Gene Expression Patterns in Visceral and Subcutaneous Adipose Depots in Rats are Linked to Their Morphologic Features

Mariona Palou, Teresa Priego, Juana Sánchez, Ana M. Rodríguez, Andreu Palou and Catalina Picó

Molecular Biology, Nutrition and Biotechnology (Nutrigenomics), University of the Balearic Islands (UIB), and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Palma de Mallorca

Key Words

Adipose tissue • Adiposity • Gene expression • Lipid metabolism • Visceral fat

Abstract

The aim was to characterize the expression pattern of genes involved in lipid metabolism in internal (retroperitoneal, mesenteric) and subcutaneous (inguinal) adipose tissue depots in rats and their relation with site-specific morphological- and metabolic-features. Gene expression by RT-qPCR, western blot and morphometric analyses were performed. Lipogenesis-related genes (PPAR γ 2, SREBP1c, ACC1, GPAT, LPL, CD36, GLUT4) showed higher mRNA levels in the retroperitoneal depot versus the mesenteric and the inguinal depots; the expression of PPAR γ 2, ACC1, CD36, and GLUT4 in the mesenteric depot was also higher than in the inguinal depot. HKII was similarly expressed in the retroperitoneal and mesenteric depots and higher than in the inguinal one. The expression of lipolysis-related genes (HSL, ATGL) was higher in the retroperitoneal than in the mesenteric and inguinal depots, while the expression of fatty-acid oxidation-related genes (PPAR α , CPT1) was lower in the

retroperitoneal depot compared with the mesenteric and the inguinal depots. Thus, a higher expression of lipogenesis- and lipolysis-related genes and lower expression of fatty-acid oxidation-related genes in internal depots (particularly in the retroperitoneal, which also presents the largest adipocyte size) can explain its higher triacylglyceride turnover rates and hence account for the differential behavior of fat depots in physiological situations and its involvement in obesity-linked metabolic disorders.

Copyright © 2009 S. Karger AG, Basel

Introduction

White adipose tissue is regarded as the main site of energy storage in the form of triacylglycerides (TG), which are mobilized when needed through the lipolysis process to provide fuel for other organs. However, adipose tissue is not a single homogeneous compartment, but rather a tissue with specific regional depots which have different cellular size, structural organization and biological functions [1, 2]. Differential lipid-metabolism gene expression in the different depots is, presumably,

responsible of their specific functionalities but only sparse studies on that have been previously reported.

Increased adipose tissue mass is the primary phenotypic characteristic of obesity. The amount and distribution of adipose tissue is associated with many of the adverse consequences of obesity, such as cardiovascular disease and type 2 diabetes [3, 4]. The distribution of body fat appears to be even more important than the total amount of fat [2, 5]. Visceral adiposity has been strongly linked to insulin resistance, type 2 diabetes, hypertension and dyslipidemia, leading to increased risk of cardiovascular disease, whereas such associations seem to be much less consistent with respect to subcutaneous fat mass [1, 3, 6]. Support for a specific detrimental role of visceral fat accumulation also comes from studies in insulin-resistant humans and rats, in which surgical removal of visceral fat was followed by a marked improvement [7-9].

The adverse metabolic impact of visceral fat has been attributed to distinct biological properties of adipocytes in this region compared with other adipose tissue depots. Indeed, variations in fat cell metabolism depending on the anatomical location have been observed, especially in regard to regulation of lipolysis [2, 10]. A higher turnover rate of TG has been shown in abdominal versus gluteo-femoral fat in humans by isotopic studies *in vivo* [11]. In studies on isolated fat cells, human visceral adipocytes also showed higher lipolytic rates in response to catecholamines [12] and lower sensitivity to the anti-lipolytic effects of α_2 -adrenergic receptor agonists [13] as compared with subcutaneous derived fat cells.

In vivo studies by microdialysis also showed that the epididymal adipose tissue is more responsive to β -adrenergic lipolysis stimulation than is the subcutaneous fat pad from the inguinal region [14]. Gene expression studies performed in rats have also showed that internal depots (particularly the retroperitoneal one) is more responsive to fasting conditions concerning the induction of the lipolytic enzyme adipose triglyceride lipase (ATGL) compared with the subcutaneous depot [15]. Thus, the increased visceral fat observed in central obesity would be associated with greater mobilization of free fatty acids into the portal circulation, impairing liver metabolism and leading to glucose intolerance, insulin resistance, and dislipidemia [2].

In addition to higher lipolytic activity, different studies have also shown that internal adipose tissue depots have higher rates of lipid synthesis compared with subcutaneous depots. For example, in rats, Jamdar et al [16] found higher rates of lipid formation using [^{14}C]glycerol 3-phosphate,

[^{14}C]glucose or [^{14}C]acetate as substrates for TG synthesis in perirenal and gonadal adipose tissue compared with the subcutaneous fat-tissue. The concept of higher metabolic activity of visceral compared with subcutaneous adipocytes has also been demonstrated in humans by analyzing *in vivo* glucose uptake by isotope labeling and positron emission tomography techniques [17]. In both normal weight and obese subjects, insulin-stimulated glucose uptake was found to be higher in visceral compared with subcutaneous fat, in the absence of significant differences in tissue blood flow [17].

Although it has been established that adipocytes from different anatomical localizations have different biological properties, for practical reasons most studies on adipose tissue metabolism - particularly those dealing with gene expression - have been carried out on a single fat pad, usually epididymal or subcutaneous white adipose tissue. However, given the clinical importance of the differences in regional fat distribution, studies aiming at defining different properties of adipose tissues from distinct anatomical sites, particularly concerning expression of genes involved in lipid metabolism, may be interesting since it could provide insights into the capacity to regulate body fat content and distribution, which is of great interest in the prevention/treatment of metabolic-related disorders. Thus, the aim of this study was to characterize the expression pattern of selected genes involved in lipid metabolism in different fat depots, including two internal/visceral depots (the retroperitoneal and mesenteric) and the inguinal subcutaneous one, and its relation with morphological features (adipocyte size and cellularity) of these depots. The genes studied were the key ligand-activated transcription factors sterol regulatory element binding protein 1c (SREBP1c), peroxisome proliferator activated receptor α (PPAR α) and peroxisome proliferator activated receptor γ 2 (PPAR γ 2), and other selected genes related with lipogenesis [acetyl-coenzyme A carboxylase α (ACC1), fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), lipoprotein lipase (LPL), fatty acid translocase (FAT/CD36), glucose transporter 4 (GLUT4), and hexokinase II (HKII)], lipolysis [hormone sensitive lipase (HSL) and ATGL], and fatty acid oxidation [carnitine palmitoyltransferase 1a (CPT1) and acyl-coenzyme A oxidase 1 (ACOX1)]. To assess whether differences in gene expression were translated into changes in protein levels, western blot analysis were performed for 2 key proteins related with lipogenesis and lipolysis - ACC1 and ATGL, respectively.

Table 1. Nucleotide sequences of primers and cycling conditions used for PCR amplification.

GENE	FORWARD PRIMER (5' to 3')	REVERSE PRIMER (5' to 3')	CYCLING CONDITIONS	AMPLICON SIZE (bp)
<i>ACC1</i>	TGCAGGTATCCCCACTCTTC	TTCTGATTCCCCTCCCTCCT	95°C for 2s 60°C for 6s 72°C for 10s	212
<i>ACOX1</i>	CACGCAATAGTTCTGGCTCA	ACCTGGGCGTATTTTCATCAG	95°C for 2s 60°C for 6s 72°C for 10s	221
<i>ATGL</i>	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGACATCTCT	95°C for 2s 60°C for 6s 72°C for 10s	271
<i>CD36</i>	GTCCTGCTGTGTGA	GCTCAAAGATGCTCCATTG	95°C for 2s 60°C for 6s 72°C for 14s	318
<i>CPT1</i>	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	95°C for 2s 60°C for 6s 72°C for 10s	250
<i>FAS</i>	CGGCGAGTCTATGCCACTAT	ACACAGGGACCGAGTAATGC	95°C for 2s 60°C for 6s 72°C for 10s	222
<i>GLUT4</i>	GGCATGGGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATG	95°C for 2s 60°C for 6s 72°C for 10s	233
<i>GPAT</i>	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG	95°C for 2s 60°C for 6s 72°C for 10s	194
<i>HKII</i>	CAGCCTAGACCAGAGCATCC	CGCATCTCTTCCATGTAGCA	95°C for 2s 60°C for 6s 72°C for 10s	246
<i>HSL</i>	TCACGCTACATAAAGGCTGCT	AGTTCCCTCTTTACGGGTGG	95°C for 2s 60°C for 6s 72°C for 7s	169
<i>LPL</i>	TATGGCACAGTGGCTGAAAG	CTGACCAGCGGAAGTAGGAG	95°C for 2s 60°C for 6s 72°C for 8s	157
<i>PPARα</i>	TGTCGAATATGTGGGGACAA	AAACGGATTGCATTGTGTGA	95°C for 2s 60°C for 6s 72°C for 8s	215
<i>PPARγ2</i>	GATCCTCCTGTTGACCCAGA	TCAAAGGAATGGGAGTGGTC	95°C for 2s 60°C for 6s 72°C for 10s	164
<i>SREBP1c</i>	AGCCATGGATTGCACATTG	GGTACATCTTTACAGCAGTG	95°C for 2s 60°C for 6s 72°C for 10s	260
18S	CGCGTTCTATTTTGTGGT	AGTCGGCATCGTTTATGGTC	95°C for 2s 60°C for 6s 72°C for 10s	219

Materials and Methods

Animals and tissue isolation

Three-month-old male Wistar rats (Charles River Laboratories España, SA, Barcelona, Spain), housed at 22°C with a period of light/dark of 12 h (lights on from 0800 to 2000) and fed with a standard chow diet (Panlab, Barcelona, Spain) were used (n = 6). All animals were killed by decapitation under *ad libitum* feeding conditions during the first h after the beginning of the light cycle. The retroperitoneal, mesenteric and inguinal white adipose tissue depots were rapidly removed and weighed. Samples for RNA and protein analysis and for DNA quantification were immediately frozen in liquid nitrogen and stored at -70 °C until analysis. For microscopic and morphometric analysis, fresh dissected adipose tissue samples were used.

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of the University, following its guidelines for the use and care of laboratory animals.

Morphometric analysis

Fresh dissected retroperitoneal, mesenteric and inguinal adipose tissue samples (n= 4) were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Samples were then washed in phosphate buffer and dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin blocks. Five-micrometer-thick sections of the tissues were stained with hematoxylin and eosin to assess morphology.

Images from light microscopy were digitalized and the diameter of at least 100 adipocytes of each section was determined using Axio Vision software (Carl Zeiss Imaging Solutions).

Quantification of DNA levels

For quantification of DNA levels, 0.3 g of adipose tissue was homogenized in 0.6 ml of PBS (dilution 1:3, w:v) using a polytron homogenizer, and was then centrifuged at 500 g for 10 min; the supernatant was collected and used for DNA quantification by a fluorometric method that uses 3,5 diaminobenzoic acid [18].

Table 2. Features of different adipose tissue depots. Animals were killed by decapitation under *ad libitum* feeding conditions. The retroperitoneal, mesenteric and inguinal white adipose tissue depots (rWAT, mWAT, iWAT) were rapidly removed and weighed. DNA content and adipocyte diameter were measured as described in the material and methods section. Results are means $\pm$ SEM. Mass and DNA content was measured in 6 animals and adipocyte diameter in 4 animals. $a \neq b$ ($p < 0.05$, one-way ANOVA and LSD post-hoc test).

	rWAT	mWAT	iWAT
Mass (g)	3.31 ± 0.35 (a)	2.26 ± 0.17 (b)	3.62 ± 0.20 (a)
DNA content ($\mu\text{g/g}$ wet tissue)	203 ± 13 (a)	801 ± 20 (b)	844 ± 84 (b)
Adipocyte diameter (μm)	62.9 ± 3.5 (a)	40.3 ± 1.3 (b)	43.3 ± 1.3 (b)

Fig. 1. Resin-embedded, 5- μm -thick sections of retroperitoneal, mesenteric, and inguinal white adipose tissue stained with hematoxylin and eosin. Animals were killed by decapitation under *ad libitum* feeding conditions. Samples were processed as described in the material and methods section. Bar=12 μm .

RNA extraction

Total RNA was extracted from retroperitoneal, mesenteric and inguinal adipose tissue samples by Tripure Reagent (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nadrop Technologies Inc., Wilmington, Delaware USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR) analysis

Real-time polymerase chain reaction was used to measure mRNA expression levels of ACC1, ACOX1, ATGL, CPT1, CD36, FAS, GPAT, GLUT4, HKII, HSL, LPL, PPAR α , PPAR γ 2, and SREBP1c in the different adipose tissue depots.

Total RNA was reversed transcribed to cDNA as previously described [19]. RT qPCR was completed using the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic GmbH, Mannheim, Germany). Each PCR was performed as previously described [19]. The primers and cycling conditions used are described in Table 1. All primers were obtained from Sigma (Madrid, Spain).

Relative quantification of gene expression was made based on efficiency and the crossing point deviation of an unknown sample *vs* a control, and expressed in comparison to a reference gene (18S), as previously described [19]. 18S was previously validated as a suitable internal control in the conditions of this study, and no differences were found in CT values among the different adipose tissue depots.

Western blot analysis

ACC1 and ATGL analysis in the different adipose tissue depots was performed by Western blot. Tissue was homogenized at 4 °C in 1:2 (w:v) of PBS in a Teflon glass homogenizer. The homogenate was centrifuged at 500 g for 10

min at 4 °C and the supernatant used for total protein and ACC1 and ATGL analysis. Total protein content was measured by the method of Bradford [20]. For ACC1 analysis, 100 μg of protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 20% 2-mercaptoethanol. Total protein was fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 6% polyacrylamide) and electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-ACC1 antibody (Cell Signaling Technology, Inc., MA, USA, ref. number 3662), diluted 1:1000, and then with the secondary biotinylated anti-rabbit IgG antibody conjugated to a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Barcelona, Spain) diluted 1:5000. For ATGL analysis, 15 μg of protein was solubilized and boiled for 3 min in Laemmli sample buffer containing 20% 2-mercaptoethanol. Total protein was fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) and electrotransferred onto a PVDF membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, the membrane was incubated with the primary rabbit polyclonal anti-ATGL antibody (Cayman Chemical, Ann Arbor, MI, USA, ref. number 10006409), diluted 1:1000, and then with the secondary biotinylated anti-rabbit IgG antibody conjugated to a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences, Barcelona, Spain) diluted 1:5000. The immunocomplexes were revealed using an ECLplus (Amersham Biosciences, Barcelona, Spain). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). The films were scanned and the bands were quantified as previously described [21].

Fig. 2. Expression of lipogenesis related genes in the retroperitoneal, mesenteric, and inguinal white adipose tissue depots (rWAT, mWAT, iWAT) of rats under *ad libitum* feeding conditions. mRNA levels were measured by real-time PCR and expressed relative to the maximum value for each gene in the different depots. Data are means $\pm$ SEM (n = 6). Genes determined were: peroxisome proliferator activated receptor gamma 2 (PPAR γ 2), sterol regulatory element binding protein 1c (SREBP1c), acetyl-coenzyme A carboxylase alpha (ACC1), fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), lipoprotein lipase (LPL), fatty acid translocase (CD36), glucose transporter 4 (GLUT4), and hexokinase II (HKII). a $\neq$ b $\neq$ c (p<0.05, one-way ANOVA and LSD post-hoc test).

Genes determined were: peroxisome proliferator activated receptor gamma 2 (PPAR γ 2), sterol regulatory element binding protein 1c (SREBP1c), acetyl-coenzyme A carboxylase alpha (ACC1), fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), lipoprotein lipase (LPL), fatty acid translocase (CD36), glucose transporter 4 (GLUT4), and hexokinase II (HKII). a $\neq$ b $\neq$ c (p<0.05, one-way ANOVA and LSD post-hoc test).

Statistical Analysis

All data are expressed as the mean $\pm$ SEM. One-way ANOVA was used to assess differences between depots. Least significance differences test was used for post-hoc comparisons. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at p< 0.05.

Results

Morphological features of different fat depots

Two different depots, the retroperitoneal and the mesenteric ones, were chosen as representative of internal/visceral adipose tissue, while the inguinal depot was selected as representative of subcutaneous adipose tissue. The mean weight, adipocyte diameter and DNA content of these adipose depots in three-month-old male Wistar rats (weighing 366 ± 3 g) is shown in Table 2. The size of the retroperitoneal and inguinal depots in these animals was similar and larger than the size of the mesenteric depot. The measure of DNA content, determined to estimate cellularity, showed that the retroperitoneal depot had a lower (approximately 4 times less) DNA content per g wet tissue than the mesenteric and inguinal depots. In addition, the measure of adipocyte diameter (Table 2 and Figure 1), determined in representative sections of these depots, showed that the retroperitoneal also had a larger adipocyte size (50% more) compared with the mesenteric and inguinal depots.

Comparison of the mRNA expression levels of lipogenesis related genes in different fat depots

The pattern of mRNA expression of lipogenesis related genes is shown in Figure 2. The expression of the

transcription factors involved in lipogenesis, PPAR γ 2 and SREBP1c, as well as the expression of genes encoding other proteins involved in lipogenesis, such as the lipogenic enzymes ACC1 and GPAT, or proteins providing fatty acids or glucose to adipocytes, such as the LPL enzyme and the CD36 and GLUT4 transporters, showed maximum levels of expression in the retroperitoneal adipose tissue depot, and significantly higher than the expression levels in the mesenteric and the inguinal depots. In addition, the expression levels of PPAR γ 2, ACC1, CD36 and GLUT4 were also significantly higher in the mesenteric than in the inguinal depot. In the case of GPAT and LPL, their levels of expression did not significantly differ between both depots (mesenteric and inguinal), while levels of SREBP1c were significantly higher in the inguinal depot. HKII was similarly expressed in the retroperitoneal and mesenteric depots, and in both of them the expression was higher than in the inguinal depot. The pattern of expression of FAS was different to that of the other lipogenic genes studied, since maximum expression levels were found in the inguinal depot, followed by the retroperitoneal (although differences were not statistically significant between both depots), and lastly by the mesenteric depot.

Comparison of the mRNA expression levels of lipolysis and fatty acid oxidation related genes in different fat depots

The expression of the genes encoding enzymes involved in lipid mobilization, HSL and ATGL, was significantly higher in the retroperitoneal than in the mesenteric and inguinal depots (Figure 3A). No significant differences were found concerning the expression of both genes between the mesenteric and the inguinal depots, although levels of expression were slightly higher in the

Fig. 3. Expression of lipolysis (A) and fatty acid oxidation (B) related genes in the retroperitoneal, mesenteric, and inguinal white adipose tissue depots (rWAT, mWAT, iWAT) of rats under ad libitum feeding conditions. mRNA levels were measured by real-time PCR and expressed relative to the maximum value for each gene in the different depots. Data are means $\pm$ SEM (n = 6). Genes determined were: hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), peroxisome proliferator activated receptor alpha (PPAR α), carnitine palmitoyltransferase 1a (CPT1) and acyl-coenzyme A oxidase 1 (ACOX1). $a \neq b \neq c$ ($p < 0.05$, one-way ANOVA and LSD post-hoc test).

Fig. 4. Protein levels obtained by western blot analysis of lipogenesis (A) and lipolysis (B) related proteins in the retroperitoneal, mesenteric, and inguinal white adipose tissue depots (rWAT, mWAT, iWAT) of rats under ad libitum feeding conditions. Protein levels are expressed relative to the maximum value for each gene in the different depots. Data are means $\pm$ SEM (n = 5). Proteins determined were: (A) acetyl-coenzyme A carboxylase alpha (ACC1) and (B) adipose triglyceride lipase (ATGL). $a \neq b$ ($p < 0.05$, one-way ANOVA and LSD post-hoc test).

mesenteric than in the inguinal depot. A strong positive correlation was found between the pattern of expression of both genes in the different depots (Pearson's correlation coefficient $r = 0.832$, $p < 0.01$). In addition, the pattern of expression of both genes was also positively correlated with the patterns of the lipogenic transcription factors PPAR γ 2 and SREBP1c, as well as with the patterns of other lipogenesis related genes (ACC1, GPAT, LPL, CD36 and GLUT4) ($p < 0.05$).

The expression of genes encoding proteins related with fatty acid oxidation, particularly the transcription factor PPAR α and the enzymes CPT1 and ACOX1, showed different patterns in the different tissues (Figure 3B). PPAR α showed the maximum expression levels in the mesenteric depot, followed by the inguinal depot and lastly by the retroperitoneal one; CPT1 showed higher expression levels in the inguinal and mesenteric depots and lower expression levels in the retroperitoneal depot, while ACOX1 showed a tendency to higher expression levels in the retroperitoneal and mesenteric depots and lower expression levels in the inguinal depot, although differences were not statistically significant. The pattern

of PPAR α expression in the different depots correlated negatively with that of SREBP1c, LPL, HSL and ATGL ($p < 0.05$).

Comparison of protein levels of ACC1 and ATGL in different fat depots

To ascertain whether changes at the mRNA level among the different fat depots are associated with changes at the protein level, protein levels of ACC1, as representative of proteins involved in lipogenesis, and ATGL, as representative of lipolysis, were determined by western blot.

Figure 4A shows the specific protein levels of ACC1 in the three adipose depots studied. The mesenteric depot showed significantly higher ACC1 levels than the other ones. No significant differences were found between the retroperitoneal and the inguinal depots, although ACC1 levels tended to be higher in the retroperitoneal than in the inguinal depot.

Specific levels of the ATGL protein in the different adipose tissue depots studied (Figure 4B) followed the same tendency as their mRNA levels. The retroperitoneal

depot was the one with the highest levels of ATGL, followed by the mesenteric one (without statistical differences between them) and finally by the inguinal depot, which showed significantly lower levels than those of the internal depots studied.

Discussion

Adipose tissue is considered as a heterogeneous organ distributed in different anatomical sites, with different features and functions, which may be associated to a different pattern of expression of genes involved in lipid metabolism and handling [1, 2]. Here we have characterized the patterns of expression of key genes involved in lipogenesis, lipolysis and fatty acid oxidation and found different patterns of expression, which are related to the morphologic and metabolic differences between adipose tissue depots.

In the rats used in this study (3-month-old rats, weighing 366 ± 3 g), the inguinal was the largest adipose depot of those studied (representing approx 1% of the rat total weight), followed by the retroperitoneal (0.9%) and finally by the mesenteric (0.6%). These data are coherent with previously published results [22, 23]. On the other hand, the retroperitoneal depot was the one with the largest adipocyte size and with the lowest DNA content per g tissue, while no differences were found in these parameters when comparing the mesenteric and the inguinal depots. These results highlight the important morphologic differences between fat depots, even when comparing internal depots.

Moreover, it is well established that there are differences in fat cell metabolism depending on the anatomical location. *In vivo* and *in vitro* studies have demonstrated that internal adipose tissue depots have a higher metabolic rate and higher turnover rate of TG than subcutaneous depots, which has been related with the adverse metabolic impact of visceral fat accumulation [2, 10]. Here we have found that the described higher rates of lipid synthesis in internal depots compared with subcutaneous depots can be explained by higher expression levels of lipogenic factors.

It is known that lipogenesis in the adipose tissue is regulated by a set of transcription factors, with a main role of PPAR γ and SREBP1c [24]. PPAR γ consists of two protein isoforms, PPAR γ 1 and PPAR γ 2, with the former being expressed in a wide variety of tissues and the latter being preferentially expressed in adipose tissue [25]. In addition to its role orchestrating the process of

adipocyte differentiation, PPAR γ may promote the storage of fatty acids in mature adipocytes by acting on several steps including the induction of LPL, fatty acid binding protein (aP2), CD36, acyl-CoA synthase, phosphoenolpyruvate carboxykinase (PEPCK) and GLUT4 [24, 26, 27]. SREBP1c is responsible for the regulation of fatty acid biosynthetic genes such as FAS, acetyl CoA carboxylases [(ACC) ACC1 and ACC2], stearoyl CoA desaturase-1 (SCD1), GPAT, as well as HKII [28-30]. We found that both lipogenic transcription factors, PPAR γ 2 and SREBP1c, were highly expressed in the retroperitoneal depot compared with the mesenteric and the inguinal depots. The levels of PPAR γ 2 expression were also higher in the mesenteric than in the inguinal depot, while the pattern in these two depots was opposite concerning SREBP1c. Previous studies in rats have also reported higher expression levels of PPAR γ in the retroperitoneal adipose tissue compared with the inguinal depot [31]. In addition, higher expression of PPAR γ 2 and SREBP1c, as well as of the CCAAT enhancer-binding protein alpha (C/EBPalpha) - another key adipogenic transcription factor - has been previously described in gonadal (visceral) adipose tissue compared with inguinal adipose tissue in rats [32], and this has been related with a higher increase in the size of this fat depot under induction of obesity by cafeteria diet exposure [32]. Moreover, the expression of PPAR γ 2 in the retroperitoneal adipose tissue in rats has been reported to be very responsive to nutritional conditions, since its mRNA expression levels have been found to have already decreased after only 4 h fasting as well as increased after a short-term refeeding period of 3 h following 8 h fasting [33]. In contrast, no significant changes concerning PPAR γ 2 expression have been described in the mesenteric and inguinal depots under fasting conditions [15], indicative of the different metabolic flexibility of the fat depots.

In addition to PPAR γ 2 and SREBP1c, the expression of their downstream-regulated genes LPL, CD36 and GLUT4 (all three regulated by PPAR γ), and ACC1 and GPAT (both regulated by SREBP1c) in the different adipose tissues also followed similar patterns and were positively correlated with those of both PPAR γ 2 and SREBP1c ($p < 0.05$). The pattern of expression of HKII, regulated by SREBP1c, followed a different pattern to that of SREBP1c. The pattern of expression of these lipogenic related genes in the different fat depots agrees with the different features of visceral and subcutaneous adipose tissue depots. On the one hand, the activity of the insulin-activated glucose transporter GLUT4, the fatty

acid transporter CD36, and the LPL enzyme - which catalyses the hydrolysis of circulating TG to fatty acids - are important to provide substrates (glucose and fatty acids) for TG synthesis and storage in the adipose tissue. The higher expression levels of these three genes found in the retroperitoneal depot followed by the mesenteric and by the inguinal depot agrees with the higher capacity of this internal depot to store TG, and hence with its larger adipocyte size. On the other hand, the activities of ACC1, HKII, and GPAT enzymes are important for *de novo* fatty acid biogenesis and TG synthesis. ACC1 catalyzes the carboxylation of acetyl-CoA to malonyl CoA, which is the substrate for fatty acid biosynthesis, as well as the suppressor of fatty acid beta-oxidation [34]; HKII catalyses the first step of glucose oxidation [35]; and GPAT catalyses the first committed step in glycerolipid biosynthesis, and is regarded as the rate-limiting enzyme for the glycerol-3-phosphate biosynthetic pathway [36]. The expression of these genes in the different depots studied also showed similar patterns to the aforementioned genes, with maximum expression levels in internal depot and lower expression levels in the inguinal adipose depot, thus indicating the higher capacity of the formers for substrate uptake and *de novo* fatty acid and TG synthesis, and hence for TG accretion. Although these results are mainly based on gene expression, analysis by western blot of ACC1 showed that the mesenteric depot had higher levels of this protein compared with the other depots studied; conversely the inguinal depot showed the lowest levels of this protein. This tendency within internal depots is not exactly the same as found for mRNA expression levels (since ACC1 mRNA levels in the retroperitoneal depot were higher than in the mesenteric) but agrees with the higher lipogenic capacity of internal depots compared with the subcutaneous one. As a whole, these results may explain the differences described in regional fat accumulation in rats under high-fat diet feeding, being more marked in internal depots than in the inguinal subcutaneous fat depot [37].

In addition to ACC1, FAS is also a key lipogenic enzyme since it catalyses the conversion of acetyl-CoA and malonyl-CoA to palmitate. This enzyme plays a central role in *de novo* fatty acid synthesis and in the long-term regulation of lipogenesis [38]. The pattern of FAS expression between internal and subcutaneous followed different characteristics to that of ACC1 and GPAT, and to that of other lipogenic genes, since the maximum expression levels were found in the inguinal depot and the lowest levels in the mesenteric depot. Levels of expression in the retroperitoneal depot did not differ

statistically to those of the inguinal depot, although they tended to be lower. Since in this study we have not determined enzyme activity, it becomes difficult to speculate the resulting capacity of fatty acid and TG synthesis, however, the patterns of expression of other enzymes involved in these processes, ACC1 and GPAT, as well as other genes related with substrate supply for TG synthesis such as LPL, CD36 and GLUT4, as well as HKII, agree with a higher lipogenic capacity of internal depots, particularly the retroperitoneal depot, compared with the inguinal subcutaneous depot. Other authors [39] have also reported lower FAS expression in visceral (mesenteric) fat compared with subcutaneous fat. According to these authors [39], a possible reason could lie in the high lipolytic activity of visceral depots resulting in high free fatty acid levels, which are known to inhibit FAS expression.

Concerning lipid mobilization, we found that the pattern of expression of genes encoding both proteins mainly involved in TG mobilization (HSL and ATGL) in the different fat depots studied - with higher mRNA levels in the retroperitoneal depot, followed by the mesenteric and the inguinal depots - were positively correlated with the patterns of the main genes involved in lipogenesis, thus suggesting that tissues which have the main rate of lipid synthesis and storage also have the main rate of lipid mobilization. Depot related differences at the protein level for the ATGL were essentially related with changes at the mRNA level, but no differences were found within the two internal depots studied. These results, suggesting that internal depots, and particularly the retroperitoneal one, have higher capacity to mobilize stored TG are consistent with previous results showing that ATGL mRNA levels increase after 8 h of fasting in the retroperitoneal depot but remain unaltered in the inguinal depot [15]. However internal depots, and particularly the retroperitoneal adipose tissue, may have, in turn, a lower capacity of mitochondrial fatty acid oxidation, as evidenced by lower mRNA expression levels of CPT1 - which encodes for the enzyme that catalyses the limiting rate for mitochondrial β -oxidation which constitutes the major pathway by which fatty acids are oxidized to generate energy [40]. This would suggest that free fatty acids resulting from TG mobilization in the retroperitoneal depot are mainly released into blood circulation, while the higher mRNA expression levels of CPT1 in the inguinal and mesenteric depots would suggest that free fatty acids released by these depots can be oxidized in a relatively higher proportion in the same depots through mitochondrial β -oxidation.

However, unlike mitochondrial fatty acid oxidation, no significant differences were found concerning the expression of ACOX1 gene - which encodes the first, rate-limiting enzyme in the peroxisomal β -oxidation of very-long-chain saturated and unsaturated fatty acids [41] - and even a tendency to higher levels of expressions was found in the internal depots compared with the subcutaneous depot. Nevertheless, under normal circumstances, peroxisomal β -oxidation is a minor pathway of fatty acid oxidation relative to the mitochondrial system [41].

These results showing that the retroperitoneal internal depot has a higher lipid mobilization capacity together with a lower fatty acid oxidation capacity in terms of RNA expression, agree with previously published results showing regional variations in the metabolic activity of fat cells, especially in regard to regulation of lipolysis [11, 12]. In fact, the adverse metabolic impact of visceral fat [1, 6] has been attributed to distinct biological properties of adipocytes in this depot, particularly the higher fatty acid turnover capacity of internal fat compared with other adipose tissue depots, which would be associated with greater mobilization of free fatty acids into the portal circulation, impairing liver metabolism and leading to glucose intolerance, insulin resistance, and dislipidaemia [2].

Taken together, these results demonstrate differences in the expression patterns of genes involved in lipid metabolism between different adipose tissue depots that are in accordance with site-specific differences in adipocyte metabolism and function. As a general trend, lipogenesis and lipolytic related genes are highly expressed in the retroperitoneal depot, which is also the depot with larger adipocytes, followed by the mesenteric and latterly by the inguinal depot. Levels of representative proteins encoded by these genes generally reinforced the results obtained by mRNA analysis, although with some differences particularly for the ACC1 within internal depots. On the other hand, the inguinal and the mesenteric

depots have a predominant expression of genes related with mitochondrial fatty acid oxidation, particularly CPT1, and of the lipolytic transcription factor PPAR α . These differences may have a strong impact on mitochondrial and lipid metabolism and, as such, could also explain the differential behavior of adipose depots in physiological and pathological situations such as metabolic disorders linked to obesity and lipodystrophies. Moreover, these results also show differences concerning adipocyte features and gene expression patterns that go beyond the simple classification of adipose tissue as internal/visceral and subcutaneous depots.

Abbreviations

ACC1 (acetyl-coenzyme A carboxylase alpha); ACOX1 (acyl-coenzyme A oxidase 1); ATGL (adipose triglyceride lipase); FAT/CD36 (fatty acid translocase); CPT1 (carnitine palmitoyltransferase 1a); FAS (fatty acid synthase); GLUT4 (glucose transporter 4); GPAT (glycerol-3-phosphate acyltransferase); HKII (hexokinase II); HSL (hormone-sensitive lipase); LPL (lipoprotein lipase); PPAR α (peroxisome proliferator activated receptor alpha); PPAR γ 2 (peroxisome proliferator activated receptor gamma 2); SREBP1c (sterol response element binding protein 1c).

Acknowledgements

This work was supported by the Spanish Government (grant AGL2006-04887/ALI). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, EU Contract: n $^{\circ}$ FP6-506360). The CIBER Fisiopatología de la obesidad y nutrición is an initiative of the ISCIII. We thank Enzo Ceresi for technical assistance in morphometric analysis.

References

- Despres JP, Nadeau A, Tremblay A, Ferland M, Moorjani S, Lupien PJ, Theriault G, Pinault S, Bouchard C: Role of deep abdominal fat in the association between regional adipose tissue distribution and glucose tolerance in obese women. *Diabetes* 1989;38:304-309.
- Giorgino F, Laviola L, Eriksson JW: Regional differences of insulin action in adipose tissue: insights from in vivo and in vitro studies. *Acta Physiol Scand* 2005;183:13-30.
- Evans DJ, Hoffmann RG, Kalkhoff RK, Kissebah AH: Relationship of body fat topography to insulin sensitivity and metabolic profiles in premenopausal women. *Metabolism* 1984;33:68-75.
- Brochu M, Poehlman ET, Ades PA: Obesity, body fat distribution, and coronary artery disease. *J Cardiopulm Rehabil* 2000;20:96-108.

- 5 Weiss R: Fat distribution and storage: how much, where, and how? *Eur J Endocrinol* 2007;157 Suppl 1:S39-45.
- 6 Despres JP: Health consequences of visceral obesity. *Ann Med* 2001;33:534-541.
- 7 Gabriely I, Ma XH, Yang XM, Atzmon G, Rajala MW, Berg AH, Scherer P, Rossetti L, Barzilai N: Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process? *Diabetes* 2002;51:2951-2958.
- 8 Gabriely I, Barzilai N: Surgical removal of visceral adipose tissue: effects on insulin action. *Curr Diab Rep* 2003;3:201-206.
- 9 Thorne A, Lonnqvist F, Apelman J, Hellers G, Arner P: A pilot study of long-term effects of a novel obesity treatment: omentectomy in connection with adjustable gastric banding. *Int J Obes Relat Metab Disord* 2002;26:193-199.
- 10 Jensen MD: Lipolysis: contribution from regional fat. *Annu Rev Nutr* 1997;17:127-139.
- 11 Martin ML, Jensen MD: Effects of body fat distribution on regional lipolysis in obesity. *J Clin Invest* 1991;88:609-613.
- 12 Rebuffe-Scrive M, Andersson B, Olbe L, Bjorntorp P: Metabolism of adipose tissue in intraabdominal depots of nonobese men and women. *Metabolism* 1989;38:453-458.
- 13 Vikman HL, Savola JM, Raasmaja A, Ohisalo JJ: Alpha 2A-adrenergic regulation of cyclic AMP accumulation and lipolysis in human omental and subcutaneous adipocytes. *Int J Obes Relat Metab Disord* 1996;20:185-189.
- 14 Portillo MP, Villaro JM, Torres MI, Macarulla MT: In vivo lipolysis in adipose tissue from two anatomical locations measured by microdialysis. *Life Sci* 2000;67:437-445.
- 15 Palou M, Sanchez J, Priego T, Rodriguez AM, Pico C, Palou A: Regional differences in the expression of genes involved in lipid metabolism in adipose tissue in response to short- and medium-term fasting and refeeding. *J Nutr Biochem* 2009.
- 16 Jamdar SC: Glycerolipid biosynthesis in rat adipose tissue. Influence of adipose-cell size and site of adipose tissue on triacylglycerol formation in lean and obese rats. *Biochem J* 1978;170:153-160.
- 17 Virtanen KA, Lonnroth P, Parkkola R, Peltoniemi P, Asola M, Viljanen T, Tolvanen T, Knuuti J, Ronnema T, Huupponen R, Nuutila P: Glucose uptake and perfusion in subcutaneous and visceral adipose tissue during insulin stimulation in nonobese and obese humans. *J Clin Endocrinol Metab* 2002;87:3902-3910.
- 18 Kissane JM, Robins E: The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J Biol Chem* 1958;233:184-188.
- 19 Pico C, Oliver P, Sanchez J, Miralles O, Caimari A, Priego T, Palou A: The intake of physiological doses of leptin during lactation in rats prevents obesity in later life. *Int J Obes (Lond)* 2007;31:1199-1209.
- 20 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.
- 21 Fuster A, Pico C, Sanchez J, Oliver P, Zingaretti MC, Murano I, Morroni M, Hoeller U, Goralczyk R, Cinti S, Palou A: Effects of 6-month daily supplementation with oral beta-carotene in combination or not with benzo[a]pyrene on cell-cycle markers in the lung of ferrets. *J Nutr Biochem* 2008;19:295-304.
- 22 DiGirolamo M, Fine JB, Tagra K, Rossmanith R: Qualitative regional differences in adipose tissue growth and cellularity in male Wistar rats fed ad libitum. *Am J Physiol* 1998;274:R1460-1467.
- 23 Deveaud C, Beauvoit B, Salin B, Schaeffer J, Rigoulet M: Regional differences in oxidative capacity of rat white adipose tissue are linked to the mitochondrial content of mature adipocytes. *Mol Cell Biochem* 2004;267:157-166.
- 24 Rosen ED, Spiegelman BM: PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. *J Biol Chem* 2001;276:37731-37734.
- 25 Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W: From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog Lipid Res* 2006;45:120-159.
- 26 Wu Z, Xie Y, Morrison RF, Bucher NL, Farmer SR: PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. *J Clin Invest* 1998;101:22-32.
- 27 Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N: Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. *J Biol Chem* 1998;273:16710-16714.
- 28 Horton JD, Goldstein JL, Brown MS: SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125-1131.
- 29 Oh SY, Park SK, Kim JW, Ahn YH, Park SW, Kim KS: Acetyl-CoA carboxylase beta gene is regulated by sterol regulatory element-binding protein-1 in liver. *J Biol Chem* 2003;278:28410-28417.
- 30 Gosmain Y, Dif N, Berbe V, Loizon E, Rieusset J, Vidal H, Lefai E: Regulation of SREBP-1 expression and transcriptional action on HKII and FAS genes during fasting and refeeding in rat tissues. *J Lipid Res* 2005;46:697-705.
- 31 Laplante M, Festuccia WT, Soucy G, Gelinas Y, Lalonde J, Berger JP, Deshaies Y: Mechanisms of the depot specificity of peroxisome proliferator-activated receptor gamma action on adipose tissue metabolism. *Diabetes* 2006;55:2771-2778.
- 32 Rodriguez E, Ribot J, Rodriguez AM, Palou A: PPAR-gamma2 expression in response to cafeteria diet: gender- and depot-specific effects. *Obes Res* 2004;12:1455-1463.
- 33 Palou M, Priego T, Sanchez J, Villegas E, Rodriguez AM, Palou A, Pico C: Sequential changes in the expression of genes involved in lipid metabolism in adipose tissue and liver in response to fasting. *Pflugers Arch* 2008;456:825-836.
- 34 Kim KH: Regulation of mammalian acetyl-coenzyme A carboxylase. *Annu Rev Nutr* 1997;17:77-99.
- 35 Wilson JE: Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol* 2003;206:2049-2057.
- 36 Coleman RA, Reed BC, Mackall JC, Student AK, Lane MD, Bell RM: Selective changes in microsomal enzymes of triacylglycerol phosphatidylcholine, and phosphatidylethanolamine biosynthesis during differentiation of 3T3-L1 preadipocytes. *J Biol Chem* 1978;253:7256-7261.
- 37 Priego T, Sanchez J, Pico C, Palou A: Sex-differential Expression of Metabolism-related Genes in Response to a High-fat Diet. *Obesity (Silver Spring)* 2008;16:819-826.
- 38 Semenkovich CF: Regulation of fatty acid synthase (FAS). *Prog Lipid Res* 1997;36:43-53.
- 39 Li Y, Bujo H, Takahashi K, Shibasaki M, Zhu Y, Yoshida Y, Otsuka Y, Hashimoto N, Saito Y: Visceral fat: higher responsiveness of fat mass and gene expression to calorie restriction than subcutaneous fat. *Exp Biol Med (Maywood)* 2003;228:1118-1123.
- 40 Kerner J, Hoppel C: Fatty acid import into mitochondria. *Biochim Biophys Acta* 2000;1486:1-17.
- 41 Hashimoto T: Peroxisomal beta-oxidation enzymes. *Neurochem Res* 1999;24:551-563.