Secretory Patterns of Leptin and Luteinizing Hormone in Food-Restricted Young Female Sheep

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ABSTRACT

Leptin, the product of the ob gene, has been proposed as a metabolic signal that regulates the secretion of GnRH/LH. This may be critical during prepubertal development to synchronize information about energy stores and the secretion of GnRH/LH. This study aimed to assess the effect of food restriction on the episodic secretion of leptin and LH in young female sheep. Five 20-week-old prepubertal females were fed a low-level diet for 10 weeks to maintain the body weight. Control females of the same age received food ad libitum.

Blood samples were collected at 10-min intervals for six hours at 20, 26, and 30 weeks of age, and plasma leptin, LH, insulin and cortisol concentrations were measured. In the control group, no changes were found in pulsatile LH secretion characteristics. Mean LH concentrations and LH amplitude were lower in the food-restricted group than in the control group at 26 and 30 weeks of age. In the control group, pulsatile leptin secretion did not change. When compared to control lambs of the same age, the food-restricted group showed lower mean plasma leptin concentrations, pulse amplitude and plasma insulin levels, after 6 weeks of restriction (week 26), although by week 30, plasma leptin concentrations and plasma insulin rose to those of the control group. Leptin pulse frequency did not change, nor did mean plasma levels of insulin in the control group at any age studied. Mean plasma concentration of cortisol did not change within or between groups.

These data suggest that plasma leptin concentrations may not be associated with the onset of puberty under regular feeding and natural photoperiod in lambs. Prolonged food restriction, however, induces metabolic adaptations that allow an increase of leptin during the final period, probably related to the development of some degree of insulin resistance.

Key terms: female sheep, food restriction, leptin, LH, puberty.

INTRODUCTION

The initiation of puberty in sheep depends upon a complex neuroendocrine interplay. Despite considerable information gained in understanding the mechanisms controlling the sexual maturation process, many questions remain unanswered, specifically the way in which nutrition and puberty are linked. It has been proposed that the neuroendocrine system of the growing female recognizes signals reflecting the metabolic status either to reinitiate or to postpone the secretion of GnRH. Such signals would probably be translated into a cascade of events culminating in the increase of GnRH/LH secretion.

Many blood-borne substances have been proposed as metabolic signals, such as glucose (Nagatani et al., 1996; Murahashi et al., 1996), amino acids (Recabarren et al., 1996a, Recabarren et al., 1996b), free fatty acids (Rutter et al., 1983), insulin (Steiner et al., 1983; Hileman et al., 1993), IGF-I (Hiney et al., 1991), and recently leptin (Mantzoros et al., 1997; Vogel, 1996). Leptin partially fulfills the criteria to be considered as a metabolic signal to stimulate the initiation of puberty. One of these criteria is that the substance increases before puberty and
accelerates the normal events leading to the onset of puberty (Foster and Nagatani, 1999). Studies in humans, pigs, and rodents have demonstrated prepubertal rises in serum leptin (Mantzoros et al., 1997; Clayton et al., 1997; Qian et al., 1999; Ahima et al., 1998). The administration of leptin accelerates the initiation of puberty in normal female mice (Ahima et al., 1997; Chehab et al., 1997) and can reverse the delay in the onset of puberty in severely food-restricted female rats, despite the weight loss and the increase in energy expenditure (Cheung et al., 1997). Leptin administration to fasting female rats (Nagatani et al., 1998; Ahima et al., 1996) and female mice (Ahima et al., 1996) reestablished the LH secretion diminished by fasting. In prepubertal heifers, short-term fasting lowered plasma LH concentrations and plasma leptin concentrations, concomitant with a reduction in the leptin gene expression (Amstalden et al., 2000), thus providing a sustainable basis for the role of leptin as an intermediary between energy status and gonadotropin secretion in prepubertal females.

Most of the interventions to study the influence of energy on leptin secretion have included a short fast (Nagatani et al., 1998; Ahima et al., 1996; Amstalden et al., 2000). It is not well known whether the control of leptin secretion during fasting is similar to that obtained during a prolonged food-restriction period either in adult or growing females. Knowledge obtained in women suffering of anorexia nervosa may not be comparable to the physiological effect produced by food restriction, because anorexia nervosa is very complex and its underlying mechanisms are mainly psychological. The growth-limited female under food restriction may be a good model to provide information regarding the pattern of leptin secretion during a long-term energy limitation. It is possible to expect that plasma levels of leptin decrease during food restriction, and therefore the onset of puberty would be delayed.

This research aimed to assess the episodic fluctuations of circulating LH and leptin during normal and delayed prepubertal development due to food restriction in young female sheep. Studies of pulsatile LH and leptin secretion were evaluated at 20, 26 (early prepubertal), and 30 (peripubertal) weeks of age to give approximate information about the pattern of leptin secretion during the final stages of development. Plasma insulin and cortisol were also determined to assess the effect of food-restriction on these 2 metabolic hormones and the probable appearance of stress.

**MATERIAL AND METHODS**

**General procedures**

Ten spring-born Suffolk female lambs were used. They were born at the Sheep Production Unit of the Universidad de Concepción, at the Chillán Campus, weaned at 8 weeks, and then moved to the facilities of the Faculty of Veterinary Medicine. They were maintained in pasture and given a supplement of pelleted food twice a day. Starting at 16 weeks, they were fed only pelleted food containing 16 % de protein, 14 % crude fiber, 2 % fat, and 2450 Kcal of energy. At 20 weeks, the young females were divided into 2 groups. The control group (n=5), continued receiving the pelleted food ad libitum, and the food-restricted group began receiving the same pelleted food in an amount equivalent to the 2 % of the body weight to prevent growth (Recabarren et al., 1998a, 2000a). This scheme of feeding was continued for 10 weeks (until 30 weeks of age), at which time the food-restricted group was allowed to eat the pelleted food ad libitum. Females were weighed at 5-day intervals. The procedures were reviewed and approved by the local ethics committee in the care and use of animal research.

**Experimental procedures**

Patterns of circulating leptin and LH were studied at 20, 26, and 30 weeks of age. Four to five days before each time point, the young females were moved to an animal experimentation room and placed in individual crates with free access to pelleted food and water according to the feeding protocol. An indwelling jugular
vein catheter placed under local anesthesia was used to collect blood samples as described elsewhere (Recabarren et al., 1995). Blood samples were collected at regular intervals 2-3 days prior to the experiment to allow lambs to become accustomed to the blood collection procedures and to minimize stress.

The study of episodic leptin and LH secretion consisted of collecting blood samples from the jugular vein for six hours at 10-min intervals, beginning at 10:00 AM. Blood samples were received in heparinized tubes kept on ice, centrifuged at 1000 g for 15 minutes, and the plasma was stored frozen at -20°C until later hormone measurements. Plasma LH and leptin were determined in each sample. Plasma insulin and cortisol concentrations were measured in hourly samples to define the effect of food restriction in these two metabolic hormones and the probable development of stress.

From 26 to 36 weeks of age, blood samples were obtained at 5-day intervals from all lambs to determine plasma progesterone concentrations in order to define the onset of puberty. Plasma progesterone concentrations higher than 1 ng/mL, in one single or in two consecutive samples, were considered to represent prior ovulation and therefore the onset of puberty.

**Hormone assays**

Plasma LH concentrations were determined by RIA, using ovine radioiodinated LH (LER 1374-A), ovine antiserum CSU-204 and ovine LH standard oLH-S25 (provided by the NIADDK, USA.) in 200 µL duplicates, following procedures described elsewhere (Recabarren et al., 1996b). The intra- and interassay coefficients of variation were 5 % and 12 %, respectively. The minimal detectable dose of LH, defined as 90 % of buffer control, was 0.1 ng/mL.

Plasma leptin concentrations were determined by RIA, using the Multi-species RIA kit from Linco Research Co. This kit has been used to determine plasma leptin concentrations in farm animals such as pigs (Qian et al., 1999), cows (Chilliard et al., 1998a,b) and sheep (Chilliard et al., 1998a,b; Bocquier et al., 1998; Nagatani et al., 2000a). The intra- and interassay coefficients of variation were 7.2 and 12.5 % respectively. The minimal detectable dose of leptin, defined as 90 % of buffer control, was 0.92 ng/mL. This value was given in cases when concentrations were lower for statistical purposes.

Plasma concentration of insulin was measured by RIA, using commercial kits (Insulin Coat-A-Count, DPC, USA) validated for sheep plasma. The intra- and interassay coefficients of variation of coefficient were 2 % and 5 %, respectively. The minimal detectable dose of insulin, defined as 90 % of control, was 1.5 µIU/mL.

Plasma concentration of cortisol was measured by RIA, using commercial kits (Cortisol Coat-A-Count, DPC, USA) validated for sheep plasma. The intra- and interassay coefficients of variation of coefficient were 4 % and 11 %, respectively. The minimal detectable dose of cortisol, defined as 90 % of control, was 0.35 µg/dL.

Plasma progesterone concentrations were measured by RIA, using commercial kits (DPC, USA). DPC kits are routinely used to measure plasma progesterone in animals and have been validated for sheep plasma. The intra- and interassay coefficients of variation of coefficient were 2 % and 5 %, respectively. The minimal detectable dose of progesterone, defined as 90 % of control, was 0.1 ng/mL.

**Pulse analysis and statistical evaluation**

For pulse analysis, the computerized version of the cluster pulse algorithm was used (Veldhuis and Johnson, 1986). A cluster configuration of 1x2 (one sample for the test peak and two for the test nadir), and a t-value of 2.14/2.14 to reduce the possibilities of false positive pulse determination <5 % was selected. The following mean properties of leptin and LH pulsatile concentrations were analyzed: transversal mean (ng/mL/6h), pulse frequency (number of significant peaks/6h), pulse amplitude (ng/mL), and nadir (ng/mL).

The transversal mean, pulse frequency, pulse amplitude, and nadir of both hormones were studied by analyzing the variance for repeated measures, with
treatment as the main factor and age as the repeated measure factor using the GB-Stat v6.5 statistical program. Pairwise post-hoc comparisons were made by the Newman-Keuls’ test.

The horizontal means of hourly plasma concentrations of insulin and cortisol representing the mean of six samples were calculated (ng/mL/6h) and examined with analysis of variance for repeated measures with age as the repeated factor using the GB-Stat v6.5 statistical program. Pairwise post-hoc comparisons were made by the Newman-Keuls’ test. Differences were considered statistically significant at a level of P< 0.05. Results are shown as mean ± SEM.

RESULTS

Body weight of control lambs increased from 26.8 ± 0.3 kg. at 20 weeks to 34.3±1.5 kg. at 30 weeks of age, while in food-restricted lambs, body weight decreased from 26.8±1.9 kg. to 22.8±1.1 kg between both ages respectively. None of the food-restricted lambs exhibited a rise in progesterone indicative of ovulation prior to 30 weeks of age, while 2 of the control ewe lambs had their first rise at 29 weeks (Fig. 1). At 36 weeks, when progesterone measurement ended, all control lambs were cycling, while only 2 of the 5 food-restricted lambs had had a rise in progesterone by that age (Fig 1).

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Figure 1. Plasma progesterone concentrations profiles in individual female sheep. Blood samples were taken from 26 to 36 weeks of age to compare the initiation of puberty. A. Normal growing female sheep (control group). B. Food-restricted female sheep. Food restriction was initiated at 20 weeks of age and lasted for 10 weeks.
Table I shows the summary of LH pulsatile secretion characteristics in normal growing and in food-restricted female lambs at 20, 26, and 30 weeks of age. In the control group, mean plasma LH concentrations, mean pulse amplitude, and mean frequency did not change from 20 to 30 weeks of age. Mean LH concentrations and mean LH amplitude decreased with food restriction and were lower than in the control group at 26 and 30 weeks of age (P<0.05). Figures 2 and 3 show plasma LH profiles in three normal growing female sheep and in 3 food-restricted sheep. The impact of food restriction is clearly recognized in females of 26 and 30-weeks of age in the food-restricted group after 6 and 10 weeks of food restriction.

Table II shows the summary of characteristics of pulsatile leptin secretion in normal growing and in food-restricted female lambs at 20, 26, and 30 weeks of age. In the control group, an increase of 50 % in mean plasma leptin concentrations (ng/mL/6h) was observed between lambs of 20 and 26 weeks of age. No statistical difference was found between lambs of 20 and 30 weeks of age or 26 and 30 weeks of age in mean plasma leptin concentrations. Mean pulse amplitude and pulse frequency did not change between 20 and 30 weeks of age. In the food-restricted group, mean plasma leptin concentrations and mean pulse amplitude were decreased by 26 weeks of age, after 6 weeks of food restriction, and they were lower (P<0.05) than those exhibited by control lambs of the same age. In many samples, the plasma concentration of leptin was below the level of detectability of the assay. At 30 weeks of age, after 10 weeks of food-restriction, plasma leptin concentrations recovered and reached the levels observed in the control group at the same age. Leptin pulse frequency did not change within a group or between the groups. Figures 4 and 5 show individual plasma leptin profiles in 3 normal growing females and in 3 food-restricted female sheep.

The horizontal mean of plasma insulin concentrations did not change in the control group in the three ages studied while in the food-restricted group a significant decrease (P< 0.05) in plasma insulin concentrations was observed at 26 weeks of age, after 6 weeks of food restriction (Fig 6). Thereafter, at 30 weeks of age, plasma insulin levels increased to values obtained before the food-restriction began.

### Table I

Characteristics of the pulsatile luteinizing hormone secretion in control normal growing and in food-restricted female sheep

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Food-restricted</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean(ng/mL/6h)</td>
<td>Nº of significant pulses/6h</td>
</tr>
<tr>
<td>20 weeks of age</td>
<td>0.31±0.1</td>
<td>4.6±0.9</td>
</tr>
<tr>
<td>26 weeks of age</td>
<td>0.46±0.06</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>30 weeks of age</td>
<td>0.46±0.1</td>
<td>4.6±0.4</td>
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</table>

* significantly different from controls females of the same age, Newman-Keuls’s test P<0.05
Figure 2. Individual profiles of plasma LH concentrations in 3 representative normal growing female sheep. A. 20 weeks of age. B. 26 weeks of age. C. 30 weeks of age.
**Figure 3.** Individual profiles of plasma LH concentrations in 3 representative food-restricted female sheep. A. 20 weeks of age. B. 26 weeks of age. C. 30 weeks of age.
TABLE II
Characteristics of the pulsatile leptin secretion in control normal growing and in food-restricted female sheep

<table>
<thead>
<tr>
<th></th>
<th>Control Mean (ng/mL/6h)</th>
<th>Nº of significant pulses/6h</th>
<th>Amplitude of pulses/6h</th>
<th>Nadir (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 weeks of age</td>
<td>1.6±0.1</td>
<td>5.8±0.5</td>
<td>5.8±0.5</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>26 weeks of age</td>
<td>2.3±0.1</td>
<td>5.8±0.4</td>
<td>2.7±0.2</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>30 weeks of age</td>
<td>2.1±0.3</td>
<td>6.2±0.6</td>
<td>2.5±0.3</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td><strong>Food-Restricted</strong></td>
<td></td>
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</tr>
<tr>
<td>20 weeks of age</td>
<td>1.56±0.2</td>
<td>5.0±0.5</td>
<td>1.96±0.2</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>26 weeks of age</td>
<td>1.1±0.1*</td>
<td>6.2±0.6</td>
<td>1.6±0.1*</td>
<td>0.9±0.1*</td>
</tr>
<tr>
<td>30 weeks of age</td>
<td>1.9±0.1*</td>
<td>4.8±0.9</td>
<td>2.5±0.2</td>
<td>1.4±0.1</td>
</tr>
</tbody>
</table>

* significantly different from control females of 26 weeks of age, Newman Keuls’s test P<0.05

The horizontal mean of plasma cortisol concentrations did not change in both the control group and the food-restricted group in the three ages studied (Fig 6).

DISCUSSION

The results of the present study show that in normal growing ewe lambs, LH secretion did not change between 20 and 30 weeks of age. Mean plasma leptin concentrations increased between lambs of 20 and 26 weeks of age. In food-restricted ewe lambs, the onset of puberty is delayed, LH secretion is diminished, and leptin secretion exhibits a biphasic pattern in plasma concentrations, with an initial decrease in circulating leptin after 6 weeks of food-restriction and a recovery of plasma concentrations after 10 weeks of food restriction.

The absence of a significant leptin increase before puberty has been described in male monkeys and female and male rats (Plant and Durrant, 1997, Cheung et al., 2001). However, in other species (Qian et al., 1999), including humans (Clayton et al., 1997; Mantzoros et al., 1997), an increase in plasma leptin concentration has been observed before puberty, leading to the hypothesis that plasma leptin may be a metabolic signal for the onset of puberty (Cheung et al., 1997). This raises the question of when the increase in plasma leptin should take place in order to be considered as a cue signal associated with the onset of puberty. In male monkeys, it has been proposed that this increase in leptin is very early in the prepubertal development, preceding the nocturnal increase in LH concentrations (Suter et al., 2000). In female rats, the nocturnal increase in leptin precedes puberty by 8 days (Nagatani et al., 2000b). In the normally fed control lambs of the present study, leptin tended to increase from 20 weeks of age to 26 weeks of age. This increase of nearly 50% could be high enough to indicate that the female sheep is already metabolically competent at that age to initiate puberty, and that female lambs are only waiting the photoperiodic information to stimulate the increase in GnRH secretion (Foster, 1994; Wood and Foster, 1998) and that further increases in leptin are not necessary. This notion is supported by the fact that 2 lambs initiated puberty at 29 weeks of age and the remaining 3 lambs, around 32 weeks of age.
Figure 4. Individual profiles of plasma leptin concentrations in 3 representative normal growing female sheep. A. 20 weeks of age. B. 26 weeks of age. C. 30 weeks of age.
Figure 5. Individual profiles of plasma leptin concentrations in 3 representative food-restricted female sheep. A. 20 weeks of age. B. 26 weeks of age. C. 30 weeks of age.
Foster and Nagatani (1999) have proposed that metabolic signals, among them leptin, may regulate the GnRH neurons in a cascade of neuroendocrine events. According to this proposal, plasma leptin variations could be a condition to modify the availability of glucose or other substances for the GnRH neurons. The results of our study do not show how leptin regulates GnRH/LH secretion. However, preliminary results of our laboratory suggest that ewe lambs may develop insulin resistance before puberty, with a tendency to high insulin concentrations between 20 and 30 weeks of age. In consequence, a higher availability of insulin, along with over-threshold levels of leptin and other blood-borne substances such as IGF-1, could act in concert to regulate GnRH secretion before puberty.

Figure 6. A. Horizontal mean (± standard error) of insulin concentrations (µIU/mL/6h) in normal growing (control group) and in food-restricted female sheep of 20, 26, and 30 weeks of age. There were no statistical differences between control females at the three ages studied, while in the food-restricted female sheep of 26 weeks (r-26) there was a significant decrease compared to 20 (r-20) and 30 weeks (r-30). Anova for repeated measurements and post-hoc Newman Keuls’s test. B. Horizontal mean (± standard error) of cortisol concentrations (µIU/mL/6h) in normal growing (control group) and in food-restricted female sheep of 20, 26, and 30 weeks of age. There were no statistical significant differences between treatment groups and between ages. Anova for repeated measurements and post-hoc Newman Keuls’s test.
It has been established that plasma leptin concentrations are related to the amount of body fat and the body mass index in humans (Weigle et al., 1997). However, during fasting, the reduction in plasma leptin levels exceeds that expected because of the weight loss and fat loss, suggesting that other regulators, such as insulin, control leptin secretion from adipocytes (Weigle et al., 1997). Insulin promotes the release of leptin from adipocytes (Bradley and Cheatham, 1999), and leptin regulates the release of insulin (Kieffer et al., 1997), establishing a metabolic feedback system between the adipocyte and the pancreatic beta cell. The reduction in circulating leptin during fasting has been attributed to the reduction in plasma insulin levels because both decrease in parallel with the reduction in body weight, independent of changes in adiposity (Havel et al., 1996). This may account for the decrease in leptin concentrations in the first part of the experiment, because there was also a decrease in plasma insulin concentrations. However, despite the continuation of the food restriction, the increase in circulating leptin observed from 26 to 30 weeks of age may be due to another relationship between insulin and leptin. It has been observed that insulin resistance, independent of adiposity, is associated with high circulating levels of leptin (Segal et al., 1996). Although it is not known whether our lambs developed insulin resistance in response to the prolonged food-restriction, unpublished results from our laboratory show that prepubertal female sheep after six weeks of food-restriction developed insulin resistance measured with the euglicemic hyperinsulinemnic clamp, showing at the same time hyperinsulinemia compared to control normal growing female sheep (Recabarren et al submitted). Additionally, fasting for 8 days in ewe lambs is accompanied by insulin resistance, probably as a homeostatic adaptation to fasting (Recabarren et al., 2000b). Although the metabolic consequences of fasting may not be completely comparable to those of food-restriction, it is highly possible that food-restricted lambs may have developed insulin resistance, and that the hyperinsulinemia secondary to it increased plasma leptin concentrations. Although in the present study insulin did not reach plasma levels that could be defined as hyperinsulinemic after 10 weeks of food restriction, insulin nevertheless increased significantly above the levels obtained in blood after 6 weeks of food-restriction, mirroring the pattern of leptin concentrations.

Glucocorticoids may be another source of stimulation for the adipocytes. Studies in humans and rats have shown that glucocorticoids stimulate leptin release (Wabitsch et al., 1996; Tan et al., 1998). However, in the present study, plasma levels of cortisol did not change during food-restriction. These results are not different from other observations in prepubertal sheep during food restriction, as well as during fasting in lambs (I’Anson et al., 1994; Recabarren et al., 1999), strongly suggesting that fasting or food-restriction may not be considered stressful for sheep. It is then unlikely that cortisol would be involved in the regulation of leptin secretion during episodes of food-restriction. On the other hand, the mechanisms by which the amount of energy is sensed by the adipocyte to modify the leptin gene expression and secretion during long or short periods of food shortage are not completely understood. The difference in the way information is conveyed to the adipocyte during fasting or long periods of food restriction could explain the escape in the leptin secretion due to the metabolic adaptation originated by the prolonged food restriction.

It seems surprising that there was no increase in LH pulse frequency between 20- or 26-week-old females and 30-week-old females. The onset of puberty is preceded by an increase in LH pulsatility in a matter of days (Huffman et al., 1987). However, other studies from our laboratory (Recabarren et al., 1998b) have not detected this pattern of secretion, probably due to the sampling methodology used. Our studies of LH pulsatility are separated by intervals of many weeks (6 and 10 weeks), and it is highly possible that we could have missed the increase in LH pulsatility that characterizes the initiation of the LH surge in sheep.
In summary, our study shows that LH concentrations in blood do not change between 20 and 30 weeks of age in normal growing prepubertal female sheep, while plasma leptin concentrations increase between 20 and 26 weeks of age. Whether this increase may be considered a true metabolic signal directed to regulating the onset of puberty remains to be determined. On the other hand, in females of the same age placed under food restriction, parameters of LH secretion were clearly modified, where leptin concentrations initially decreased, with a subsequent recovery during the last part of the food restriction period.

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