Regulation of serum leptin and its role in the hyperphagia of lactation in the rat

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Abstract

The factors regulating serum leptin concentration and its relationship to the hyperphagia of lactation have been investigated in rats. Lactation results in hypoleptinaemia and loss, or at least marked attenuation, of the nocturnal rise in serum leptin. Litter removal resulted in a fall in food intake and restoration of the nocturnal rise in serum leptin. Returning the litter to the mother after a 48-h absence increased food intake and began to reinstate milk production, but the nocturnal serum leptin levels were still increased at 48 h after litter restoration. Adjusting litter size to four, eight, ten or fourteen pups at parturition resulted in different rates of litter growth and food intake during the subsequent lactation, but had no effect on the degree of hypoleptinaemia. Reducing litter size from ten to four pups at mid-lactation resulted in a transient increase in both serum leptin and pup growth rate, while food intake fell to a level found in rats suckling four pups

throughout lactation. Reducing milk production by injection of bromocriptine increased serum leptin, but did not restore the nocturnal rise in serum leptin; food intake decreased, but remained much higher than in non-lactating rats. Feeding a varied, high-energy diet resulted in a decrease in the weight of food ingested, but no change in calorie intake, and had no effect on the hypoleptinaemia. These studies suggested that the hypoleptinaemia of lactating rats is due to negative energy balance, but the loss of the nocturnal rise in serum leptin is due to the suckling stimulus. The negative energy balance of lactation does not appear to be caused by a physical constraint on food intake. While the hypoleptinaemia should facilitate the hyperphagia of lactation, other orexigenic signals must also be involved.

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Introduction

Lactation increases greatly the nutrient needs of an animal; in the rat, for example, the demands of the mammary gland can exceed those of the rest of the body (Wade & Schneider 1992, Barber *et al.* 1997). This increase in nutrient requirement is usually met primarily by increased food intake (Chilliard 1986, Wade & Schneider 1992, Barber *et al.* 1997); in rats, food intake typically increases fourfold to over 60 g/day at peak lactation. Additionally, there are adaptations to improve the metabolic efficiency of the animal and to favour nutrient utilization by the mammary gland; for example, rats show decreased thermogenesis in brown adipose tissue (Trayhurn 1989), decreased lipogenesis in white adipose tissue, hypothyroidism and hypoinsulinaemia (Williamson & Lund 1994, McNamara 1995, Vernon & Pond 1997). Nevertheless, laboratory rats are usually in a modest degree of negative energy balance during lactation, mobilizing about 1 g adipose tissue lipid per day to supplement nutritional needs (Barber *et al.* 1997).

The causes of hyperphagia in lactation are not well understood. One possibility is a decreased secretion of the adipocyte hormone, leptin (Zhang *et al.* 1994), which acts on various hypothalamic nuclei to decrease appetite (Ahima 2000, Ahima & Flier 2000, Ahima *et al.* 2000, Havel 2000, Spiegelman & Flier 2001). Indeed, the daytime serum leptin concentration has been reported to decrease by 20–75% during lactation in the rat (Kawai *et al.* 1997, Pickavance *et al.* 1998, Terada *et al.* 1998, Woodside *et al.* 1998, 2000, Brogan *et al.* 1999, Herrera *et al.* 2000), although others found no change (Chien *et al.* 1997, Carmen-Garcia *et al.* 2000). Rats, however, normally consume 80% or more of their food at night (Kimura *et al.* 1970, Bruckdorfer *et al.* 1974) and this pattern of eating is retained during lactation (Strubbe & Gorissen 1980, Munday & Williamson 1983, Pickavance *et al.* 1996, 1998). Both adipose tissue leptin mRNA expression (Saladin *et al.* 1995, Pickavance *et al.* 1998, Xu *et al.* 1999) and serum leptin concentration (Pickavance *et al.* 1998, Xu *et al.* 1999, Nagatani *et al.* 2000, Nishiyama *et al.* 2000, Mastronadi *et al.* 2000, Pu *et al.* 2000,

Johnstone & Higuchi 2001, Kalsbeek *et al.* 2001) show a diurnal rhythm in fed rats, increasing at night. The nocturnal increase in leptin is dependent on food intake, as the rise in leptin mRNA in adipocytes was not observed in fasted rats (Saladin *et al.* 1995). Furthermore, restricting feeding to a 6-h period during the day caused the rise in serum leptin to occur during the day, concurrently with food consumption (Xu *et al.* 1999). However, a subsequent study suggested that food intake is not the only factor, and implicates the suprachiasmatic nucleus, possibly acting via the sympathetic nervous system, in regulating the diurnal rhythm of leptin secretion (Kalsbeek *et al.* 2001).

The precise function of the nocturnal rise in serum leptin is unclear, but it may act to put some constraint on food intake, or perhaps to increase energy expenditure. Whatever, the nocturnal rise in serum leptin found in fed, non-lactating rats is lost during lactation (Pickavance *et al.* 1998, Johnstone & Higuchi 2001), and so may be an important factor facilitating the hyperphagia.

Leptin may also have a role in adaptations that increase metabolic efficiency during lactation. Leptin administration prevents both the fall in thyroid hormone secretion (Ahima 2000, Ahima & Flier 2000, Ahima *et al.* 2000, Flier *et al.* 2000) and the decrease in brown adipose tissue thermogenesis (Ahima & Flier 2000, Ahima *et al.* 2000, Havel 2000, Spiegelman & Flier 2001) which occur during fasting.

The objectives of this study were to clarify further the role of leptin in the hyperphagia of lactation and to identify factors responsible for the hypoleptinaemia. While it has been suggested that the decreased daytime serum leptin during lactation is due to the energy demands of the mammary gland, rather than the suckling stimulus (Brogan *et al.* 1999, Woodside *et al.* 2000), the factors responsible for the attenuation of the nocturnal rise in leptin have not been defined. We have therefore used a variety of manipulations designed to induce changes in energy balance and suckling intensity. These comprised changing the intensity of the suckling stimulus by removing the litter and varying litter size, feeding a varied, high-energy diet (to try to increase energy intake), and suppression of prolactin secretion and hence milk production using bromocriptine (Cowie *et al.* 1980). The findings provide evidence for negative energy balance being a key factor responsible for the daytime hypoleptinaemia of lactation, whereas the attenuation of the nocturnal rise in leptin is due to the suckling stimulus. Furthermore, leptin appears to facilitate rather than directly cause the hyperphagia of lactation.

Materials and Methods

Animals

Wistar rats (original stock from A Tuck & Sons Ltd, Rayleigh, Essex, UK) were raised in-house at the Hannah

Research Institute, Ayr, UK. Rats were fed a standard chow *ad libitum* (CRM diet; Labsure, Poole, Dorset, UK), which contained 3.2 kcal/g. In one experiment, rats were given a high-energy diet, which comprised standard chow as before, plus cheese crackers (5.3 kcal/g), chocolate (5.3 kcal/g) and peanuts (6.0 kcal/g); all components were given *ad libitum*. Animals were maintained on 12 h light:12 h darkness schedules, with the light period from 0700 h to 1900 h or from 1900 h to 0700 h. At the time of experimentation, rats had been exposed to their specific light:darkness regimens for a minimum of 4 weeks. Animals in the dark between 0700 h and 1900 h were exposed to minimal disturbance. Animals were mated at 2–3 months of age, and the number of pups per litter was adjusted to ten within 24 h after parturition, unless stated otherwise. In one experiment, pups were removed from their mothers for 48 h; during this period the pups were kept with foster mothers. Rats were accustomed to frequent handling, daily weighing and food intake measurements prior to the experimental period. Control, virgin rats were randomly cycling, and were of the same age as lactating rats. When given, bromocriptine (500 µg; Sigma-Aldrich Co. Ltd, Poole, Dorset, UK) was injected subcutaneously twice daily at 0900 h and 1700 h.

Rats were killed by cervical dislocation at 0900–1000 h and trunk blood was collected. In some experiments, blood samples (no more than 0.5 ml) were obtained from the tail vein. For this, rats were lightly anaesthetized using halothane (2–3% in air). The tail was cut with a sharp scalpel no more than 2 mm from the tip, and blood collected. Following this, light pressure was applied to the wound until bleeding ceased. Rats were sampled no more than three times by this procedure and no more than once in a 24-h period.

Further details of individual experiments are given in the Figure and Table legends. All procedures were approved by the Ethical Review Committee of the Hannah Research Institute.

Serum hormone assays

Serum leptin, insulin and thyroxine (T_4) levels were determined by radioimmunoassay using kits from Linco Research Inc., MO, USA (distributed by Biogenesis, Poole, Dorset, UK), and for T_4 from IDS Ltd (Boldon, Tyne and Wear, UK).

Statistical analyses

Data are expressed as means $\pm$ S.E.M. When appropriate, results were analysed by ANOVA; factors and their interactions are given in Figure and Table legends. In some cases results were analysed by Student's *t*-test. A *P* value of 0.05 or less was considered as significant.

Figure 1 Diurnal changes in (a) food intake and (b) serum leptin in lactating (shaded columns/solid squares) and non-lactating (open columns/open circles) rats. Two groups of lactating and non-lactating rats were used; one group of each was exposed to a light period from 0700 h to 1900 h and the other group from 1900 h to 0700 h. Blood was obtained from rats by tail bleeding. Each rat was sampled at 0900 h, 1300 h and 1700 h; each sample was taken on a different day over a 3-day period. Studies were performed between 11 and 14 days of lactation. Food intake was measured over 4-h periods, hence the period 0500 h to 0900 h includes both a light and a dark phase. Results were analysed by ANOVA with state, period (light or dark) and time of sampling and their interactions as factors. Results are expressed as means $\pm$ S.E.M. of eight observations.

Results

Diurnal changes

A similar diurnal pattern of food intake was found in both non-lactating and lactating rats, with over 80% being

consumed during the dark period in both states but, at all times, intake was greater ($P < 0.001$) in lactating than non-lactating rats (Fig. 1a). Serum leptin concentration was increased during the dark period in both non-lactating ($P < 0.001$) and lactating ($P < 0.05$) rats, but the increase

Figure 2 Effect of litter removal on (a) food intake and (b) serum leptin levels at night (2100 h; shaded bars) and morning (0900 h; open bars). The light period was 0700 h to 1900 h. Eight animals had their pups removed at 2100 h. Food intake was measured over 24-h periods (2100–2100 h), starting 24 h before removing the litter. Blood samples were obtained by tail bleeding at 2100 h each day. A second group of five rats had their pups removed at 0900 h; food intake was measured over 24-h periods (0900–0900 h) and blood was sampled each day at 0900 h. All rats were killed 96 h after pup removal, and a further blood sample obtained. Results are expressed as means $\pm$ S.E.M. of thirteen values (food intake), five values for daytime and eight values for night-time leptin.

was markedly smaller in lactating rats (43%) than in non-lactating rats (80%, $P < 0.005$) (Fig. 1b).

Effects of litter removal and replacement

Removal of the litter at day 11 of lactation resulted in a rapid decrease ($P < 0.001$) in food intake, with levels

returning to those of non-lactating animals after 24 h of removal (Fig. 2a). The normal nocturnal rise in serum leptin, which was completely attenuated during lactation, was restored by 24 h after litter removal (Fig. 2b). Litter removal had no effect on daytime serum leptin concentrations (Fig. 2b).

Table 1 Effects of litter removal for 48 h and litter restoration for 48 h, on food intake, maternal weight, pup weight gain and nocturnal serum hormone concentrations. Pups were removed at 2100 h on day 13 of lactation, and fostered by other mothers prior to their return to their respective mothers 48 h later. Food intake was measured over each 24-h period (2100–2100 h). The light period was from 0700 h to 1900 h. Maternal blood samples were obtained by tail bleeding at 2100 h. Results were analysed by ANOVA and are means $\pm$ S.E.M. of seven values unless otherwise stated. Results for five, age-matched control, non-lactating rats are also included; these values were compared with corresponding values for mothers using Student's *t*-test

	Lactating			Non-lactating
	Control	Pups removed (48 h)	Pups replaced (48 h)	Control
Rat weight (g)	296.8 $\pm$ 4.8 ^a	267.6 $\pm$ 4.8 ^b	283.0 $\pm$ 4.8 ^{ab}	234.6 $\pm$ 7.2 ^c
Pup weight gain (g/day)	1.72 $\pm$ 0.18 ^a		0.26 $\pm$ 0.18 ^b	
Food intake (g/day)	58.0 $\pm$ 1.84 ^a	18.3 $\pm$ 1.84 ^c	38.9 $\pm$ 1.84 ^b	14.5 $\pm$ 1.79 ^c
Leptin (ng/ml)	2.55 $\pm$ 0.20 ^a	4.11 $\pm$ 0.20 ^{bc}	3.64 $\pm$ 0.20 ^b	4.76 $\pm$ 0.38 ^c
Insulin (ng/ml)	1.76 $\pm$ 0.65 ^a	3.81 $\pm$ 0.73 ^b	3.85 $\pm$ 0.73 ^b	3.62 $\pm$ 0.44 ^b
	(n = 5)	(n = 4)	(n = 4)	
T ₄ (nmol/l)	23.3 $\pm$ 2.40 ^a	49.3 $\pm$ 2.40 ^b	27.6 $\pm$ 2.40 ^a	47.8 $\pm$ 4.48 ^b
	(n = 4)	(n = 4)	(n = 4)	

Values in a row without the same letter (a, b, c) differ significantly ($P < 0.05$).

If the litter was restored 48 h after removal from the mother, lactation was resumed and maternal food intake increased (Table 1). However, 48 h later, while litters were gaining weight (indicative of some milk production), both litter growth and food intake were much lower than before removal (Table 1). A substantial nocturnal increase in serum leptin concentration was apparent 48 h after litter removal, and was still present 48 h after re-initiation of suckling (Table 1). Both serum insulin and T₄ increased after litter removal to levels found in non-lactating rats (Table 1). Serum T₄ fell to levels found in lactating rats by 48 h after re-initiation of suckling, whereas serum insulin concentration remained at the higher levels found after litter removal (Table 1).

Effects of litter size

Litter size was altered within 24 h of parturition to four, eight, ten and fourteen pups. Pup weight gain per day

between days 10 and 13 of lactation was lower for litters of ten and fourteen pups than for smaller litters (Table 2). Total litter weight gain and maternal food intake both varied in proportion with litter size, being least in mothers suckling four pups and most in mothers suckling fourteen pups (Table 2). Food intake varied linearly with litter growth rate (Table 2). However, nocturnal serum leptin concentration did not vary consistently with litter size, and was significantly lower than in virgin control rats in all lactating groups (Table 2). Serum T₄ concentrations were lower than those of virgin rats regardless of litter size and did not vary consistently with litter size (Table 2).

When litter size was reduced from ten to four pups on day 11 of lactation, food intake fell within 24 h to a level found in rats suckling only four pups throughout lactation (Fig. 3a and Table 2). There was also a transient increase in nocturnal serum leptin concentration 12 h after reduction of litter size, with the concentrations returning to the level found before litter size reduction by 36 h (Fig. 3b).

Table 2 Food intake, litter weight gain, maternal weight, nocturnal serum leptin and T₄ concentrations of lactating rats suckling litters of different numbers of pups. Pup number was adjusted at birth to four ($n = 4$), eight ($n = 4$), ten ($n = 3$) or fourteen pups ($n = 5$). Results for age-matched, non-lactating, control rats ($n = 4$) are also included. Litter weight gain, food intake and maternal weight were measured daily over 3 days (days 11–14 of lactation) and mean values determined. Blood samples were taken by tail bleeding at 2100 h on day 11 of lactation. Statistical analysis was performed using ANOVA, with litter size as a factor. Results from the non-lactating group were compared with corresponding values for mothers using Student's *t*-test. Results are expressed as means $\pm$ S.E.M.

	Non-lactating	Four pups	Eight pups	Ten pups	Fourteen pups
Litter weight gain (g/day)	—	9.81 $\pm$ 0.82 ^a	17.5 $\pm$ 0.82 ^b	18.6 $\pm$ 0.95 ^b	24.7 $\pm$ 0.74 ^c
Rat weight (g)	228 $\pm$ 9.60 ^a	260 $\pm$ 5.36 ^b	305 $\pm$ 5.36 ^c	292 $\pm$ 6.18 ^c	301 $\pm$ 4.79 ^c
Food intake (g/day)	11.7 $\pm$ 0.78 ^a	36.5 $\pm$ 1.60 ^b	57.5 $\pm$ 1.60 ^c	58.8 $\pm$ 1.84 ^c	69.7 $\pm$ 1.43 ^d
Leptin (ng/ml)	3.89 $\pm$ 0.20 ^a	1.79 $\pm$ 0.23 ^{bc}	1.36 $\pm$ 0.23 ^b	1.56 $\pm$ 0.27 ^{bc}	2.03 $\pm$ 0.21 ^c
T ₄ (nmol/l)	47.9 $\pm$ 2.83 ^a	33.2 $\pm$ 3.03 ^b	25.6 $\pm$ 3.03 ^{bc}	23.3 $\pm$ 3.49 ^c	25.2 $\pm$ 2.71 ^{bc}

Values in a row without the same letter (a, b and c) differ significantly ($P < 0.05$).

Figure 3 (a) Food intake (shaded bars) and pup growth (solid bars) and (b) nocturnal serum leptin concentrations following litter size reduction from ten pups to four pups at 0900 h on day 12 of lactation. Daily food intake and pup weight change values are from 0900 h to 0900 h. Blood samples were collected every night at 2100 h by tail bleeding; the last value was obtained after killing the rats. All values are expressed as means $\pm$ S.E.M. ($n=6$). Statistical analyses were performed using ANOVA, for each variable, values (bars) without the same letter, a, b and c, differ significantly ($P<0.05$).

Pup weight gain also rose transiently during the first 24 h after reducing litter size from ten to four pups (Fig. 3a). Interestingly, the increase in weight of the whole litter of four pups over the 24 h after litter reduction (20 g/day) was similar to that for the ten-pup litter over the previous day (22 g/day). However, during the second day after reducing litter size, litter weight increased by only 11 g/day.

Effects of giving a varied, high-energy diet

Rats given a high-energy 'cafeteria diet' (cheese crackers, chocolate and peanuts plus standard chow) from parturition onwards consumed a similar weight of food over the first 4 days of lactation as rats fed standard chow. From day 5 onwards, the weight of food eaten per day was less in the rats fed the high-energy diet than in those fed standard

Figure 4 (a) Calorie intake (kcal/day) and (b) food intake (g/day) of lactating rats given, *ad libitum*, either a standard chow diet (◆) or a high-energy diet (○) composed of standard chow, cheese crackers, chocolate and peanuts. The amount of each individual component consumed over each period (parturition to day 4 of lactation, day 4 to day 7, day 7 to day 10 and day 10 to day 13) was determined, and hence the total calorie intake was calculated. Values are expressed as means $\pm$ S.E.M. of eight observations and were analysed by ANOVA, with diet and time after parturition as factors. *Significantly different ($P < 0.05$) from that for rats fed standard chow.

chow (Fig. 4a), but daily energy intake was the same irrespective of diet (Fig. 4b). Pup growth was not altered by giving mothers the high-energy diet (results not shown). The high-energy diet had no significant effect on daytime or night-time serum leptin (Table 3). Neither daytime serum T_4 concentration nor its normal nocturnal fall were altered by giving a high-energy diet (Table 3). However, giving a high-energy diet abolished the nocturnal rise in serum insulin concentration (Table 3).

Effects of bromocriptine treatment

Twice daily injections of bromocriptine for 2 days resulted in a decrease in pup weight gain (Table 4). Food intake also decreased with bromocriptine treatment but, after 2 days, intake remained more than double that of non-lactating rats (Table 4). Bromocriptine treatment increased

Table 3 Effects of feeding a high-energy diet on serum hormone concentrations in lactating rats. Rats were fed on either a standard chow diet or a high-energy diet from parturition. The light period was from 0700 h to 1900 h. Blood was collected at 0900 h or 2100 h by tail bleeding on day 12 of lactation and after killing on day 14 of lactation. Half of the rats on each feeding regimen were tail-bled at 0900 h on day 12 and then were killed at 2100 h on day 14 and the other half bled at 2100 h on day 12 and killed at 0900 h on day 14. ANOVA was performed with diet, time of the day and their interactions as factors. Values are expressed as means $\pm$ S.E.M. of eight observations (leptin and insulin) or four observations (T_4)

	Time	Chow diet	Cafeteria diet
Hormone			
Leptin (ng/ml)	Day	2.18 $\pm$ 0.26 ^a	2.33 $\pm$ 0.26 ^{ab}
	Night	2.42 $\pm$ 0.26 ^{ab}	3.06 $\pm$ 0.26 ^b
Insulin (ng/ml)	Day	1.36 $\pm$ 0.62 ^a	1.27 $\pm$ 0.62 ^a
	Night	5.00 $\pm$ 0.62 ^b	1.66 $\pm$ 0.62 ^a
T_4 (nmol/l)	Day	27.79 $\pm$ 1.61 ^b	26.43 $\pm$ 1.61 ^{ab}
	Night	23.17 $\pm$ 1.61 ^a	22.75 $\pm$ 0.61 ^a

Values without the same letter (a, b) differ significantly ($P < 0.05$).

serum leptin concentration during both the dark and light periods, but the diurnal rhythm in serum leptin was not restored after 2 days of treatment (Table 4). Bromocriptine also increased serum insulin concentrations, but had no significant effect on serum T_4 concentrations in lactating rats (Table 4). These effects of bromocriptine on food intake and serum leptin concentrations in lactating rats were confirmed in a second study in which the same dosage as before was continued for 3 days. After 3 days, pup growth had decreased to zero (0.0 ± 0.1 g/day) but, despite this, maternal food intake was still 41.9 ± 3.5 g/day (values are means $\pm$ S.E.M. of eight observations). Serum leptin concentration in both the light and dark periods was increased by 3 days of bromocriptine treatment ($P < 0.01$), but the nocturnal rise in serum leptin was still absent (leptin values: light, 1.55 and 2.46 ng/ml for saline and bromocriptine-treated rats; dark, 1.38 and 2.46 ng/ml for saline and bromocriptine-treated rats; results are means of four observations; S.E.D. 0.32).

In non-lactating rats bromocriptine treatment resulted in a small decrease in food intake and serum leptin, but did not have any effect on the nocturnal rise in serum leptin (Table 4). Bromocriptine treatment decreased daytime serum T_4 in non-lactating rats, but had no effect on night-time levels (Table 4).

Discussion

Lactating rats are usually in marginal negative energy balance, mobilizing about 1 g adipose tissue lipid per day (Barber *et al.* 1997). Consistent with this, most studies have shown that serum leptin concentrations are decreased to some extent during lactation in rats (Kawai *et al.* 1997,

Table 4 Effect of bromocriptine treatment on food intake, pup weight gain, serum leptin, T₄ and insulin concentrations in lactating and non-lactating rats. Groups of ten non-lactating rats and eight lactating rats were exposed to a light period from 0700 h to 1900 h or from 1900 h to 0700 h. Half of each group was treated with saline and the other half with bromocriptine at 0900 h and 1700 h for 2 days. All animals were killed after 48 h of treatment, between 0900 h and 1000 h and trunk blood collected. Food intake and pup weight gain were measured over the 24 h before killing; values were analysed using Student's *t*-test. Values for serum hormones were analysed using ANOVA with state, time and treatment and their interactions as factors (serum leptin and T₄) or state and treatment and their interactions (serum insulin). Results are expressed as means ± S.E.M.

	Lactating		Non-lactating	
	Saline	Bromocriptine	Saline	Bromocriptine
Food intake (g/day)	59.9 ± 2.90 ^a	47.7 ± 1.47 ^b	15.8 ± 0.30 ^a	13.1 ± 0.38 ^b
Pup weight gain (g/day)	1.82 ± 0.11 ^a	0.81 ± 0.12 ^b	—	—
Serum leptin (ng/ml)				
Day	1.56 ± 0.27 ^a (<i>n</i> = 4)	2.45 ± 0.27 ^{bc} (<i>n</i> = 4)	2.78 ± 0.24 ^c (<i>n</i> = 5)	1.96 ± 0.24 ^{ab} (<i>n</i> = 5)
Night	1.82 ± 0.27 ^{ab} (<i>n</i> = 4)	2.83 ± 0.27 ^c (<i>n</i> = 4)	3.91 ± 0.24 ^d (<i>n</i> = 5)	3.18 ± 0.24 ^c (<i>n</i> = 5)
T ₄ (nmol/l)				
Day	36.6 ± 2.66 ^b (<i>n</i> = 4)	34.7 ± 2.66 ^b (<i>n</i> = 4)	45.5 ± 2.38 ^{ac} (<i>n</i> = 5)	35.9 ± 2.38 ^b (<i>n</i> = 5)
Night	26.7 ± 2.66 ^a (<i>n</i> = 4)	32.8 ± 2.66 ^{ab} (<i>n</i> = 4)	41.8 ± 2.66 ^c (<i>n</i> = 4)	39.2 ± 2.38 ^{bc} (<i>n</i> = 5)
Insulin (ng/ml)				
Night	0.77 ± 0.62 ^a (<i>n</i> = 4)	4.15 ± 0.62 ^b (<i>n</i> = 4)	2.18 ± 0.50 ^{ac} (<i>n</i> = 5)	3.40 ± 0.50 ^{bc} (<i>n</i> = 5)

For each variable, values without the same letter (a, b, c and d) differ significantly (*P* < 0.05).

Pickavance *et al.* 1998, Terada *et al.* 1998, Woodside *et al.* 1998, 2000, Brogan *et al.* 1999, Herrera *et al.* 2000, Johnstone & Higuchi 2001). In addition, the nocturnal rise in serum leptin is usually completely lost during lactation, with a proportionally greater fall in serum leptin than seen during the daytime (Pickavance *et al.* 1998, Johnstone & Higuchi 2001). The daytime hypoleptinaemia during lactation has been attributed to the drain on nutrients by the mammary gland, rather than being due to suckling (Brogan *et al.* 1999, Woodside *et al.* 2000); the present study supports this conclusion. In addition, we have shown that the attenuation of the nocturnal rise in serum leptin involves the suckling stimulus.

Energy balance

Varying energy balance in lactating rats usually involves altering suckling intensity, which complicates the interpretation of findings. However, our experiments, in which lactating rats were induced into positive energy balance, resulted in increased serum leptin concentrations, even though in most cases suckling continued. Litter removal results in the immediate cessation of suckling and, although food intake decreases, rats move into positive energy balance, as shown by increased serum insulin and increased lipogenesis in white adipocytes (Agius *et al.* 1979, Flint *et al.* 1981). The diurnal rhythm in serum leptin is rapidly restored on litter removal. An increase in serum leptin on litter removal was found

previously (Brogan *et al.* 1999, López-Soriano *et al.* 1999), but in these studies only daytime values were reported. Milk production can be fully restored after litter removal by return of the pups; this takes 3 to 4 days in mice (Sorensen & Knight 1997). Restoration of suckling 48 h after litter removal resulted in a substantial increase in food intake, and while levels found in rats with uninterrupted lactation were not achieved (at least by 48 h after litter replacement), intake was much greater than required for the low rate of pup growth. This suggests that these rats were in positive energy balance; consistent with this, serum insulin remained high, but serum T₄ had returned to the low level found during lactation. A significant nocturnal rise in serum leptin occurred, achieving levels found in rats whose litters had been removed. Thus when rats are in substantial positive energy balance, serum leptin is elevated, despite a suckling stimulus. Reducing litter number from ten to four pups resulted in a transient increase in nocturnal leptin. Again, this is probably due to the mothers moving briefly into positive energy balance as food intake remained high during the first day after litter reduction. Bromocriptine treatment suppresses prolactin secretion and milk production in rats (Cowie *et al.* 1980). Milk production did not stop completely, even after 3 days of treatment with bromocriptine (if it had, pups would have lost weight). However, such treatment only slightly decreased food intake, which greatly exceeded the estimated need (from Table 2) for the decreased rate of pup

growth. This implies that the mothers moved into positive energy balance. Consistent with this, serum insulin levels were increased as found previously (Agius *et al.* 1979, Flint *et al.* 1981). Other studies have shown that bromocriptine treatment increases adipocyte lipogenesis in lactating rats (Agius *et al.* 1979, Flint *et al.* 1981), again indicating a move into positive energy balance. Serum leptin concentration increased during bromocriptine treatment, but the diurnal rhythm of leptin secretion was not restored. The effect of bromocriptine on serum leptin was not due to a fall in serum prolactin, as prolactin increases leptin secretion (Gualillo *et al.* 1999).

Thus, in several situations where mothers move into positive energy balance, serum leptin concentration increases, although the diurnal rhythm of leptin secretion is not always restored. These findings support the view (Brogan *et al.* 1999, Woodside *et al.* 2000) that altered energy balance is a major cause of the daytime hypoleptinaemia of lactation. Differences in the degree of negative energy balance could account for why the effects of lactation on serum leptin are quantitatively very variable (0–75% decrease) depending on the study (Chien *et al.* 1997, Kawai *et al.* 1997, Pickavance *et al.* 1998, Terada *et al.* 1998, Woodside *et al.* 1998, 2000, Brogan *et al.* 1999, Carmen-Garcia *et al.* 2000, Herrera *et al.* 2000, Johnstone & Higuchi 2001).

We also tried to induce positive energy balance by giving mothers a varied, high-energy diet to facilitate increased energy intake. However, after a brief adjustment period, the mothers decreased the weight of food eaten relative to others given just the chow diet, but both groups consumed a similar number of calories. We conclude that in rats the negative energy balance of lactation is not due to a physical constraint on intake, as those given the high-energy diet could clearly have consumed more food. Consistent with this, in the study in which litter size was varied, rats suckling ten pups could have increased their intake (59 g/day) to the level found in those suckling fourteen (70 g/day), which would probably be enough to counteract the energy deficit.

Suckling stimulus

Food intake in lactating rats varies with litter size and hence growth rate (Ota & Yokoyama 1967, Issler *et al.* 1984), but the signals linking intake and mammary output have not been defined. There are afferent nerves from the nipple to various regions of the brain and hypothalamus, including the paraventricular nucleus, which mediate the effects of suckling on oxytocin and prolactin secretion (Grosvenor & Mena 1974). These nerves could also transmit signals to regulate intake, for the paraventricular nucleus is a key centre co-ordinating signals regulating appetite (Williams *et al.* 2001). In addition, there may also be chemical signals from the mammary gland to centres of appetite control. Cutting of the galactophores (milk ducts) or removal of the nipples results in a decrease in food

intake in lactating animals (Flemming 1976, Mann *et al.* 1983, Woodside *et al.* 2000). This effect could be due to severance of afferent nerves to the hypothalamus or accumulation of milk within the gland leading to altered signalling by chemical mediators. In the present study, food intake remained relatively high in mothers treated with bromocriptine, even though milk production was markedly reduced; pups would be hungry and so would be expected to maintain a strong suckling stimulus. Similarly, restoration of litters after they had been removed from their mothers for 48 h increased food intake more than was required for the amount of milk being produced. These observations suggest that suckling can increase appetite in lactating rats, independent of milk production, probably by transmission of a signal from the nipple to the hypothalamus via the nervous system.

While suckling is an important stimulant of food intake, it does not seem to have an effect on daytime serum leptin levels, or at least any effects are easily counteracted by positive energy balance. Thus bromocriptine treatment resulted in an increase in daytime serum leptin in lactating rats, despite continued suckling. Cutting the galactophores increased daytime serum leptin in lactating rats (Woodside *et al.* 2000); as in the present study, there was a rise in serum insulin and also an increase in fat pad weight, indicating that the rats had moved into positive energy balance (Woodside *et al.* 2000). Similarly, litter restoration failed to decrease daytime leptin levels in rats in which milk production was not reinitiated; again the high serum insulin levels suggest that these rats were in positive energy balance (Brogan *et al.* 1999).

Although bromocriptine treatment increased serum leptin levels, such treatment did not restore the diurnal rhythm of leptin secretion. This suggests that the loss of rhythm is due in part at least to the suckling stimulus. The failure to restore the nocturnal rise is not an artifact of bromocriptine treatment itself, as the drug did not attenuate the diurnal rhythm of leptin secretion in non-lactating rats. However, litter restoration for 48 h after removal, while achieving similar litter growth and food intake as bromocriptine treatment for 48 h, did not prevent the diurnal rhythm of leptin secretion. This could be due to the mechanism not being fully restored 48 h after litter replacement.

The suckling stimulus was also varied by altering litter size, but this had no effect on the low nocturnal leptin level, which was similar in rats suckling from four to fourteen pups. Possibly the suckling stimulus induced by just four pups was sufficient to achieve a maximum effect on the serum leptin level.

Leptin and the hyperphagia of lactation

Lactation results in increased expression of both neuropeptide Y (Ciofi *et al.* 1991, Pelletier & Tong 1992, Smith 1993, Malabu *et al.* 1994, Pape & Tramu 1996, Wilding

et al. 1997, Li *et al.* 1998, Chen *et al.* 1999) and agouti-related peptide (Chen *et al.* 1999), and decreased expression of pro-opiomelanocortin (Smith 1993, Pape & Tramu 1996). These changes in hypothalamic neuro-peptide gene expression are consistent with the hypoleptinaemia and should promote hyperphagia (Ahima 2000, Ahima & Flier 2000, Ahima *et al.* 2000, Havel 2000, Spiegelman & Flier 2001, Williams *et al.* 2001). However, leptin would not appear to have a direct role in causing the hyperphagia, because varying litter size resulted in changes in food intake without any apparent change in serum leptin. Furthermore, suckling can increase food intake despite an increase in serum leptin. Thus the hypoleptinaemia of lactation appears to have a permissive role, facilitating the hyperphagia, rather than being a direct cause.

Leptin and T_4

In addition to its effects on appetite, leptin also modulates the secretion of pituitary hormones (Ahima 2000, Ahima & Flier 2000, Ahima *et al.* 2000, Flier *et al.* 2000). Treatment with leptin partly prevented the fall in serum T_4 on fasting (Ahima 2000). Thyroid hormones increase basal metabolic rate (Shetty 1990) and may also enhance brown adipose tissue thermogenesis (Silva 1993). Undernutrition results in decreased energy expenditure, which is at least partly due to the concomitant hypothyroidism (Shetty 1990). Thus the hypoleptinaemia of lactation could contribute to the hypothyroidism, and hence increased metabolic efficiency of the state. Indeed, with one exception (litter restoration), serum leptin and T_4 changed in parallel in the present study. Notably, varying litter size from four to fourteen pups, which altered food intake, had no effect on the degree of hypoleptinaemia or hypothyroidism. In general, changes in energy balance were accompanied by changes in serum T_4 and leptin levels.

Conclusions

The hypoleptinaemia of lactation may facilitate the hyperphagia, but the suckling signal, and possibly other signals from the mammary gland, would appear to be more important determinants of appetite. Curiously, while there is a clear relationship between milk production and food intake, lactating rats are usually in slight negative energy balance; this is not due to a physical constraint on intake. This negative energy balance is a major factor causing the hypoleptinaemia of lactation, and differences in the degree of negative energy balance could explain why the degree of daytime hypoleptinaemia varies quite markedly between reported studies. By contrast, the suckling stimulus is involved in the suppression of the nocturnal rise in leptin. The hypoleptinaemia probably not only facilitates the hyperphagia, but may also be the cause of the hypothyroidism of lactation and hence the increased metabolic efficiency of the state.

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