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Lactation Suppresses Diurnal Rhythm of Serum Leptin

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Rats consume most of their daily food intake at night; serum leptin levels and adipose tissue leptin mRNA content are elevated at night in non-lactating rats fed *ad libitum*. Lactation induces massive hyperphagia with most food still consumed at night, but the nocturnal increase in leptin secretion was not observed in lactating rats. Thus the link between nocturnal food intake and increased serum leptin is broken during lactation and the hypoleptinaemia may be an important factor promoting the hyperphagia of lactation.

During lactation the nutrient demands of the mammary gland often exceed those of the whole body of the non-lactating animal (1, 2). This increased requirement for nutrients is met primarily by increased food intake, although there is some mobilisation of reserves, especially adipose tissue lipid, and adaptation of certain metabolic processes (eg lipogenesis) to favour preferential use of nutrients by the mammary gland (2, 3).

Non-lactating rats consume about 85% of their daily food intake at night (4-6). During lactation food consumption increases from 15-20 g/day to 60-70 g/day (1-3), but about 80% of food is still consumed at night (6, 7). The factors responsible for this massive increase in appetite during lactation remain poorly defined, but we and others have found evidence for enhanced activity of specific hypothalamic neurones that express neuropeptide Y (NPY), an extremely potent stimulator of feeding (7-10). The discovery of leptin (11), a peptide produced and released by adipocytes, which is thought to have a major role in appetite regulation and energy expenditure (12, 13), provides another possible factor which could be involved in the increased appetite of lactation. Leptin acts on the central nervous system to inhibit feeding by a mechanism which may be mediated, at least in part, by inhibition of hypothalamic NPY neurones (12, 13). The possibity that changes in circulating leptin concentration contribute to the hypophagia of lactation has been investigated in the present study.

MATERIALS AND METHODS

Animals. Female Wistar rats were bred at the Hannah Research Institute from stock originally obtained from A Tuck and Son, Rayleigh, Essex, UK. They were fed standard pelleted chow *ad libitum* (CRM diet, Labsure, Poole, UK). Rats were divided into two equal groups of 12 animals: one group was maintained on a light-dark schedule of light from 8.00 h to 20.00 h and the other on light from 20.00 h to 8.00 h. One half of each group was mated at about 10 weeks of age, while the other was kept as unmated controls. Unmated, control rats and lactating rats (days 12-15 of lactation), from both light-dark schedules, were anaesthetised between 9.30 and 10.30 h by an intraperitoneal injection of 0.6 ml of Sagatal (RMB Animal Health Ltd, Dagenham, Essex, UK) containing 44 μl of Hypnorm (Janssen Pharmaceuticals, Oxford, UK) Samples of blood and parametrial (white) adipose tissue were removed and the rat then killed by exsanguination. At the time of sampling, rats had been exposed to their specific light-dark regimens for a minimum of 4 weeks. Animals in the dark between 8.00 h and 20.00 h were exposed to minimal disturbance during this period.

Serum hormones and metabolites. Serum was prepared and stored at −40°C prior to assay as described previously (7). Serum leptin was determined using a mouse leptin RIA kit with an intra-assay co-efficient of variation of 7% and a limit of sensitivity of 0.2 ng/ml (LINCO Research, Biogenesis, Poole, Dorset) (14). Serum insulin and glucose were measured using an RIA kit with an intra-assay co-efficient of variation of 5.6% and sensitivity of <2 μU/ml (Pharmacia and Upjohn Diagnostics, Lewes, Sussex), and a D-glucose UV-test kit (Boehringer Mannheim, Milton Keynes, Bucks.), respectively. Serum non-esterified fatty acid (NEFA) concentration was determined by a procedure based on that of Itaya and Ui (15). One hundred μl of serum was diluted with 330 μl of 10 mM NaH_2PO_4, pH 6.5. One ml chloroform was added and tubes shaken for 90 sec and then left to stand for 25 min. Upper phase was removed and discarded; 0.5 ml of 0.9 M triethanolamine containing 0.1 M acetic acid and 6.45% (w/v) Cu $(NO_3)_2$ added to the remaining lower phase. Tubes were shaken for 30 sec and then allowed to stand for 25 min. Upper phase was removed and discarded; 0.5 ml of lower phase was removed and mixed with 25 μl of 0.1% (w/v) sodium diethyl-dithiocarbamic acid in butan-l-ol following which the A_{440} was determined. Palmitic acid, 20 to 240 nM, was used as a standard.

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TABLE 1

Effect of Lactation on Diurnal Changes in Body Weight and Serum Leptin, Insulin, Glucose and Nonesterified Fatty Acid Concentrations

	Non-lactating		Lactating		
Variable	Light	Dark	Light	Dark	SED
Maternal body weight (g)	250[a]	263[a]	293[b]	317[c]	9
Litter weight (g)	—	—	312[a]	297[a]	22
Serum leptin (ng/ml)	2.6[a]	8.7[b]	1.4[a]	1.8[a]	1.0
Serum insulin (ng/ml)	20.6[ab]	32.0[b]	8.6[a]	17.6[a]	6.5
Serum glucose (μM)	5.6[a]	4.9[ac]	3.5[bc]	2.6[b]	1.0
Serum nonesterified fatty acid (μM)	424[a]	206[b]	410[a]	383[a]	78

Note. Values are means of 6 observations and were analyzed by ANOVA: SED is standard error of difference. a, b, c, values in a row without the same suffix (a, b, c) differ significantly ($P < 0.05$).

Adipocyte metabolism. Adipocytes were prepared by collagenase digestion; adipocyte mean cell volumes and rate of incorporation of [U-^{14}C] glucose into fatty acids were determined as described previously (16).

Measurement of leptin mRNA. Leptin mRNA levels were measured in white adipose tissue collected from the parametrial fat pad. Tissue was snap-frozen in liquid nitrogen and stored at −80°C. Total RNA extraction and processing by Northern blotting, followed by chemiluminescent detection of leptin mRNA was performed as described previously (14). The amount of 18S rRNA was also determined (14), and the amount of leptin mRNA was expressed as the leptin mRNA/18S rRNA ratio.

Statistical analysis. Results were analysed by analysis of variance (ANOVA) with physiological state, photoperiod and their interaction as factors.

RESULTS

Analysis of variance showed that body weight was increased in lactating rats ($P<0.001$) and was greater during the dark phase ($P<0.01$) than during the light phase (Table 1), probably due to increased amount of food in the gastrointestinal tract. Pup weight, however, was the same during both phases (Table 1).

In non-lactating rats, serum leptin concentrations, showed a marked diurnal variation, increasing over 3-fold (P 0.05) during the dark period (Table 1). ANOVA showed that serum leptin concentrations were diminished in lactating rats, and, in addition, there was no diurnal variation, concentrations in the dark period being the same as those found in the light period (Table 1). Lactation also decreased serum insulin concentrations ($P<0.01$, from ANOVA). Serum insulin exhibited diurnal variation, being higher during the dark period ($P<0.05$, from ANOVA) in both lactating and non-lactating groups (Table 1). Serum glucose concentration was lowered by 40-50% during lactation ($P<0.01$; ANOVA), but showed no diurnal variation. In non-lactating rats, serum NEFA concentration was higher ($P<0.05$) during the light than during the dark phase (Table 1). By contrast, serum NEFA concentrations showed no diurnal variation in lactating rats, levels in both phases being similar to the elevated serum NEFA concentrations found in non-lactating rats during the light phase (Table 1).

Adipocyte mean cell volume was decreased by lactation but did not exhibit diurnal variation in either lactating or non-lactating rats (Table 2). The rate of fatty acid synthesis (lipogenesis) of adipocytes was decreased ($P<0.01$) by lactation. The rate of lipogenesis was highest during the dark phase in non-lactating rats, tending to fall in the light phase (Table 1). Leptin mRNA levels (expressed as the ratio to 18S rRNA levels) of adipose tissue showed a diurnal rhythm in non-lactating rats, with levels during the dark period being higher ($P<0.05$) than during the light period (Table 2). No diurnal change in the amount of leptin mRNA of adipose tissue was found in lactating rats, leptin mRNA levels in both dark and light phases being similar to those found in non-lactating rats during the light phase (Table 2).

DISCUSSION

The present study suggests that a fall in circulating leptin levels contributes to the massive hyperphagia of lactation. The study also emphasises the importance of diurnal variation in serum leptin concentrations and also the need to determine serum leptin concentrations during the night (dark phase) when the concentration is maximum.

A previous study (17) showed diurnal changes in the amount of leptin mRNA of adipose tissue in male rats fed *ad libitum*, with maximum levels at night and minimum levels during the day. Fasting, however, resulted in a loss of the nocturnal rise in adipose tissue leptin mRNA content (17); refeeding or insulin treatment of fasted rats increased adipose tissue leptin mRNA levels, suggesting that the noctural rise was due to feeding and mediated, at least in part, by serum insulin (17). In the present study we show that serum leptin as well as adipose tissue leptin mRNA concentration shows a

TABLE 2

Effect of Lactation on Adipocyte Mean Cell Volume, Fatty Acid Synthesis and Adipose Tissue Leptin mRNA Concentration

	Non-lactating		Lactating		
Variable	Light	Dark	Light	Dark	SED
Adipocyte mean cell volume (pl)	437^{a}	446^{a}	270^{b}	287^{b}	64
Rate of fatty acid synthesis (nmole glucose incorporated/2 h per 10^6 cells)	242^{ac}	336^{a}	176^{bc}	116^{b}	58
Leptin mRNA:18S rRNA ratio	0.26^{a}	1.18^{b}	0.23^{a}	0.34^{ab}	0.42

Note. Results are means of 6 observations (*5 observations) and were analyzed by ANOVA: SED is standard error of difference from ANOVA. a, b, c, values in a row without the same suffix (a, b, c) differ significantly ($P < 0.05$).

diurnal rhythm with a maximum at night in *ad libitum* fed rats. As leptin inhibits feeding, the nocturnal rise in serum leptin may serve as a form of negative feed-back control to limit intake.

Despite the massive hyperphagia, *ad libitum* fed lactating rats still consume over 70% of their intake at night; we have confirmed this observation in a subset of our own rats (13.1 ± 2.6 and 59.2 ± 2.4g food consumed during the day and during the night, respectively, at day 14 of lactation; $n = 3$). During lactation, however, there is no concurrent rise in serum leptin. Thus the link between nocturnal leptin secretion and food intake is broken during lactation; a loss of leptin-induced, negative feed-back control may then be a major factor promoting the hyperphagia of this state.

Previous studies of serum leptin and adipose tissue leptin mRNA in lactating rodents have all involved sampling during the day-time. Several studies have shown that serum leptin concentration is elevated during pregnancy in rats (18, 19) and mice (20, 21), returning to levels found in virgin, control animals by early lactation (18, 21). However, in one study (19) serum leptin fell during early lactation to levels below those found in virgin rats. Moreover, a decrease in adipose tissue leptin mRNA concentration was found in lactating rats in one study (19), but not in another (22). These apparent discrepancies probably arise from different times of sampling and, particularly, how far the non-lactating animals were into the post-prandial period.

Despite increasing their food intake several fold, lactating rats still usually mobilise some adipose tissue lipid (2, 3). This was observed in the present study, with lactation resulting in a fall in adipocyte size, due in part to a decrease in lipogenesis and also to an increase in lipolysis as evinced by the elevated serum NEFA concentration. The rate of lipogenesis tended to increase in the dark period in non-lactating rats as found previously (23) while lipolysis was greatest during the light period (5). This is consistent with accumulation of lipid when non-lactating rats are eating, followed by mobilisation during the post-prandial period; this diurnal cycle is lost in lactation, with lipogenesis apparently being permanently reduced and lipolysis permanently elevated.

The mechanism responsible for the diminished leptin secretion during lactation is unclear, but both neural and hormonal factors could contribute. The pattern of changes in serum leptin concentration with lactation and photoperiod are the inverse of those in serum NEFA concentration. Leptin secretion is inhibited (24), whereas lipolysis is stimulated, by catecholamines, so both could be due to enhanced sympathetic nervous activity to white adipose tissue. Little is known about the effects of lactation on the sympathetic nervous system, but there is some evidence for increased sympathetic activity in white adipose tissue in this state (25). Insulin stimulates leptin production (12, 13, 24) and insulin also shows a pattern of diurnal change in non-lactating rats similar to that of leptin (26, 27). However, although serum insulin levels are decreased during lactation (2, 3), the diurnal rhythm is retained (6, 28), so changes in serum insulin alone cannot account for the loss of the diurnal rhythm in circulation in serum leptin during lactation. Insulin stimulation of leptin release by adipocytes appears to depend on stimulation of glucose uptake and metabolism (29), and the ability of insulin to stimulate glucose utilisation is markedly attenuated during lactation contributing to the decreased rate of lipogenesis (2, 3). This insulin resistance of adipocytes rats may thus contribute to the hypoleptinaemia and also the loss of diurnal rhythm in circulating leptin during lactation.

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