"Developmental and nutritional changes of ob and PPAR
gamma 2 gene expression in rat white adipose tissue."

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Abstract
The ob gene encodes leptin, a hormone which induces satiety and increases
energy expenditure. The peroxisome proliferator-activated receptor gamma 2
isoform (PPAR gamma 2) gene encodes a transcription factor which controls
adipocyte differentiation and expression of fat-specific genes. We have studied
the regulation of these two genes in white adipose tissue (WAT) during the
suckling-weaning transition. Suckling rats ingest a high-fat diet (milk). Fat-pad
weight barely varied during the last week of suckling. ob mRNA levels, which
were very low in 15-day-old rats, rose approximately 6-fold until weaning at 21
days. When the rats were weaned on to a standard (high-carbohydrate) laboratory
chow, epididymal WAT enlarged approximately 7-fold, and ob mRNA kept
increasing progressively and doubled between 21 and 30 days. This evolution
contrasted with that of fatty acid synthase (FAS) mRNA, which increased sharply,
but only after weaning. To distinguish between the influence of development...

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Developmental and nutritional changes of \( ob \) and PPAR\( \gamma \)2 gene expression in rat white adipose tissue

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INTRODUCTION

Adipocytes may function as endocrine cells and play an active role in the regulation of energy homoeostasis. The \( ob \) gene is selectively expressed in fat-cells. It encodes leptin, a newly identified hormone which induces satiety and increases energy expenditure, thereby regulating the size of body fat (lipostat model) (reviewed in [1]). Morbidly obese mice, which are homozygous for the \( ob \) mutation, fail to produce leptin, but lose weight when injected with the hormone (reviewed in [1]). One target tissue of leptin is the hypothalamus, where specific receptors have been identified [2,3]. The evidence that adipocytes can communicate directly with the brain has aroused considerable interest in the origin and fate of fat-cells and, thence, in a member of the peroxisome proliferator-activated receptor subfamily of nuclear hormone receptor, PPAR\( \gamma \)4. It is the first adipocyte-specific transcription factor identified so far, and, in vitro at least, it appears to control the expression of several other fat-specific genes and adipocyte differentiation itself (i.e. adipose-cell number). Forced expression of this gene in fibroblasts initiates a transcription cascade that leads to conversion in cells of the adipose lineage [4,5].

The suckling–weaning transition is a physiological situation during which dramatic nutritional changes occur spontaneously. At weaning, suckling rats, which had been ingesting a high-fat (HF) diet (milk), are switched to a standard (high-carbohydrate, HC) laboratory chow. These nutritional changes, and the ensuing hormonal and metabolic modifications, underlie the marked increase in size and lipid content that normally occurs in white adipose tissue (WAT) at weaning [6]. They also contribute to the development of overt obesity in susceptible animals [6,7].

In the present work, we have studied the regulation of \( ob \) and PPAR\( \gamma \)2 genes in WAT during the suckling–weaning transition. To distinguish between the influence of ontogenic and nutritional factors, a group of rats was weaned on to an HF diet. The expression of genes coding for fatty acid synthase (FAS) and phosphoenolpyruvate carboxykinase (PEPCK), two enzymes which are mainly regulated by variations of the nutritional state [6,8], was also measured for comparison.

MATERIALS AND METHODS

Animals and research design

Wistar rats, bred in the Faculty animal house, were used. On the day of delivery, the litter size was standardized to eight pups per mother. The studies were performed on male rats only, at the ages of 15 and 21 days (suckling rats) and 23, 25 and 30 days (weaned rats). At 21 days, one group of rats was weaned to a semi-synthetic HC diet composed of (%) of total gross energy: 62 carbohydrate (cornstarch/dextrose, 1:3), 12 fat (corn oil/colza oil/lard, 1:1:3), 26 protein (casein). The other group was weaned to an HF diet composed of (%) of total gross energy: <1 carbohydrate, 72 fat (corn oil/colza oil/lard, 1:1:3), 26 protein.
(casein) (U.A.R., Villemoisson, France). Because suckling rats begin to nibble the food of their mothers around 15 days of age [8], the mothers were fed an HF diet from day 14 of lactation until weaning to avoid any consumption of carbohydrates by the pups during the last week of lactation [8]. At weaning, the young rats were housed individually. Pups destined to be killed at 30 days were housed in metabolism cages permitting accurate measurements of food and gross energy intake. All rats were maintained at a constant temperature (22°C) with a 12 h/12 h light (07.00–19.00 h)/dark cycle.

The animals were killed by decapitation in the morning (08.30–10.30 h). Blood was collected, and plasma kept at −20°C until glucose, non-esterified fatty acid (NEFA) and insulin measurements. Pairs of epididymal and inguinal fat-pads were immediately removed, weighed, frozen in liquid nitrogen, and stored at −70°C for subsequent RNA extraction or morphometric determinations.

**RNA extraction and Northern-blot analysis**

Total RNA was isolated from the fat tissue of each individual rat (30 days) or from pooled tissue of two or three rats (younger animals), with an acid guanidinium thiocyanate/phenol/chloroform mixture as previously described [9]. The concentration of RNA was determined by absorbance at 260 nm. For Northern-blot analysis, 30 µg of total RNA was denatured in a solution containing 2.2 mM formaldehyde and 50% (v/v) formaldehyde by heating at 95°C for 2 min. RNA was then size-fractionated by 1% agarose-gel electrophoresis, transferred to a Hybond-N membrane (Amersham International, Amersham, Bucks., U.K.) and cross-linked by UV irradiation. The integrity and relative amounts of RNA were assessed by Methylene Blue staining of the blot.

The ob cDNA probe was prepared as described in [9]. The PPARγ2 [5] and β2-adrenoceptor (β2-AR) [10] cDNA probes were prepared as follows. Products of 391 bp and 434 bp were obtained respectively for PPARγ2 and β2-AR after reverse-transcription PCR on total RNA from rat adipose tissue [sense primers: 5'-TATGGAGCCTAAGTTTGAGT-3' (PPARγ2), 5'-TGGGACTCCTCAGTGGCACCAC-3' (β2-AR); antisense primers: 5'-GCAAATCTAGGAAGAACAC-3' (PPARγ2), 5'-CCTGCAGCATAGACAGACATATAC-3' (β2-AR)]. The FAS and PEPCK cDNA probes were kindly supplied by Dr. A. G. Goodridge [11] and Dr. R. W. Hanson [12] respectively. Hybridizations with the different radiolabelled probes and subsequent washings of the membranes were performed as reported in [9]. The filters were then exposed to Kodak X-OMAT AR films for 4–72 h at −70°C with intensifying screens. The same filters were successively hybridized with the different probes. The intensities of the RNA bands on the blots and of 18 S RNA on membrane were quantified by scanning densitometry (Sharp Scanner JX 325 combined with Image Master Software; Pharmacia) and expressed as absorbance (A). Ribosomal 18 S RNA levels were similar in all groups.

**Microscopic determination of fat-cell size and estimation of fat-cell number**

In 15- and 21-day-old pups, microscopic measurements could not be done on the same samples as mRNAs, because of the small size of the fat-pads. Two groups of pups matched for body weight and fat-pad weight were therefore used. In older rats the fat-pad size was sufficient to allow both measurements.

Epididymal adipose tissue was allowed to thaw for 3–4 min at room temperature, fixed in Bouin’s solution, embedded in paraffin and stained with Hemalun/Eosin. Diameter profiles of fat-cells were measured by planimetry with a semi-automatic image analyser (Videoplan, Kontron Bildanalyse, Eching, Germany) and treated by the Wicksell transformation, to obtain the actual size of the cells [13]. Accurate determination of cell size required analysis of ~ 100 cells per sample, as shown by the lack of change in the coefficient of variation when n was further increased. Fat-cell number was calculated by dividing the adipose-tissue mass by the mean cell volume and the density of fat tissue (0.94 g/ml at 37°C).

**Analytical procedures**

Plasma glucose, insulin and NEFA were measured as previously described [9].

**Presentation of the results**

Results are means ± S.E.M. for the indicated number of rats. Comparisons between groups were carried out by an unpaired Student’s t test or analysis of variance followed by the Newman–Keuls test for multiple comparisons, when appropriate. Differences were considered statistically significant at P < 0.05.

**RESULTS**

The body-weight gain of rats weaned on to an HF diet was slightly lower (~ 14% at day 30; P < 0.01) than that of control rats weaned on to a more standard (HC) laboratory chow (Figure 1). This slight decrease can probably be ascribed to a 10% reduction in the daily food consumption: over the last 7 days of the study, i.e. when rats were fully adapted to solid food and individual housing in metabolism cages, the average calorie intake (based on gross energy intake) was 144.8 ± 4.2 kJ (34.6 ± 1.0 kcal)/day for HF versus 161.1 ± 4.2 kJ (38.5 ± 1.0 kcal)/day for HC and 143.2 ± 8.2 kcal (34.4 ± 1.0 kcal)/day for control (Figure 1).

**Figure 1 Changes in body weight and food intake during the suckling–weaning transition**

Suckling (S) rats were weaned to a high-carbohydrate (HC) or high-fat (HF) diet at 21 days of age. The animals were housed in individual metabolism cages from weaning onward. Values are means ± S.E.M. for ten rats in each group. Note: 1 kCal = 4.184 kJ (the S.I. unit).
Table 1 Fat-pad weight, adipocyte volume and number during the suckling–weaning transition

(a) Weight of pairs of epididymal and inguinal fat-pads in rats weaned at 21 days of age to an HC or HF diet. (b) Adipocyte volume was measured microscopically by planimetry: ∼ 100 cells were analysed per rat. (c) Adipocyte number was calculated on the basis of the average values of the two aforementioned parameters. Values are means ± S.E.M. from seven (15 days), seven (21 days) or ten (30 days, HC) and ten (30 days, HF) rats. *P < 0.001 versus 15 days; †P < 0.001 versus 15 and 21 days.

<table>
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Figure 2 Changes in plasma glucose, insulin and NEFA levels during the suckling–weaning transition

Suckling (S) rats were weaned to a high-carbohydrate (HC) or high-fat (HF) diet at 21 days of age. Measurements were made on the blood obtained when the animals were killed. Values are means ± S.E.M. for ten individual rats in each group. mEq/L = mequiv./litre.

Figure 3 Northern-blot analysis of ob, FAS, PPARγ2 ('PPARγ'), PEPCK and β3-AR mRNA in epididymal WAT during the suckling–weaning transition

All lanes were loaded with 30 µg of total RNA. The filters were then successively hybridized with the different radiolabelled cDNA probes. This figure is representative of four (15, 21, 23 and 25 days) or ten (30 days) determinations. Spots of 18 S rRNA ('18S r') obtained after Methylene Blue staining of the membranes are shown to indicate similar loading of RNA.

/day for HC rats (n = 10; P < 0.02). Fat-pad weight and fat-cell size increased ∼ 3-fold during the last week of suckling, while cell number did not change (Table 1). After weaning, fat tissue markedly enlarged (∼ 6-fold for epididymal fat between 21 and 30 days of age) due to a 2–3-fold increase in adipocyte size and number. This development was not significantly affected by the type of diet (HC or HF) administered to weaned pups (Table 1).

The glycaemia of pups increased by about 1 mmol/l during the last week of the suckling period (Figure 2). In rats weaned on to an HC diet, a substantial further rise in glycaemia occurred within the first 2 days post-weaning. This was accompanied by a marked increase in insulinaemia and a decrease in plasma NEFA (P < 0.01 or less for these three parameters in 23- versus 21-day-old pups). Weaning on to an HF diet largely prevented these
observed in inguinal WAT of HF- versus HC-fed rats. However, average ob mRNA expression was lower in inguinal than in epididymal tissue (Figure 4A), confirming that it may be characterized by an adipose region-specificity [9,14]. This observation can be extended to the other genes studied (cf. Figure 4).

Because ob expression may be influenced by variations in body weight and/or quantitative changes in food intake [9,15–18], two subgroups of the HC and HF rats killed at 30 days were analysed to ascertain the direct impact of dietary fat. These subgroups (each comprising seven out of ten rats from the initial groups) were matched for daily food intake [average over the last 7 days of the study: HC, 153.6 ± 1.68 (36.7 ± 0.4); HF, 149.8 ± 5.02 (35.8 ± 1.2) kJ (kcal)/day] and for body weight (at 21 days: HC, 40 ± 1 g; HF, 41 ± 1 g and at 30 days: HC, 81 ± 1 g; HF, 77 ± 3 g). When these subgroups were compared, ob gene expression remained higher ($P < 0.03$ or less) in HF than in HC rats (by 104% in epididymal and 138% in inguinal fat at day 30). This indicates that HF diet specifically enhances ob gene expression.

mRNA concentrations of PPARγ2, like those of ob, increased (2-fold; $P < 0.01$) during the last week of suckling. After weaning, they increased further (by 55%; $P < 0.01$) reaching a maximum by as soon as 23 days. This evolution also differed from that of ob mRNA in being not influenced by the diet composition (Figures 3 and 4B).

Unlike ob and PPARγ2, FAS mRNA was almost undetectable during suckling. Yet, as previously described [8], FAS expression increased sharply after weaning to a HC diet, and this rise was strongly attenuated in HF rats (Figure 3).

In line with previous data [6], PEPCK mRNA did not significantly rise during the last week of the suckling period. After weaning to a HC diet, PEPCK expression fell to undetectable levels in 23-day-old pups (concomitantly with the rise in insulinemia) and re-increased slightly thereafter to pre-weaning values. By contrast, PEPCK expression was stimulated by weaning to a HF diet (3.5-fold in 30- versus 21-day-old pups), and the levels reached at each time point of this period were higher than the corresponding values in HC rats ($P < 0.05$ or less in epididymal or inguinal WAT) (Figure 3).

β3-AR mRNA increased during the suckling-weaning transition. A transient decrease of β3-AR expression occurred after weaning to an HC diet ($P < 0.05$; Figures 3 and 4C), but there was no significant difference in β3-AR mRNA concentrations between HC and HF groups from day 25 onwards (Figures 3 and 4C).

**DISCUSSION**

The present study shows that ob mRNA levels markedly increase during the suckling–weaning transition, and that this effect is accentuated by an HF diet. ob gene expression is thus submitted to both developmental and nutritional regulatory mechanisms.

In control rats, a large increase in ob gene expression was observed during the last week of suckling. ob expression kept increasing after weaning, but at a lower rate. Later in life, ob mRNA has been reported to increase slowly with the size of epididymal WAT [15,19]. These observations apparently fit with the lipostat theory which suggests that leptin serves as a signal reflecting the state of energy stores and is produced in proportion to total body-fat mass, cell size or number (reviewed in [20]). ob mRNA increased 6.7-fold during the last week of suckling, whereas adipocyte number changed little and cell size enlarged 3-fold. This supports the hypothesis that ob expression is stimulated in response to hypertrophy rather than hyperplasia [21]. However, after weaning to a standard diet, adipocyte size similarly increased (2.6-fold) between 21 and 30 days, and ob mRNA only...
doubled. Yet, as previously suggested [21], filling of small adipocytes could have a greater influence on ob expression than filling of larger adipocytes. Changes in fat-cell size and/or number could thus be involved in developmental changes in ob expression. On the other hand, ob expression was higher in HF than in HC rats, although there were no differences in epididymal and inguinal fat-pad weights, and in fat cell size and number. Thus, in agreement with our and other previous findings [9,22,23], the present results indicate that there is no tight relationship between ob expression and adipocyte size and number during certain acute phases of metabolic changes.

Our observations also show that ob expression is regulated not only by quantitative (i.e. fasting/refeeding) [9,16,17] but also by qualitative alterations in diet. The greater ob expression under an HF diet is consonant with that observed in FBV mice fed a so-called ‘western diet’ [24]. This previous study did not clearly establish, however, the exact contribution of increased dietary fat on ob expression because of several confounding factors [24,25]. First, the western diet was enriched not only with fat (41% of calories) but also with sucrose (30% of calories), and it was not possible to determine which component was responsible for ob overexpression. Second, obesity develops in FBV mice chronically offered the western diet (as it may occur in some susceptible inbred strains of rodents), and overt obesity itself (genetic or acquired forms) often results in increased ob mRNA and protein levels [22–24]. Our study unambiguously shows that an HF diet may specifically and rapidly upregulate ob gene expression, independently of any increase in body weight.

The mechanisms underlying the nutritional and developmental changes of ob expression during the suckling–weaning transition remain unknown. The possible role of changes in adipose-tissue mass or cell size/number has already been discussed. Several other factors may be involved. First, glucocorticoids increase ob gene expression in vivo [26] and in most [27,28], though not all [29], in vitro studies. The increase in free plasma corticosterone between days 12–14 and day 19 [6] is likely to stimulate ob expression during the last week of suckling. Whether the subtle increase in corticosterone levels or responsiveness reported under an HF diet [30,31] suffices to explain the post-weaning difference between the two diets remains unsettled. Secondly, several lines of evidence suggest that insulin stimulates ob gene expression in vivo. ob mRNA and protein levels were decreased by fasting and re-induced by refeeding, concomitantly with the changes in insulinemia [9,16,17,22]. Moreover, high insulin levels during euglycaemic–hyperinsulinaemic clamps up-regulated ob gene expression [14,17,32]. In the present study, the rise in insulin occurring within the first 2 days of weaning to an HC diet was accompanied by a sharp increase of FAS mRNA levels and a decrease of PEPC mRNA levels, as expected from their known regulation and/or potentiation by insulin [33,34]. The concomitant increase in ob expression was only marginal (11%) and smaller than in HF-weaned rats, whose diet prevented the rise in insulinemia. Thus the observed changes in ob gene expression are probably not mediated by insulin. Thirdly, besides hormones, nutrients themselves could be involved in ob gene regulation. Hyperglycaemic-clamp studies have shown that glucose does not, by itself, modulate ob expression [17]. However, NEFA are stimulatory in vitro (B. Reul and S. M. Brichard, unpublished work). It is thus possible that, together with glucocorticoids, they contribute to the higher ob expression in HF-weaned rats and to the rise in ob mRNA during the last week of suckling.

Recent studies have shown that leptin secretion is regulated at pre-translational levels [22,23]. The changes in plasma leptin should thus vary in parallel with those in ob mRNA. If this assumption is correct, plasma leptin levels were probably higher in HF- than in HC-weaned rats. One may therefore wonder whether, besides lesser palatability and rewarding effect of the diet, high leptin levels could contribute to the slight decrease in food intake and body-weight gain of these HF rats. However, when two subgroups of HC- and HF-weaned rats (n = 7 for each) were matched for daily food intake and body weight, ob gene expression remained elevated in the latter. Thus, in a substantial subgroup of HF rats, food intake was not decreased in spite of presumably high leptin levels. This would suggest, at variance with the hypothesis expressed above, that sensitivity to leptin may actually be reduced in some HF pups, as reported for mice fed the western diet and for obese rodents or humans in general [15,23,24]. This would also imply that fat-induced resistance to leptin may precede overt obesity or occur independently of it. Since insulin resistance is a common characteristic of obese subjects [35] and HF pups [36], it is possible that resistance to leptin and insulin develop concomitantly.

In vitro studies with adipocyte-cell lines indicate that PPARγ2 may play a key role in the development of the adipose lineage, and that its transcriptional activation may be potentiated by a variety of lipids or lipid-like compounds [4], including naturally occurring polyunsaturated fatty acids. The developmental and nutritional regulation of the PPARγ2 gene has not yet been characterized in vivo. Our study clearly shows that PPARγ2 mRNA increased during suckling, but rapidly reached a plateau after weaning and no longer changed thereafter. In particular, PPARγ2 expression was not influenced by the diet composition. The receptor, therefore, appears to be submitted to developmental, but not to qualitative nutritional regulatory mechanisms.

PPARγ2 is an adipocyte-specific, nuclear transcription factor that is activated by endogenous ligands, among which are arachidonate metabolites of the prostaglandin J2 group [37]. It has been proposed to regulate the expression of the ob gene [38]. However, we show here that the expression of PPARγ2 differed from that of ob in reaching a maximum by as soon as 2 days after weaning. Moreover, it remains to be determined how the ligands of PPARγ2 change during the suckling–weaning transition.

Several lines of evidence indicate that the β2-AR may play a pivotal role in obesity.β2-specific agonists have potent anti-obesity and anti-diabetic effects in both animals and humans; expression of the receptor is impaired in rodent models of obesity, and a missense mutation in the gene may lead to visceral obesity in man (reviewed in [39,40]). Moreover, recent in vivo and in vitro studies have proposed that the sympathetic system and β2-agonists may modulate ob expression [28,41], though this point is still controversial [18]. Our study demonstrates a developmental rise in β2-AR mRNA during the suckling–weaning transition. The most striking nutritional effect was produced by the HC diet and consisted in a very transient (day 23) decrease of β2-AR mRNA. It is possible that this inhibition results from the concomitant rise in insulinemia, since insulin rapidly lowered β2-AR mRNA and protein levels in 3T3-F442A adipocytes [41].

In conclusion, we have shown that ob expression markedly increases during the suckling–weaning transition, and that this effect is accentuated by an HF diet. PPARγ2 also increased during suckling, but rapidly reached a plateau after weaning and no longer changed thereafter. Unlike ob, PPARγ2 was not influenced by the diet composition.

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REFERENCES