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Serum leptin and insulin levels in lactating protein-restricted rats: implications for energy balance

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The present study analysed the effect of protein restriction on serum insulin and leptin levels and their relationship with energy balance during lactation. Four groups of rats received isocaloric diets containing 170 g protein/kg or 60 g protein/kg from pregnancy until the 14th day of lactation: control non-lactating, control lactating (both fed a control diet), low-protein non-lactating and low-protein lactating. Energy intake, body composition, energy balance, serum insulin and leptin concentrations and the relationship between these hormones and several factors related to obesity were analysed. Low-protein-intake lactating rats exhibited hypoinsulinaemia, hyperleptinaemia, hypophagia and decreased energy expenditure compared with control lactating rats. The protein level in the carcasses was lower in the low-protein lactating group than in the control lactating group, resulting in a higher fat content in the first group compared with the latter. Body fat correlated inversely with serum insulin and positively with serum leptin level. There was a significant negative correlation between serum leptin and energy intake, and a positive relationship between energy intake and serum insulin level in lactating rats and in the combined data from both groups. Energy expenditure was correlated positively with serum insulin and negatively with serum leptin in lactating rats and when data from control non-lactating and lactating rats were pooled. Lactating rats submitted to protein restriction, compared with lactating control rats, showed that maternal reserves were preserved owing to less severe negative energy balance. This metabolic adaptation was obtained, at least in part, by the hypoinsulinaemia that resulted in increased insulin sensitivity favouring enhanced fat deposition, hyperleptinaemia and hypophagia.

Low-protein diet: Serum leptin: Serum insulin: Energy balance: Lactation

In adult animals and human subjects, body weight tends to remain within a relatively narrow range despite large day-to-day fluctuations in the amount of the food consumed. In spite of these observations, it has been noted that the incidence of obesity has increased worldwide. Prevalence studies in the Brazilian adult population have shown an epidemic increase in obesity, mostly among women from lower social strata (Instituto Nacional de Alimentação e Nutrição, 1991; Coitinho, 1998; Florêncio, 1998; Monteiro & Mondini, 1998). Although an improvement in economic conditions can partly explain such trends, other factors may also be important (Popkin & Bisgrove, 1988).

Low dietary protein intake can be associated with hyperphagia (Colombo *et al.* 1992; White *et al.* 1998), enhanced body fat content and plasma leptin levels (White *et al.* 1998; Du *et al.* 2000) and a number of adaptations, such as an enhanced BMR (Tulp *et al.* 1979), brown adipose tissue thermogenesis (Rothwell & Stock, 1987) and lipogenesis of white adipose tissue (Heard & Turner, 1967). Dramatic feeding behaviour and metabolic changes are also seen in lactation, as hyperphagia (Denis *et al.* 2003) and a decrease in BMR (Shetty, 1990), brown adipose tissue thermogenesis (Trayhurn, 1989) and lipogenesis of white adipose tissue (McNamara, 1995; Vernon & Pond, 1997), and act to meet the high energy demand for the production of milk.

The feeding pattern and metabolic changes verified in protein restriction and lactation correlate with equally profound alterations in the circulating levels of insulin and leptin (Vernon, 1989; Terada *et al.* 1998), two hormones involved in the regulation of food intake and energy expenditure, and might promote obesity. Therefore, in the present study, we evaluated whether protein restriction affected serum insulin and leptin levels and the role of these hormones on energy balance at peak lactation. To address this issue, we used lactating rats submitted to protein restriction during pregnancy and lactation, and examined the relationship between insulin and leptin levels and some predictory variables of obesity (energy intake and energy expenditure).

Abbreviations: CL, control lactating; CNL, control non-lactating; HOMA-IR, homeostasis model assessment of insulin resistance; LPL, low-protein lactating; LPNL, low-protein non-lactating.

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Materials and methods

Animals and diets

The present experiment followed the Brazilian College of Animal Experimentation adopted by the Federal University of Mato Grosso. Thirty-one female Wistar rats (90 d old) were supplied by the animal central care facility of the Federal University of Mato Grosso (Cuiabá, Brazil). Mating was performed by housing females with males overnight, and pregnancy was confirmed by the presence of sperm in vaginal smears. Virgin and pregnant females were separated at random and maintained from the first day of pregnancy until the 14th day of lactation with isocaloric diets containing 60 g protein/kg (low-protein diet) or 170 g protein/kg (control diet), as described in Table 1. Rats were divided in four groups on the first day of lactation: control non-lactating (CNL), control lactating (CL), low-protein non-lactating (LPNL) and low-protein lactating (LPL).

The experimental period was from the first day until the 14th day of lactation, when the rats were given free access to food and water. They were kept under standard lighting conditions (12 h light–dark cycle; lights on 06.00 h) at a temperature of 24 ± 1°C. Food intake and body weight were monitored three times a week. Spontaneous delivery took place on day 22 of pregnancy, after which, at 3 d of age, large litters were reduced to eight pups, ensuring a standard litter size per mother.

Sample collection and analyses

At the end of the experimental period (day 14 of lactation), the rats were killed. Blood samples were collected, serum was obtained by centrifugation, and aliquots were used to measure serum glucose level by the oxidase–peroxidase method (Trinder, 1969), total serum protein by the biuret modified method (Wolfson *et al.* 1948) and serum albumin by the bromocresol green method (Doumas *et al.* 1971).

Serum insulin concentration were determined by RIA using rat insulin as standard, ^{125}I-labelled bovine insulin as the radioactive tracer and guinea-pig anti-porcine insulin serum as the antibody (Scott *et al.* 1981). The intra-assay and inter-assay variations of this method are 8 % and 14 %, respectively. The processed detect rat serum insulin between 0·039 to 5·0 ng/ml.

Table 1. Composition (g/kg) of the normal and low-protein diets

Ingredient	Control diet (170 g protein/kg)	Low-protein diet (60 g protein/kg)
Casein (840 g protein/kg)	202·0	71·5
Cornstarch	397·0	480·0
Dextrinised cornstarch	130·5	159·0
Sucrose	100·0	121·0
Soyabean oil	70·0	70·0
Fibre	50·0	50·0
Mineral mix (AIN-93G)†	35·0	35·0
Vitamin mix (AIN-93G)†	10·0	10·0
L-cystine	3·0	1·0
Choline bitartrate	2·5	2·5

† For the detailed composition, see Reeves *et al.* (1993).

Serum leptin concentrations were analysed by ELISA assay (Kits Cristal Chem. Inc., Chicago, IL, USA). The intra-assay and interassay precisions of the rat leptin ELISA kit were 5·4 % and 6·9 %, respectively. The ELISA kit has a minimum rat leptin detection level of 200 pg/ml and a measurable range of concentration of 200–12 800 pg/ml.

The physiological index of insulin resistance used was homeostasis model assessment of insulin resistance (HOMA-IR), assessed from fasting glucose and fasting insulin concentrations using the following formula:

$$\text{HOMA} - \text{IR} = (\text{fasting insulin}\,[\text{ng/ml}])\,\% \\ (\text{fasting glucose}\,[\text{mg/dl}])/22{\cdot}5.$$

The retroperitoneal and gonadal white adipose tissue, and brown adipose tissue, was quickly removed to determine the fresh weight.

Measurements of carcass composition and energy intake

The carcasses were eviscerated, weighed and stored at −20°C for posterior composition analysis. Carcass water was measured by oven-drying at 80°C until a constant weight was reached. By subtracting the dry from the wet carcass weight, body water was determined. Fat carcass was measured by extraction in petroleum ether using a Soxlet continuous fat extractor (Fanen, São Paulo, Brazil). Fat content was then calculated by subtracting the fat-free dry mass from the dry carcass weight. Ash content was estimated, following combustion at 550°C until constant weight. Protein content was determined by subtracting the water, fat and ash content from the wet carcass weight.

To calculate carcass energy and energy intake, we assumed the energy content of protein and carbohydrate to be 16·74 kJ/g and that of fat to be 37·7 kJ/g (Du *et al.* 2000). To calculate the carcass composition at the beginning of lactation (initial carcass energy), we used data from female rats under similar dietary treatments and the same physiological status at day 1 of lactation. From the difference between the final carcass composition and the calculated initial carcass composition, we calculated the residual lipid balance, protein balance, energy balance, energy efficiency (ratio of energy gain to energy balance) and energy expenditure (difference between energy intake and energy balance).

Statistical analyses

The results are expressed as the means and their standard errors for the number of rats indicated. Initially, Levene's test for homogeneity of variance was used to check the fit of the data to the assumptions for parametric analysis of variance. When necessary, a Box–Cox transformation was used to correct variance heterogeneity or non-normality (Sokal & Rohlf, 1995). All data were subsequently analysed by two-way ANOVA (nutritional status and physiological status) followed by the Tukey–HSD test for individual differences between groups when necessary. The correlation coefficient was used to examine the relationship between serum leptin or insulin levels and some predictory variables of obesity.

In all comparisons, statistical significance was accepted at $P \leq 0{\cdot}05$. Statistical analysis was performed using the Statistic Software package (StatSoft, Inc., Tulsa, OK, USA).

Results

Biochemical and hormonal factors

At day 14 of lactation, serum total protein and glucose concentration did not differ between the groups. In both nutritional statuses, lactating rats had a lower albumin level than non-lactating rats ($F_{1,27} = 15{\cdot}71$, $P < 0{\cdot}001$). Serum insulin was influenced by nutritional status ($F_{1,27} = 15{\cdot}00$, $P < 0{\cdot}001$), but not by physiological status ($F_{1,27} = 3{\cdot}27$, $P = 0{\cdot}083$) and had interaction between both statuses ($F_{1,27} = 26{\cdot}55$, $P < 0{\cdot}001$). No difference was observed between the LPL and LPNL groups, and the serum insulin of CL rats was significantly higher than that of the LPL, LPNL and CNL groups ($P < 0{\cdot}01$). The serum leptin concentration was influenced only by nutritional status ($F_{1,27} = 33{\cdot}52$, $P < 0{\cdot}001$). An interaction was observed between the two effects ($F_{1,27} = 13{\cdot}71$, $P < 0{\cdot}001$) in that results in the LPL and LPNL groups were similar, and values were lower in the CL group than in the CNL, LPL and LPNL groups ($P < 0{\cdot}01$; Table 2). HOMA-IR was calculated and had a lower value in the LPL (1·57) than the CL (3·96; $P = 0{\cdot}02$) rats.

Body weight and food intake

Lactation significantly increased total food intake and consequently the total energy and protein intakes in both nutritional statuses. These values were, however, lower in LPL than CL rats ($P < 0{\cdot}001$). When expressed per gram body weight, food intake did not differ between the LPL and LPNL rats but was greater in the CL group than the CNL group ($P < 0{\cdot}001$; Table 3).

The relationship between energy intake and serum insulin concentration, energy intake and leptin levels are shown in Fig. 1A and 1B, respectively. In lactating rats, there was a significant positive correlation between serum insulin level and energy intake ($r = 0{\cdot}82$, $P < 0{\cdot}001$) and a negative relationship between serum leptin concentration and energy intake ($r = -0{\cdot}83$, $P < 0{\cdot}001$). These correlations were absent in non-lactating animals for serum insulin ($r = -0{\cdot}15$, $P = 0{\cdot}606$) and leptin ($r = 0{\cdot}04$, $P = 0{\cdot}879$) levels. When data for non-lactating and lactating rats were pooled, energy intake was directly correlated with serum insulin concentration ($r = 0{\cdot}44$, $P < 0{\cdot}02$) but not with serum leptin concentration ($r = -0{\cdot}32$, $P < 0{\cdot}09$). Significant correlations were not observed when the groups were separated in the low-protein and control diet groups.

Carcass composition and energy balance

Independently of nutritional status, lactating rats had a body weight higher at the beginning of the experimental period ($F_{1,27} = 19{\cdot}58$, $P < 0{\cdot}001$) and a lower weight at day 14 of lactation compared with non-lactating rats ($F_{1,27} = 4{\cdot}91$, $P < 0{\cdot}03$). No effects were observed in nutritional status (initial weight, $F_{1,27} = 1{\cdot}23$, $P = 0{\cdot}276$; final weight, $F_{1,27} = 1{\cdot}58$, $P = 0{\cdot}219$), and there was no interaction between nutritional status and physiological status (initial weight, $F_{1,27} = 0{\cdot}00$, $P = 0{\cdot}981$; final weight, $F_{1,27} = 0{\cdot}01$, $P = 0{\cdot}907$).

Carcass fresh weight and carcass protein content were also lower in the lactating rats than in the non-lactating rats for both nutritional statuses ($F_{1,27} = 8{\cdot}79$, $P < 0{\cdot}006$ and $F_{1,27} = 5{\cdot}70$, $P < 0{\cdot}024$, respectively). No effect occurred in nutritional status (carcass fresh weight, $F_{1,27} = 1{\cdot}13$, $P = 0{\cdot}295$; carcass protein content, $F_{1,27} = 0{\cdot}00$, $P = 0{\cdot}946$), and there was no interaction between these factors (carcass fresh weight, $F_{1,27} = 0{\cdot}11$, $P = 0{\cdot}740$; carcass protein content, $F_{1,27} = 0{\cdot}22$, $P = 0{\cdot}642$).

The fat content of the carcass in absolute and relative values was significantly affected by lactation ($F_{1,27} = 18{\cdot}81$, $P < 0{\cdot}001$ and $F_{1,27} = 13{\cdot}06$, $P < 0{\cdot}001$, respectively) and by dietary protein content ($F_{1,27} = 24{\cdot}39$, $P < 0{\cdot}001$ and $F_{1,27} = 27{\cdot}41$, $P < 0{\cdot}001$, respectively), but there was no interaction between these factors ($F_{1,27} = 1{\cdot}97$, $P = 0{\cdot}172$ and $F_{1,27} = 2{\cdot}778$, $P = 0{\cdot}107$, respectively). Thus, the carcass fat content of lactating rats was lower than that of non-lactating rats and higher in the protein-deprived groups than in the control group.

The carcass water and ash contents were not influenced by nutritional status ($F_{1,27} = 2{\cdot}20$, $P = 0{\cdot}150$ and $F_{1,27} = 0{\cdot}25$, $P = 0{\cdot}621$, respectively) or by physiological status ($F_{1,27} = 0{\cdot}65$, $P = 0{\cdot}423$ and $F_{1,27} = 3{\cdot}02$, $P = 0{\cdot}093$, respectively), nor was there any interaction between the groups ($F_{1,27} = 0{\cdot}04$, $P = 0{\cdot}834$ and $F_{1,27} = 0{\cdot}01$, $P = 0{\cdot}89$, respectively). There was also no difference between groups when

Table 2. Serum concentration of glucose, albumin, total protein, insulin and leptin of non-lactating and lactating groups of rats maintained with control (CNL, CL, respectively) or low-protein (LPNL, LPL, respectively) diets

(Values are means and their standard errors for the number of rats indicated)

	Groups											
	CNL			CL			LPNL			LPL		
	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Glucose (mg/l)	820	100	8	650	71·7	8	800	110	8	670	100	7
Albumin (g/l)	33·7	1·6	8	26·2***	1·5	8	33·4	3·8	8	23·9***	1·4	7
Total protein (g/l)	62	5·7	8	55·7	4·0	8	59·2	4·0	8	47·8	4·9	7
Insulin (ng/ml)	0·69[b]	0·09	7	1·38[a]	0·12	7	0·82[b]	0·12	7	0·49[b]	0·09	7
Leptin (pg/ml)	4530[b]	700	7	2251[c]	531	7	5620[ab]	368	7	7208[a]	604	7

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (Tukey HSD test; $P \leq 0{\cdot}05$).
Mean values were significantly different in relation to non-lactating rats; *** $P < 0{\cdot}001$ (two-way ANOVA).

Table 3. Absolute and relative food intake, total energy food intake and total protein intake, of non-lactating and lactating groups of rats maintained with control (CNL, CL, respectively) or low-protein (LPNL, LPL, respectively) diets

(Values are means with their standard errors for the number of rats indicated)

	Groups							
	CNL (*n* 8)		CL (*n* 8)		LPNL (*n* 8)		LPL (*n* 7)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Absolute food intake (g)	180	5·28^{c}	319	5·28^{a}	196	6·42^{c}	270	8·60^{b}
Relative food intake (g/100 g)	66	2·80^{c}	101	2·80^{a}	68	1·86^{bc}	79	3·85^{b}
Total energy intake (kJ)	2827	85^{c}	5012	84^{a}	3091	104^{c}	4253	127^{b}
Total protein intake (g)	31	0·92^{b}	54	0·91^{a}	12	0·40^{d}	16	0·53^{c}

^{a,b,c} Means with unlike superscript letters were significantly different (Tukey HSD test; $P \leq 0{\cdot}05$).

the carcass protein and ash contents were expressed relative to body weight. The proportion of water in the carcass was affected by the physiological ($F_{1,27} = 16{\cdot}46$, $P < 0{\cdot}001$, respectively) and nutritional ($F_{1,27} = 29{\cdot}56$, $P < 0{\cdot}001$) status, but there was no interaction between these factors ($F_{1,27} = 2{\cdot}024$, $P = 0{\cdot}166$); hence, lactating rats exhibited a lower proportion of fat and a higher proportion of water in their carcass than did non-lactating rats. In contrast, protein-deprived rats had a higher percentage of fat and a lower proportion of water in their carcass than control rats (Table 4).

The retroperitoneal and gonadal fat pad weights were significantly affected by lactation ($F_{1,27} = 8{\cdot}90$, $P < 0{\cdot}005$ and $F_{1,27} = 33{\cdot}16$, $P < 0{\cdot}001$, respectively) and by protein deprivation ($F_{1,27} = 4{\cdot}90$, $P < 0{\cdot}03$ and $F_{1,27} = 15{\cdot}33$, $P < 0{\cdot}001$) in absolute values but not in terms of the two-way interaction nutritional status % physiological status ($F_{1,27} = 0{\cdot}003$, $P = 0{\cdot}953$ and $F_{1,27} = 0{\cdot}28$, $P = 0{\cdot}603$, respectively). Similar results were observed in relative values for lactation ($F_{1,27} = 6{\cdot}24$, $P < 0{\cdot}019$ and $F_{1,27} = 42{\cdot}24$, $P < 0{\cdot}001$, respectively) and protein deprivation ($F_{1,27} = 4{\cdot}84$, $P < 0{\cdot}03$ and $F_{1,27} = 20{\cdot}68$, $P < 0{\cdot}001$, respectively) and in the two-way interaction nutritional status % physiological status ($F_{1,27} = 0{\cdot}30$, $P = 0{\cdot}582$ and $F_{1,27} = 0{\cdot}04$, $P = 0{\cdot}831$, respectively). Thus, the fat pad weights of lactating rats were lower than those of non-lactating rats, and higher in the protein-deprived groups than in the control groups.

Brown adipose tissue weight was modified only by nutritional status in absolute values ($F_{1,26} = 14{\cdot}02$, $P < 0{\cdot}001$) and in relative values ($F_{1,26} = 10{\cdot}42$, $P < 0{\cdot}001$). Protein-deprived rats exhibited an absolute brown adipose tissue

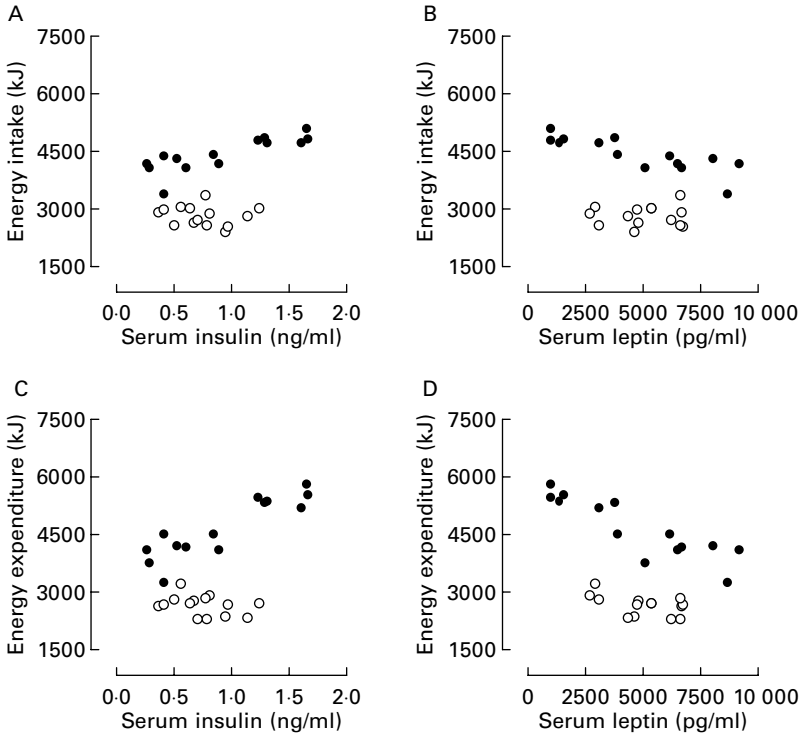

Fig. 1. Correlation between energy intake and serum insulin levels (A), energy intake and serum leptin levels (B), energy expenditure and serum insulin concentration (C) and energy expenditure and serum leptin concentration (D) of lactating (○) and non-lactating (●) rats. (A) Lactating, $r = 0{\cdot}82$, $P < 0{\cdot}001$; non-lactating, $r = -0{\cdot}15$, $P = 0{\cdot}606$. (B) Lactating, $r = -0{\cdot}83$, $P < 0{\cdot}001$; non-lactating, $r = 0{\cdot}04$, $P = 0{\cdot}879$. (C) Lactating, $r = 0{\cdot}89$, $P < 0{\cdot}001$; non-lactating, $r = -0{\cdot}33$, $P = 0{\cdot}248$. (D) Lactating, $r = -0{\cdot}87$, $P < 0{\cdot}001$; non-lactating, $r = -0{\cdot}54$, $P = 0{\cdot}066$.

Table 4. Carcass composition of non-lactating and lactating groups of rats maintained with control (CNL, CL, respectively) or low-protein (LPNL, LPL, respectively) diets

(Values are means with their standard errors for the number of rats indicated)

	Groups							
	CNL (*n* 8)		CL (*n* 8)		LPNL (*n* 8)		LPL (*n* 7)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body weight (g)	274	10	310	9·43***	283	5·28	319	10***
Final body weight (g)	280	10	262	9·43*	292	4·53	272	11*
Fresh carcass weight (g)	204	6·80	187	6·42**	208	4·15	194	4·50**
Protein (g)	50	1·90	47	2·48*	51	1·99	46	1·93*
Lipid (g)	28	2·69	15	2·26***	35	1·95†††	29	2·18***†††
Water (g)	121	3·67	119	4·15	117	2·77	113	4·08
Ash (g)	5	0·57	6	0·32	5	0·61	6	0·38
Carcass weight								
Protein (g/100 g)	25	0·55	25	0·65	24	0·70	24	0·55
Lipid (g/100 g)	13	0·93	8	1·12***	17	0·89†††	15	1·28***†††
Water (g/100 g)	59	0·73	64	0·83***	56	0·93†††	58	1·03***†††
Ash (g/100 g)	3	0·28	3	0·22	3	0·27	3	0·19

Mean values were significantly different in relation to non-lactating rats: *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ (two-way ANOVA).
Mean values were significantly different in relation to rats fed with the control diet: †$P < 0·05$, ††$P < 0·01$, †††$P < 0·001$ (two-way ANOVA).

weight higher than that of control rats, independent of physiological status. This was confirmed when values were expressed as a percentage of body weight (Table 5).

Serum leptin concentration was directly correlated with lipid carcass content ($r = 0·63$, $P<0·001$) and the weights of the retroperitoneal fat pads ($r = 0·55$, $P<0·002$), gonadal fat pads ($r = 0·50$, $P<0·007$) and brown adipose tissue ($r = 0·43$, $P<0·022$). In contrast, there was an inverse relationship between serum insulin level and fat carcass content ($r = -0·62$, $P<0·001$), retroperitoneal fat ($r = -0·54$, $P<0·001$) and weight of gonadal fat ($r = -0·56$, $P<0·002$). Serum insulin levels were not correlated with weight of brown adipose tissue ($r = -0·35$, $P=0·070$).

LPL rats had a lower energy intake but a higher carcass energy than CL rats. The energy balance was influenced by nutritional status ($F_{1,27} = 30·86$, $P<0·001$) and lactation ($F_{1,27} = 113·04$, $P<0·001$), as well as by the interaction between the two factors ($F_{1,27} = 8·82$, $P<0·006$). There was thus a negative energy balance in lactating rats, less severe in the LPL than in the CL group. Energy balance correlated negatively with energy intake in lactating rats ($r = -0·81$, $P<0·001$) and when the data from the two groups were combined ($r = -0·87$, $P<0·001$). This correlation was weaker only in non-lactating rats ($r = 0·44$, $P=0·117$).

A considerable carcass energy loss as lipid was observed in the LPL and CL groups, but this was less severe in the former group ($P<0·001$). Independent of nutritional status, lactating rats showed a reduction in carcass energy as protein in relation to the non-lactating rats ($F_{1,27} = 28·13$, $P<001$).

Energy expenditure, calculated by the energy-balance method, was significantly affected by the nutritional ($F_{1,27} = 31·08$, $P<0·001$) and physiological ($F_{1,27} = 443·13$, $P<0·001$) status, as well as by the interaction between these factors ($F_{1,27} = 39·16$, $P<0·001$). Thus, energy expenditure was significantly higher in the lactating rats, but lower in the LPL than the CL group ($P<0·001$). Energy efficiency was significantly modified by lactation ($F_{1,27} = 90·01$, $P<0·001$) and by nutritional status ($F_{1,27} = 20·78$,

Table 5. Absolute and relative weights of retroperitoneal and gonadal white adipose tissue and brown adipose tissue of non-lactating and lactating groups of rats maintained with control (CNL, CL, respectively) or low-protein (LPNL, LPL, respectively) diets

(Values are means with their standard errors for the number of rats indicated)

	Groups							
	CNL (*n* 8)		CL (*n* 8)		LPNL (*n* 8)		LPL (*n* 7)	
Retroperitoneal (g)	4·83	30·37	3·57	0·61**	5·64	0·35†	4·58	0·14**†
Gonadal (g)	10	0·69	6·77	0·97***	13	0·52†††	8·99	0·42***†††
Brown adipose (g)	0·29	0·02	0·26	0·02	0·39	0·03†††	0·35	0·03†††
Retroperitoneal (g/100 g)	1·74	0·09	1·34	0·20*	1·95	0·11†	1·70	0·10*†
Gonadal (g/100 g)	3·67	0·13	2·54	0·29***	4·53	0·12†††	3·32	0·17***†††
Brown adipose (g/100 g)	0·11	0·01	0·10	0·01	0·14	0·01†††	0·12	0·01†††

Mean values were significantly different in relation to non-lactating rats: *$P < 0·05$, ***$P < 0·01$, ***$P < 0·001$ (two-way ANOVA).
Mean values were significantly different in relation to rats fed with the control diet: †$P < 0·05$, ††$P < 0·01$, †††$P < 0·001$ (two-way ANOVA).

$P<0{\cdot}001$), but no effect of the interaction of these factors was verified ($F_{1,27}=2{\cdot}15$, $P=0{\cdot}154$). Hence, energy efficiency was reduced by lactation and increased by protein deprivation (Table 6).

The relationship between energy expenditure and serum insulin level was positive ($r=0{\cdot}89$, $P<0{\cdot}001$), and that between energy expenditure and serum leptin level negative ($r=-0{\cdot}87$, $P<0{\cdot}001$) in lactating rats. These correlations were weaker in non-lactating rats (Fig. 1C, 1D). When data from non-lactating and lactating rats were pooled, energy expenditure correlated directly with serum insulin concentration ($r=-0{\cdot}53$, $P<0{\cdot}004$) and inversely with serum leptin concentration ($r=-0{\cdot}46$, $P<0{\cdot}012$).

Discussion

Rats fed with a low-protein diet did not exhibit the typical features of protein malnutrition such as low body weight and hypoalbuminaemia. However, the 'adiposity signals' (serum insulin and serum leptin concentrations) were different in response to dietary protein content. In LPL and LPNL rats, the insulinaemia was similar, whereas in CL rats, the serum insulin concentration was higher than in CNL rats.

In addition, serum leptin concentration did not differ between LPL and LPNL rats, and in CL rats the leptinaemia was reduced by 50 % compared with CNL rats. In consequence, the serum leptin concentration was higher in LPL than in CL rats. Although some researchers have found no change in serum leptin in lactating rats (Chien *et al.* 1997; Carmen-Garcia *et al.* 2000), several studies have shown that there is a 20–75 % decrease in serum leptin in the daytime during lactation (Brogan *et al.* 1999; Herrera *et al.* 2000; Woodside *et al.* 2000).

There are several possible explanations for the increase in serum leptin concentration verified in lactating rats submitted to protein restriction in the present study. First, lactating rats fed protein-restricted diet have a low rate of milk production, as already described (Grigor *et al.* 1987) and verified in the present study (data not shown), and in situations in which milk production is decreased, there is an elevation in serum leptin concentration (Cowie *et al.* 1980; Denis *et al.* 2003).

The second explanation is the decreased clearance rate of leptin due to a reduced cardiac output and reduced blood flow and/or milk yield (Sakanashi *et al.* 1987). This supposition is reinforced by the fact that the clearance rate of leptin is increased in normal lactating rats, which is compatible with the high cardiac output during lactation (Williamson, 1980), and that leptin is also secreted into the milk (Bonnet *et al.* 2002).

Finally, the major explanation is the increased synthesis of hormone by white adipose tissue since leptin is synthesised predominantly by this tissue, proportionally to body fat stores (Frühbeck, 2001; Harvey & Ashford, 2003). In the present study, despite the fact that rats fed with a low-protein diet ate less than control rats, a significant increase in weight of the retroperitoneal and gonadal fat pads, as well as higher carcass fat content, was verified. Moreover, serum leptin concentrations were directly correlated with carcass fat, gonadal and retroperitoneal adipose tissue fresh weight, explaining the increase in the synthesis of leptin in malnourished rats.

The higher adiposity verified in the present study in rats submitted to protein restriction may be the consequence of lower milk production and thus energy expenditure. It could also be the result of a higher sensitivity of adipocytes to insulin, with a consequent increase in the synthesis of lipids and an inhibition of their degradation. This hypothesis is supported by a lower HOMA-IR value and a higher rate constant for the disappearance of serum glucose during insulin tolerance test (data not shown) seen in LPL rats compared with CL rats. In accordance, our results showed an inverse correlation between insulinaemia and fat carcass content and retroperitoneal and gonadal fat pad weights.

A high sensitivity of adipocytes to insulin could also contribute to increase leptin secretion and biosynthesis. Studies *in vitro* showed that insulin acts directly at the level of the adipocytes, increasing leptin mRNA level and secretion (Hardie *et al.* 1996; Barr *et al.* 1997), perhaps due to increased glucose transport and metabolism (Müller *et al.* 1998). On the other hand, the enhanced peripheral sensitivity to insulin

Table 6. Residual energy balance of non-lactating and lactating groups of rats maintained with control (CNL, CL, respectively) or low-protein (LPNL, LPL, respectively) diets

(Values are means with their standard errors for the number of rats indicated)

	Groups							
	CNL (*n* 8)		CL (*n* 8)		LPNL (*n* 8)		LPL (*n* 7)	
Final carcass energy (kJ)‡	1886	115	1353	94***	2190	75†††	1866	62***†††
Initial carcass energy (kJ)	1835	67	2271	76†††	1954	46	2176	47†††
Energy balance (kJ)§	51	45[a]	−918	94[c]	236	75[a]	−310	62[b]
Lipid balance (kJ)	32	10[ab]	−781	85[c]	204	74[a]	−155	82[b]
Protein balance (kJ)	19	9·01	−137	41***	32	13	−155	32***
Calculated energy expenditure (kJ)‖	2776	92[c]	5930	145[a]	2855	82[c]	4563	167[b]
Energy efficiency¶	1·80	0·25	−18	1·79***	7·63	2·16†††	−7·29	1·38***†††

[a,b,c] Mean values within a row with unlike superscript letters were significantly different (Tukey HSD test; $P\leq 0{\cdot}05$).

* Mean values were significantly different in relation to non-lactating rats: ***$P<0.001$ (two-way ANOVA).

† Mean values were significantly different in relation to rats fed with control diet: †††$P<0{\cdot}001$ (two-way ANOVA).

‡ Carcass energy was calculated from the energy content of protein and carbohydrate to be 16·74 kJ/g and that of fat to be 37·7 kJ/g.

§ Energy balance was calculated as the difference between energy intake and energy expenditure.

‖ Energy expenditure was calculated by the balance method (energy expenditure = energy intake − energy balance).

¶ Energy efficiency was calculated as the (energy balance/energy intake) × 100.

might be a consequence of higher leptin levels, since the administration of leptin either centrally or peripherally increased insulin-stimulated glucose use by peripheral tissues in normal rats (Barzilai *et al.* 1997; Lin *et al.* 2002).

Finally, the negative energy balance shown by lactating rats in the present study maintained with a low-protein diet could be involved. The negative energy balance appears to be the major factor responsible for the hypoleptinaemia of lactation (Vernon *et al.* 2002). Confirming this supposition, we observed that leptinaemia was directly related to energy balance only in lactating rats. Moreover, LPL rats that showed higher levels of this hormone also had a negative energy balance, although this was less accentuated.

Hypoleptinaemia during lactation may drive, or at least facilitate, hyperphagia (Vernon *et al.* 2002). In this study, LPL rats, which had higher serum leptin and lower serum insulin levels, consumed less food (in absolute values and also when expressed per gram of body weight) than the CL rats with lower serum leptin and higher serum insulin levels. In lactating rats, there was a significant negative correlation between serum leptin and energy intake, but this correlation was absent in non-lactating rats and when the data for non-lactating and lactating rats were pooled. The relationship between energy intake and serum insulin level was not significant in non-lactating rats alone; there was, however, a positive correlation in lactating rats and in the combined data from the two groups. Furthermore, energy intake was negatively related to energy balance in lactating rats.

These findings suggest the following. First, in normal lactation, peripheral insulin resistance occurs (Vernon *et al.* 1990) that contributes to negative energy balance, leading to hypoleptinaemia. Together with hypothalamic insulin resistance, this results in hyperphagia. Second, protein restriction during lactation reduces serum insulin levels, favouring an enhanced peripheral sensitivity to hormones that contributes to greater maternal fat deposition and hyperleptinaemia. The high serum leptin levels associated with the increase in the hypothalamic sensitivity to insulin decreased the food intake.

Studies have showed that the high cost of lactation in energy terms is paid by the increase in food intake associated with use of the stored fat (Naismith *et al.* 1982), and by conserving metabolic fuels owing to a reduction in brown fat thermogenesis (Trayhurn & Richard, 1985). In the reduction of dietary protein level, although hyperphagia occurs, there is an accompanying decrease in food efficiency and an increase in energy expenditure in an apparent attempt to dissipate excess energy (Specter *et al.* 1995). These changes in metabolic efficiency appear to be the result of adaptive diet-induced thermogenesis associated with an increased activity of brown adipose tissue (Rothwell & Stock, 1987). In this study, LPL rats had decreased energy expenditure and a lower depletion of the reserves of fat carcass compared with CL rats.

Curiously, the groups maintained with a low-protein diet exhibited better energy efficiency, possibly due to hypophagia that resulted in a decrease of energy expenditure in an attempt to conserve energy. This change in metabolic efficiency did not appear to be the result of adaptive diet-induced thermogenesis, as judged by the higher brown adipose tissue weight in rats maintained with a low-protein diet, an indication of greater metabolic activity in this tissue (Rothwell & Stock, 1987). Nor did this result from a decrease in BMR as this change is related, among others factors, to a low thyroid hormone concentration. In our case, hypothyroidism could be discounted, at least in the lactation, once this alteration is associated to hypoleptinaemia (Ahima, 2000).

As well as hypophagia, hypoinsulinaemia is another candidate to explain the reduced energy expenditure as the latter was directly correlated to serum insulin level and negatively correlated to serum leptin. A low serum insulin concentration could determine increased insulin sensitivity in the adipocytes, raising the rate of lipogenesis in the white adipose tissue.

Thus, rats submitted to protein restriction during pregnancy and lactation showed, at peak lactation, a preservation of maternal reserves owing to negative energy balance, although this was less severe when compared with lactating control rats. This metabolic adaptation was obtained, at least in part, by the hypoinsulinaemia that resulted in increased insulin sensitivity favouring enhanced fat deposition, hyperleptinaemia and hypophagia.

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References

Ahima RS (2000) Leptin and the neuroendocrinology of fasting. *Front Horm Res* **26**, 42–56.

Barr VA, Malide D, Zarnowski MJ, Taylor SI & Cushman SW (1997) Insulin stimulates both leptin secretion and production by rat white adipose tissue. *Endocrinology* **138**, 4463–4472.

Barzilai N, Wang J, Massilon D, Vuguin P, Hawkins M & Rossetti L (1997) Leptin selectively decreases visceral adiposity and enhances insulin action. *J Clin Invest* **100**, 3105–3110.

Bonnet M, Delavaud C, Laud K, Gourdou I, Leroux C, Djiane J & Chilliard Y (2002) Mammary leptin synthesis, milk leptin and their putative physiological roles. *Reprod Nutr Dev* **42**, 1–15.

Brogan RS, Mitchell SE, Trayhurn P & Smith MS (1999) Suppression of leptin during lactation: contribution of the suckling stimulus versus milk production. *Endocrinology* **140**, 2621–2627.

Carmen-Garcia MD, Casanueva FF, Dieguez C & Senaris RM (2000) Gestational profile of leptin messenger ribonucleic acid (mRNA) content in the placenta and adipose tissue in the rat, and regulation of the mRNA levels of the leptin receptor subtypes in the hypothalamus during pregnancy and lactation. *Biol Reprod* **62**, 698–703.

Chien EK, Hara M, Rouard M, Yano H, Phillippe M, Polonsky KS & Bell GI (1997) Increase in serum leptin and uterine leptin receptor messenger RNA levels during pregnancy in rats. *Biochem Biophys Res Commun* **237**, 476–480.

Coitinho DC (1998) A influência da história reprodutiva no índice de massa corporal de mulheres brasileiras. (The influence of childbearing on the body mass index in Brazilian women.). PhD Thesis, Universidade de São Paulo, Brazil.

Colombo JP, Cervantes H, Kokorovic M, Pfister U & Perritaz R (1992) Effects of dietary protein on the distribution of amino acids in plasma, liver and brain in the rats. *Ann Nutr Metabol* **36**, 23–33.

Cowie AT, Forsyth IA & Hart IC (1980) Hormonal control of lactation. *Monogr Endocrinol* **15**, 165–168.

Denis RGP, Williams G & Vernon RG (2003) Regulation of serum leptin and its role in the hyperphagia of lactation in the rat. *J Endocrinol* **176**, 193–203.

Doumas BT, Watson WA & Biggs HG (1971) Albumin standards and measurements of serum albumin with bromocresol green. *Clin Chim Acta* **31**, 87–96.

Du F, Higginbotham DA & White BD (2000) Food intake, energy balance and serum leptin concentrations in rats fed low-protein diets. *J Nutr* **130**, 514–521.

Florêncio TT (1998) Obesity in adult population residing in a "sem teto"– homeless encampment in the northeast of Brazil. *Intern J Obes* **22**, 245S.

Frühbeck G (2001) A heliocentric view of leptin. *Proc Nutr Soc* **60**, 301–318.

Grigor MR, Allan JE, Carrington JM, Carne A, Geursen A, Young D, Thompson MP, Hayness EB & Coleman RA (1987) Effect of dietary protein and food restriction on milk production and composition, maternal tissues and enzymes in lactating rats. *J Nutr* **117**, 1247–1258.

Hardie LJ, Guilhot N & Trayhurn P (1996) Regulation of leptin production in cultured mature white adipocytes. *Horm Metab Res* **28**, 685–689.

Harvey J & Ashford MLJ (2003) Leptin in the CNS: much more than a satiety signal. *Neuropharmacology* **44**, 845–854.

Heard CRC & Turner MR (1967) Glucose tolerance and related factors in dogs fed diets of suboptimal protein value. *Diabetes* **16**, 96–107.

Herrera E, Lasunción MA, Huerta L & Martín-Hidalgo A (2000) Plasma leptin levels in rat mother and offspring during pregnancy and lactation. *Biol Neonate* **78**, 315–320.

Instituto Nacional de Alimentação e Nutrição (1991) INAN Pesquisa Nacional de Saúde e Nutrição Condições nutricionais da população brasileira: adultos e idosos. (Nutritional conditions of adult and elderly Brazilian population.). Brasilia: INAN/Ministério da Saúde.

Lin CY, Higginbotham DA, Judd RL & White BD (2002) Central leptin increases insulin sensitivity in streptozotocin-induced diabetic rats. *Am J Physiol Endocrinol Metab* **282**, E1084–E1091.

McNamara JP (1995) Role and regulation of metabolism in adipose tissue during lactation. *Nutr Biochem* **6**, 120–129.

Monteiro CA & Mondini L (1998) The changing nature of nutrition disorders in the developing countries: the case of Brazil. *Br J Nutr* **86**, 277–283.

Müller WM, Gregoire FM, Stanhope KL, Mobbs CV, Mizuno TM, Warden CH, Stern JS & Havel PJ (1998) Evidence that glucose metabolism regulates leptin secretion from cultured rat adipocytes. *Endocrinology* **139**, 551–558.

Naismith DJ, Richardson DP & Pritchard AE (1982) The utilization of protein and energy during lactation in the rat, with particular regard to the utilization of fat accumulated during pregnancy. *Br J Nutr* **48**, 433–441.

Popkin BM & Bisgrove ES (1988) Urbanization and nutrition in low-income countries. *Food Nutr Bull* **10**, 3–23.

Reeves PG, Nielsen FH & Fahey GC Jr (1993) AIN 93 purified diets for laboratory rodents: report of the American Institute of Nutrition ad hoc Working Committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.

Rothwell NJ & Stock MJ (1987) Influence of carbohydrate and fat intake on diet-induced thermogenesis and brown fat activity in rats fed low protein diets. *J Nutr* **117**, 1721–1726.

Sakanashi TM, Brigham HE & Rasmussen KM (1987) Effect of dietary restriction during lactation on cardiac output, organ blood flow and organ weights of rats. *J Nutr* **117**, 1469–1474.

Scott AM, Atwater I & Rojas E (1981) A method for the simultaneous measurement of insulin released and B-cell membrane potential in single mouse islets of Langerhans. *Diabetologia* **21**, 470–475.

Shetty PS (1990) Physiological mechanisms in the adaptive response of metabolic rates to energy restriction. *Nutr Res Rev* **3**, 49–74.

Sokal RR & Rohlf FJ (1995) Assumptions of analysis of variance. In *Biometry: The Principles and Practice of Statistics in Biological Research*, pp. 392–450 New York: W. H. Freeman.

Specter SE, Hamilton JS, Stern JS & Howitz BA (1995) Chronic protein restriction does not alter energetic efficiency of brown adipose tissue thermogenic capacity in genetically obese (fa/fa) Zucker rats. *J Nutr* **125**, 2183–2193.

Terada Y, Yamakawa K, Sugaya A & Toyoda N (1998) Serum leptin levels do not rise during pregnancy in age-matched rats. *Biochem Biophys Res Commun* **253**, 841–844.

Trayhurn P (1989) Thermogenesis and the energetics of pregnancy and lactation. *Can J Physiol Pharm* **67**, 370–375.

Trayhurn P & Richard D (1985) Brown adipose tissue thermogenesis and the energetics of pregnancy and lactation in rodents. *Biochem Soc Trans* **13**, 826–828.

Trinder P (1969) Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. *J Clin Path* **22**, 158–161.

Tulp OL, Krupp PP, Danforth E & Horton ES (1979) Characteristics of thyroid function in experimental protein malnutrition. *J Nutr* **109**, 1321–1332.

Vernon RG (1989) Endocrine control of metabolic adaptation during lactation. *Proc Nutr Soc* **48**, 23–32.

Vernon RG, Denis RGP, Sorensen A & Williams G (2002) Leptin and the adaptations of lactation in rodents and ruminants. *Horm Metab Res* **34**, 678–685.

Vernon RG, Faulkner A, Hay WWJ, Calvert DT & Flint DJ (1990) Insulin resistance of hind-limb tissues in vivo in lactating sheep. *Biochem J* **290**, 783–786.

Vernon RG & Pond CM (1997) Adaptations of maternal adipose tissue to lactation. *J Mamm Gland Biol Neoplasia* **2**, 231–241.

White BD, Dean RG & Martin RJ (1998) An association between low levels of dietary protein, elevated NPY gene expression in the basomedial hypothalamus and increased food intake. *Nutr Neurosci* **1**, 173–182.

Williamson DH (1980) Integration of metabolism in tissues of the lactating rat. *FEBS Letts* **117**, K93–K105.

Wolfson WQ, Chon C, Calvary F & Ichiba F (1948) Studies in serum proteins 5. A rapid procedure for the estimation of total protein, true albumin, total globulin, alpha globulin, and gamma globulin in 1·0ml of serum. *Am J Clin Path* **18**, 723–730.

Woodside B, Abizaid A & Walker C-D (2000) Changes in leptin levels during lactation: implications for lactational hyperphagia and anovulation. *Horm Behav* **37**, 353–365.