

# Up-regulation of a thermogenesis-related gene (UCP1) and down-regulation of PPAR $\gamma$ and aP2 genes in adipose tissue: possible features of the antiobesity effects of a $\beta_3$ -adrenergic agonist

Javier Margareto, Eider Larrarte, Amelia Marti, J. Alfredo Martinez\*

*Department of Physiology and Nutrition, University of Navarra, Pamplona, Spain*

Received 19 May 2000; accepted 4 December 2000

## Abstract

A number of experiments have demonstrated the antiobesity effects of  $\beta_3$ -adrenergic receptor stimulation by promoting thermogenesis and/or lipolysis. While many studies have been performed in order to develop  $\beta_3$ -adrenergic agonists as a novel strategy in the management of obesity, more information is needed about the mechanisms involved in thermogenesis and the actions of these drugs on adipocyte differentiation. To address this, the possible thermogenic and antiadipogenic properties of Tertatolol, a  $\beta_3$ -adrenergic agonist, in a diet-induced obesity model has been tested. Animals fed on a high-fat diet gained more weight and fat mass as compared with control and high-fat fed animals treated with Tertatolol. A RT-PCR was carried out in white adipose tissue specific genes involved in thermogenesis such as uncoupling proteins (UCPs) and adipogenesis such as peroxisome proliferator-activated receptor (PPAR $\gamma$ 2), retinoid receptors (RXR $\alpha$ /RAR $\alpha$ ), and fatty acid binding protein (aP2). Levels of UCP1 mRNA were augmented in the Tertatolol-treated group as compared to non-treated high-fat fed animals, while the  $\beta_3$ -adrenergic agonist treatment significantly decreased the expression levels of aP2 and transcription factors such as PPAR $\gamma$ 2 and the ratio RXR $\alpha$ /RAR $\alpha$  as compared to obese rats. Altogether these data suggest that the antiobesity effects of  $\beta_3$ -adrenergic agonists are not limited to the promotion of thermogenesis and/or lipolysis and support the implication that these  $\beta_3$ -adrenergic agonists also affect fat deposition by impairing adipogenesis in white adipose tissue (WAT). © 2001 Elsevier Science Inc. All rights reserved.

**Keywords:**  $\beta_3$ -Adrenergic agonist; Thermogenesis; Adipogenesis; Uncoupling proteins; Peroxisome proliferator-activated transcription factor; Retinoid X receptors; aP2; Diet-induced obesity

## 1. Introduction

Obesity is defined as a state of pathological increased adipose mass [1], which results from an imbalance between energy intake and energy expenditure [2]. Current trends for obesity management involve multiple tactics including behavioural therapies, exercise programmes, diets to decrease energy and fat intake, as well as pharmacological strategies [3]. In this sense,  $\beta_3$ -adrenergic receptors ( $\beta_3$ AR) are

known to play an important role in the regulation of energy balance, promoting energy expenditure by triggering thermogenesis in brown adipose tissue (BAT), and/or lipolysis in white (WAT) and brown adipose tissues (BAT) [4]. Selective  $\beta_3$ -adrenergic agonists are considered as potential antiobesity drugs, since some of them have been successfully applied in animal models and a number of companies and laboratories are looking for new molecules with affinity for  $\beta_3$ -adrenergic receptors for the management of excessive fat accumulation in humans. Molecules with affinity for  $\beta_3$ -adrenoceptors have been associated with increased energy expenditure by inducing UCPs [5], lipolysis and decreased food intake [4,6] as the most likely mechanisms contributing to reverse or to delay diet induced obesity (DIO) in animals [7,8]. Little is known about the effects of  $\beta_3$ -adrenergic agonists on the adipocyte differentiation process and the expression of related genes. In this context, Tertatolol is an aryloxy-propanolamine with high affinity

\* Corresponding author. Tel.: +34-948-425600 x6424; fax: +34-948-425.649.

E-mail address: jalfmtz@unav.es (J.A. Martinez).

**Abbreviations:** AP2, adipocyte specific fatty acid binding protein; BAT, brown adipose tissue; DIO, diet-induced obesity; HFD, high-fat diet; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; RXR $\alpha$ , retinoid X receptor alpha; RAR $\alpha$ , retinoid acid receptor alpha; UCP, uncoupling protein; WAT, white adipose tissue; RT-PCR, reverse transcription-polymerase chain reaction; M-MLV, Moloney-Murine Leukemia Virus

for  $\beta_3$  adrenergic receptors, but also with  $\beta_1$  and  $\beta_2$  antagonist properties [9,10].

Adipogenesis regulation involves different transcription factors which function cooperatively to transactivate adipocyte genes bringing about adipocyte differentiation [11]. The transcription factor, PPAR $\gamma$ 2, a member of the peroxisome proliferator-activated receptor subfamily of nuclear hormone receptors [12], heterodimerizes with retinoid X receptors (RXR) resulting in the promotion of gene transcription by binding to DNA recognition motifs of specific genes. The PPAR $\gamma$ 2-RXR $\alpha$  heterodimer plays a critical role in the adipocyte differentiation process and in the maintenance of the fully differentiated adipocyte phenotype by enhancing the expression of mature adipocyte marker genes such as aP2 [11,13]. Accordingly, the activation of PPAR $\gamma$ 2 constitutes an important part of the mechanism behind the adipogenic effect of overfeeding and high fat diet [14]. The focus of this study is on the molecular effects of a  $\beta_3$ -adrenergic agonist (Tertatolol) on WAT mRNA expression of UCP1, UCP2, UCP3, aP2, PPAR $\gamma$ 2 and the RXR $\alpha$ /RAR $\alpha$  ratio in a diet-induced obesity model, which is known to promote adipocyte hypertrophy [8] and PPAR $\gamma$ 2 expression [14].

Therefore, current work evaluates the proposal that the antiobesity effects of the  $\beta_3$ -adrenergic agonists (Tertatolol), involve modifying the expression of adipogenic transcription factors in addition to promote UCP expression. Furthermore, potential associations between PPAR $\gamma$ 2, aP2 and UCPs were also investigated in order to find potential interaction mechanisms.

## 2. Materials and methods

### 2.1. Animals and diets

Twenty-six female Wistar rats, supplied by the Applied Pharmacobiology Centre (CIFA-Pamplona, Navarra, Spain), weighing about 150 g, were housed at  $25 \pm 1^\circ$  with a 12-hr light/dark cycle (08:00 to 20:00) and assigned into three experimental groups. One group of rats was fed on 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 30 days (named Lean-Control group, with  $N = 10$ ). This lean-control group was intraperitoneally injected with saline every day. Another two groups were fed on a fat-rich high-energy diet (High-Fat diet, HFD), whose components were pate, bacon, chips, cookies, chocolate, and chow with proportions 2:1:1:1:1:1, which was given to each rat per day as published elsewhere [9,15]. The macronutrient composition of the high-fat diet was 103 g of protein, 357 g of carbohydrates, and 330 g of lipids, which provided 19614 kJ/kg of diet. All animals had free access to water and food during 30 days. One HFD group was daily intraperitoneally injected with saline (named High-Fat Control group;  $N = 8$ )

and the other (High-Fat +  $\beta_3$ ;  $N = 8$ ) was intraperitoneally injected every day with Tertatolol (1 mg/kg/day), which is a new  $\beta_3$ -adrenergic agonist with  $\beta_1$  and  $\beta_2$  antagonist properties [9] for the whole experimental time. After 30 days, rats were starved for 12 hr, euthanized by cervical dislocation, and their trunk blood was collected. Immediately, the whole abdominal white adipose tissue (retro and intraperitoneal, omental and mesenteric sites) was carefully excised, weighed, frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis. Rectal temperature was also recorded by a rectal probe (Yellow Springs Instruments) connected to a Panlab thermometer pb 0331 (Panlab).

All experimental procedures were performed according to national and institutional guidelines for animal care and use at the University of Navarra.

### 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. Glucose was measured with the commercial kit Unimate 7 PAP (Roche) and free fatty acids by the NEFA C ACS-ACOD Method (Waco Chemicals USA, Inc.). The glucose and fatty acid kits were adapted for a COBAS MIRA (Roche) equipment. Serum insulin was measured with a commercially available RIA kit (Rat Insulin [ $^{125}\text{I}$ ] assay system, Amersham) as indicated by the supplier.

### 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) from 150 mg of fat tissue. After 30-min treatment with 10 units of RNase-free Dnase I (Boehringer Mannheim), 1.5  $\mu\text{g}$  of RNA were used to synthesis first-strand complementary DNA (c-DNA). The RT reaction was carried out in a volume of 30  $\mu\text{L}$  containing 50 mmol/L Tris HCl (pH 8.3), 75 mmol/L KCl, 3 mM  $\text{MgCl}_2$ , 10 mmol/L dithiothreitol, 100 ng of random hexamers (Boehringer Mannheim), 1 mmol/L each DTP (Bioline), 20 units of Rnase inhibitor (Promega, Madison, USA), 200 units of M-MLV RT (Gibco BRL, Life Technologies, Gaithersburg, MD, USA), and incubated at  $37^\circ$  for 60 min. The enzyme was inactivated by heating at  $95^\circ$  for 5 min. Four microliters from the RT reaction were amplified in a 50- $\mu\text{L}$  reaction mixture containing 40 ng of each primer, 16 mmol/L  $(\text{NH}_4)_2\text{SO}_4$ , 67 mmol/L Tris HCl (pH 8.8), 2 mmol/L  $\text{MgCl}_2$ , 0.1% Tween-20, 0.2 mmol/L 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'-TA AAGCAGTTCTACACCAA-GGG-3' (sense, 308–329)

Table 1  
Specific conditions used for the amplification of each gene studied

	Annealing (s)	Elongation (s)	No of cycles
UCP1	58°–30	72°–30	33
UCP2	61°–30	72°–30	26
UCP3	55°–45	72°–60	35
PPAR $\gamma$ 2	53°–35	72°–50	44
aP2	60°–30	72°–30	29
RXR $\alpha$	57°–30	72°–40	40
RAR $\alpha$	60°–30	72°–30	35
$\beta$ -actin	59°–30	72°–30	27

and 5'-CGAAGGCAGAAGTGAAGTGG-3' (antisense, 648–667); UCP3 (GenBank U92069) 5'-GGAAGCTGGAG GCGAGAGGAA-3' (sense, 577–596) and 5'-TTTGTA GAAGGCTGTGGGGC-3' (antisense, 926–945); PPAR $\gamma$ 2 (GenBank Y12882) 5'-TCTGATTATGGGTGAAACTC-3' (sense 43–62) and 5'-TTTCTACTCTTTT-TGTGGATC-3' (antisense 592–612); aP2 (GenBank K02109) 5'-AACAC CGAGATTT-CCTTCAA-3' (sense 178–197) and 5'-TCACGCCTTTCATAACACAT-3' (antisense 351–370); RXR $\alpha$  (GenBank M84817) 5'-CACATTGGGCTTCGG GACT-3' (sense 359–367) and 5'-ACTCCACCTCGT TCT-CATTC-3' (antisense 755–774); RAR $\alpha$ 2 (GenBank U15211) 5'-GACCGAAACAAA-AAGAAGAAAGA-3' (sense 712–734) and 5'-GCAATGGTGAGGGTGGT GAA-3' (antisense 976–995);  $\beta$ -actin (GenBank J00691) 5'-TCTACAATGAGCTGCGTGTG-3' (sense 1599–1618) and 5'-GGTCAGGATCTTCAT-GAGGT-3' (antisense, 2357–2376). Primers for all genes were designed using the Oligo 4.05 Primer Analysis Software (National Bioscience, Inc.). 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° 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 [16]. Amplifications were linear under conditions shown in table 1 and were carried out in a GeneAmp PCR System 2400 (Perkin Elmer). 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  $\beta$ -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).

The use of PCR is a specific strategy to evaluate mRNA expression, which is supported by the following points: linearity of the PCR reactions was tested by amplification of 200 ng of first strand cDNA per reaction from 20 to 40 cycles; the data obtained concerning PPAR $\gamma$ 2, aP2 retinoid receptors and UCP mRNA are comparable to values in the

literature using the RT-PCR and other methods (Northern Blot) under different experimental treatments and because it is generally assumed that RT-PCR determinations tend to reduce the apparent differences between samples. Furthermore, a number of articles published in highly rated journals have accepted this methodology [16–22] in order to measure mRNA expression.

#### 2.4. Statistical analysis

All results are expressed as mean and standard error of the mean (SEM). Once normality was positively confirmed by the Kolmogorov-Smirnov test, those data were analysed using a one-way ANOVA followed by orthogonal contrasts. The Kruskal-Wallis test followed by a Mann-Whitney U test multiple comparisons was used when normality was not confirmed by the Kolmogorov-Smirnov test. The Pearson correlation coefficient was computed to analyze association between two variables. The calculations were performed using the SPSS/Windows version 7.5 (SPSS Inc.) statistical package. A *P* value lower than 0.05 was considered to be statistically significant.

### 3. Results

#### 3.1. Body weight rectal temperature and serum determinations

High-fat fed animals significantly increased body and abdominal fat weights as compared to the controls at the end of experimental time (Table 2). High-fat fed animals receiving the  $\beta_3$ -adrenergic agonist showed a significant reduction in body weight as compared to high-fat fed animals, although they did not achieved the weight of the lean-controls. Abdominal fat weight, however, was not significantly different between  $\beta_3$ -adrenergic agonist injected animals and lean controls (Table 2).

In regard to serum determinations, glucose levels were significantly elevated in both high-fat fed groups (either  $\beta_3$ -adrenergic agonist treatment or saline injected) as compared to lean controls. Although the administration of the  $\beta_3$ -adrenergic agonist significantly reduced serum leptin levels with respect to the high-fat fed groups, leptin levels in the Tertatolol injected group were significantly greater than in the lean control animals. No statistical differences were found among experimental groups concerning serum fatty acid (FA) and insulin serum levels (Table 2). Moreover, while rectal temperature was slightly higher in the  $\beta_3$ -adrenergic agonist treated group, it did not reach statistical relevance.

#### 3.2. White adipose tissue (WAT) UCP mRNA expression levels

The UCP1 mRNA levels in WAT were hardly detectable in the lean control group, whereas a significant overexpres-

Table 2  
Weight and serum determinations

	Control	High-Fat	High-Fat + $\beta_3$	P value
Final body weight (g)	266.3 ± 10.9 <sup>a</sup>	308.0 ± 20.6 <sup>b</sup>	284.2 ± 21.5 <sup>c</sup>	p < 0.01
Abdominal fat weight (g)	7.4 ± 1.7 <sup>a</sup>	18.9 ± 5.8 <sup>b</sup>	12.3 ± 5 <sup>a</sup>	p < 0.01
Rectal temperature (°C)	37.6 ± 0.4	37.4 ± 0.2	38.2 ± 0.2	p = 0.15
Serum fatty acids (mmol/L)	0.83 ± 0.1	0.72 ± 0.05	0.71 ± 0.03	n.s.
Serum insulin (ng/mL)	0.88 ± 0.14	1.22 ± 0.18	1.02 ± 0.05	n.s.
Serum glucose (mg/dL)	80.5 ± 2.1 <sup>a</sup>	93.3 ± 3.7 <sup>b</sup>	90.9 ± 3.8 <sup>b</sup>	P < 0.05
Serum leptin (ng/mL)	50.1 ± 6.5 <sup>a</sup>	121.9 ± 7.4 <sup>b</sup>	80 ± 12.1 <sup>c</sup>	P < 0.01

Data are mean ± SEM. Rats were assigned into the following groups: Control (control fed and saline injected), High-fat (fed on a high fat diet and saline injected) and High-fat +  $\beta_3$  (fed on a high-fat diet and  $\beta_3$ -adrenergic agonist injected).

<sup>a,b,c</sup> Data not sharing a common superscript are significantly different after statistical analysis.

sion was found in the high-fat  $\beta_3$ -adrenergic agonist injected group with respect to the high-fat fed saline injected animals (Table 3).

Levels of UCP2 and UCP3 mRNA were more than two-fold increased in the high-fat fed group as compared to lean controls, while the  $\beta_3$ -adrenergic agonist treatment did not induce statistically significant changes in UCP2 and UCP3 mRNA expression with respect to high-fat fed animals (Table 3).

### 3.3. White adipose tissue PPAR $\gamma$ 2, aP2 expression, and RXR $\alpha$ /RAR $\alpha$ ratio

PPAR $\gamma$ 2 expression was significantly increased after high-fat feeding as compared to control fed animals. The  $\beta_3$ -adrenergic agonist treatment (Fig. 1) reduced such elevation as compared to the high-fat fed group. Similarly, aP2 mRNA expression levels were significantly elevated in high-fat fed group with respect to the control group, while Tertatolol treated animals showed statistically significant lower aP2 levels as compared to the other high-fat fed group (Fig. 1). The RXR $\alpha$ /RAR $\alpha$  ratio showed significant higher ratios in high-fat fed groups as compared to control fed animals, whereas the administration of the  $\beta_3$ -adrenergic agonist significantly decreased the RXR $\alpha$ /RAR $\alpha$  ratio with respect to the high-fat fed animals (Fig. 2).

### 3.4. Associations body fat content, PPAR $\gamma$ 2, aP2, RXR $\alpha$ , and UCPs mRNA

Statistically significant associations (Fig. 3) were found after correlation analyses between PPAR $\gamma$ 2 and white fat weight ( $r = 0.46$ ,  $P < 0.01$ ), PPAR $\gamma$ 2 and aP2 ( $r = 0.81$ ,  $P < 0.01$ ), PPAR $\gamma$ 2 and UCPs ( $r = 0.62$ ,  $P < 0.01$ ) and, also between PPAR $\gamma$ 2 and RXR $\alpha$  ( $r = 0.55$ ,  $P < 0.01$ ). Also, a statistically significant correlation between aP2 and fat weight ( $P < 0.05$ ) was found (Plot not shown).

## 4. Discussion

The discovery of the  $\beta_3$ -adrenergic receptor as an adipocyte  $\beta$ -adrenoceptor subtype, with thermogenic and lipolytic properties [7] has resulted in the proposal for  $\beta_3$ -adrenergic agonists as one of the possible strategies to increase energy expenditure [3]. The role of the  $\beta_3$ -adrenoceptor stimulation in human obesity treatment is still discussed [23] and numerous groups are seeking additional  $\beta_3$ -adrenergic agonists with high affinity and specificity for the human  $\beta_3$ -adrenoceptor for the possibility of treating obese individuals. However, the implication of such adrenoceptor stimulation in the adipocyte differentiation process is scarcely studied.

High-fat diets have been used to generate diet induced obesity models (DIO) and to promote adipocyte differentiation in a number of studies [11,14]. In this context, the administration of the  $\beta_3$ -adrenergic agonist (Tertatolol) was

Table 3  
UCPs mRNA expression levels in WAT

	Control	High-Fat	High-Fat + $\beta_3$	P value
UCP1 mRNA levels	0.01 ± 0.01 <sup>a</sup>	0.23 ± 0.14 <sup>b</sup>	0.64 ± 0.31 <sup>b</sup>	P < 0.05
UCP2 mRNA levels	0.05 ± 0.009 <sup>a</sup>	0.12 ± 0.02 <sup>b</sup>	0.11 ± 0.03 <sup>b</sup>	P < 0.01
UCP3 mRNA levels	0.06 ± 0.01 <sup>a</sup>	0.14 ± 0.01 <sup>b</sup>	0.09 ± 0.03 <sup>a,b</sup>	P < 0.05

Data are mean ± SEM of the ratio between each gene and  $\beta$ -actin. Rats were assigned into the following groups: Control (control fed and saline injected), High-fat (fed on a high-fat diet and saline injected) and High-fat +  $\beta_3$  (fed on a high fat diet and  $\beta_3$ -adrenergic agonist injected).

<sup>a,b,c</sup> Data not sharing a common superscript are significantly different after statistical analysis.

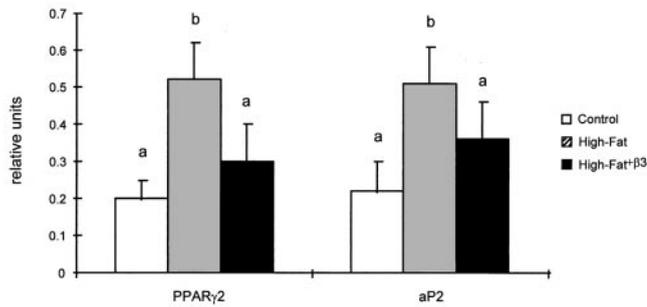


Fig. 1. PPAR $\gamma$ 2 and aP2 mRNA expression levels in white adipose tissue in lean, control-fed rats (Control; □), high-fat fed rats (HF; ▨), high-fat fed administered with  $\beta_3$ -adrenergic agonist rats (high-fat fed +  $\beta_3$ ; ■). Data are mean  $\pm$  SEM of the ratio between each gene and  $\beta$ actin. Bars represent Standard Error of each Mean (SEM). <sup>a,b,c</sup> Data not sharing a common superscript are significantly different after statistical analysis.

able to delay fat accumulation in rats fed on a high-fat diet (HFD). Since Tertatolol has antagonist properties on  $\beta_1$  and  $\beta_2$  adrenoceptors, which are known to be involved in lipid mobilisation, it appears as a good strategy to study the role of  $\beta_3$ -adrenergic agonists as antiobesity agents. The low serum leptin levels observed after the Tertatolol treatment suggest a down-regulation of leptin by  $\beta_3$ -adrenergic agonists through the sympathetic nervous system, as previously published [24]. Nevertheless, other reports [25,26] indicate that leptin as an adiposity signal, is lowered after the lipid mobilisation produced by the  $\beta_3$ -adrenergic agonist treatment. In this sense, acute treatment with  $\beta_3$ -adrenergic agonists may inhibit *ob* gene expression, while long-term  $\beta_3$ -adrenergic agonist administration induces a reduction of fat deposition, which may further decrease adipocyte leptin secretion [27].

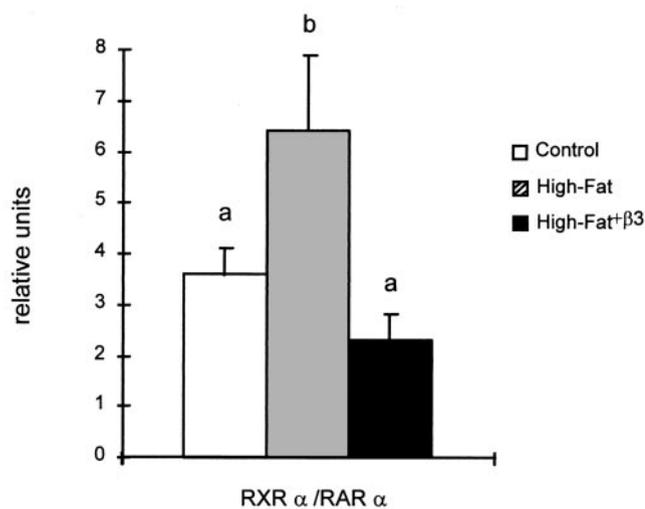


Fig. 2. RXR $\alpha$ /RAR $\alpha$  mRNA expression levels in white adipose tissue in lean, control fed rats (Control; □), high-fat fed rats (HF; ▨), high-fat fed  $\beta_3$  injected rats (high-fat fed +  $\beta_3$ ; ■); Data are mean  $\pm$  SEM of the ratio between each gene and  $\beta$ actin. Bars represent Standard Error of each Mean (SEM). <sup>a,b,c</sup> Data not sharing a common superscript are significantly different after statistical analysis.

Long periods of  $\beta_3$ -adrenergic agonist treatment have been reported to promote fatty acid oxidation inside the adipocyte, thus reducing serum fatty acids levels [16,28] as it is observed in the current work, although statistical significance was not achieved. The lower levels of fatty acids in high-fat fed animals may also be explained by the fact that this diet induces fat accumulation at the expense of circulating fatty acids [16].

The  $\beta_3$ -adrenergic agonists are known to promote energy expenditure by inducing UCP expression in a variety of tissues, such as brown adipose tissue [7], white adipose tissue and skeletal muscle [28,29]. Nevertheless, regulation of each UCP might be differently accomplished by distinct tissues. In this sense UCP1, primarily expressed in BAT [12], is strongly induced in WAT after  $\beta_3$ -adrenergic agonist treatment [8]. This expression has been attributed to brown adipocyte appearance among typical white adipose depots in response to sympathetic stimulation. UCP1 overexpression may be responsible for the marginal increase in body temperature observed after the treatment with the  $\beta_3$ -adrenergic agonist, thus supporting the thermogenic effect of  $\beta_3$ -adrenergic agonist. UCP2, another uncoupling protein with 55% homology of aminoacid sequence of UCP1, is widely distributed in different tissues, including WAT, BAT, skeletal muscle, liver, kidney, lung and the immune system [1]. While high-fat diet consumption and adiposity significantly increased UCP2 mRNA expression in WAT, as previously reported [30,31], the administration of the  $\beta_3$ -adrenergic agonist failed to induce any additional overexpression in high-fat fed animals [28,32]. Consistent with this idea, dietary fat or an adiposity sensor, such as leptin, may contribute to the regulation of UCP2 expression in WAT [33] rather than sympathetic signals. The third member of the uncoupling protein family is UCP3, which is expressed in skeletal muscle, BAT and, at low levels, in heart and WAT [31,33]. High-fat feeding for long periods increased WAT UCP3 mRNA levels, whereas Tertatolol treatment slightly reduced such elevation.

In order to investigate the potential implication of the  $\beta_3$ -adrenergic receptor stimulation in adipogenesis, the expression of PPAR $\gamma$ 2, which is known to regulate adipocyte differentiation, was analysed after Tertatolol treatment. PPAR $\gamma$ 2 is one of the most important transcription factor regulating fat cell differentiation [11], mediating high-fat diet induced adipocyte hypertrophy [34]. As some other nuclear receptors, it heterodimerizes with the retinoid X receptor (RXR) to regulate adipogenesis [12] as well as to control the expression of adipocyte specific genes such as aP2 involved in the maintenance of adipocyte phenotype [11–13]. In this sense, measuring PPAR $\gamma$ 2 mRNA expression may provide insights on how dietary fat intake and  $\beta_3$ -adrenergic agonist could affect the adipocyte differentiation process.

High-fat feeding induced a significant increase in fat pad weights, which is probably due to adipocyte hypertrophy [8]. Such effect may be mediated by PPAR $\gamma$ 2 [34] as

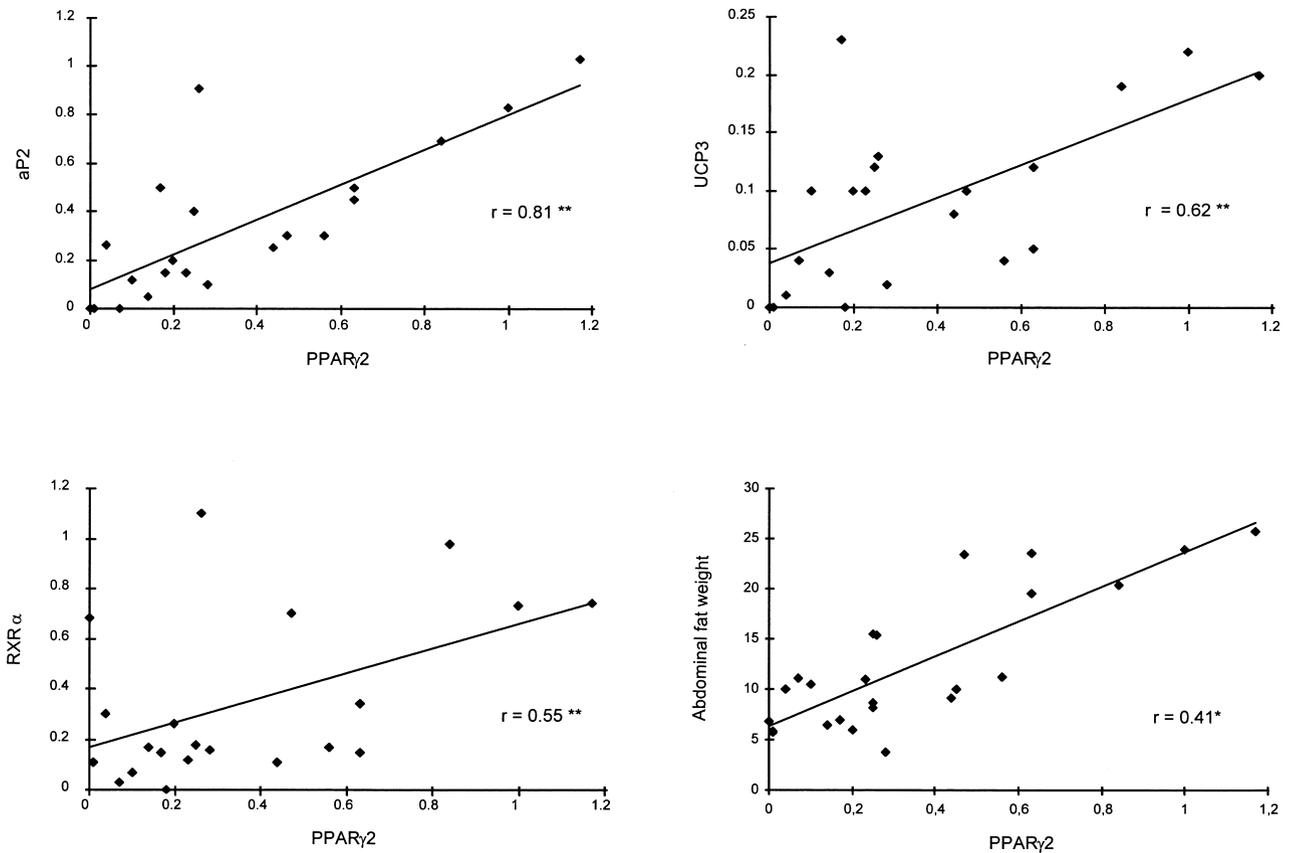


Fig. 3. Statistical associations. (a) PPAR $\gamma$ 2 with aP2; (b) PPAR $\gamma$ 2 with UCP3; (c) PPAR $\gamma$ 2 with RXR $\alpha$  in WAT; (d) PPAR $\gamma$ 2 with abdominal fat weight. Analyses were developed using the Pearson correlation test.  $^{**}P < 0.01$ ;  $^{*}P < 0.05$ .

suggested by the significantly elevated mRNA levels and the positive significant correlation found with the body fat content of animals. Furthermore, the high-fat feeding induced increase in PPAR $\gamma$ 2 mRNA levels was blunted in Tertatolol treated animals, resulting in only a minor elevation of fat pad weight. A similar expression pattern was also observed when aP2 mRNA levels were analysed. These results suggest that Tertatolol may exert a negative effect on PPAR $\gamma$ 2, and aP2 mRNA expression. In this sense, the present data are consistent with the hypothesis that  $\beta_3$ -adrenergic agonists are able to impair adipogenesis probably by decreasing mRNA expression of genes specifically involved in adipocyte differentiation and phenotype maintenance.

Retinoic acid receptors are nuclear receptors involved in adipocyte differentiation, which includes RXR $\alpha$  and RAR $\alpha$ . RXR $\alpha$  transcription factor induces adipogenesis by heterodimerizing with PPAR $\gamma$ 2, while RAR $\alpha$  blocked adipocyte differentiation because it binds to RXR $\alpha$ , avoiding RXR $\alpha$ -PPAR $\gamma$ 2 heterodimerization [35]. In this sense, the RXR $\alpha$ /RAR $\alpha$  ratio was calculated because high ratios indicate that RXR $\alpha$  is promoted over RAR $\alpha$  expression, thus favouring adipogenesis, whereas low ratios mean the oppo-

site, indicating that adipogenesis is inhibited [36]. Our results confirm this hypothesis, since the RXR $\alpha$ /RAR $\alpha$  ratio in the high-fat fed animals are significantly higher than the ratio obtained from control fed animals. Furthermore, Tertatolol treatment significantly reduced the RXR $\alpha$ /RAR $\alpha$  ratio, suggesting that differentiation is promoted by high-fat diet with respect to the other groups, whereas the  $\beta_3$ -adrenergic agonist appears to delay or block the adipogenic effect of the high-fat diet.

In addition, statistically significant associations between PPAR $\gamma$ 2 with RXR $\alpha$  and aP2 were found, as might be expected because the close physiological function of these genes in adipocytes. Interestingly, the significant association found between PPAR $\gamma$ 2 and UCP3 may suggest a possible regulatory role of PPAR $\gamma$ 2 on the UCP3 expression. The identification of a functional peroxisome proliferator responsive element (PPRE) in the promotor region of UCP3 [37] supports this hypothesis.

In summary, the high-fat diet intake promoted adipocyte growth, thus increasing total body weight and expression of adipogenic transcription factor. The  $\beta_3$ -adrenergic agonist, Tertatolol, appears to delay the development of a diet-induced obesity by increasing energy expenditure and by

impairing adipogenesis. This is supported by the findings that  $\beta_3$ -adrenergic agonist administration reduced the RXR $\alpha$ /RAR $\alpha$  ratio, the adipocyte specific gene, aP2, and PPAR $\gamma$  mRNA levels, which negatively affect adipogenesis. This information is of interest in biochemical pharmacology and for the development of new molecules with specific application in human obesity.

## Acknowledgments

Thanks are given to Dr M. José Calasanz from the Biostatistics Unit at the University of Navarra for statistical advise as well as to Line Special (LE/97) and Government of Navarra for financial support. Also, Dr B di Fanti is gratefully acknowledged for helpful discussions.

## References

- [1] Flier JS, Bradford BL. Obesity research springs a proton leak. *Nat Genet* 1997;15:223–4.
- [2] Gong DW, He Y, Karas M, Reitman ML. Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone,  $\beta_3$ -adrenergic agonists, and leptin. *J Biol Chem* 1997;272:24129–32.
- [3] Bray GA, Greenway FL. Current and potential drugs for treatment of obesity. *Endocr Rev* 1999;20:805–75.
- [4] Kumar MV, Moore RL, Scarpace PJ.  $\beta_3$ -adrenergic regulation of leptin, food intake, and adiposity is impaired with age. *Pflügers Arch* 1999;438:681–8.
- [5] Boss O, Hagen T, Lowell BB. Uncoupling proteins 2 and 3. *Diabetes* 2000;49:143–56.
- [6] Savontaus E, Pesonen U, Rouru J, Huupponen R, Koulu M. Effects of ZD7114, a selective  $\beta_3$ -adrenoceptor agonist, on neuroendocrine mechanisms controlling energy balance. *Eur J Pharmacol* 1998;347:265–74.
- [7] Himms Hagen J, Cui J, Danforth E, Taatjes DJ, Lang SS, Waters BL, Claus TH. Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *Am J Physiol* 1994;266:R1371–82.
- [8] Ghorbani M, Claus TH, Himms-Hagen J. Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with  $\beta_3$ -adrenoceptor agonist. *Biochem Pharmacol* 1997;54:121–31.
- [9] Corbalán MS, Margareto J, Martínez JA, Marti A. High-fat feeding reduced muscle uncoupling protein 3 expression in rats. *J Physiol Biochem* 1999;55:67–72.
- [10] Pietri-Rouxel F, Drumare MF, Strosberg AD. Adrenoceptors. Structure, Function and Pharmacology. Luxembourg: Harwood Academic Publisher, 1997.
- [11] Gregoire FM, Smas CM, Sul HS. Understanding adipocyte differentiation. *Physiol Rev* 1998;78:783–809.
- [12] Spiegelman BM. PPAR- $\gamma$ : adipogenic regulator and thiazolidindione receptor. *Diabetes* 1998;47:507–14.
- [13] Lowell BB. PPAR $\gamma$ : An essential regulator of adipogenesis and modulator of fat cell function. *Cell* 1999;99:239–42.
- [14] Vidal Puig A, Jimenez Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. *J Clin Invest* 1996;97:2553–61.
- [15] Berraondo B, Bonafonte A, Fernández-Otero MP, Martínez JA. Effects on energy utilization of a  $\beta_3$  adrenergic agonist in rats fed on a cafeteria diet. *Eating Weight Disord* 1997;2:130–7.
- [16] Berraondo B, Marti A, Duncan JS, Trayhurn P, Martínez JA. Up-regulation of the muscle UCP2 gene expression by a new  $\beta_3$ -adrenoceptor agonist, trectadrine, in obese (cafeteria) rodents, but down-regulation in lean animals. *Int J Obes* 2000;24:156–64.
- [17] Shimabukuro M, Zhou YT, Lee Y, Unger RH. Induction of uncoupling protein-2 mRNA by troglitazone in the pancreatic islets of Zucker diabetic fatty rats. *Biochem Biophys Res Commun* 1997;237:359–61.
- [18] Shimabukuro M, Wang MY, Zhou YT, Newgard CB, Unger RH. Protection against lipoapoptosis of beta cells through leptin-dependent maintenance of Bcl-2 expression. *Proc Nat Acad Sci USA* 1998;95:9558–61.
- [19] Zhou YT, Shimabukuro M, Lee Y, Koyama K, Trieu F, Unger RH. Leptin normalizes the impaired response of proinsulin mRNA to long chain fatty acids in heterozygous Zucker diabetic fatty rats. *J Biol Chem* 1997;272:25648–51.
- [20] Parry GCN, Erlich JH, Carmeliet P, Luther T, Mackman N. Low levels of tissue factor are compatible with development and hemostasis in mice. *J Clin Invest* 1998;101:560–9.
- [21] Harte RA, Kirk EA, Rosenfeld ME, LeBoeuf RC. Initiation of hyperinsulinemia and hyperleptinemia is diet dependent in C57BL/6 mice. *Horm Metab Res* 1999;31:570–75.
- [22] Adli H, Bazin R, Perret CY. Interaction of amiodarone and triiodothyronine on the expression of  $\beta$ -adrenoceptors in brown adipose tissue of rat. *Br J Pharmacol* 1999;126:1455–61.
- [23] Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD. Molecular characterization of the human  $\beta_3$ -adrenoceptor. *Science* 1989;245:1118–21.
- [24] Trayhurn P, Duncan JS, Rayner DV, Hardie LJ. Rapid inhibition of the *ob* gene expression and circulating leptin levels in lean mice by the  $\beta_3$ -adrenoceptor agonists BRL 35135A and ZD2079. *Biochem Biophys Res Commun* 1996;228:605–10.
- [25] Takahashi Y, Ide T. Effects of dietary fats differing in degree of unsaturation on gene expression in rat adipose tissue. *Ann Nutr Metab* 1999;43:86–97.
- [26] Liuzzi A, Savia G, Tagliaferri M, Lucantoni R, Berselli ME, Petroni ML, Medici C, Viberti GC. Serum leptin concentration in moderate and severe obesity: relationship with clinical, anthropometric and metabolic factors. *Int J Obes Relat Metab Disord* 1999;23:1066–73.
- [27] Collins S, Surwit RS. Pharmacologic manipulation of *ob* expression in a dietary model of obesity. *J Biol Chem* 1996;271:9437–40.
- [28] Yoshitomi H, Yamazaki K, Abe S, Tanaka I. Differential regulation of mouse uncoupling proteins among brown adipose tissue, white adipose tissue, and skeletal muscle in chronic beta 3 adrenergic receptor agonist treatment. *Biochem Biophys Res Commun* 1998;253:85–91.
- [29] Yoshida T, Umekawa T, Kumamoto K, Sakane N, Kogure A, Kondo M, Wakabayashi Y, Kawada T, Nagase I, Saito M.  $\beta_3$ -adrenergic agonist induces a functionally active uncoupling protein in fat and low-twitch muscle fibers. *Am J Physiol* 1998;274:E469–75.
- [30] Gong DW, He Y, Reitman ML. Genomic organization and regulation by dietary fat of the uncoupling protein 3 and 2 genes. *Biochem Biophys Res Commun* 1999;256:27–32.
- [31] Matsuda J, Hasoda K, Itoh H, Son C, Doi K, Nakao K. Cloning of rat UCP3 and UCP2: their gene expression in rats fed high-fat diet. *FEBS Lett* 1997;418:200–4.
- [32] Savontaus E, Rouru J, Boss O, Huupponen R, Koulu M. Differential regulation of uncoupling proteins by chronic treatments with beta 3-adrenergic agonist BRL 35135 and metformin in obese fa/fa Zucker rats. *Biochem Biophys Res Commun* 1998;246:899–904.
- [33] Larkin S, Mull E, Miao W, Pittner R, Albrandt K, Moore C, Young A, Denaro M, Beaumont K. Regulation of the third member of the uncoupling protein family, UCP3, by cold and thyroid hormone. *Biochem Biophys Res Commun* 1997;240:222–7.
- [34] Kubota N, Terauchi Y, Miki H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S,

- Kadowaki T. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* 1999;4:597–609.
- [35] Xue JC, Schwarz EJ, Chawla A, Lazar ML. Distinct stages in adipogenesis revealed by retinoid inhibition of differentiation after induction of PPAR $\gamma$ . *Mol Cell Biol* 1996;16:1567–75.
- [36] Villarroya F, Giralt M, Iglesias R. Retinoids and adipose tissues: metabolism, cell differentiation and gene expression. *Int J Obes Relat Metab Disord* 1999;23:1–6.
- [37] Acin A, Rodriguez M, Rique H, Canet E, Boutin JA, Galizzi JP. Cloning and characterization of the 5' flanking region of the human uncoupling protein 3 (UCP3) gene. *Biochem Biophys Res Commun* 1999;258:278–83.