Leptin Is a Metabolic Gate for the Onset of Puberty in the Female Rat

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ABSTRACT

The timing of puberty onset in mammals is tightly coupled to the animal’s nutritional and metabolic state. We conducted two experiments to test the hypothesis that leptin acts as a metabolic signal for the onset of puberty. In the first experiment, we administered leptin (6.3 µg/g twice daily) to a group of normal pubertal female rats and compared their rate of sexual maturation to that of two control groups. The group of leptin-treated animals and one group of control animals were allowed to eat ad lib, while the other group of control animals was pair-fed to the leptin-treated group. Food intake in the leptin-treated group was reduced to approximately 80% of the ad lib-fed control group, resulting in retarded growth in both leptin-treated and pair-fed animals. All measured indices of pubertal maturation—age at vaginal opening, age at first estrus, ovarian weight, ovulatory index (corpora lutea/ovarian section), uterine weight, and uterine cross-sectional area—were significantly delayed in the pair-fed group but not different between the leptin-treated group and ad lib-fed controls. The second experiment was similar to the first, except that both the leptin-treated group and the pair-fed group were fed at 70% of the ad lib-fed controls. Under these conditions, leptin only partially reversed the delay in sexual maturation, as reflected by the age at vaginal opening and first estrus. These results suggest that leptin is not the primary signal that initiates the onset of puberty but that instead, it acts in a permissive fashion, as a metabolic gate, to allow pubertal maturation to proceed—if and when metabolic resources are deemed adequate; moreover, these observations suggest that other metabolic factors, besides leptin, influence the timing of puberty onset under conditions of more severe dietary stress.

PUBERTY in mammals is physiologically gated by the energy resources of the body (1). The classical studies of Kennedy and Mitra and the work of Frisch and her co-workers established that the timing of sexual maturation is associated with body weight and composition (2, 3). Alterations in diet and exercise exert a powerful influence on pubertal maturation in many species—including the rat, sheep, and human (4-7). While it is acknowledged that the onset of puberty and the maintenance of reproductive function in the adult is physiologically coupled to nutrition and energetics, we don’t understand how this linkage is accomplished at the cellular and molecular level. We know that the brain receives and processes metabolic cues and that sexual maturation is initiated only when energy reserves are adequate to meet the caloric demands of mating, pregnancy, and lactation (8).

Leptin is a protein product of the obese (ob) gene, which is secreted as a hormone from adipocytes and plays an important role in the regulation of body weight and metabolism (9-12). Plasma levels of leptin are correlated with the degree of adiposity and are regulated by feeding and fasting (13-15). Mutant mice that are unable to produce leptin or its receptor (ob/ob and db/db mice, respectively) fail to undergo normal sexual maturation and remain infertile throughout life (16-19). Reproductive failure in these animals has been attributed to their inability to produce sufficient quantities of gonadotropins (17, 20, 21). Administering leptin to ob/ob mice stimulates all aspects of their reproductive endocrine system and rescues their fertility (22, 23). Because plasma levels of leptin are linked both to metabolism and reproduction, it seems conceivable that the normal onset of puberty may reflect, in part, the activation of the neuroendocrine reproductive axis by leptin. If leptin were the primary signal for initiating puberty onset, one would predict that animals experiencing a premature rise in plasma leptin levels would show precocious puberty. We tested this hypothesis by administering leptin to prepubertal female rats and comparing their rate of sexual maturation to groups of control animals that were either fed ad lib or fed identically to the leptin-treated group.

Materials and Methods

Animals. Female Sprague-Dawley rats (18 days of age), along with lactating mothers, were purchased (B & K Universal, Kirkland, WA) and housed in the animal care facilities at the University of Washington, under the auspices of the Department of Comparative Medicine. The animals were weaned at 21 days of age, maintained on standard rodent chow, given water ad lib, and housed individually on a 14:10 light/dark cycle with lights off at 2000 h. All procedures were approved by the Institutional Animal Care Committee of the University of Washington.

Experiment 1. At 23 days of age, the animals were divided into three groups. Two of the three groups were ad lib fed and received ip injections of either the vehicle only (50 mM sodium borate, pH 8.0; n = 8), or leptin in the vehicle (6.3 µg/g body weight; n = 8). The third group was pair-fed to the leptin-treated animals and injected with the vehicle alone (n = 8). The injections were performed at 0900-1000 h and 1200-2000 h; the animals and their food were weighed at the same times. For pair-feeding, just before the lights were turned off, the animals were given an amount of food equivalent to that eaten by the leptin-treated animals. The vagina of each animal was inspected daily to determine the date of vaginal opening, and subsequently, vaginal smears were taken daily. Animals were sacrificed on the day immediately following the day they showed a fully cornified vaginal smear, at approximately 1610-1700 h. Those that did not show vaginal opening by 38 days of age were sacrificed on day 38 and assigned a vaginal opening and estrus date of 39 days. Trunk blood was collected. Ovaries and uterus were dissected, weighed, and fixed in Bouin’s solution (Sigma, St. Louis, MO). (One ad lib-fed animal that had a balloononed uterus was excluded from the analysis of the uterine data.)

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Experiment 2. At 23 days of age, animals were divided into three groups. The first group was fed ad lib and received injections of the vehicle twice daily (n = 9 each). The other two groups were fed approximately 70% of ad lib food intake and received twice daily injections of either leptin in the vehicle (6.3 μg/g body weight; n = 8) or the vehicle only (n = 8). Because it was predicted that ad lib-fed animals would show puberty (and be sacrificed) sooner than the food-restricted animals, an additional group of ad lib-fed animals (n = 9) was used to determine the amount of food that would constitute 70% of ad lib food intake, once the majority of the “experimental” ad lib-fed animals were sacrificed. Other details of this experiment were the same as in Experiment 1, except that animals that did not show vaginal opening were sacrificed on day 43 and assigned a vaginal opening and estrus day of 44. (One ad lib-fed and one leptin-treated animal that had ballooned uteri were excluded from the analysis of the uterine data.)

Leptin. Recombinant full-length human leptin protein was produced in Saccharomyces cerevisiae, purified to near homogeneity (>95%) by analytical HPLC, and quantified by mass spectroscopy.

Histology. The uteri and ovaries were dehydrated in a graded ethanol series, cleared with xylene, then infiltrated and embedded in paraffin. Cross sections of uteri and longitudinal sections of ovaries were stained with hematoxylin-eosin and analyzed by light microscopy.

Statistical analysis. Data are presented as mean ± SEM. ANOVA followed by Fisher's PLSD test was used to analyze data on food intake, daily weight gain, uterine and ovarian weight, and uterine histology. The nonparametric Kruskal-Wallis test, followed by the Mann-Whitney U Test for two group comparisons was used to analyze the data on vaginal opening, estrus, and corpora lutea. Fisher's Exact Test was used to analyze the percentage of animals showing vaginal opening and estrus. A probability of greater than 0.05 was considered not statistically significant (NS).

Results

Experiment 1

Food intake and body weight. Leptin treatment significantly decreased both food intake and weight gain. As shown in Fig. 1, animals receiving leptin consumed 20% less food and showed retarded growth compared to animals not receiving leptin and fed ad lib (Fig 1a; p<0.001 and Fig 1b; p<0.001). In contrast, there were no differences in either food intake or average daily weight gain between leptin-treated and pair-fed animals (Fig 1; NS).

Vaginal opening and first estrus. Although leptin-treated animals had lower food intake and body weights than the ad lib-fed animals, their average age at vaginal opening and first estrus was indistinguishable from the ad lib-fed animals (Table 1). By day 38, 7 of 8 leptin-treated and 7 of 8 ad lib-fed animals had already shown vaginal opening; likewise, 7 of 8 ad lib-fed and 6 of 8 leptin-treated animals had shown estrus.

**TABLE 1. Vaginal opening and first estrus (Experiment 1)**

<table>
<thead>
<tr>
<th></th>
<th>Percent showing vaginal opening by 38 days of age</th>
<th>Average age at vaginal opening (day) a</th>
<th>Percent showing estrus by 38 days of age</th>
<th>Average age at first estrus (day) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad lib</td>
<td>87.5% (7/8)</td>
<td>32.0 ± 1.1</td>
<td>87.5% (7/8)</td>
<td>32.1 ± 1.0</td>
</tr>
<tr>
<td>Leptin</td>
<td>87.5% (7/8)</td>
<td>33.6 ± 1.3</td>
<td>75.0% (6/8)</td>
<td>33.9 ± 1.3</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.0% (0/8) b</td>
<td>&gt;38.0 b</td>
<td>0.0% (0/8) b</td>
<td>&gt;38.0 c</td>
</tr>
</tbody>
</table>

a For statistical purposes, animals not showing either vaginal opening or estrus by 38 days of age were assigned a value of 39.

b p<0.005 compared to ad lib and pair-fed animals

c p<0.005 compared to ad lib and p<0.05 compared to leptin-treated animals

In contrast, the age of vaginal opening was delayed in the pair-fed animals, none of which showed vaginal opening by day 38 (Table 1; p<0.005). This was true, despite the fact that the mean body weight of the pair-fed animals on day 38 was nearly identical to that of the leptin-treated and ad lib-fed animals when they were sacrificed on their first diestrus (ad lib=130 ± 3 g, leptin=127 ± 6 g, pair fed=129 ± 2 g; NS).

Ovarian and uterine indices. Ovarian and uterine weights were indistinguishable between leptin-treated and ad lib-fed animals (Fig 2a and b). Both leptin-treated and ad lib-fed animals had ovarian and uterine weights that were significantly greater than those of pair-fed animals (Fig 2a and b; p<0.0001 in all cases). Confirming ovulation and the onset of puberty, histological analysis of the ovaries showed the presence of corpora lutea in both leptin-treated and ad lib-fed animals, and the number of corpora lutea did not differ between these two groups (Table 2). Corpora lutea were completely absent in the pair-fed animals, suggesting that they did not reach full sexual maturity (Table 2). Histological analysis of the uteri showed that although the cross-sectional areas did not differ significantly between leptin-treated and ad lib-fed animals (Table 2), the uteri of pair-fed females were significantly smaller in cross sectional area (Table 2) and had reduced epithelial height (data not shown) compared to both the ad lib-fed and leptin-treated groups (p<0.005).

Light- and dark-phase food intake. Leptin affected food consumption primarily during the light phase. Although leptin was administered in both the morning and evening, there was no significant difference in food consumption during the light phase between the leptin-treated and ad lib-fed animals (4.1 ± 0.4 g and 4.4 ± 0.5 g, respectively);
TABLE 2. Ovarian and Uterine Histology

<table>
<thead>
<tr>
<th></th>
<th>Number of Corpora Lutea</th>
<th>Uterine Cross-Sectional Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad lib</td>
<td>3.50 ± 0.76 b</td>
<td>1.55 ± 0.13 c</td>
</tr>
<tr>
<td>Leptin</td>
<td>3.17 ± 0.60 c</td>
<td>1.80 ± 0.13 c</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>0.00 ± 0.00</td>
<td>0.91 ± 0.77</td>
</tr>
</tbody>
</table>

a NS for all parameters between leptin and ad lib animals; all parameters were measured in one cross section from each animal.
b p<0.05 compared to pair-fed.
c p<0.005 compared to pair-fed.

Discussion

We have shown here that modest growth retardation imposed by food restriction delays the onset of puberty in the female rat—confirming well-known observations (8). We have also shown that leptin is able to reverse this effect, restoring the normal timing of sexual maturation—provided that growth retardation is not too severe (Tables 1 and 3). Moreover, in the dosages used in these experiments, leptin did not advance the onset of puberty beyond that which occurs in animals fed a normal laboratory diet ad lib (Table 1). Possible targets for the action of leptin on the reproductive system include the brain and the ovaries, where leptin receptors are expressed (24, 25); however, its site of action cannot be determined more precisely on the basis of the work conducted here.

The fact that leptin did not further advance the onset of puberty relative to non-leptin-treated, ad lib-fed animals suggests that leptin is not the rate-limiting determinant for puberty onset—but is instead, only a permissive factor that allows puberty to proceed, when its plasma threshold has been attained. If this were the case, one would predict that leptin levels below threshold would prevent the onset of puberty, whereas elevated leptin levels above threshold would not advance the normal physiological timing of puberty. This model is consistent with the concept that leptin acts to alert the reproductive system to the metabolic status of the animal is adequate to support reproduction and allows other rate-limiting factors to control the precise timing of the event. This interpretation is also consistent with the results of studies in adult, leptin-deficient animals demonstrating that reproductive competence is dependent upon achieving adequate circulating levels of leptin (22, 23). If leptin has the same permissive role in the peripubertal animal, then pubertal activation of the reproductive system should occur only when leptin levels are above the putative threshold.

A permissive role for leptin in the control of the onset of puberty makes even more sense considering the natural conditions facing animals in the wild. Most animals live on the fragile edge of starvation—nothing similar to their experience in the laboratory being fed an all-you-can-eat diet. Perhaps the rate of puberty onset under ad lib-fed conditions reflects the greatest rate possible, and under these circumstances, prematurely elevated titers of leptin cannot further advance this process. In the wild, however, the normal rate of puberty onset is highly variable, reflecting the unpredictable availability of food resources (8). Under these circumstances plasma levels of leptin may be monitored by the reproductive axis and used as a physiological gate to either permit (in its presence) or block (in its absence) the procession of puberty.

It is possible that circulating levels of leptin rise over the course of normal development and that at some threshold, leptin permits the activation of the reproductive system. At this writing, there are no reports of plasma leptin measurements across pubertal development (in any species); however, increased plasma levels of leptin are correlated with body weight and adiposity in the rodent, and leptin mRNA levels in rat adipose tissue rise over development (13, 14, 26), providing some basis for this hypothesis. The results

TABLE 3. Vaginal opening and first estrus (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>Percent showing vaginal opening by 43 days of age</th>
<th>Average age at vaginal opening (day)</th>
<th>Percent showing estrus by 43 days of age</th>
<th>Average age at first estrus (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad lib</td>
<td>100.0% (9/9)</td>
<td>35.0 ± 0.9 c</td>
<td>100.0% (9/9)</td>
<td>35.2 ± 0.9 c</td>
</tr>
<tr>
<td>Leptin</td>
<td>62.5% (5/8)</td>
<td>41.8 ± 1.0</td>
<td>50.0% (4/8)</td>
<td>42.4 ± 1.0</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.0% (0/8)</td>
<td>&gt;43.0 b</td>
<td>0.0% (0/8)</td>
<td>&gt;43.0 b</td>
</tr>
</tbody>
</table>

a Animals not showing either vaginal opening or estrus by 43 days of age were assigned a value of 44.
b p<0.05 compared to leptin-treated animals
c p<0.005 compared to leptin-treated animals

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of our second experiment, in which animals were restricted to ~70% of their ad lib dietary intake and given leptin, are consistent with the theory that plasma leptin levels must rise to some threshold before the reproductive system can become activated. When the availability of food is restricted, circulating leptin levels should rise at a lower rate, delaying the time at which the threshold is reached. Under these conditions, it should be possible to advance the onset of puberty by artificially increasing leptin levels. Indeed, we found pubertal maturation in food-restricted, leptin-treated animals to be significantly advanced relative to animals fed comparably but not receiving leptin (Table 3). The fact that leptin did not restore the normal timing of puberty to that of ad lib-fed controls in the more severely restricted group (Exp 2) suggests that the dose of leptin we administered, when added to rising endogenous levels, was not sufficient to elevate circulating leptin concentrations to threshold levels until approximately day 42. If this interpretation is correct, then higher doses of leptin should be more effective in advancing pubertal maturation; this remains to be tested.

We have shown here that leptin advances the onset of puberty in animals whose food intake is less than normal. This is consistent with the premise that leptin is an important link between metabolic status and reproductive competency during puberty. How leptin achieves this linkage is unknown. Leptin reduces food intake and alters metabolism, which in turn induces changes in plasma levels of metabolic hormones such as insulin and glucocorticoids, as well as altering the secretion of leptin itself (27-29). A more precise determination of the physiology role of leptin in the rat will depend on careful monitoring and control of metabolically active hormones that are influenced by leptin, as well as the development of an assay to measure circulating concentrations of leptin in the rat.

Acknowledgements

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References

1. Darwin C 1896 The variation of animals and plants under domestication, D. Appleton and Company, New York