Leptin secretion and hypothalamic neuropeptide and receptor gene expression in sheep

ANNETTE SORENSEN,1 CLARE L. ADAM,2 PAT A. FINGLAY,2 MICHEL MARIE,2 LOUISE THOMAS,2 MAUREEN T. TRAVERS,1 AND RICHARD G. VERNON1

1Hannah Research Institute, Ayr KA6 5HL; and 2Appetite and Energy Balance Division, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, United Kingdom

Received 28 September 2001; accepted in final form 12 December 2001

Leptin secretion and hypothalamic neuropeptide and receptor gene expression in sheep. Am J Physiol Regulatory Integrative Comp Physiol 282: R1227–R1235, 2002; 10.1152/ajpregu.00595.2001.—Peripheral and hypothalamic mechanisms underlying the hyperphagia of lactation have been investigated in sheep. Sheep were fed ad libitum and killed at 6 and 18 days of lactation; ad libitum-fed nonlactating sheep were killed as controls. Despite increased food intake, lactating ewes were in negative energy balance. Lactation decreased plasma leptin and adipose tissue leptin mRNA concentrations. OB-Rb gene expression, determined by in situ hybridization, was increased in the hypothalamic arcuate nucleus (ARC) and ventromedial hypothalamic nucleus (VMH) at both stages of lactation. Neuropeptide Y (NPY) was increased by lactation in the ARC and dorsomedial hypothalamus (DMH), although increased gene expression in the DMH was only apparent at day 18 of lactation. Gene expression was decreased for cocaine- and amphetamine-regulated transcript (CART) in the ARC and VMH and for proopiomelanocortin in ARC during lactation. Agouti-related peptide gene expression was increased in the ARC, and melanocortin receptor expression was unchanged in both the ARC and VMH with lactation. Thus the hypo leptinemia of lactation may activate NPY orexigenic pathways and attenuate anorexigenic melanocortin and CART pathways in the hypothalamus to promote the hyperphagia of lactation.

NUTRIENT REQUIREMENTS ARE markedly increased during lactation to meet the demands for milk production (7, 70). In most mammals, these additional requirements are met primarily by increasing food intake (7, 20, 70), but factors regulating the hyperphagia of lactation are not well understood. In addition, in many species, the increased intake is insufficient to meet the metabolic demands, resulting in a state of negative energy balance during early lactation when body lipid reserves are mobilized (7, 69). In some species such as the rat, the degree of negative energy balance is usually slight, with animals mobilizing ~1 g fat/day (7). Domestic ruminants, however, usually exhibit a greater degree of negative energy balance during early lactation (7, 11), which can impact on their welfare and productivity (28), yet the regulatory mechanisms have not been elucidated.

It is postulated that leptin, a peptide hormone secreted by adipose tissue (75), could play a role in the hyperphagia of lactation. Leptin acts on hypothalamic neuronal systems to regulate energy balance and neuroendocrine function; in particular, a reduction in circulating leptin is a potent signal of negative energy balance, activating compensatory orexigenic pathways in the hypothalamus (3–5, 35, 64). Key targets are neurons in the hypothalamic arcuate nucleus (ARC) expressing the signaling form of the leptin receptor (OB-Rb); these are the orexigenic peptides neuropeptide Y (NPY) and agouti-related peptide (AGRP) and the anorexigenic peptides proopiomelanocortin (POMC, precursor for melanocortins) and cocaine- and amphetamine-regulated transcript (CART) (4, 5, 31, 62, 64, 72). Adipose leptin gene expression and serum leptin concentrations are reported to be reduced during lactation in the monogastric rat in most (16, 36, 39, 56, 65, 74), but not all (17, 19, 61), studies. NPY (18, 21, 43, 44, 54, 55, 63, 71) and AGRP (18) are upregulated, but not all (17, 19, 61), studies. NPY (18, 21, 43, 44, 54, 55, 63, 71) and AGRP (18) are upregulated, and POMC is downregulated (54, 63) in the hypothalamus during lactation in the rat. However, there are no reports of gene expression for CART or for the hypothalamic melanocortin receptor (MC3-R) during lactation.

Little is known about the leptin-hypothalamic axis in lactating ruminants, despite their markedly different nutritional physiology and greater degree of lactational negative energy balance compared with laboratory rats. This study examined the hypothesis that leptin signaling plays a role in the hyperphagia of lactation in a ruminant. Specifically, we investigated the effects of lactation in sheep on leptin gene expression in adipose tissue, plasma leptin concentrations, and the gene expression of leptin-sensitive hypothalamic neuropeptides and receptors.

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MATERIALS AND METHODS

Animals. Twenty-four multiparous Finn × Dorset Horn crossbred ewes from the same flock were used. Eight had not been mated and were nonlactating, nonpregnant controls, whereas the remaining 16 sheep suckled either two or three lambs. Animals were given a fixed daily ration of concentrates comprising 18% protein, 12.5 MJ metabolizable energy (ME)/kg dry matter (DM) (Davidson Brothers, Lanarkshire, UK) two times daily at 0700 and 1600, at 1.2 kg/day for lactating sheep and 0.5 kg/day for controls. Sheep were also fed “hay saver” pellets ad libitum; these comprised sodium hydroxide-treated straw and dried grass (9.4 MJ ME/kg DM; N. S. Milling, Heck Hall Farm, East Yorkshire, UK). Ewes were killed on day 6 or day 18 of lactation and on close calendar dates for the controls (n = 8/group). The number of animals suckling three lambs was equally distributed between the two groups. Food intake was measured daily throughout lactation and daily for 1 wk before killing for controls. Body weight of ewes and their lambs was measured at days 2, 6, 10, 14, and 18 of lactation and 1 wk before and immediately before the control animals were killed. All procedures involving animals were approved by the Hannah Research Institute Ethical Review Committee.

Blood sampling. Blood samples were collected via a jugular catheter at 1200, 1400, and 1600 (just before feeding) on the day before killing and at 0900 immediately before the animal was killed. Plasma was prepared and stored at −20°C until further analysis.

Measurement of hormones and metabolites. Plasma glucose concentrations were determined in deproteinized samples as described by Bergmeyer (14). Free fatty acid (FFA) concentrations were measured colorimetrically (38). Insulin and thyroxine were determined by RIA using commercial kits (ICN Pharmaceuticals, Hampshire, UK, and IDS, Tyne and Wear, UK). Plasma leptin concentrations were determined by RIA (47), with a detection limit of 0.45 ng/ml and intraclass coefficients of variation 12 and 16%, respectively.

Adipose tissue. Sheep were anesthetized with 30 ml pentobarbital sodium (Sagatal; Rhone Merieux) and were killed by exsanguination. Subcutaneous adipose tissue samples were removed from the flank fat pads anterior to the hindlimbs. Tissue for leptin RNA determination was snap-frozen in liquid N2 and stored at −80°C before assay. Adipose tissue for measurement of lipogenesis and mean cell volume was placed in medium 199 with Earle’s salt and 25 mM HEPES, pH 7.3 (Life Technologies, Paisley, UK), with 2 mM acetate and antibiotics (57) at 37°C for transportation to the laboratory (~5 min).

The rate of fatty acid synthesis was measured in adipose explants by the incorporation of [14C]acetate into total lipid, as described by Vernon and Finley (67). Adipocytes were removed by collagenase digestion, and the adipocyte mean cell volume and number of adipocytes per gram adipose tissue were determined (57).

RNase protection assay. A 252-bp ovine leptin cDNA in pCRII-TOPO (a kind gift from R. Ehrhardt and Y. Boisclair, N. S. Milling, Heck Hall Farm, East Yorkshire, UK) was linearized with HindIII and transcribed using SP6 RNA polymerase and antisense transcripts with [α-32P]CTP and T7 RNA polymerase (41, 52).

Tissue samples were powdered in a mortar and pestle using liquid N2 and were homogenized in 5 M guanidinium isothiocyanate and 100 mM EDTA (pH 7.0) using a constant tissue-to-volume ratio. Aliquots (40 μl) of these were then hybridized to the antisense ovine leptin cRNA and subsequently digested using RNase A/RNase T1 and proteinase K, as described by Firestein et al. (32). In addition, a standard amount of sense transcript was hybridized to the antisense transcript as a positive control. After extraction with phenol and chloroform, the samples were precipitated two times and rinsed with 80% (vol/vol) alcohol. Samples were then dried, resuspended, and, after denaturing in formamide loading buffer at 85°C, resolved on a 6% (wt/vol) acrylamide/7 M urea sequencing gel (45). After being dried, the gels were exposed to a Kodak phosphor screen overnight. The resulting images were scanned using a Molecular Dynamics PhosphorImager 445 SI, and the volume of the individual bands was obtained using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The weight of the tissue-to-volume ratio of the homogenates was constant, so, using the number of adipocytes per gram tissue, the results were expressed as arbitrary units per 106 adipocytes.

Hypothalamic gene expression. Brains were removed and immediately frozen in isopentane and were stored at −80°C until further analysis. Coronal brain sections (20 μm) were collected through the hypothalamus and mounted on gelatin-coated slides. A standard number of leptin-sensitive neuropeptides and receptors were determined by semiquantitative in situ hybridization using methods described in detail by Adam et al. (1) and Mercer et al. (49). Riboprobes for the leptin receptor (OB-Rb) and CART were generated from cloned sheep cDNAs as described by Williams et al. (73) and Barrett et al. (9), respectively. NPY was generated from a rat cDNA kindly provided by Dr. S Sabol and validated on sheep hypothalamic sections by Adam et al. (1). AGRP and POMC riboprobes were generated from Siberian hamster cDNAs (51) and validated on sheep sections (C. L. Adam, Z. A. Archer, and P. A. Findlay, unpublished observations). The MC3-R riboprobe was generated from cloned human cDNA fragments and initially validated on Siberian hamster tissues (2). A preparative investigation showed that hybridization to the heterologous MC3-R riboprobe occurred with localization in the ovine hypothalamus similar to that seen in the Siberian hamster after identical RNase digestion and high-stringency washing protocols. The sense probe showed no hybridization with ovine hypothalamic tissue.

Three representative sections from the rostral, mid, and caudal regions of the hypothalamus from each animal were selected, fixed in 4% paraformaldehyde, and acetylated before hybridization. Using 35S-labeled riboprobes at a concentration of 1 × 105 counts-min−1·ml−1, slides were hybridized overnight at 58°C. After incubation, slides were treated with RNase A, desalted in saline-sodium citrate with increasing stringency, dried, and apposed to Hyperfilm β-max (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Autoradiographic images were quantified using the Image-Pro Plus software system, which measures the intensity and area of the hybridization signal on the basis of set parameters. A standard curve was generated using [3H]microscales (Amersham Pharmacia Biotech), and the integrated intensity of the hybridization signal was then corrected against this standard curve. CART, OB-Rb, and MC3-R were expressed in both the ARC and ventromedial hypothalamus (VMH; adjacent regions of the hypothalamus). Subdivision of the hypothalamus into ARC and VMH was made by a comparison with other sections from the same region from the same sheep but was hybridized for POMC, AGRP, and NPY, which are expressed in the ARC but not in the VMH.

Statistical analysis. All data were analyzed by ANOVA using genstat (Genstat 5 Release 4.1; Lawes Agricultural Trust, Rothamsted Experimental Station); factors were animal and physiological state and time of day. Where no effect...
was found for “time of day,” the average for the four plasma samples taken at different times was calculated, and the data were reanalyzed using animal and physiological state as factors.

RESULTS

Total DM intake (concentrates plus hay saver pellets) increased ($P < 0.01$) over the first 2 wk of lactation and then appeared to plateau (Fig. 1). Food intake of ewes at 6 and 18 days of lactation was greater than that of nonlactating sheep ($P < 0.01$); in terms of ME, controls consumed ~20 MJ/day, whereas lactating ewes consumed ~27 MJ/day at day 6 and ~29 MJ/day at day 18. The body weight of control ewes was maintained at 81 ± 2.8 kg for the week before killing. The body weight of the two lactating groups at day 2 postpartum was 70.0 ± 2.1 and 73 ± 3.0 kg for the groups killed on day 6 and day 18 of lactation, respectively, and in both cases was significantly ($P < 0.05$) less than that of controls. The body weight at time of death was 68.7 ± 2.0 and 70.9 ± 2.6 kg for ewes killed on day 6 and day 18; in both cases, there was a tendency for body weight to decrease between day 2 postpartum and the day of killing ($P = 0.10$ and 0.11, respectively, for day 6 and day 18 lactating ewes). In the early lactating group, the lambs gained 2.8 ± 0.37 kg between days 2 and 6 of lactation, whereas in the peak lactating group the lambs gained 7.5 ± 0.84 kg between days 2 and 18 of lactation.

Plasma insulin concentrations were lower at day 6 of lactation compared with control animals, whereas lactation had no effect on plasma thyroxine concentration (Table 1). There was no difference in the concentration of plasma glucose between any of the groups (Table 1). There was no difference in the concentration of adipose tissue was significantly higher ($P < 0.001$) in lactating animals than nonlactating animals (Fig. 2). The time of day affected plasma levels of FFA and concentrations were lowest 2 h after the concentrate meal given at 0700 and increased until the next meal of concentrates given at 1600. Furthermore, the plasma concentration of FFA was higher at day 6 of lactation than day 18, although this difference was only significant at 0900 and 1200 (Fig. 2). Plasma leptin concentration was four to five times higher ($P < 0.001$) in nonlactating than in lactating animals (Fig. 3). No difference was evident in leptin concentration between day 6 and day 18 of lactation, and there was no effect of time of day.

The mean cell volume of subcutaneous adipocytes was significantly lower during lactation ($P = 0.01$), but no difference was found between day 6 and day 18 of lactation (Table 2). The rate of fatty acid synthesis was markedly reduced during lactation ($P < 0.001$), and again no significant difference was evident between day 6 and day 18 of lactation (Table 2). Leptin mRNA concentration of adipose tissue was significantly reduced during lactation ($P < 0.001$; Table 2), and there was a tendency for the concentration to be lower at day 6 than at day 18 of lactation ($P = 0.07$). Leptin plasma levels correlated significantly with leptin mRNA levels in adipose tissue ($R = 0.72, P < 0.001$); the relationship was not altered by lactation.

Hypothalamic neuropeptides and receptor gene expression. OB-Rb gene expression in the hypothalamus was localized in the ARC and VMH. Lactating animals

Table 1. Plasma concentrations of thyroxine, insulin, and glucose from day 6 and day 18 lactating sheep and nonlactating control sheep

<table>
<thead>
<tr>
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<th>Day 6 of Lactation</th>
<th>Day 18 of Lactation</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>Thyroxine, ng/ml</td>
<td>$68 \pm 7.4^*$</td>
<td>$82 \pm 7.3^*$</td>
<td>$84 \pm 7.2^*$</td>
</tr>
<tr>
<td>Insulin, µIU/ml</td>
<td>$24 \pm 1.6^+$</td>
<td>$39 \pm 13.3^+$</td>
<td>$51 \pm 9.2^+$</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>$2.8 \pm 0.24^*$</td>
<td>$3.5 \pm 0.24^*$</td>
<td>$3.3 \pm 0.21^*$</td>
</tr>
</tbody>
</table>

Values are means ± SE of 8 observations. Within a row, values that do not share a common superscript are significantly different, $P < 0.05$, ANOVA.
tended to have higher expression of OB-Rb in both ARC (P < 0.08) and VMH (P < 0.10) compared with nonlactating control animals, and no difference was found between days 6 and 18 of lactation (Fig. 4). Values for lactating ewes were very variable; when results were log transformed and then reanalyzed by ANOVA, a significant increase was revealed in OB-Rb expression in the ARC and VMH of lactating ewes (P = 0.002 and 0.049, respectively).

POMC and AGRP mRNAs were localized to the ARC, whereas mRNA for MC3-R was localized in the ARC and VMH (Fig. 5). POMC gene expression was down-regulated (P < 0.001), whereas AGRP mRNA was significantly upregulated by lactation (P = 0.05; Fig. 6); no difference was found in expression between day 6 and day 18 of lactation. Expression of MC3-R mRNA was not affected by lactation in either the ARC or VMH (Fig. 6).

NPY mRNA was detected in the ARC and dorsomedial hypothalamus (DMH; Fig. 7). Compared with nonlactating controls, NPY gene expression was increased significantly in the ARC of lactating animals but was not affected by the stage of lactation; NPY expression also increased in the DMH but only on day 18 of lactation (Fig. 4).

CART mRNA was expressed in the ARC, VMH, and paraventricular nucleus (PVN). CART gene expression tended to decrease with lactation in both the ARC (P = 0.1) and VMH (P = 0.1; ANOVA; Fig. 6). No effect was found for the stage of lactation on CART gene expression in any of the hypothalamic nuclei. Comparison of control values with pooled values for sheep at both stages of lactation showed that lactation caused a significant decrease in CART gene expression in both the ARC and VMH (P < 0.05).

**DISCUSSION**

The effects of pregnancy and lactation on Finn × Dorset Horn sheep have been studied in some detail previously. The empty carcass weight decreases during late pregnancy, mostly because of loss of adipose tissue lipid (58, 59); hence, the decreased body weight of the ewes at day 2 postpartum was not unexpected. Food intake increases after birth, reaching a plateau between 2 and 3 wk postpartum (24, 60). Nevertheless, ewes suckling two or more lambs lose ~5 kg in body weight, mostly because of loss of fat from adipose tissue during the first 5–6 wk of lactation (22, 24, 23, 24, 60). Ewes suckling a single lamb do not appear to lose adipose tissue lipid, whereas ewes suckling three lambs lose an amount of adipose tissue lipid similar to those suckling two lambs (66).

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Table 2. Mean cell volume of subcutaneous adipocytes, rate of fatty acid synthesis from acetate, and leptin mRNA concentration in adipose tissue in day 6 and day 18 lactating sheep and nonlactating control sheep

<table>
<thead>
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<th>Day 6 of Lactation</th>
<th>Day 18 of Lactation</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte mean cell volume, pl</td>
<td>555 ± 34*</td>
<td>517 ± 49*</td>
<td>776 ± 87†</td>
</tr>
<tr>
<td>Rate of incorporation of acetate into fatty acid, μmol·h⁻¹·10⁷ cells⁻¹</td>
<td>0.044 ± 0.01*</td>
<td>0.040 ± 0.013*</td>
<td>10.84 ± 2.89†</td>
</tr>
<tr>
<td>Leptin mRNA, arbitrary units/10⁶ cells</td>
<td>17 ± 3.8*</td>
<td>20 ± 2.1*</td>
<td>76 ± 15.2†</td>
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Values are means ± SE of 8 observations. Within a row, values that do not share a common superscript are significantly different, P < 0.05, ANOVA.
tissue lipid indicates that the ewes suckling two or more lambs were in negative energy balance during the first 5–6 wk of lactation. Consistent with this, serum nonesterified fatty acid concentrations are elevated during the first 3 wk of lactation, then decline gradually to levels found in fed, nonlactating ewes by ~6 wk after birth (23, 66). The changes in body weight, food intake, adipocyte size, and plasma nonesterified fatty acid concentration found with lactation in the present study are thus consistent with previous findings. Furthermore, the elevated serum nonesterified fatty acid levels and the very low rates of fatty acid synthesis of the adipocytes from the lactating ewes show that adipocytes are in a catabolic state. Hence the ewes were in negative energy balance for at least the first 18 days after birth, despite having food available ad libitum.

Plasma leptin concentrations and adipocyte leptin mRNA concentrations were both markedly decreased by lactation in sheep. A very recent study suggests that serum leptin concentrations were not decreased in Karakul ewes suckling a single lamb (30). However, although the lactating ewes in the study were fed ad libitum, food intake of the control, nonlactating ewes was restricted to maintain a constant body weight over the previous 24 wk (30), which could have decreased their serum leptin concentrations (26, 47). Previous studies with rats have also shown that serum leptin is decreased by lactation (16, 36, 39, 56, 65, 74). However, in the daytime at least, the fall in leptin concentration is much smaller in rats than in sheep. This species difference may reflect a greater degree of negative energy balance during lactation in sheep than in rats. Rats differ from sheep in that they are mainly nocturnal feeders, and this pattern of intake is reflected in an increase in serum leptin at night, which is markedly suppressed during lactation (56). Fed sheep show no evidence for a circadian rhythm of plasma leptin but do exhibit low-amplitude postprandial peaks (47) that could have remained undetected in the present trial given the low sampling frequency. Undernutrition or fasting in sheep results in a fall in serum leptin (26, 47) and adipose tissue leptin mRNA concentration (42), as in other species (3, 5, 37, 68). Thus the hypoleptinemia of lactating sheep suckling two or more lambs is not surprising in view of the negative energy balance of the animal and would be expected to increase appetite and so contribute to the hyperphagia of lactation.

Leptin acts on a number of neuropeptides and receptors in the hypothalamus to regulate appetite and energy balance; systems involved include the NPY, CART, and melanocortin pathways (3, 4, 31, 62, 64, Fig. 5. Typical autoradiograph showing hypothalamic localization of melanocortin receptor (MC3-R) mRNA by in situ hybridization of antisense riboprobe to a 20-μm coronal section from a day 18 lactating ewe. Sense probe showed no hybridization. 3V, third ventricle. Magnification, ×9.6.

Fig. 6. Gene expression of proopiomelanocortin (POMC), MC3-R, agouti-related peptide (AGRP), and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamic ARC, ARC and VMH, VMH, respectively, of nonlactating (filled bar), day 6 lactating (gray bar), and day 18 lactating (open bar) ewes. Values are means ± SE of 8 observations. For each mRNA, the mean value for nonlactating ewes was standardized to 100 arbitrary units, and other values were adjusted accordingly. Within columns, values that do not share a common superscript (a, b) are significantly different, P < 0.05, ANOVA.

Fig. 7. Typical autoradiograph showing hypothalamic localization of neuropeptide Y (NPY) mRNA by in situ hybridization of antisense riboprobe to a 20-μm coronal section from a day 18 lactating ewe. Sense probe showed no hybridization. 3V, third ventricle. Magnification, ×9.6.
72). In this study, we examined the effect of lactation on four key neuropeptides (NPY, CART, POMC, and AGRP) and also on OB-Rb receptor and MC3-R. In our study, gene expression of OB-Rb in both the ARC and VMH increased during lactation in sheep. In the rat, there are complex changes in OB-Rb expression with lactation, with an increase in expression in the supraoptic nucleus, a decrease in expression in the VMH, and no change in the ARC, DMH, or PVN (15). By contrast, fasting or feed restriction increases OB-Rb expression in the ARC and VMH in both rats (9, 13) and sheep (29), suggesting that leptin suppresses expression of its receptor in the ARC and VMH. Our data for lactating sheep are consistent with this reciprocal relationship between leptin and OB-Rb expression. The reason for the rather different findings for the lactating rat (15) may be because of lactation inducing a more modest degree of negative energy balance and a smaller decrease in serum leptin in rats than in sheep. The increased gene expression of OB-Rb also suggests that the hypothalamus may become more sensitive to leptin during lactation, as shown during fasting in rats (10).

Leptin increases expression of the anorexigenic neuropeptides POMC and CART and decreases expression of the orexigenic neuropeptides NPY and AGRP in the hypothalamus (3, 4, 31, 62, 64, 72). The increased gene expression for NPY and AGRP and the decreased gene expression for POMC and CART found during lactation in the present sheep are thus consistent with the concurrent hypoleptinemia and should act to drive hyperphagia. A rise in gene expression for NPY (18, 21, 43, 44, 54, 55, 63, 71) and AGRP (18) and a fall in POMC gene expression (54, 63) have been reported previously for lactating rats, but this is the first report of decreased CART gene expression during lactation. In keeping with these findings, negative energy balance induced by fasting in sheep also decreases serum leptin concentrations (26, 47), increases hypothalamic gene expression for NPY and AGRP, and decreases expression of POMC and CART (C. L. Adam, Z. A. Archer, and P. A. Findlay, unpublished observations).

Lactation increased expression of NPY mRNA in both the ARC and the DMH in sheep; however, the increased expression in the DMH developed more slowly, being apparent at 18 but not 6 days of lactation. NPY gene expression in the rat is also increased in both the ARC and DMH around peak lactation (43, 44). However, the regulation of NPY neuronal activity apparently differs between the ARC and DMH; for example, NPY neurons in the DMH are not activated under conditions of food restriction, which activate NPY neurons in the ARC of rats (48). Conversely, in both MC4-R knockout mice and agouti mice, which lack a functional MC4-R because of functional blockade by endogenous agouti protein, NPY is upregulated in the DMH but not in the ARC, suggesting that signaling via MC4-R acts to attenuate NPY expression in the DMH (40). Li et al. (43) showed that, in lactating rats, NPY expression in the DMH could be induced within 3 h by the suckling stimulus, whereas 24 h of suckling were required to increase NPY expression in the ARC. During lactation, signaling through MC4-R is likely to be markedly suppressed by the large increase in the ratio of AGRP to POMC in both rats and sheep, and this may facilitate increased gene expression for NPY in the DMH in sheep, at least at 18 days of lactation. It is possible that the lack of upregulation of NPY mRNA in the DMH at 6 days as opposed to 18 days of lactation in sheep may be because of a lower suckling stimulus at this time. It is not known if NPY expression is increased in the rat DMH during early lactation.

The melanocortin system is a major anorexic system of the hypothalamus; the system is stimulated by α-melanocyte-stimulating hormone, which is derived from POMC, and is inhibited by AGRP. These neuropeptides act via MC3-R in the ARC and VMH and MC4-R in the PVN (6, 53). In the ARC, MC3-R mRNA is expressed in both AGRP and POMC neurons, which has led to the suggestion that MC3-R mediates the antagonistic interaction between AGRP and POMC (6). Localization of MC3-R mRNA in the ovine ARC and VMH is consistent with its hypothalamic localization in rodents (2, 6). As described above, expression of POMC is decreased, whereas that of AGRP is increased, during lactation in both sheep and rats, and this, by diminishing signaling through the melanocortin system, should promote the hyperphagia of lactation. However, despite the changes in AGRP and POMC gene expression, MC3-R gene expression was not affected in either the ARC or VMH in lactating sheep. There are no published data available on MC3-R gene expression in lactating rodents. In contrast to lactating sheep, food-restricted hamsters, with reduced circulating leptin, exhibit decreased gene expression for both MC3-R and POMC in the ARC and increased MC3-R gene expression in the VMH (50). It is open to speculation whether these differences are attributable to a species difference in sensitivity of the melanocortin pathway to negative energy balance or to a difference in response to negative energy balance induced by food restriction as opposed to lactation.

Thus lactation in sheep suckling two or more lambs induced a state of negative energy balance, low circulating leptin, and changes in hypothalamic gene expression consistent with responses to the diminished leptin feedback: the orexigenic NPY pathway was activated, and the activities of the anorexic melanocortin and CART pathways were downregulated. These findings are consistent with reduced leptin signaling playing a role in the hyperphagia of lactation in ruminants. The changes in gene expression of leptin-sensitive hypothalamic neuropeptides were very similar to those observed during fasting or food restriction. However, during lactation, the negative energy balance is not the result of a lack of availability of food (the sheep in the present study were fed ad libitum); rather it was because of an inadequate appetite. Lactating rats with ad libitum food also show negative energy balance (7), and, for this species at least, it would appear that physical constraints such as gut size are not responsible for limiting intake (27). Further-
more, high-yielding dairy cows during early lactation were induced to increase both milk yield and food intake by increased milking frequency (8); this suggests that a physical constraint on appetite is also unlikely to be responsible for the negative energy balance that occurs during early lactation in dairy cows. Indeed, a period of negative energy balance appears to be a widely used strategy, possibly to increase metabolic efficiency, during lactation (69). Resolving the molecular basis for this self-imposed period of negative energy balance during lactation should provide useful insight into the regulation of appetite control in sheep and other species.

It is important to note, of course, that lactation can occur in animals in energy balance, so other factors must also drive the hyperphagia in addition to “negative energy balance.” The suckling stimulus is important for hyperphagia in rats (33, 46, 74), but the mechanism is unresolved. Suckling increases secretion of both prolactin and oxytocin (25). Prolactin can increase food intake in nonlactating rats, and hypothalamic prolactin receptor mRNA expression is increased during lactation in the rat (34). Nevertheless, the role of prolactin in the hyperphagia of lactation is still unresolved (34). Oxytocin secretion is also increased during lactation, but oxytocin in fact decreases food intake in the rat (12). Thus the mechanisms driving the hyperphagia of lactation remain unresolved. Hypoleptinemia, probably arising from a self-induced state of negative energy balance, would appear to be an important stimulus of hyperphagia during the initial stages of lactation at least in sheep, as also appears to be the case in rats.

We thank Dr. N. Hoggar for help in preparing the ovine leptin antiserum and Drs R. A. Ehrlhardt, Y. R. Bousilclair, S. Sabel, J. Mercer, A. Ross, P. Barrett, and N. Hoggar for ribprobes. We thank I. Nevison (Biological and Biomathematical Sciences, Scotland) for statistical advice and the Scottish Executive Environment and Rural Affairs Department for financial support. Present address for M. Marie: Sciences Animales, ENSAIA-INPL, BP 172, 54505, Vandoeuvre le’s Nancy, France.

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AJP-Regulatory Integrative Comp Physiol • VOL 282 • APRIL 2002 • www.ajpregu.org


