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Changes in UCP mRNA expression levels in brown adipose tissue and skeletal muscle after feeding a high-energy diet and relationships with leptin, glucose and PPARγ

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Abstract

Brown adipose tissue and skeletal muscle are known to be important sites for nonshivering thermogenesis. In this context, it is accepted that uncoupling proteins (UCPs) are involved in such process, but little is known about the physiological regulation of these proteins as affected by the intake of a high-energy (cafeteria) diet inducing fat deposition. In this study, the UCP messenger RNA (mRNA) expression in interscapular brown adipose tissue (iBAT) and skeletal muscle was assessed to evaluate the influence of a dietary manipulation on energy homeostasis regulation. We report a statistically significant increase in mRNA levels of iBAT UCP1 and UCP3 and a statistical marginal rise in skeletal muscle UCP3 mRNA expression after feeding a high-energy diet, whereas no changes in UCP2 expression were found in either tissue. Furthermore, significant positive associations between iBAT UCP1 and UCP3 mRNA levels with serum leptin were found. Although the expression of the β_3 adrenoceptor (β_3AR) was about 50% in the lean controls compared with the obese group in iBAT, no statistically significant changes were observed concerning peroxisome proliferator-activated receptor γ2 (PPARγ2) mRNA levels in muscle or iBAT. We conclude that feeding a diet inducing weight and fat gain produces different outcomes on iBAT and skeletal muscle UCP mRNA expression, revealing a tissue-dependent response for the three UCPs. Results suggest that the regulation of UCP expression in both tissues under these specific dietary conditions may be related to leptin circulating levels.

Keywords: Uncoupling proteins; β_3-Adrenergic receptor; Peroxisome proliferator-activated receptor γ2; High-energy diet; Skeletal muscle; Brown adipose tissue

1. Introduction

Obesity is a disorder of the energy balance in which energy intake is greater than energy expenditure [1]. The second factor of the energy equation involves the maintenance of the basal metabolic rate, physical activity, growth and thermogenesis. In this context, diet-induced thermogenesis (DIT) is a metabolic pathway to burn/oxidize the excess of energy ingested to match energy intake and needs in order to prevent excessive weight gain [2].

Brown adipose tissue (BAT) is an important site of facultative (nonshivering) thermogenesis [3], which is acutely induced by thyroid hormone administration [4], sympathetic nervous system (SNS) stimulation [5] and cold, as well as by food intake [6]. Skeletal muscle is another important site for energy expenditure, but muscle thermogenesis seems not to be modulated by the SNS in response to diet or cold [2], as occurs with BAT. In this sense, the discovery of new molecular mechanisms underlying thermogenesis such as the uncoupling proteins (UCPs) in the adipose tissue and skeletal muscle may explain the increasing interest in understanding the role of both tissues in energy homeostasis.

Mitochondrial UCPs (UCP1, UCP2 and UCP3) appear to be involved in heat loss by generating a dissipation of the proton electrochemical gradient across the inner-mitochondrial membrane [7]. Brown fat seems to be the only tissue in which the three UCP genes are coexpressed. UCP2 is widely distributed in different tissues such as white adipose tissue (WAT), BAT, skeletal muscle, liver and spleen, and UCP3 is mainly expressed in skeletal muscle, although it is also expressed in WAT and BAT [8]. Gene regulation of these UCPs is quite different and apparently tissue dependent. For instance, the UCP2 and UCP3 mRNA expression

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closely follows that of UCP1 in interscapular brown adipose tissue (iBAT) because sympathetic stimulation [9], either induced by β_3-agonists [10] or cold [11,12], and thyroid hormone administration [11] up-regulates the three UCPs. On the other hand, fasting, free fatty acids, exercise and thyroid hormone participate in the up-regulation of UCP2 and specially UCP3 in skeletal muscle [13,14]. Nevertheless, these UCPs seem not to be affected by cold or sympathetic stimulation in the muscle [2,11,15]. Furthermore, leptin and glucose have been hypothesized as possible mediators of the dietary effects on UCP mRNA expression in both tissues [16,17].

On the other hand, the β_3-adrenergic receptors (β_3AR), which are expressed predominantly in adipocytes, are known to play a significant role in adipocyte metabolism by promoting lipolysis and inducing UCP expression. In this sense, one important physiological regulator of β_3AR mRNA expression in BAT is SNS stimulation. Thus, sympathetic activation by cold exposure dramatically down-regulates β_3AR mRNA expression [18], whereas SNS inhibition by surgical denervation up-regulates β_3AR mRNA levels in BAT [18,19]. Thus, this study investigated the eventual mediation of the adrenergic system after a period of feeding a high-energy diet in the BAT UCP regulation by measuring the β_3AR mRNA expression levels.

Other potential regulators affecting UCP expression appear to be the peroxisome proliferator-activated receptors (PPARs) [3]. These transcription factors belong to a nuclear receptor superfamily that is involved in the adipocyte differentiation process by controlling the expression of several genes such as UCP mRNA expression [14,20]. In this sense, some studies have linked UCP mRNA expression with such receptors in adipose tissues and/or skeletal muscle on different strains of mice or during treatments with PPAR agonists (thiazolidinediones) [3,21–23]. Nevertheless, little is known about the regulation of UCP by PPARs, especially PPARγ2, under different nutritional conditions such as high-energy diet intake.

The main objective of this work was to study the influence of a dietary manipulation (cafeteria diet intake) increasing fat deposition (overweightness) on UCP mRNA expression levels in BAT and skeletal muscle. In addition, plasma glucose, leptin and β_3AR gene expression were assessed. Finally, PPARγ was searched as a potential transcription factor involved in the regulation of UCP mRNA expression after an experimentally induced high-energy intake.

2. Methods and materials

2.1. Animals and diets

Female Wistar rats, supplied by the Applied Pharmacobiology Center (CIFA-Spain), weighing about 150 g, were housed at 22 ± 1°C with a 12-hour light cycle (8:00 AM to 8:00 PM) and assigned to two dietary categories. One group of animals was fed on a standard 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 (15,204 kj/kg) for 8 days (control group, $n = 8$). To obtain a diet-induced obesity model, another group was fed a fat-rich high-energy diet (cafeteria diet), whose components were paté, bacon, chips, cookies, chocolate and chow with proportions 2:1:1:1:1:1. This experimental diet is nutritionally balanced and was offered to induce overweightness, which is widely accepted despite the differences in the nutrient content [13,24,25]. The macronutrient composition of the cafeteria diet was 103 g of protein, 357 g of carbohydrates and 330 g of lipids, which provided 19,614 kjoules per kg of diet. This group was offered this diet for 8 days (high-energy diet, $n = 8$). All animals had ad libitum access to water and food during the experimental trial. After 8 days of feeding, animals were fasted for 12 h, killed by decapitation and the trunk blood was collected. iBAT and skeletal muscle from the leg (gastrocnemius muscle) 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, Pamplona, Navarra, Spain.

2.2. Serum measurements

Serum leptin was determined with a correlate-EIA kit (rat leptin Enzyme Immunometric Assay Kit, Assay Designs, Inc.) following standard procedures. Free fatty acids were measured by the NEFA C ACS-ACOD method (Waco Chemicals USA, Inc., Richmond, VA, USA). Glycerol and triacylglycerol were determined with the RANDOX kit for glycerol and triacylglycerol diagnostic, respectively (Randox Laboratories LTD, Ardmore Road, UK) and glucose was measured with the commercial kit Unimate 7 PAP (Roche, Basilea, Switzerland). The glycerol, triacylglycerol, glucose and fatty acid kits were adapted for COBAS MIRA (Roche, Basilea, Switzerland) equipment.

2.3. Extraction of total RNA and semiquantitation by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by the Ultraspec-II RNA Isolation System (Biotecx Laboratories, Houston, TX, USA) from 100 mg of brown fat and skeletal muscle. 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 (cDNA). The RT reaction was carried out in a volume of 30 μl containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mM each DTP (Bioline, London, UK), 20 units of RNase inhibitor (Promega, Madison, USA), 200

Table 1
Weight-related measurements and serum determinations in control and high-energy-fed rats

	BAT			Muscle		
	Annealing	Elongation	No. cycles	Annealing	Elongation	No. cycles
UCP1	58°30″	72°30″	21			
UCP2	61°30″	72°30″	30	61°30″	72°30″	30
UCP3	61°30″	72°30″	33	63°30″	72°30″	33
PPARγ2	52°30″	72°40″	45	53°45″	72°50″	47
β-actin	59°30″	72°30″	27	59°30″	72°30″	27
β_3AR	58°30″	72°30″	30			

units of M-MLV RT (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and incubated at 37°C for 60 min. The enzyme was inactivated by heating at 95°C for 5 min. Four μl from the RT reaction were amplified in a 50-μl reaction mixture containing 40 ng of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl (pH 8.8), 2 mM $MgCl_2$, 0.1% Tween-20, 0.2 mM each dNTP and 1 unit of BioTaq polymerase (Bioline). Primers used to amplify UCP1 cDNA (GenBank M11814) were 5′-GTCTTAGGGACCATCACCA-3′ (sense, 351–369) and 5′-CCAGTGTAGCGGGGTTT-3′ (antisense, 629–646); UCP2 (GenBank AF039033) 5′-TAAAG CAGTTCTACACCAAGGG-3′ (sense, 308–329) and 5′-CGA AGGCAGAAGTGAAGTGG-3′ (antisense, 648–667); UCP3 (GenBank U92069) 5′-GGAACTGGAGGCGAGAGGAA-3′ (sense, 577–596) and 5′-TTTGTAGAAGGC-TGTGGGGC-3′ (antisense, 926–945); PPARγ2 (GenBank Y12882) 5′-TCT GATTA-TGGGTGAAACT-C-3′ (sense 43–62) and 5′-TTTC TACTCTTTTTGTGGATC-3′ (antisense 592–612); β_3AR (GenBank S56481) 5′-CTTCTACCTTCCCCTCCTT-3′ (sense, 638–656) and 5′-CTTCATAGCCATCAAAC-CTG-3′ (antisense, 1172–1191); β-actin (GenBank J00691) 5′-TCTACAAT GAGCTGCGGTG-3′ (sense 1599–1618) and 5′-GGTCAG GATCTTCATGAGGT-3′ (antisense, 2357–2376). Primers for all genes were designed using the Oligo 4.05 Primer Analysis Software (National Bioscience, Inc., Plymouth, MN, USA). cDNA was amplified using the parameters shown in Table 1.

A first step of denaturation was applied (95° for 30 s) and a final extension step for all primers at 72°C for 7 min in all cases. To ensure the linearity of PCR reactions and to validate the cDNA quantitation, adequate controls and standard curves were carried out by amplifications of 200 ng of first strand cDNA per reaction from 20 to 40 cycles, as previously reported [25]. Amplifications were linear under the conditions shown in Table 1 and were carried out in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). The amplified products were resolved in a 1.5% agarose gel with ethidium bromide. Levels of 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 fluorescent gel documentation system and Molecular Analyst 1.4.1 software for quantitation of images (Bio-Rad, Hercules, CA, USA). Furthermore, data obtained concerning UCP, PPARγ2 and β_3AR mRNA were comparable to values in the literature using RT-PCR and other methods (Northern blot) under different experimental treatments.

2.4. Statistical analysis

All results are expressed as means ± standard error of the mean (S.E.M.). Data were analyzed using the two-tailed unpaired *t* test or U Mann-Whitney in case of a nonparametric distribution. The Pearson correlation coefficient was computed to analyze correlations between two variables. The calculations were performed using the SPSS/Windows version 6.1.3 (SPSS Inc., Chicago, IL, USA) statistical package [24]. A *P* value lower than .05 was considered statistically significant.

3. Results

3.1. Diet-induced overweightness

Overweight was induced in rats by offering a palatable high-energy diet (Table 2). Outcome of feeding the high-energy diet was confirmed by the significant increase ($P < .01$) in body weight exhibited by the group of rats fed the high-energy diet with respect to the control group. Also, BAT weight was significantly higher in obese animals than in control rats (50% increase, $P < .01$), whereas no

Table 2
Weight-related measurements and serum determinations in control and high-energy-fed rats

	Control	High-energy
Weight increase (g)	24.4 ± 2.4	44.8 ± 4.6**
BAT weight (g)	0.1 ± 0.01	0.2 ± 0.02**
Muscle weight (g)	1.2 ± 0.05	1.2 ± 0.05
Serum glucose (mg/dl)	78 ± 4.3	95 ± 3.4*
Leptin (ng/ml)	29 ± 8.6	56 ± 7.2**
Fatty acids (meq/l)	1.3 ± 0.04	0.9 ± 0.05
Triacilglycerol (mg/dl)	112 ± 8.7	59 ± 7.8**
Glycerol (mmol/l)	362 ± 37.7	270 ± 30.3*

Data are means ± S.E.M. ($n = 8$ in all cases). All comparisons were performed by two-tailed unpaired *t* tests. Asterisks denote statistically significant differences (* $P < .05$, ** $P < .01$) between both experimental groups.

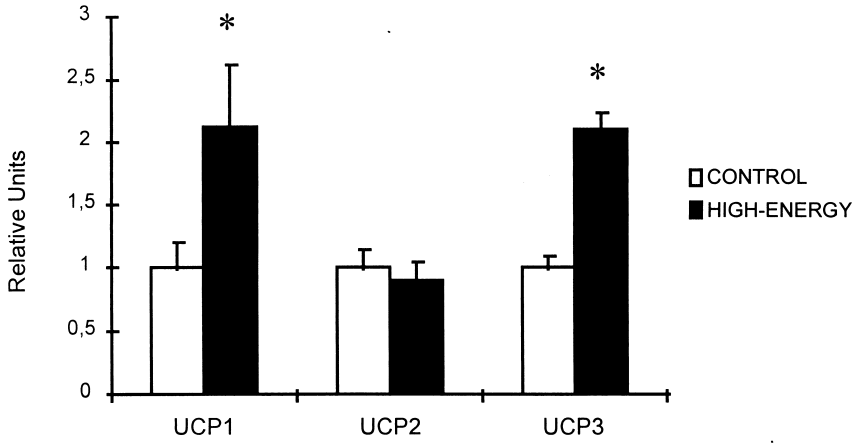

Fig. 1. UCP1, UCP2, UCP3 mRNA levels in iBAT in control and high-energy-fed rats. Data are means ± S.E.M. of the ratio between each gene and β-actin. Expression of each gene was related to the respective control. An asterisk indicates significant differences (*$P < .05$) between control and high-fat diet in each experimental group.

changes were observed in gastrocnemius muscle weights after the experimental feeding period.

3.2. Effects of high-energy intake on serum markers

Serum fatty acids were slightly, but not statistically, modified after the high-energy diet, whereas serum glucose was significantly ($P < .05$) increased in the obese. Furthermore, serum triacylglycerol and glycerol levels were significantly decreased ($P < .01$ and $P < .05$, respectively) in the obese group compared with control animals. On the other hand, serum leptin levels were significantly higher ($P < .01$) in the obese group than in the lean group.

3.3. Brown adipose mRNA levels and statistical associations with other determinations

UCP mRNA levels in BAT were differently affected by the intake of a high-energy diet (Fig. 1). Thus, UCP1 mRNA levels were significantly increased ($P < .05$) in the group fed the high-energy diet compared with the control group, as well as UCP3 mRNA levels ($P < .05$), whereas no changes were observed in UCP2 mRNA. The β_3AR mRNA expression levels were significantly increased ($P < .05$) in the group of animals eating the high-energy diet compared with the control group (Fig. 2a). On the other hand, the high-energy diet seemed to produce no effects on PPARγ2 mRNA expression levels in this tissue (Fig. 2b). Statistically significant associations were achieved between serum leptin with UCP1 ($r = .665$, $P < .01$) and UCP3 ($r = .496$, $P < .05$) in BAT (Fig. 4a and 4b, respectively).

3.4. Skeletal muscle mRNA levels and statistical associations with other variables

The possible effects of high-energy diet on the expression of some skeletal muscle genes such as PPARγ2, UCP2 and especially UCP3 were assessed (Fig. 3a and 3b). Opposite to BAT, no statistically significant differences depending on dietary patterns on PPARγ2, UCP2 and UCP3 mRNA expression levels were detected. Nevertheless, UCP3 mRNA levels were marginally increased ($P < .06$, one-tail Student's t test) in the obese group when compared with controls (Fig. 3a). Furthermore, PPARγ2 mRNA anal-

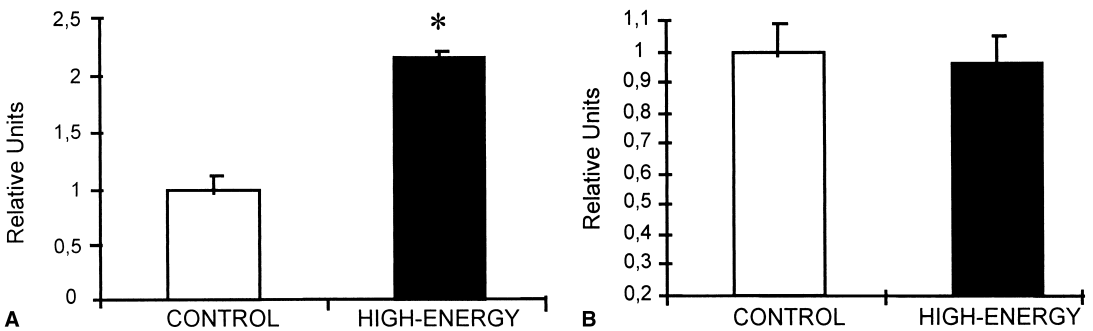

Fig. 2. (a) iBAT β_3AR mRNA levels and (b) PPARγ2 mRNA levels in control and high-energy fed rats. Data are means ± S.E.M. of the ratio between each gene and β-actin. Expression of each gene was related to the respective control. An asterisk indicates significant differences (*$P < .05$) between control and high-fat diet in each experimental group.

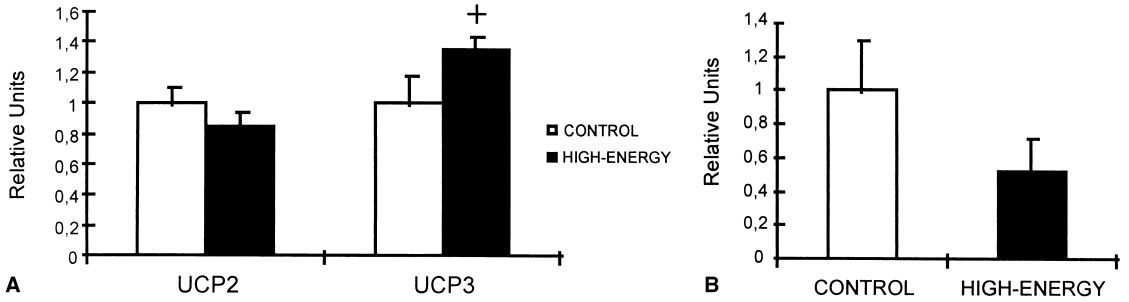

Fig. 3. (a) Skeletal muscle UCP2 and UCP3 mRNA levels and (b) PPARγ2 mRNA levels in control and high-energy fed rats. Data are means ± S.E.M. of the ratio between each gene and β-actin. Expression of each gene was related to the respective control. A plus indicates $P = .06$.

ysis showed no statistically significant differences after the period feeding the high-energy diet (Fig. 3b). When associations were analyzed in this tissue, a positive statistically significant relationship was obtained between muscle UCP3 and serum glucose ($r = .613$, $P < .05$), whereas a negative correlation for muscle UCP3 with PPARγ2 mRNA levels ($r = -.728$; $P < .01$) was found (Fig. 4c and 4d, respectively).

4. Discussion

High-energy diets are widely used and accepted in nutritional experiments as a good strategy to induce overweightness and fat deposition in animals [25,26]. Thus, the assayed period of high-energy feeding induced a marked weight increase in rats. The low levels of circulating fatty acid and other lipid metabolism markers may be due to a physiological response in order to store the fatty acid (energy) excess when animals are fed ad libitum on this experimental diet. Furthermore, this model of diet-induced obesity has been demonstrated to affect oxygen consumption in different tissues and the respiratory quotient [24].

In this context, despite the known specific effects of long-chain fatty acids on fatty acid oxidation and mitochondrial oxidative phosphorylation [27,28], it is well documented that high-energy diets may produce gene expression and composition changes in BAT [1,29]. Thus, in this dietary trial, high-energy diet intake significantly increased iBAT UCP1 and UCP3 mRNA levels as previously reported [11,30], which suggests that mechanisms controlling UCP1 and UCP3 expression may be similar. Also, several studies carried out to clarify UCP regulation under different experimental circumstances revealed that β_3-adrenergic agonists [10,31], leptin infusion [32,33], thyroid hormone administration [4,11] and cold exposure [11,32] induced a significant increase in UCP1 and UCP3 mRNA levels. The results of these studies suggested that the gene expression of both UCPs were similarly modified by those factors that occur after feeding a high-energy diet. In this context, it has been shown that UCP2 mRNA levels were increased after β_3-agonist treatment [10] and cold exposure [12,34]. However, in contrast to UCP1 and UCP3, the expression levels of UCP2 in iBAT were not modified after feeding a high-energy diet. As a whole, these data reveal that the mechanisms controlling UCP1 and UCP3, but not those of UCP2, may be influenced by high-energy-yielding diets, thus promoting DIT after high-energy feeding, which suggest that at least in iBAT, different mechanisms may be involved concerning UCP regulation.

The sympathetic stimulation through β_3AR is one of the most important mechanisms regulating iBAT UCPs [9]. Thus, β_3AR are involved in energy expenditure regulation by triggering lipolysis in BAT and WAT and thermogenesis in BAT [10,35]. In this sense, it has been reported that β_3AR mRNA in iBAT is negatively correlated with the SNS activity. Thus, high β_3AR mRNA levels are observed under low SNS activity [35,36], whereas stimulation of the SNS down-regulates β_3AR mRNA levels [19]. Our results suggest that other additional mechanisms apart from adrenergic stimulation should be involved in the up-regulation of UCP1 and UCP3, as already published [16], because the high β_3AR mRNA levels may indicate that a high-energy diet intake can decrease the SNS activation [35]. Other possible mechanisms that could be involved in iBAT UCP regulation in response to dietary manipulations may include the nuclear receptor PPARγ and the adipocyte hormone leptin [16,37].

The assessment of PPARγ2 mRNA expression in BAT as a possible molecular mediator in the up-regulation of both UCPs (UCP1 and UCP3) produced no significant differences between both groups. Although an overexpression of these nuclear receptors has been reported to induce UCP expression in BAT [3,37], high-energy feeding apparently did not promote such PPARγ2 overexpression and, therefore, no role for such a transcriptional factor in mediating thermogenesis in iBAT after high-energy feeding could be ascribed.

Another potential factor implicated in the regulation of UCP in iBAT is serum leptin, which is known to play a major role in energy homeostasis [38]. Increasing fat stores induced by high-energy diet intake produce an elevation of serum leptin levels, which suppresses food intake and increases energy dissipation, probably by promoting UCP

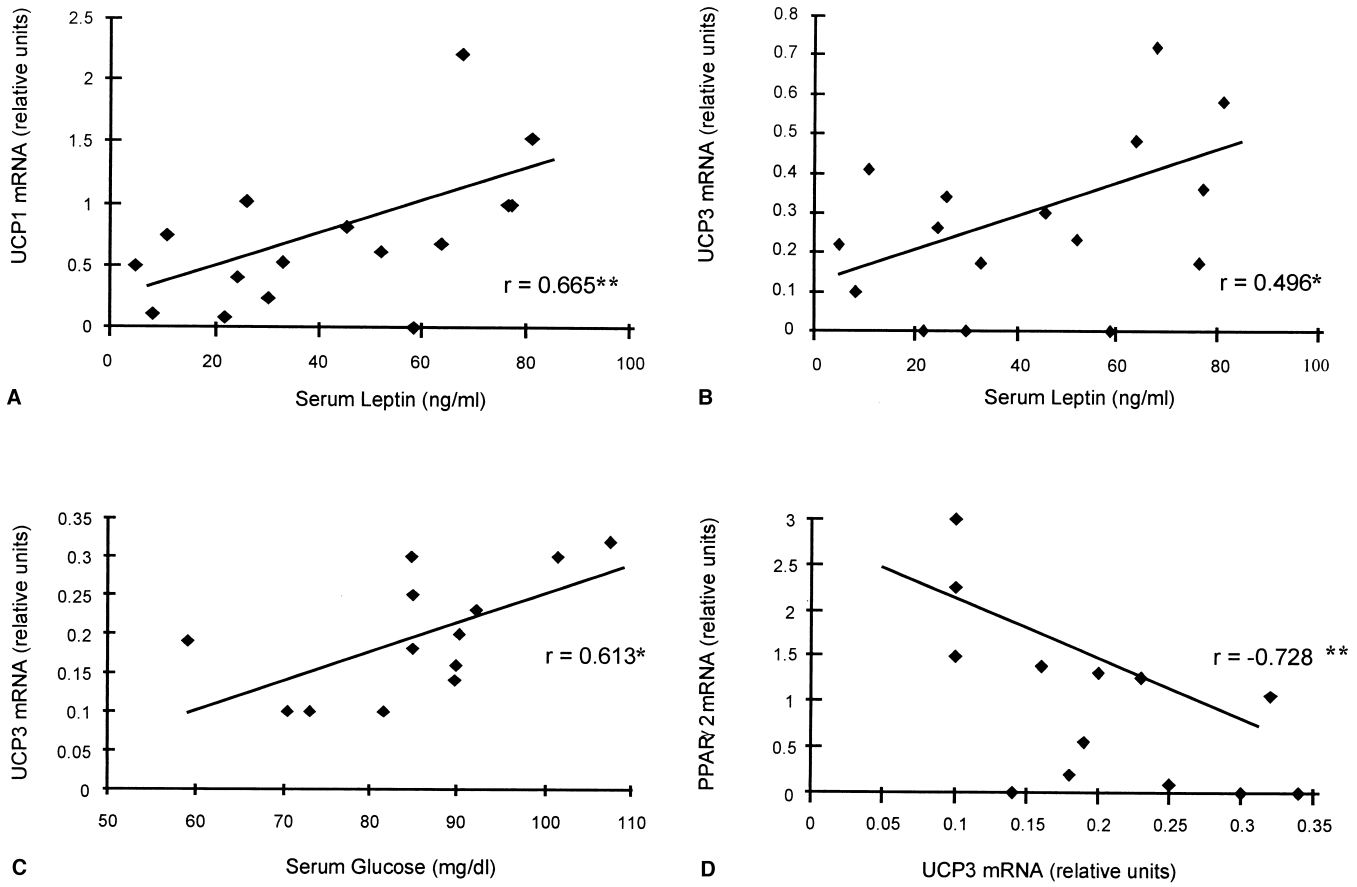

Fig. 4. Associations between (a) iBAT UCP1 mRNA levels with serum leptin; (b) iBAT UCP3 mRNA levels with serum leptin; (c) muscle UCP3 mRNA levels with serum glucose; (d) UCP3 mRNA levels with PPARγ2 mRNA levels in skeletal muscle. Asterisks indicates significant association ($*P < .05$ and $**P < .01$) between variables.

expression in iBAT, as previously reported [16,32,33]. This is in good agreement with the statistically significant relationships obtained between both iBAT UCPs (UCP1 and UCP3) with serum leptin.

On the other hand, skeletal muscle is another tissue involved on energy dissipation (thermogenesis) via UCPs [39,40]. Thus, the occurrence of a tissue-dependent regulation of these mitochondrial proteins has been reported under different dietary, pharmacological and physiological circumstances [2,11,12,40]. A marginal increase ($P < .06$) in muscle UCP3 mRNA levels was found in the high-energy-fed group, whereas no changes were observed for skeletal muscle UCP2 mRNA levels. A number of studies have been carried out under different situations showing that both muscle UCPs are similarly regulated, but UCP3 seemed to be much more sensitive to changes. Thus, leptin [40], fasting [2] and exercise [41] increase expression of both proteins, whereas refeeding after fasting [2] and β_3 adrenergic agonist administration [10] decrease their levels. Our results showed a significant increase in mRNA UCP3 levels in animals fed a high-calorie diet, and are in accordance with previous data [7,15]. Also, muscle UCP3 mRNA levels were statistically associated with serum glucose levels, as has been reported in a previous study with hyperglycemic mice [23]; this supports the possibility that muscle UCP3 function may be related to glucose metabolism and insulin resistance [42], probably through the glucose transporter GLUT4 [17].

Because no dietary effects were observed on muscle UCP2 mRNA expression levels, it can be suggested that muscle UCP2 is apparently not sensitive to nutritional changes as occurs for WAT UCP2, confirming different regulatory mechanisms concerning UCPs. On the other hand, a number of studies have focused on assessing a potential regulatory role for PPARγ2 in the muscle UCP mRNA expression in rats, but there are no clear conclusions because controversial results have been reported [3,21,23, 39]. In this sense, our results showed that high-energy diet intake lowered muscle PPARγ2 mRNA levels, but not in a statistically significant manner. Nevertheless, a significant negative relation was found between PPARγ2 expression with muscle UCP3 (Fig. 4d), suggesting that muscle UCP3 mRNA expression may be negatively affected by PPARγ2 under fat-feeding conditions, as previously reported after the administration of a PPAR agonist [23].

In summary, this study provided information about the

differential physiological changes of some genes related to thermogenesis after a period of high-energy feeding, producing an increase weight gain in a nongenetically obese animal strain. Results show that UCPs may follow different tissue-dependent regulatory mechanisms that may be linked to leptin circulating levels. Thus, BAT UCP1 and UCP3 seem to respond in the same way to a high-energy diet, whereas expression patterns exhibited by UCP3 in skeletal muscle under the same conditions are more difficult to explain than in iBAT. This experiment suggests that skeletal muscle gene expression is less sensitive to nutritional changes than iBAT.

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