Abstract

We investigated the effects of lactation on diurnal changes in serum leptin and hypothalamic expression of the leptin receptor isoforms, Ob-Ra, -Rb, -Rc, -Re and -Rf in rats. In non-lactating rats, serum leptin concentration was increased at night while hypothalamic mRNA levels of Ob-Rb, -Rc and -Re decreased; by contrast, expression of Ob-Ra and Ob-Rf was unchanged at night. There were significant negative correlations between serum leptin and mRNA expression of Ob-Rb (P < 0.001) and Ob-Re (P < 0.05), which were independent of time of day. In lactating rats, the nocturnal rise in serum leptin was attenuated. Daytime hypothalamic Ob-Rb mRNA levels were significantly lower than in non-lactating controls, and the normal nocturnal decreases in expression of Ob-Rb, -Rc and -Re were lost. The relationship between serum leptin and Ob-Re expression was not changed by lactation. Lactation had no effect on the expression of Ob-Ra mRNA in the hypothalamus. Decreased daytime Ob-Rb expression led to reduced hypothalamic sensitivity to leptin, and thus contribute to increased daytime appetite in lactating rats. Moreover, maintaining high levels of Ob-Re expression could, by increasing hypothalamic leptin-binding protein concentration and reducing local leptin bioavailability, further accentuate hyperphagia. Thus, selective changes in expression of specific isoforms of the leptin receptor may contribute to the hyperphagia of lactation in rats.

Introduction

Lactation is a state of high energy demand, which, in rodents, is met primarily by increasing food intake several-fold (Wade & Schneider 1992, Barber et al. 1997). Various other metabolic and hormonal changes serve to increase metabolic efficiency, both by channelling nutrients preferentially to the mammary gland and by decreasing energy expenditure in some tissues. Thus, for example, brown adipose tissue (BAT) thermogenesis is decreased (Trayhurn 1989) and animals also become hypothyroid and hypoinsulinaemic (Vernon & Pond 1997). Nevertheless, lactating rats enter and remain in a state of negative energy balance, mobilising about 1 g fat/day up to the peak of lactation (Barber et al. 1997).

The factors that regulate the hyperphagia of lactation have not been resolved. The discovery of leptin, a 16 kDa peptide produced and released by adipocytes (Zhang et al. 1994), introduced an additional and potentially important candidate. Leptin acts via receptors in the hypothalamus to decrease appetite and increase energy expenditure (Friedman & Halaas 1998, Ahima 2000, Ahima et al. 2000, Havel 2000, Spiegelman & Flier 2001, Williams et al. 2001). Serum leptin concentrations decrease during periods of negative energy balance (Friedman & Halaas 1998, Houseknecht et al. 1998, Ahima 2000, Vernon et al. 2001), and this would be predicted to increase hunger while decreasing energy expenditure.

Leptin receptors (Ob-R) are highly expressed in the hypothalamus (Fei et al. 1997). Six leptin receptor isoforms (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf), generated by alternative splicing, have been discovered to date (Ahima & Flier 2000, Ahima et al. 2000, Meister 2000). All share a common extracellular ligand-binding domain at the amino terminus and all except Ob-Re have transmembrane domains, but differ in their intracellular carboxyl-terminal domains (Ahima & Flier 2000, Ahima et al. 2000, Meister 2000). Only Ob-Rb contains all the intracellular motifs required for effective signalling via the JAK-STAT pathway, and is thought to be most important in modulating appetite (Ahima & Flier 2000, Ahima et al. 2000, Meister 2000). Ob-Re, which lacks both transmembrane and intracellular domains, circulates as a soluble...
leptin-binding protein (Li et al. 1998, Huang et al. 2001). Ob-Ra is thought to be important for the transport of leptin into the brain (Ahima & Flier 2000, Ahima et al. 2001, Kastin & Pan 2000). The functions of the other small isoforms (Ob-Rc, Ob-Rd and Ob-Rf) remain to be clarified, but they may interact with Ob-Rb (White et al. 1997).

Rats consume most food at night (Kimura et al. 1970, Bruckdorfer et al. 1974) and this pattern of behaviour is retained during lactation (Strubbe & Gorissen 1980, Munday & Williamson 1983, Pickavance et al. 1998).

There are increases in adipocyte leptin mRNA and serum leptin levels at night (Saladin et al. 1995, Pickavance et al. 1998, Xu et al. 1999, Ahrén 2000, Mastronardi et al. 2000, Nagatani et al. 2000, Nishiyama et al. 2000, Pu et al. 2000), which are dependent on food intake (Saladin et al. 1995, Xu et al. 1999, Kalbbeck et al. 2001). The function of the nocturnal rise in leptin is uncertain as it should act to attenuate food intake. Most studies with lactating rats suggest that serum leptin concentration is decreased during the daytime (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, Johnstone & Higuchi 2001, Denis et al. 2003), while the normal nocturnal increase in serum leptin is abolished or markedly attenuated (Pickavance et al. 1998, Johnstone & Higuchi 2001, Denis et al. 2003). The decrease in serum leptin during lactation would be predicted to stimulate food intake and perhaps to decrease BAT thermogenesis.

Hypothalamic Ob receptors are clearly crucial in modulating leptin’s effects on feeding, and there is some inconsistent evidence that lactation may affect daytime levels of certain Ob isoforms. Brogan et al. (2000) found decreased Ob-Rb mRNA in the ventromedial hypothalamus (VMH) with lactation, with no significant changes in the arcuate nucleus (ARC), paraventricular nucleus or dorsomedial hypothalamus. By contrast, del Carmen Garcia et al. (2000) found an increase in hypothalamic Ob-Rf mRNA expression at peak lactation, whereas expression of other isoforms was unchanged.

Here, we investigated further the impact of lactation on hypothalamic levels of the mRNAs of five isoforms of the leptin receptor in the rat. As changes in serum leptin during lactation are more pronounced at night than during the day, animals were studied during both light and dark periods.

Materials and Methods

Animals

Female Wistar rats were raised at the Hannah Research Institute from stock obtained originally from A Tuck and Sons, Ltd (Rayleigh, Essex, UK). They were fed standard pelleted chow freely (CRM diet; Lubsure, Poole, Dorset, UK) and had free access to water. One group (n=16) was maintained on a light–darkness schedule with lights on from 0700 h to 1900 h, and the other (n=16) with light from 2200 h to 1000 h. Eight from each group were mated at about 10 weeks of age, while the others were kept as non-lactating controls. Litter size of lactating rats was adjusted to ten pups within 24 h of parturition. After at least 4 weeks’ exposure to the specific light–darkness regime, non-lactating and lactating (day 14 of lactation) rats were killed by cervical dislocation at 2 h into the light or dark phase. Food intake was measured over the last 24 h before killing.

Blood was collected and allowed to clot; serum was separated by centrifugation and stored at −20 °C for later analysis of leptin concentrations. Parametrial white adipose tissue and brain were collected. The brain was frozen in liquid nitrogen and the hypothalamus later dissected en bloc under a binocular microscope, using consistent landmarks (Williams et al. 1988). The hypothalamic block was removed from a frontal slice of brain tissue between the optic chiasm and the mammillary bodies, using cuts made immediately below the anterior commissure and through the perihypothalamic sulci. The block was kept frozen in liquid nitrogen until mRNA extraction as described below.

All studies were conducted according to the UKCCR guidelines for the care and use of laboratory animals.

Serum leptin

Serum leptin concentration was determined by RIA using a commercial kit (Linco Research Inc., St Louis, MO, USA; distributed by Biogenesis, Poole, Dorset, UK).

Quantitative RT-PCR

RT-PCR was used to analyse the expression of isoform-specific Ob-Rb, Ob-Ra, Ob-Rc, Ob-Re and Ob-Rf mRNAs. Total RNA was extracted from frozen dissected hypothalami, using TriReagent (Sigma, Poole, Dorset, UK). The yield and quality of extracted RNA were assessed by the 260/280 nm optical density ratio and electrophoresis on 1·1% agarose gel. Only samples displaying satisfactory quality were used for analysis. Total RNA was treated with RNase-free DNase I (Roche, Lewes, East Sussex, UK) for 10 min at 37 °C and a further 10 min at 75 °C.

Reverse transcription was carried out using optimised amounts (1 µg) of total RNA with a Reverse-IT 1st Strand Synthesis Kit (Abgene, Epsom, Surrey, UK) in a final volume of 25 µl. First-strand cDNA was used for PCR amplification of the target genes using the following primers (obtained from Sigma-Genosys Ltd, Pampisford, Cambridge, UK): Ob-Ra, 5′-CCTATCGAGAAATATCGTTTA-3′ and 5′-TCAGAGGCTCATCTCCTCT-3′ corresponding to a 284-bp sequence (del Carmen Garcia et al. 2000, GenBank: D84126);
Ob-Rb, 5'-TATGTCATTGTACCGATAATTATT-3' and 5'-CAGAGAAGTTAGCACTGTT-3' corresponding to a 370-bp sequence (Wang et al. 1996, GenBank: AF287268); Ob-Rc, 5'-ATTGTACCGGTAATTATTTCT-3' and 5'-CTGCAACCTTAGATATCTTGG-3' corresponding to a 180-bp sequence (del Carmen Garcia et al. 2000, GenBank: AF007818); Ob-Re, 5'-GCAGAATCAGCACACACTGTT-3' and 5'-GTAAAGCACACAGTACACATACC-3' corresponding to a 297 bp sequence (del Carmen Garcia et al. 2000, GenBank: AF007819); Ob-Rf, 5'-TATGTCATTGTACCGATAATTATT-3' and GGGTACCTGCACATATGTG-3' corresponding to a 389 bp sequence (Wang et al. 1996, GenBank: U53144). Housekeeping gene HPRT (hypoxanthine-guanine phosphoribosyltransferase, Siegling et al. (1994)) was also amplified using 5'-CAGTCCCAGCGTCGTGATTA-3' and 5'-AGCAAGTCTTTCAGTCCTGTC-3' corresponding to a 138 bp sequence (GenBank: X62085).

PCR was performed with 1 µl template cDNA and 35 µl PCR mix (1·1× ReddyMix PCR master mix, 1·5 mM MgCl2, ABgene) on a thermal cycler (Hybaid, Ashford, Kent, UK). After initial denaturation at 94 °C for 5 min, the samples were subjected to cycles of amplification: denaturation at 94 °C for 30 s, annealing at 54 °C for Ob-Ra, -Rb, -Rc, -Rf and HPRT and 56 °C for Ob-Re for 30 s, and extension at 72 °C for 40 s. According to preliminary PCR experiments (data not shown) the optimised cycles of amplification within linear range were used for each gene: 24 cycles for HPRT, 36 cycles for Ob-Re and 34 cycles for the other isoforms. Final extension was performed at 72 °C for 7 min. Negative controls without reverse transcriptase were systematically performed to detect cDNA contamination. PCR products were subjected to electrophoresis on a 1·1% agarose gel (Fig. 1). Bands were visualised and quantified using a digital image analysis system (Kodak 1-D; Kodak Eastman, Rochester, NY, USA). Optical density was calculated for each Ob-R product and the results expressed as the ratio of its density to that of the HPRT product.

Adipocyte cell volume
Adipocytes were prepared by collagenase digestion and adipocyte mean cell volume was determined as described previously (Robertson et al. 1982).

Statistical analyses
Results are expressed as means ± S.E.M. and were analysed by ANOVA followed by Tukey’s test, with physiological state, light/dark phase and their interactions as factors. Degrees of linear relationship between two variables were performed using Genstat (Genstat 4·2, 5th edition; Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). A P value of 0·05 or less was considered as significant.

Results
Food intake per day was 14·3 ± 1·7 and 58·2 ± 3·8 g/rat for non-lactating and lactating rats respectively (P<0·001). Adipocyte mean cell volume did not alter with the light phase, but decreased significantly during lactation (P<0·02) (Table 1). ANOVA showed that for serum leptin concentration there was a significant state–light/dark phase interaction (P<0·05). Lactation had no significant effect on serum leptin concentration during the light phase, but the nocturnal increase (P<0·001) in leptin, which was apparent in both states, was significantly diminished (P<0·01) by lactation (Table 1).

Expression of Ob-R isoforms in the hypothalamus
Neither lactation nor light/dark phase had a significant effect on hypothalamic Ob-Ra mRNA expression.
(P=0.10 for state, P=0.31 for light/dark phase, P=0.74 for their interaction) (Fig. 2a). By contrast, hypothalamic Ob-Rb mRNA exhibited a highly significant interaction between state and light/dark phase (P<0.001) (Fig. 2b). In non-lactating rats, hypothalamic Ob-Rb mRNA levels were 50% lower during the dark phase than in the light (P<0.001). In lactating rats, hypothalamic Ob-Rb mRNA concentration was lower during the light phase than in non-lactating rats (P<0.001), and the hypothalamic Ob-Rb mRNA concentration did not differ significantly between light and dark phases. During the dark phase, hypothalamic Ob-Rb mRNA levels were significantly greater in lactating than in non-lactating rats (P<0.01).

Expression of hypothalamic Ob-Rc mRNA also showed a significant interaction between state and light/dark phase (P<0.01) (Fig. 2c). In non-lactating rats, hypothalamic Ob-Rc mRNA levels were 30% lower during the dark than in the light phase (P<0.05). In the lactating group, Ob-Rc mRNA did not differ significantly with light/dark phase.

Hypothalamic Ob-Re expression was decreased during the dark phase in non-lactating rats (P<0.05), but did not change with light/dark phase in lactating rats (Fig 2d). Ob-Re expression, was not altered by lactation in the light phase, but expression was higher during the dark phase in lactating compared with non-lactating rats (P<0.05).

ANOVA showed that there was no significant effect of light/dark phase on hypothalamic Ob-Rf expression (Fig. 2e). Ob-Rf expression in the dark phase was greater (P<0.05) in lactating compared with non-lactating rats, but light-phase expression was similar in both groups (Fig. 2e).

### Relationship of Ob-R isoform expression with leptin

In non-lactating rats, there was a significant negative correlation between serum leptin and hypothalamic Ob-Rb mRNA levels (r = -0.82, P<0.001) (Fig. 3). This correlation was absent in lactating rats (r=0.49, P=0.13); furthermore, when data for non-lactating and lactating rats were pooled, the correlation was weaker (r = -0.44, P<0.05) than within the non-lactating group alone.

Similarly, there was a significant correlation (r = -0.57, P<0.05) between serum leptin and hypothalamic Ob-Re mRNA expression in non-lactating, but not in lactating rats (Fig. 4). However, combining data from both groups strengthened the correlation between serum leptin and hypothalamic Ob-Re mRNA as compared with non-lactating rats alone (r = -0.61, P<0.001).

There were no significant correlations between serum leptin concentration and either Ob-Ra, Ob-Rc or Ob-Rf (data not shown).

### Discussion

This study shows that there are diurnal changes in the expression of specific isoforms of the leptin receptor in the hypothalamus, and that some of these changes are modulated by lactation.

#### Normal diurnal changes in serum leptin and hypothalamic leptin receptor expression

As already mentioned, fed rodents normally show nocturnal increases in adipocyte 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, Ahrén 2000, Mastronardi et al. 2000, Nagatani et al. 2000, Nishiyama et al. 2000, Pu et al. 2000), these rises being dependent on food intake (Saladin et al. 1995, Xu et al. 1999, Kalsbeek et al. 2001). This diurnal pattern was confirmed in the non-lactating female rats of the present study (Table 1).

We also found diurnal changes in the hypothalamic expression of specific Ob-R isoforms (but not others) in our non-lactating female rats. There were significant nocturnal decreases in mRNAs for Ob-Rb, -Rc and -Re, but not of Ob-Ra or Ob-Rf. A nocturnal fall in hypothalamic Ob-R mRNA levels has previously been reported in normal rats by Xu et al. (1999) and appeared to follow the night-time rise in plasma leptin; however, the Ob-R isoform(s) recognised by the probe were not identified by Xu et al. (1999), and ours is the first study to examine the major isoforms individually.

The factors responsible for the decreased expression of Ob-Rb, -Rc and -Re during the dark have not been identified, but for Ob-Rb and Ob-Re the rise in serum leptin probably contributes. Leptin appears to modulate Ob-Rb levels in various tissues, including the hypothalamus, both by inducing down-regulation of the receptor

### Table 1 Serum leptin concentrations and adipocyte mean cell volume of lactating and non-lactating rats at 2 h into the light or darkness period. Values are expressed as means ± S.E.M. from ANOVA of eight observations

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<th>Lactating</th>
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<td>Leptin (ng/ml)</td>
<td>1.55 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.79 ± 0.22&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Adipocyte volume (pl)</td>
<td>244 ± 32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>286 ± 32&lt;sup&gt;c&lt;/sup&gt;</td>
<td>311 ± 32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>379 ± 32&lt;sup&gt;c&lt;/sup&gt;</td>
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Values in a row without the same letter (a, b, c) differ significantly (P<0.05).
Figure 2 Amount of hypothalamic Ob-Ra mRNA (a), Ob-Rb mRNA (b), Ob-Rc mRNA (c), Ob-Re mRNA (d) and Ob-Rf mRNA (e) relative to HPRT mRNA in lactating and non-lactating rats in the light and dark phase. Values are means ± S.E.M., obtained from ANOVA, of seven and six observations for non-lactating and lactating rats respectively, except for Ob-Rb for lactating rats in the dark, for which the value is a mean of five observations. There were no significant differences between values for Ob-Ra; for other variables values (bars) without the same labels (a, b or c) differ significantly (P<0.05).
protein (Barr et al. 1999, Uotani et al. 1999, Martin et al. 2000) and by decreasing expression of Ob-Rb mRNA (Mercer et al. 1997, Hikita et al. 2000, Martin et al. 2000, Tena-Sempere et al. 2000, 2001). Consistent with this, the falls in serum leptin induced by fasting (Baskin et al. 1998) and cold-exposure (Mercer et al. 1997, Hardie et al. 1996), and the increase in serum leptin during pregnancy (del Carmen Garcia et al. 2000) are all associated with reciprocal changes in hypothalamic Ob-Rb expression. Baskin et al. (1998) proposed that an increase in hypothalamic Ob-Rb mRNA will result ultimately in enhanced sensitivity to leptin. If so, then the nocturnal fall in Ob-Rb expression should decrease sensitivity to the night-time rise in leptin levels, and so tend to offset the diurnal changes in plasma leptin.

Ob-Re is expressed in many tissues (Lollmann et al. 1997), but lacks both transmembrane and intracellular domains and is released into the circulation where it acts as a leptin-binding protein (Li et al. 1998). Consistent with such a role, overexpression of Ob-Re in vivo led to increased serum leptin concentrations due to decreased clearance of leptin (Huang et al. 2001). Leptin, however, decreases the serum concentration of its soluble receptor in rats (Huang et al. 2001). Also, there are reciprocal diurnal changes in serum leptin and its soluble receptor in humans (Chan et al. 2002). We found that hypothalamic Ob-Re mRNA level varies inversely with serum leptin concentration during the light and dark phases. It is possible that Ob-Re acts as a leptin-binding protein within the extracellular fluid of the hypothalamus or even the cerebrospinal fluid; this might be expected to reduce the local bioavailability of leptin, in which case the central effects of high and low leptin levels would be accentuated.

**Effects of lactation on hypothalamic leptin receptors**

Most previous studies agree that lactation decreases serum leptin and abolishes or at least markedly attenuates the normal nocturnal rise (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, Johnstone & Higuchi 2001, Denis et al. 2003), although two reports found no effect on daytime serum leptin (Chien et al. 1997, del Carmen Garcia et al. 2000). The reason for the lack of a significant decrease in daytime serum leptin during lactation in the present study is not clear. However, the decrease in adipocyte mean cell volume with lactation in the present study was less than in a previous study (Pickavance et al. 1998), in which a significant decrease in daytime leptin was observed in lactating rats. This suggests that the degree of negative energy balance of the lactating rats is less than in our previous study. Overall, hypoleptinaemia would be expected to facilitate the hyperphagia of lactation.

Ob-Rb, the long isoform thought to be crucially involved in decreasing appetite (Ahima & Flier 2000, Ahima et al. 2000, Meister 2000), shows reciprocal changes in expression following alterations in serum leptin, in various states including fasting, cold-exposure and pregnancy (see above). Intriguingly, this inverse relationship between serum leptin and hypothalamic Ob-Rb mRNA expression is lost during lactation (Figs. 2b and 3). The daytime decrease in hypothalamic Ob-Rb mRNA expression compared with non-lactating rats is consistent with the reduced expression in the VMH (and a tendency to decrease in the ARC) reported by Brogan et al. (2000), but not with the unchanged whole-hypothalamic levels described by del Carmen Garcia et al. (2000). A decrease in daytime Ob-Rb expression during lactation cannot be

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**Figure 3** Amount of Ob-Rb mRNA relative to HPRT mRNA concentration in the hypothalamus plotted against serum leptin concentration for lactating and non-lactating rats.

**Figure 4** Amount of Ob-Re mRNA relative to HPRT mRNA concentration in the hypothalamus plotted against serum leptin concentration for lactating and non-lactating rats.
obviously attributed to an increase in leptin (which is usually lower than in non-lactating rats); additional factors regulating expression of this receptor are thus implicated. If, as proposed for other states by Baskin et al. (1998), decreased hypothalamic Ob-Rb mRNA expression during lactation diminishes sensitivity to leptin during the daytime, then this might also contribute to the hyperphagia of lactation. By contrast to Ob-Rb expression, the reciprocal relationship between serum leptin and hypothalamic Ob-Re expression does not appear to be altered by lactation.

Our finding that expression of Ob-Ra (which may be involved in leptin transport into the brain) (Björbaek et al. 1998, Kastin & Pan 2000) in the hypothalamus was unaltered by lactation is consistent with that of del Carmen Garcia et al. (2000). The functions of the short isoforms, Ob-Rc and Ob-Rf, are still not clear, but are postulated to include involvement in the transport of leptin across the blood–brain barrier (Guan et al. 1997) and the modulation of leptin signalling via Ob-Rb (White et al. 1997). Expression of both isoforms showed marked diurnal rhythms similar to that of Ob-Rb, which again were lost during lactation. The lack of effect of lactation on Ob-Rc expression during the light phase is consistent with the finding of del Carmen Garcia et al. (2000), but unlike these authors, we found no increase in Ob-Rf expression during the light phase in lactation; the reason for this difference is not clear.

Conclusions

Several factors may contribute to the sustained hyperphagia of lactation, and their importance may vary with time of day. Hypoleptinaemia, especially at night when food intake is greatest, seems likely to be important; theoretically, this effect could be accentuated by reciprocal increases in Ob-Re, which may reduce leptin’s bioavailability. Moreover, the normal inverse relationship between serum leptin and hypothalamic Ob-Rb mRNA expression is lost during lactation. Diminished Ob-Rb expression during the daytime may decrease sensitivity to leptin, which should also favour the hyperphagia. Thus, hyperphagia during lactation may be accentuated by multifactorial decreases in hypothalamic leptin sensitivity as well as hypoleptinaemia. The factors responsible for hypoleptinaemia and the altered relationships between serum leptin and hypothalamic Ob-Rb mRNA expression during lactation have yet to be resolved.

Acknowledgements

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