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# Time-Dependent Effects of a High-Energy-Yielding Diet on the Regulation of Specific White Adipose Tissue Genes

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**White adipose tissue development is regulated by many factors, including the energy content of food and the genetic background. Nevertheless, little is known about possible differential effects of high-fat palatable diets when fed for short or long-time periods. Thus, the expression of certain genes involved with lipid metabolism (peroxisome proliferator-activated receptor gamma, PPAR$\gamma$2; retinoic receptors; fatty acid binding protein, aP2 and uncoupling proteins, UCP) may be affected by those dietary manipulations (high-energy-yielding diet and time duration of feeding). High-fat feeding for 8 days decreased mRNA UCP3 levels compared to control fed animals, while feeding for 30 days increased them over controls. Similar findings occurred for PPAR$\gamma$2 and aP2. Furthermore, statistically significant associations were found among PPAR$\gamma$2, aP2 and UCP3 mRNA levels. These data suggest a physiological time-dependent response seeking to prevent excessive fat deposition when animals are fed for short-term with a high amount of dietary fat, which was followed by an adaptive period to the high-energy content of diet throughout a coregulation among certain lipid metabolism related genes: PPAR$\gamma$2, aP2, UCP3.** 

*Key Words:* **white adipose tissue; high-energy diet; thermogenesis; peroxisome proliferator-activated receptor $\gamma$ (PPAR); fatty acid binding protein (aP2); uncoupling proteins (UCP).**

Obesity is defined as a disorder in energy balance which is accompanied by an increase in adipose tissue mass (1). The management of excess fat deposition has been fraught with disappointment, in part, because the mechanisms regulating fuel homeostasis and adiposity are incompletely understood (2). In this context, several studies have been designed to investigate the effects of macronutrients or the nutritional status on gene expression in white adipose tissue (WAT), as the major energy storing tissue as well as in other tissues (3–5).

The growth of adipose tissue is known to be mediated by chronological changes in expression patterns of a number of fat tissue related genes affecting phenotype acquisition, proliferation and hypertrophy (6, 7). In addition to this age dependent changes, cell number and size could be augmented by high-carbohydrate or high-fat diets, which may, in turn, depend on gene predisposition (8, 9).

The nuclear hormone receptor peroxisome proliferator-activated receptor gamma 2 (PPAR$\gamma$2) gene is the first identified adipocyte specific transcription factor that appears to promote adipocyte differentiation and to control the expression of several fat-specific genes (10). Other adipose tissue-specific products such as the fatty acid binding protein (aP2), are known to be mature adipocyte specific genes regulated by PPAR$\gamma$ (6).

Uncoupling proteins (UCPs) are integral membrane proteins of the mitochondrial inner membrane that appear to function as proton channels or shuttles by decreasing the production of ATP and dissipating energy as heat (11). In this sense, the description of several uncoupling proteins, such as UCP1, 2 and 3, in adipose tissue (11–13) provides new molecular targets to understand adipocyte metabolism (14).

The factors controlling the expression of these proteins as well as the influence of macronutrient intake on respective gene expression are poorly understood. In this context, an experimental trial was devised in order to determine the impact of two nutritional influences (fat intake and length of dietary exposure) on WAT development and gene expression depending on the intake period (8 days vs 30 days). Thus, the aim of this work was to elucidate whether a high-energy-yielding diet induces a differential chronological regulation on the expression of specific white adipose tissue genes.

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## MATERIALS AND METHODS

*Animals and diets.* Thirty-four female Wistar rats (5 weeks old), supplied by the Applied Pharmacobiology Centre (CIFA-Pamplona, Navarra, Spain), weighing approximately 150 g, were housed at 25 ± 1°C with a 12-h light cycle (08:00 to 20:00 h) and assigned into four groups according to a 2 × 2 factorial design. Two groups of rats were fed a standard chow diet (Rodent Toxicologic Diet, B&K Universal), containing 160 g of protein, 710 g of carbohydrates and 30 g of lipid per kg of diet (15204 kj/kg), for 8 days (named Short Period Control group or SPC, with $n = 8$) or 30 days (named Long Period Control group or LPC, with $n = 10$). Another two groups were fed on a fat-rich high-energy diet, whose components were paté, bacon, chips, cookies, chocolate and chow with proportions 2:1:1:1:1:1, which was given to each rat per day as previously described (15, 16). The macronutrient composition of the high-fat diet was 103 g of protein, 357 g of carbohydrates and 330 g of lipids, per kg of diet (19614 kj/kg) of diet. One of such groups was offered this diet for 8 days (named Short Period High-Fat Diet or SPHF, with $n = 8$) and the other for 30 days (named Long Period High-Fat Diet or LPHF, with $n = 8$). All animals had free access to water and food during experiment. Following either 8 or 30 days, rats were food-deprived for 12 h and were euthanized and the trunk blood was collected. Abdominal adipose tissues were immediately excised, weighed, frozen in liquid nitrogen and stored at −80°C until analysis. All experimental procedures were performed according to national and institutional guidelines for animal care and use at the University of Navarra.

*Serum measurements.* Serum insulin was measured with a commercially available RIA kit (Rat Insulin [$^{125}$I] assay system, Amersham, Buckinghamshire, UK) as indicated by the supplier. Serum leptin was determined with a correlate-EIA kit (rat leptin Enzyme Immunometric Assay Kit, Assay Designs, Inc.) following standard procedures. Glucose was measured with the commercial kit Unimate 7 PAP (Roche, Basilea, Switzerland) and free fatty acids by the NEFA C ACS-ACOD Method (Waco Chemicals USA, Inc., Richmond, VA). The glucose and fatty acid kits were adapted for a COBAS MIRA (Roche, Basilea, Switzerland) autoanalyzer.

*Extraction of total RNA and semiquantitation by reverse transcription-polymerase chain reaction (RT-PCR).* Total RNA was isolated from 150 mg of fat tissue by the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX). After 30 min treatment with 10 units of RNase-free DNase I (Boehringer Mannheim, Barcelona, Spain), 1.5 μg of RNA were used to synthesize first-strand complementary DNA (c-DNA). The RT reaction was carried out in a volume of 30 μl containing 50 mmol/L Tris HCl (pH 8.3), 75 mmol/L KCl, 3 mM $MgCl_2$, 10 mmol/L dithiothreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mmol/L each DTP (Bioline, London, UK), 20 units of RNAse inhibitor (Promega, Madison, WI), 200 units of M-MLV RT (Gibco BRL, Life Technologies, Gaithersburg, MD) and incubated at 37°C for 60 min. The enzyme was inactivated by heating at 95°C for 5 min. Four microliters from the RT reaction were amplified in a 50 μl reaction mixture containing 40 ng of each primer, 16 mmol/L $(NH_4)_2SO_4$, 67 mmol/L Tris HCl (pH 8.8), 2 mmol/L $MgCl_2$, 0.1% Tween 20, 0.2 mmol/L each dNTP and 1 unit of BioTaq polymerase (Bioline). Primers used to amplify UCP3 cDNA (GenBank U92069) were 5′-GGAACTGG-AGGCGAGAGGAA-3′ (sense, 577–596) and 5′-TTTG-TAGAAGGCTGTGGGGC-3′ (antisense, 926–945); PPARγ2 (GenBank Y12882) 5′-TCTGATTATGGGTGAAACTC-3′ (sense 43–62) and 5′-TTTCTACTCTTTTTGTGGATC-3′ (antisense 592–612); aP2 (GenBank K02109) 5′-AACACCGAGATTTCCTTCAA-3′ (sense 178–197) and 5′-TCACGCCTTTCATAACACAT-3′ (antisense 351–370); β-actin (GenBank J00691) 5′-TCTACAATGAGCTGCGTGTG-3′ (sense 1599–1618) and 5′-GGTCAGGATCTTCATGAGGT-3′ (antisense, 2357–2376). Primers for all genes were designed using the Oligo 4.05 Primer Analysis Software (National Bioscience, Inc., Plymouth, MN). cDNA was amplified using the parameters shown in Table 1.

**TABLE 1**

Conditions Used to Amplify cDNA

| | Annealing (s) | Elongation (s) | No. cycles |
|---|---|---|---|
| UCP3 | 55°C, 45 s | 72°C, 60 s | 35 |
| PPARγ2 | 53°C, 35 s | 72°C, 50 s | 44 |
| aP2 | 60°C, 30 s | 72°C, 30 s | 29 |
| β-actin | 59°C, 30 s | 72°C, 30 s | 27 |

For all primers, a first step of denaturation was applied (95° for 30 s) along with a final extension step for 7 min at 72°C.

To ensure the linearity of PCR reactions and to validate the cDNA quantitation, adequate controls and standard curves were carried out. A linear increase in PCR product was observed when using RNA ranging between 50 and 400 ng, all subsequent PCR reactions were performed using 200 ng of RNA. A second set of PCR reactions were design to determine the appropriate number of cycles, as previously reported (17, 18). Semiquantitative estimates the relative amount of target RNA to a known housekeeping gene (β-actin) and eliminates the sample to sample variability of the RT-step, as well as the PCR step. Amplifications were linear under conditions showed in Table 1 and were carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Levels of messenger RNA (mRNA) were measured as the ratio of signal intensity for each gene relative to β-actin. PCR band intensities were determined by densitometric analysis with the Gel Doc 1000 ultraviolet (UV) fluorescent gel documentation system and Molecular Analyst 1.4.1 software for quantitation of images (Bio-Rad, Hercules, CA).

*Statistical analysis.* All results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using two-tailed unpaired *t* test. The Pearson correlation coefficient was computed to analyze correlation between two variables. The calculations were performed using the SPSS/Windows version 7.5 (SPSS Inc., Chicago) statistical package. A *P* value lower than 0.05 was considered to be statistically significant.

## RESULTS

*Weight and blood measurements.* High-fat fed rats (SPHF and LPHF) had significantly ($P < 0.001$) greater body weight and abdominal fat content than the respective control fed animals. Furthermore, high-fat feeding significantly enhanced ($P < 0.001$) serum glucose and serum leptin levels. On the other hand, serum-free fatty acids (FFA) levels were significantly reduced ($P < 0.005$) by the high-fat intake, while serum insulin remained unchanged.

Serum glucose was not modified by the length of feeding (Table 2).

*Gene expression measurements.* The UCP3 mRNA levels in LPC group were significantly reduced ($P = 0.002$) with respect to their littermates of LPHF (Fig. 1), while SPC animals showed higher UCP3 levels than high-fat fed animals over the same experimental time period, although they did not reach statistical significance (Fig. 2). Similar trends to those concerning UCP3, were observed for PPARγ2 mRNA levels, but in this case, short time period high-fat fed animals showed significantly reduced ($P = 0.01$) PPARγ2

**TABLE 2**

Body Weight and Serum Measurements in Four Experimental Groups

| | SPC | SPHF | LPC | LPHF |
|---|---|---|---|---|
| Final body weight (g) | 196.3 ± 9.0 | 223.3 ± 4.9** | 267.2 ± 4.7 | 307.3 ± 6.8** |
| Fat weight (g) | 3.7 ± 0.5 | 8.1 ± 0.8** | 7.4 ± 1.7 | 20.0 ± 5.8** |
| Serum FFAA (mmol/l) | 1.2 ± 0.04 | 1.0 ± 0.05** | 0.9 ± 0.1 | 0.7 ± 0.04** |
| Serum glucose (mg/dl) | 76.0 ± 4.3 | 95.4 ± 4.0** | 78.5 ± 1.6 | 87.2 ± 6.2** |
| Serum insulin (pmol/l) | 0.6 ± 0.1 | 0.4 ± 0.06 | 1.0 ± 0.1 | 1.16 ± 0.2 |
| Serum leptin (ng/ml) | 28.1 ± 8.6 | 60.0 ± 7.2** | 54.7 ± 1.9 | 119.7 ± 9.2** |

*Note.* Data are means ± SEM. A two-tailed unpaired *t* test was used to high-fat-fed groups with respective controls: SPC, short period control fed; SPHF, short period high-fat fed; LPC, long period control and LPHF, long period high-fat fed. Statistical significance of main effects, time, and diet, and interaction between them is provided.

**Significant differences ($P < 0.01$) following a two-tailed unpaired *t* test comparison of single groups.

mRNA levels compared to control fed animals over the same experimental time period (Fig. 2). The analysis of aP2 mRNA revealed the same expression pattern as UCP3 and PPARγ2 in experimental groups (Figs. 1 and 2) although only the values of aP2 mRNA in the SPHF fed group reached statistical significance ($P = 0.001$) when compared with SPC fed group (Fig. 2).

In this sense, statistically significant associations between UCP3 and PPARγ2 ($r = 0.65$; $P = 0.01$), aP2 and PPARγ2 ($r = 0.40$; $P = 0.05$), as well as for UCP3 and aP2 ($r = 0.73$; $P = 0.001$) were observed when the four groups were analyzed together (Figs. 3a, 3b, and 3c, respectively).

## DISCUSSION

Adipose tissue development is regulated by many factors (19), including the availability of food (energy). In this context, understanding the mechanisms that control white adipose tissue growth, development and gene expression under different dietary conditions may provide novel insight into body weight and fat stores regulation as well as the etiology of obesity (20). Some genes, such as PPARγ2, appear to play an important role in activating the adipocyte differentiation program and in the regulation of mature WAT genes (10, 21). Also, uncoupling proteins and aP2 have been studied to evaluate the potential influence of nutrients on adaptive thermogenesis and to elucidate whether dietary manipulations directly affect the gene expression pattern of adipocytes (9, 19, 22).

High-fat feeding was used to generate diet induced obesity as previously described (23, 24). Rats eating such a diet, increased energy intake, body weight and adiposity, which is in good accordance with previous data (25). While it is difficult to assess the composition of the intake, this dietary model has been repeatedly used to induce weight gain in order to evaluate the influence of high-fat and high-energy intake on lipid deposition and to extrapolate the mechanisms to obese humans [Berraondo, 1997 #51; Berraondo, 1999 #52]. In regard to serum leptin levels, the statistically significant interaction between the duration of feeding and high-fat diet suggests that both factors are involved in leptin regulation. This finding is in good accordance with reports that correlate adiposity with hyperleptinemia (26, 27), since leptin, as an adiposity

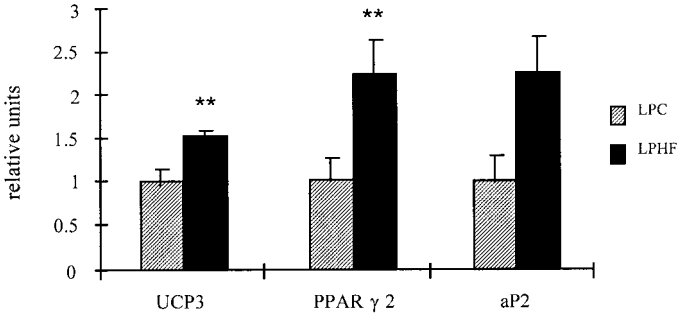


**FIG. 1.** UCP3, PPARγ2, and aP2 mRNA expression levels in white adipose tissue of long period control fed rats (LPC; ▨) and long period high-fat-fed rats (LPHF; ■). Data are means ± SEM of the ratio between each gene and β-actin. Expression of each gene were normalized to their respective control. **Significant differences ($P < 0.01$) following a two-tailed unpaired *t* test comparison of single groups.

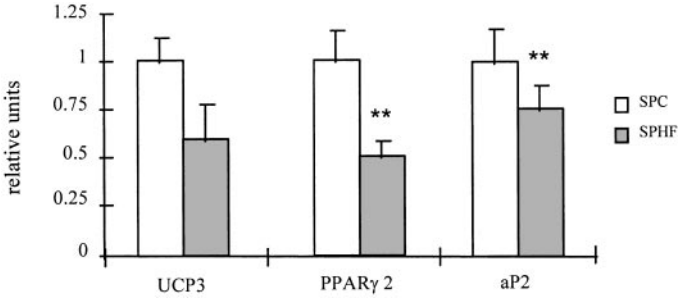


**FIG. 2.** UCP3, PPARγ2, and aP2 mRNA expression levels in white adipose tissue of short period control fed rats (SPC; □), short period high-fat-fed rats (SPHF; ▩). Data are means ± SEM of the ratio between each gene and βactin. Expression of each gene were normalized to their respective control. **Significant differences ($P < 0.01$) following a two-tailed unpaired *t* test comparison of single groups.

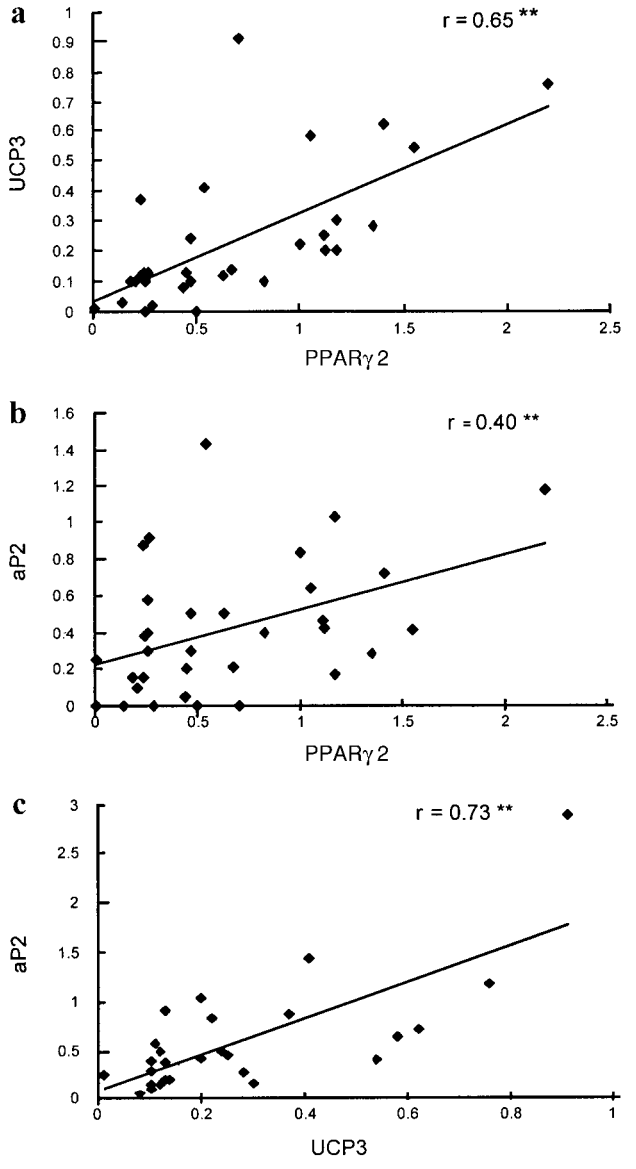


**FIG. 3.** Positive significant correlations between (a) PPARγ2 and UCP3, (b) PPARγ2 and aP2, and (c) UCP3 and aP2.

signal, is elevated as fat deposition is increased over the time in response to dietary fat manipulations (28).

To study gene expression differences, RT-PCR technique was employed to quantify mRNA expression levels of each gene in adipocytes. Data obtained by using this method are comparable to other methodologies such as Northern blot, but RT-PCR was used to measure changes on mRNA expression because of its specificity, simplicity and sensitivity to quick changes in cell machinery. Furthermore, this technology requires low levels of RNA and it gives the possibility to detect low expression genes without the utilization of radioactivity (29). In addition, to quantify mRNA expression many other articles published in highly rated journals have accepted this methodology (30–34). In this context, marked differences in gene expression were observed between dietary groups when duration of feeding was tested, suggesting a time-dependent regulation in the transcription of some WAT genes.

As already published (35), high-fat feeding for short-time periods significantly reduced UCP3 mRNA expression but, in contrast, a longer period of high-fat feeding raised UCP3 expression over the control diet. These findings evidence that UCP3 expression did not follow a similar trend as leptin, suggesting the involvement of different regulatory mechanisms. In this sense, a high level of free fatty acids (FFA) has been considered as the main factor involved in muscle UCP3 regulation (36). In this trial no association between FFA and white adipose tissue UCP3 was found, which suggests that factors and mechanisms underlying UCP3 regulation may vary among tissues; nevertheless, fatty acid implication in UCP3 regulation should not be underestimated.

Analyses of PPARγ2 mRNA showed a relationship with macronutrient intake, but in a time dependent manner. Similar expression patterns were observed with aP2 mRNA expression in each group (Fig. 1). These results may suggest different and time-dependent responses to dietary fat excess (3), since short and long period groups showed opposite gene expression pattern. Furthermore, information obtained from mRNA levels of PPARγ2 and aP2, confirms a PPARγ2 mediation in the time dependent regulation of aP2 by nutrients (37). This differential behavior to dietary manipulations over time may be considered as a physiological adaptation of WAT to dietary fat excess. That is, sudden and short periods of dietary changes (fat elevation) down-regulate PPARγ2 levels, perhaps to avoid an excess of new adipocyte appearance (38), while the maintenance of high-fat feeding over longer time periods modifies WAT, possibly via PPARγ2-induced adipocyte differentiation (39).

Furthermore, PPARγ2 was investigated as a possible transcription factor involved in white adipose tissue UCP3 regulation (40, 41), since a functional peroxisome proliferator responsive element (PPRE) has been identified in the promoter sequence of the UCP3 gene (42) and because FFA are known to be natural PPARγ2 agonists. In this sense, a very significant correlation between PPARγ2 and UCP3 mRNA expression was detected when all experimental groups were analyzed together. In addition, because aP2 facilitates intracellular mobilization of FFA (43, 44), a possible association among PPARγ2, UCP3 and aP2 in white adipose tissue may be suggested. This relationship was supported by the significant correlations found between PPARγ2, UCP3 and aP2, which is known to be modulated by PPARγ2 (37, 45). In this sense, a FFA implication in WAT UCP3 regulation throughout PPARγ2 and aP2 might be suggested, since the three genes (aP2, UCP3 and PPARγ2) are involved on lipid metab-

olism (transport, oxidation, and adipocyte differentiation, respectively) and because they were significantly correlated among each other (Figs. 3a, 3b, and 3c).

In summary, it can be concluded that feeding a high-energy-yielding diet induces an increase in body weight and adipose tissue growth, affecting WAT gene expression in a time-dependent fashion. Hence, both dietary macronutrient composition (fat content) and feeding duration (time) influenced adipose tissue metabolism following a dual pattern: an initial protective role and a further adaptation to fat deposition, which appears to be achieved via transcription factors such as PPAR$\gamma$2. Moreover, the correlation observed between PPAR$\gamma$2 and UCP3, suggests a direct role for PPAR$\gamma$2 in the regulation of UCP3.

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