus saline-treated animals

[c] $p<0.01$ versus saline-treated animals

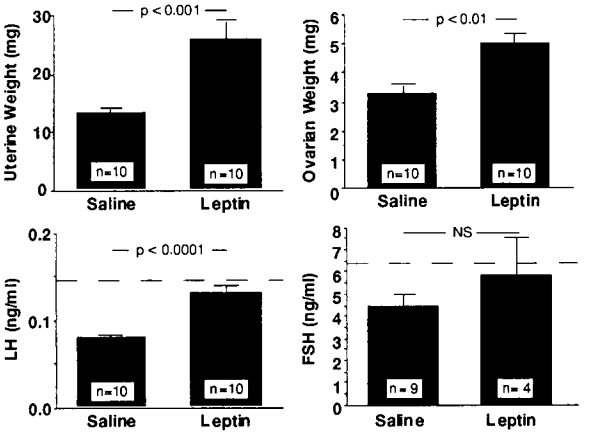

Fig. 1. Uterine weight, ovarian weight, LH levels, and FSH levels in adult female *ob/ob* mice treated with saline or leptin. The dashed lines in the LH and FSH graphs represent mean gonadotropin levels of all animals before treatment.

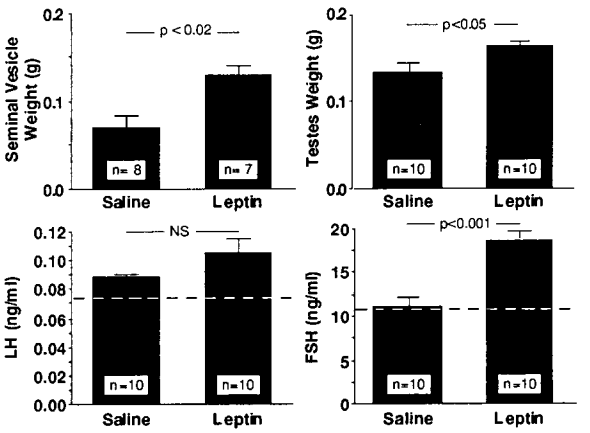

Fig. 2. Seminal vesicle weight, testes weight, LH levels, and FSH levels in adult male *ob/ob* mice treated with saline or leptin. The dashed lines in the LH and FSH graphs represent mean gonadotropin levels of all animals before treatment.

Results

As expected, leptin treatment resulted in a significant weight loss in both males and females (29% and 17%, respectively; $p<0.0001$). Control animals lost less weight (15% and 10%, respectively; data not shown), presumably reflecting the thermogenic effects of leptin (25).

Females (Fig. 1) At the end of the treatment period, serum levels of LH were significantly higher in females treated with leptin compared to controls ($p<0.001$), although leptin treatment did not produce a significant increase in LH levels over time. In saline-treated females, LH levels decreased over time, whereas in leptin-treated females, this decline did not occur. Serum levels of FSH were higher in leptin-treated animals compared to controls, although these differences were not statistically significant.

Reproductive organ weights were significantly higher in females treated with leptin. Both ovarian and uterine weight were higher in leptin-treated animals ($p<0.01$ and 0.001, respectively). Histological analysis of the ovaries showed that the number of primordial, primary, early primary, secondary, and Graafian follicles were all increased in the leptin-treated animals compared to controls; however, this difference was significant only in the number of primary and Graafian follicles ($p<0.05$ and 0.005, respectively). In addition, the total number of follicles observed in a longitudinal section of the ovary was increased significantly ($p<0.01$) compared to controls (Table 1). Histological studies of the uteri showed that uterine cross-sectional area, epithelial height, endometrial area, and glandular area all increased significantly with leptin treatment ($p<0.005$ in all cases; Table 2).

Males (Fig. 2) At the end of the treatment period, serum levels of FSH were significantly higher in males treated with leptin compared to controls ($p<0.001$). There was no significant difference in LH levels between the leptin-treated and control animals, although mean LH levels were higher in leptin-treated animals.

The weights of the testes and seminal vesicles increased significantly in the leptin-treated animals relative to controls ($p<0.05$ and 0.02, respectively). Histological analysis of the testes revealed that the saline-treated controls had significantly more abnormalities in their seminiferous tubules (i.e., decreased diameter, hypospermia, absence of germ cells, maturational arrest, intratubular sloughing; all

Table 2. Uterine histology

	Cross Sectional Area (mm^2)	Epithelial Height (μm)	Endometrial Area (mm^2)	Glandular Area (mm^2)
Saline	0.24±0.017	12.5±0.91	0.100±0.0084	0.0032±0.0009
Leptin	0.49±0.037	20.9±1.34	0.25±0.018	0.015±0.0032

$p<0.005$ for all uterine parameters. Data are presented as mean ± SEM.

Table 3. Testicular histology: number of seminiferous tubules showing abnormalities in one cross section (containing 200-300 tubules) of each testis from each animal.

	Decreased Diameter	Hypospermia	Absence of Germ Cells	Maturational Arrest	Intratubular Sloughing
Saline	9.0±3.2	41.8±26.5	1.2±0.76	29.9±15.6	3.2±1.8
Leptin	1.4±0.75	1.5±0.86	0	1.4±0.77	0

$p<0.05$ for all testicular parameters. Data are presented as mean ± SEM.

$p<0.05$) compared to leptin-treated animals (Table 3). Histological studies of the seminal vesicles showed that leptin-treated animals had greater epithelial height compared to controls (17.38±1.17 vs. 11.17±0.71μm; $p<0.005$).

Discussion

These results show that leptin stimulates the reproductive endocrine system of *ob/ob* mice—despite a profound reduction in food intake and body weight. These observations corroborate previous findings on the effects of leptin on body weight and food intake in *ob/ob* mice and provide a physiological explanation for the earlier finding that leptin can reverse the infertility in females with this mutation (10-13, 26). Moreover, our studies show that the effects of leptin on the reproductive axis extend to males and offer a unifying hypothesis suggesting that leptin may be an important mechanism by which the body's metabolic control system signals its nutritional state to the reproductive axis. While it seems clear that gonadal function in the *ob/ob* mouse is activated by leptin replacement, it is less evident whether this effect is mediated directly via the gonads or indirectly through the neuroendocrine axis.

In both male and female *ob/ob* mice, leptin unequivocally stimulated gonadal function. Ovarian and testicular weight increased following treatment with leptin, and ovarian histology indicates greater amounts of follicular development, consistent with activation of ovarian function. Similarly, testicular histology indicates that leptin stimulated cellular activity in the seminiferous tubules. In neither case was the increased weight due to pathological effects of leptin; thus, we conclude that leptin somehow influences gonadal physiology.

The trophic action of leptin on gonadal function apparently leads to an increase in sex steroid production as shown by increased uterine weight. Uterine histology revealed that the weight increase was due to proliferative growth of the uterine glands, epithelium, and endometrium, typical responses to stimulation by estrogen. In males, the increased weight of the seminal vesicles was likewise accompanied by increased epithelial height, indicating increased circulating levels of testosterone. Although leptin may conceivably act directly on the uterus and the seminal vesicles, causing proliferation of these tissues, it seems more likely that in both sexes leptin stimulates the gonads, resulting in increased sex steroid levels and thereby causing proliferation in these sex steroid-sensitive target tissues.

What is the mechanism of leptin's action on the reproductive axis? First, it seems likely that our results, at least in part, reflect a direct effect of leptin on the gonads. This inference is based on the fact that the leptin receptor (OB-R) has been localized to the ovaries and the testes (16, 18), which suggests that these organs are, in fact, targets for the action of leptin. However, it also seems likely that leptin acts on the hypothalamic-pituitary axis, although this effect may be more complex. It appears that the infertility of *ob/ob* mice is attributable to a hypothalamic/pituitary defect (21-23), although these animals are capable of exhibiting a rise in gonadotropin secretion following castration, implying that this defect is only partial (22). Our results show that the *ob/ob* control animals, which were pair-fed to the leptin-treated animals, showed an overall decline in serum levels of gonadotropins—similar to that previously described for normal rats fed a calorie-restricted diet (25). Leptin treatment blocked the fasting-induced inhibition of gonadotropin levels. LH levels in leptin-treated females and FSH levels in leptin-treated males were significantly higher than in saline-treated animals, despite the fact that these animals lost even more weight than their pair-fed controls. We conclude that whereas leptin may not actually increase circulating levels of gonadotropins over pretreatment levels in *ob/ob* mice, leptin most certainly defends against the inhibition associated with fasting. It is also possible that, because plasma levels of mouse gonadotropins are highly pulsatile (27), leptin increases the frequency or amplitude of LH and FSH pulses and that our method of blood collection (i.e., a single blood sample at the beginning and at the end of the experiment) simply did not detect this effect. Moreover, given that gonadotropin levels were significantly higher in leptin-treated animals than in controls—despite the apparent increase in circulating levels of sex steroids—it seems reasonable to conclude that the leptin treatment decreased the sensitivity of the hypothalamic-pituitary axis to the negative feedback effects of sex steroids, suggesting that leptin acts on the brain and/or pituitary.

Considerable evidence suggests that the brain is a target for leptin's action. Leptin binds strongly to the choroid plexus and leptomeninges (28). OB-R mRNA is found in the choroid plexus, leptomeninges, piriform cortex, thalamus, hippocampus, cerebellum, and hypothalamus (17). Moreover, *ob/ob* mice have unusually high levels of NPY expression in the hypothalamus, and these levels decrease dramatically when the mice are given leptin (15, 29). This effect of leptin is particularly interesting, because NPY has been widely implicated in the regulation of GnRH secretion (30), italicizing leptin's influence on neuropeptide function in the brain. In summary, it appears that leptin activates the reproductive system of *ob/ob* male and female mice—despite inducing a profound weight loss—and it is likely that these effects arise by a combined influence on the gonads and the brain.

Based on these observations, we postulate that in normal animals leptin serves as a metabolic signal to the reproductive system, informing it that sufficient fat stores are available to meet the caloric demands of reproduction. Based on our data we cannot determine whether leptin activates the reproductive axis or serves as a permissive signal that maintains reproductive function when circulating levels are above some threshold. However, in either case, metabolic stresses such as food restriction, metabolic wasting diseasses and severe exercise would be signaled to the reproductive system via low circulating levels of leptin, indicating that the resources necessary for successful reproduction are unavailable. When the animal's metabolic status improves, leptin levels increase, allowing the reproductive system to become active and restoring fertility. However, the mechanisms by which this is accomplished remain unclear.

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