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White adipose tissue genome wide-expression profiling and adipocyte metabolic functions after soy protein consumption in rats☆

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Abstract

Obesity is associated with an increase in adipose tissue mass due to an imbalance between high dietary energy intake and low physical activity; however, the type of dietary protein may contribute to its development. The aim of the present work was to study the effect of soy protein versus casein on white adipose tissue genome profiling, and the metabolic functions of adipocytes in rats with diet-induced obesity. The results showed that rats fed a Soy Protein High-Fat (Soy HF) diet gained less weight and had lower serum leptin concentration than rats fed a Casein High-Fat (Cas HF) diet, despite similar energy intake. Histological studies indicated that rats fed the Soy HF diet had significantly smaller adipocytes than those fed the Cas HF diet, and this was associated with a lower triglyceride/DNA content. Fatty acid synthesis in isolated adipocytes was reduced by the amount of fat consumed but not by the type of protein ingested. Expression of genes of fatty acid oxidation increased in adipose tissue of rats fed Soy diets; microarray analysis revealed that Soy protein consumption modified the expression of 90 genes involved in metabolic functions and inflammatory response in adipose tissue. Network analysis showed that the expression of leptin was regulated by the type of dietary protein and it was identified as a central regulator of the expression of lipid metabolism genes in adipose tissue. Thus, soy maintains the size and metabolic functions of adipose tissue through biochemical adaptations, adipokine secretion, and global changes in gene expression.

Keywords: Adipose tissue; Soy protein; Transcriptome; Leptin; Obesity

1. Introduction

An increase in adipose tissue is the main cause of the metabolic abnormalities that occur during the development of obesity [1–3]. This is because the adipocyte plays an important role in the management of dietary energy excess, releasing signaling molecules called adipokines that regulate the oxidation of metabolic fuels in several organs, including the liver, skeletal muscle, heart and pancreas [1], preventing the accumulation of lipids in non-adipose tissues avoiding lipotoxicity [4].

Several adipokines play major roles in body energy homeostasis, primarily leptin [5,6]. In addition to its function as a hypothalamic regulator of food intake [7], this hormone stimulates the leptin receptor in several tissues, mainly skeletal muscle, to increase fatty acid oxidation [8]. However, the contribution of other adipokines such as adiponectin, resistin and tumor necrosis factor alpha (TNF-α) is also important to prevent or induce lipotoxicity and insulin resistance [9]. The secretion and proportion of these adipokines are of vital importance to avoid metabolic abnormalities that occur as a consequence of obesity.

Diet is a key factor that can modify the biochemical and molecular tasks of adipocytes. The type and amount of fat and carbohydrates in the diet stimulate to various extents the accumulation of lipids in adipocytes [10]. High-fat diets promote the entrance of fatty acids from chylomicrons into adipocytes, increasing their accumulation as triglycerides. On the other hand, high carbohydrate diets stimulate adipocyte lipogenesis, also increasing the amount of triglycerides in the lipid droplets of adipocytes [11,12]. However, it has been shown that only a limited amount of triglycerides can be stored in the adipose tissue of each individual. This depends on the adipose tissue capacity to expand, which is genetically determined, and is a result of an increase in the number of metabolically functional adipocytes [13]. However, to date, there has been little evidence that dietary protein type may influence the gene expression profile and its consequences on the biochemistry and physiology of the adipose tissue, particularly during the consumption of a high-fat diet. It has been shown in rats long-term fed a Soy Protein (Soy) diet that had lower serum and hepatic

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Table 1
Composition of experimental diets

Ingredients (%)	Cas	Soy	Cas HF	Soy HF
Casein[a] or Soy Protein[b]	33.11	34.09	33.11	34.09
Dextrose	27.86	27.37	17.86	17.37
Cornstarch	27.86	27.37	17.86	17.37
Corn oil	5	5	5	5
Lard	0	0	20	20
Mineral mix[c]	5	5	5	5
Vitamin mix[d]	1	1	1	1

[a] "Vitamin-free" casein, Harlan Teklad research diets, Madison, WI.
[b] Supro 710, Solae, México City.
[c] Rogers-Harper, Harlan Teklad research diets, Madison, WI.
[d] AIN-93-VX, Harlan Teklad research diets, Madison, WI, USA: (mg/kg diet): nicotinic acid. 150; calcium pantothenate, 80; pyridoxine-HCl, 35; thiamin HCl, 30; riboflavin, 30; folic acid, 10; D-biotin, 1; vitamin B12; 0.1; DL-alpha tocopheryl acetate, (500 IU/g), 750; vitamin A palmitate (500,000 IU/g), 40; vitamin D_3 (cholecalciferol, 500,000 IU/g), 10; vitamin K (phylloquinone), 3.75.

lipids, body weight gain, carcass body fat and adipocyte size than rats fed Casein diet (Cas) [14–16].

These data suggest that consumption of a Soy diet may change the biochemical and molecular functions of adipocytes, which in turn reduces the metabolic syndrome abnormalities observed during obesity [15]. However, it is not known whether soy protein is able to modify the adipocyte transcriptome and metabolism, which could explain the phenotypic changes observed in these animals. Thus, the aim of the present study was to establish whether the consumption of a Soy diet modifies the gene expression profile of adipose tissue, the lipogenic and lipolytic capacity and endocrine functions of this tissue.

2. Methods and materials

2.1. Animals

Four week-old male Sprague Dawley rats weighing an average of 100 g were bought from Harlan Teklad (Mexico City). All rats were housed in individual cages in a controlled temperature environment with a 12-h light/12-h dark cycle, and had free access to water and food. These rats were fed four different diets for 160 days. The diets were prepared from basic ingredients as described in Table 1. High-fat diets [25% fat; Casein High-Fat (Cas HF) and Soy Protein High-Fat (Soy HF)] contained 5 kcal/g, and control diets (5% fat; Cas and Soy) contained 4 kcal/g. The diets had 30% of either soy protein or casein, and the dietary protein concentration was adjusted on the basis of protein purity (casein 90.6%, soy protein 86%). Twelve-hour fasted animals were killed with carbon monoxide and guillotine. The serum was stored at −20°C for measuring biochemical parameters, and adipose tissue was stored at −70°C for the extraction of RNA, DNA, or triglycerides. For adipocyte culture, the rats were killed as described, and the retroperitoneal fat pad was immediately minced and digested as explained below. The protocol for the present study was approved by the Animal Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán."

2.2. Serum biochemical parameters

The serum cholesterol and triglyceride concentrations were measured by colorimetric kits (DiaSys Diagnostic Systems, Holzheim, Germany). The serum free fatty acid (FFA) concentration was assayed with a FFA Half Micro Test (Roche Applied Science, Indianapolis IN). Serum leptin and adiponectin were assayed by radioimmunoassay (RIA) kits (Linco Research, St. Charles, MO, USA). TNF-α was measured by ELISA (Alpco Diagnostics, Salem, NH, USA), and insulin and interleukin-6 (IL-6) were measured by a Lincoplex kit (Linco Research, St. Charles, MO, USA).

2.3. Real-time quantitative polymerase chain reaction

Total RNA was extracted from fat tissues by Chomczynski's method using guanidine thiocyanate [17]. For integrity analysis, 15 μg of total RNA were separated by 1.0% agarose/2.2 M formaldehyde gel electrophoresis. Three hundred nanograms of total RNA from each animal were subjected to reverse transcription prior to polymerase chain reaction (PCR) amplification with the Two-Step Master Mix (Applied Biosystems, Foster City, CA, USA). Parallel non-template control (NTC) reactions were run in the absence of RNA to detect nucleic acid contamination in the reaction mixtures. TaqMan fluorogenic probes and oligonucleotide primers were designed by Applied Biosystems. TaqMan PCR assays for each gene target were carried out in triplicate in 96-well optical plates with an ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). For every PCR sample, an amplification plot was generated from the collected data, and a threshold cycle (CT) value was calculated with the software suite. With the use of the standard curve, CT values for each gene were used to calculate the initial quantity of cDNA. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an invariant control. Taqman assays for peroxisome proliferator-activated receptor alpha (PPARα; Rn00566193_m1), PPARγ (Rn00440945_m1), sterol-regulatory element binding protein-1 (SREBP-1; ATOVARPE1_PE1), HMG-CoA reductase (Rn00565598_m1), stearoyl coenzyme A desaturase (SCD-1; Rn00594894_m1), leptin (Rn00565158_m1), fatty acid synthase (FAS; Rn00569117_m1), UCP-1 (uncoupling protein-1; Rn00562126), carnitine palmitoyl transferase 1 (CPT-1; Rn00580702), glucose transporter GLUT4 (Rn00562597_m1), and HPRT (Rn01527840) were obtained from Applied Biosystems.

2.4. Microarray analysis

Total RNA from the adipose tissue of 12 rats (3 Cas, 3 Soy, 3 Cas HF and 3 Soy HF) was isolated as previously described. The RNA quality of each sample was assessed with the Bioanalyzer 2100 (Agilent Technologies). The RNA samples from each diet were pooled to prepare a single 2.5-μg sample. The four samples with one technical replicate each were labeled and hybridized to an Affymetrix Rat Expression Array 230A (RAE230A) according to the manufacturer's protocol. Briefly, 2.5 μg of pooled RNA was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly (T) oligomer. Second strand cDNA synthesis was carried out with T4 DNA polymerase, followed by an in vitro transcription, during which biotinylated dNTPs were incorporated into the generated cRNA. The cRNA products were fragmented into strands of 200 base pairs or less, and 15 μg of the fragmentation product were added to the hybridization cocktail [100 mM 2-(*n*-morpholino)-ethanesulfonic acid (MES), 1 M NaCl, 20 mM EDTA, 0.01% Tween-20, 0.1 mg/ml of herring sperm DNA, and 0.5 mg/ml acetylated bovine serum albumin]. Then 300 μl of each sample was heated to 95°C and hybridized to RAE230A (Affymetrix) for 16 h at 45°C. The samples were washed with low (6×saline-sodium-phosphate-EDTA [SSPE]) and high (100 mM MES, 0.1 M NaCl) stringency buffers and stained with streptavidin-phycoerythrin. The RAE230A array contains 15,866 probesets for the

Table 2
Weight gain, food and energy intake, serum triglyceride, cholesterol and free fatty acid concentration, and adiponectin/leptin ratio in rats fed Casein or Soy Protein containing adequate or high fat content for 160 days

Parameter	Cas	Cas HF	Soy	Soy HF	*P*		
					Protein	Fat	Protein*fat
Weight gain (g)	364±8.44	409±7.90	346±6.91	396±11.1	0.0785	<.0001	NS
Food intake (g/d)	19.5±0.1	12.8±0.7	17.8±0.7	13.9±0.4	0.0662	<.0001	NS
Energy intake (kcal/d)	75.4±3.7	78.4±3.3	77.1±3.4	74.9±2.9	0.0655	NS	NS
Retroperitoneal adipose tissue weight (g)	8.22±0.53[b]	12.9±1.7[a]	4.0±0.7[c]	6.3±0.5[b,c]	NS	NS	0.0294
Serum triglycerides (mmol/L)	0.42±0.09	0.43±0.03	0.24±0.05	0.36±0.05	0.0261	NS	NS
Serum cholesterol (mmol/L)	2.29±0.05	2.50±0.08	2.08±0.05	2.57±0.06	NS	NS	NS
Free fatty acids (mmol/L)	1.13±0.08	1.72±0.17	0.99±0.05	1.26±0.02	0.0005	<.0001	0.012
Serum leptin (μg/ml)	3.08±0.07[c]	8.07±0.24[a]	2.12±0.2[d]	4.14±0.4[b]	0.0002	<.0001	0.0052
Serum adiponectin (μg/ml)	56.1±5.3	70.2±19.5	60.8±6.9	45.7±9.7	0.033	NS	NS
Adiponectin/leptin ratio	2.28±0.12	0.88±0.01	3.54±0.58	1.38±0.25	NS	.0007	NS
Serum IL-6 (pg/ml)	135±25.7	112±32.2	65.5±18.2	133±46.4	NS	NS	NS
Serum glucose (mmol/L)	5.05±0.07	6.83±0.06	5.00±0.29	6.24±0.15	NS	<.001	NS
Serum insulin (pg/ml)	515±59.8[b]	845±195[a]	115±27.2[c]	478±55[b]	NS	NS	.05
Serum TNF-α (pg/ml)	6.28±0.60	7.27±0.63	6.51±0.37	6.29±0.57	NS	NS	NS

Values are means±S.E.M. Different superscript letters[a,b,c,d] indicate significant differences among row (P<.05). n=15 rats/per group.

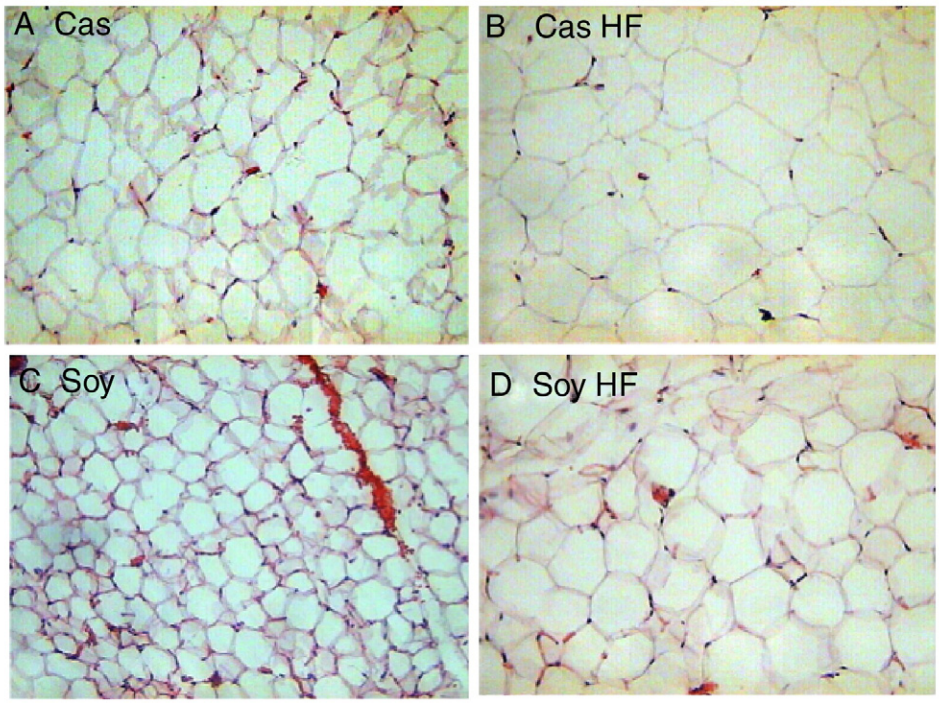

Fig. 1. Hematoxylin and eosin staining reveals decreased adipocyte size in retroperitoneal adipose tissue from rats fed Soy Protein. Adipose tissue from rats fed Cas (A), Cas HF (B), Soy (C), and Soy HF (D) for 160 days. Results represent the mean±S.E.M., $n=5$. Means without a common letter differ ($P<.05$).

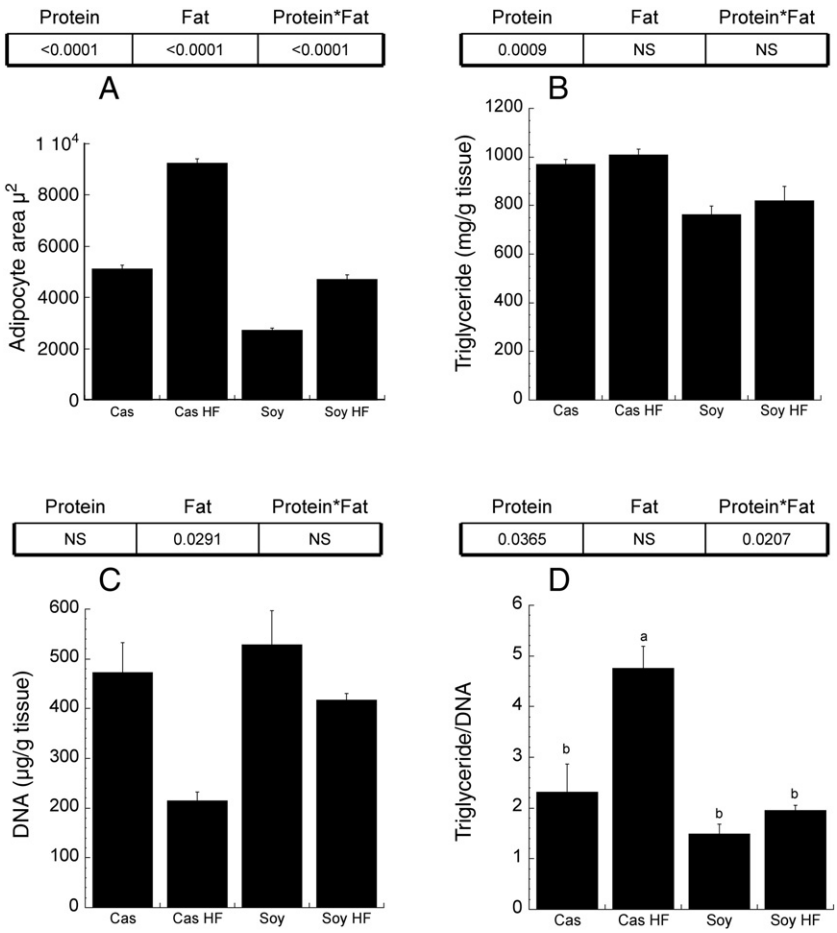

Fig. 2. Soy protein consumption prevents adipocyte hypertrophy. (A) Adipocyte area. (B) Adipose tissue triglyceride content. (C) Adipose tissue DNA content. (D) Adipose tissue triglyceride/DNA ratio from rats fed Cas, Cas HF, Soy and Soy HF for 160 days. Results represent the mean±S.E.M., $n=5$. Means without a common letter differ, $P<.05$.

analysis of approximately 14,200 genes from the UniGene (NCBI, 2002) GenBank (National Center for Biotechnology Information [NCBI], Release 129, April 2002), and RefSeq (NCBI, June 2002) rat sequence data. A GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA) was used to collect fluorescent signal, and GCOS software (Affymetrix, Santa Clara, CA, USA) was used to obtain intensity signal and quality data from the scanned arrays.

2.5. Analysis and functional interpretation of microarray data

Each microarray experiment was duplicated as a technical replicate for statistical robustness. Low-level data analysis included two normalization processes: quantile normalization [18] of technical replicates and Loess normalization applied to all microarrays to standardize the dynamic range of expression. To identify differentially expressed genes, a linear model with an empirical Bayes approach was implemented using R and the Limma package [19,20]. The selection of differentially expressed genes was based on two statistical criteria. First, genes with log-odds ≥ 3 were selected. Then a hierarchical search of genes was performed based on log-fold changes. The expression values in Tables 3, 4 and 5 are represented as fold changes (FC) between Cas vs. Soy, or Cas HF vs. Soy HF.

Biological interaction networks of regulated genes in adipose tissue from rats fed Soy versus Cas adequate or high fat diets were identified using Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA, USA). For this purpose, a data set containing significantly up- or down-regulated genes was analyzed with the Ingenuity software. Expression values of genes exhibiting significance were processed by R software (http://www.cran.r-project.org) for hierarchical clustering analysis.

2.6. Adipocyte culture

Adipocytes were obtained from retroperitoneal fat pads. Fat depots were resected under aseptic conditions, and adipocytes were isolated by collagenase digestion according to the procedure of Rodbell [21] with minor modifications as described below. The fat pads were minced in Krebs-Ringer HEPES buffer (pH 7.4) containing 5 mM D-glucose, 2% bovine serum albumin, 135 mM NaCl, 2.2 mM $CaCl_2$-$2H_2O$, 1.25 mM $MgSO_4$-$7H_20$, 0.45 mM KH_2PO_4 and 10 mM HEPES. Adipose tissue fragments were digested in Krebs-Ringer HEPES buffer with 1.25 mg/ml Type II collagenase at 37°C with gentle shaking at 80 cycles/min for 45 min. The resultant cell suspension was diluted in 13 ml of cold Krebs-Ringer HEPES buffer. For washing, cells were resuspended in 50 ml of buffer and centrifuged at 400×g for 5 min. The final wash was made with 13 ml of culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum]. Floating cells were collected as primary adipocytes, plated on 6-cm dishes, and cultured at 37°C in 5% CO_2. Each replicate of 10^5 adipocytes was incubated with 2 ml of DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin mixture and 0.5 mM sodium [^{14}C] acetate (18 dpm/pmol) for 3 h. Incorporation of [^{14}C] acetate into fatty acids and cholesterol was determined as described below.

2.7. Fatty acid and cholesterol synthesis

After 3 h of incubation with 0.5 mM [1-^{14}C] acetate (18–21 dpm/pmol), the medium was aspirated, and the cells were collected, washed with Krebs Ringer containing 10 mM HEPES pH 7.4, and lysed with 0.1 M NaOH. The medium and cell suspension were combined, and a 100-µl aliquot was removed for protein determination. For each cell suspension, 2 ml of ethanol, 1 ml 75% KOH, 10 µl [1,2 ^{3}H] cholesterol (111 000 dpm/pmol) carrier solution, and 10µl [9,10 (n) ^{3}H] oleic acid (17 760 dpm/pmol) carrier solution were added before cholesterol and fatty acid extraction. Cholesterol was extracted with 3.5 ml petroleum ether, and the resultant aqueous phase was used to extract fatty acids with 3 ml hexane. Both phases were evaporated to dryness. The residues were spotted onto silica gel thin-layer chromatography (TLC) plates and developed in chloroform for cholesterol analysis or in heptane: diethyl ether: acetic acid (90:30:1) for fatty acid analysis. Spots were visualized with iodine vapors, excised, placed in scintillation vials with scintillation solution, and then counted. The results are expressed as picomoles of [^{14}C] acetate incorporated into fatty acids or cholesterol per microgram of cellular protein.

2.8. Glycerol release from adipocytes

The measurement for glycerol content was performed according Yu and Zhu [22]. Briefly, primary adipocytes were incubated in DMEM containing fetal bovine serum for 1 h. Then the medium was collected, and free glycerol was measured by colorimetric analysis (Trig/GB Roche Diagnostics).

2.9. Determination of triglyceride and DNA in adipose tissue

The isolation and determination of triglyceride and DNA content was performed according to Okuno [23]. Briefly, 0.5 g of adipose tissue was removed and frozen at –

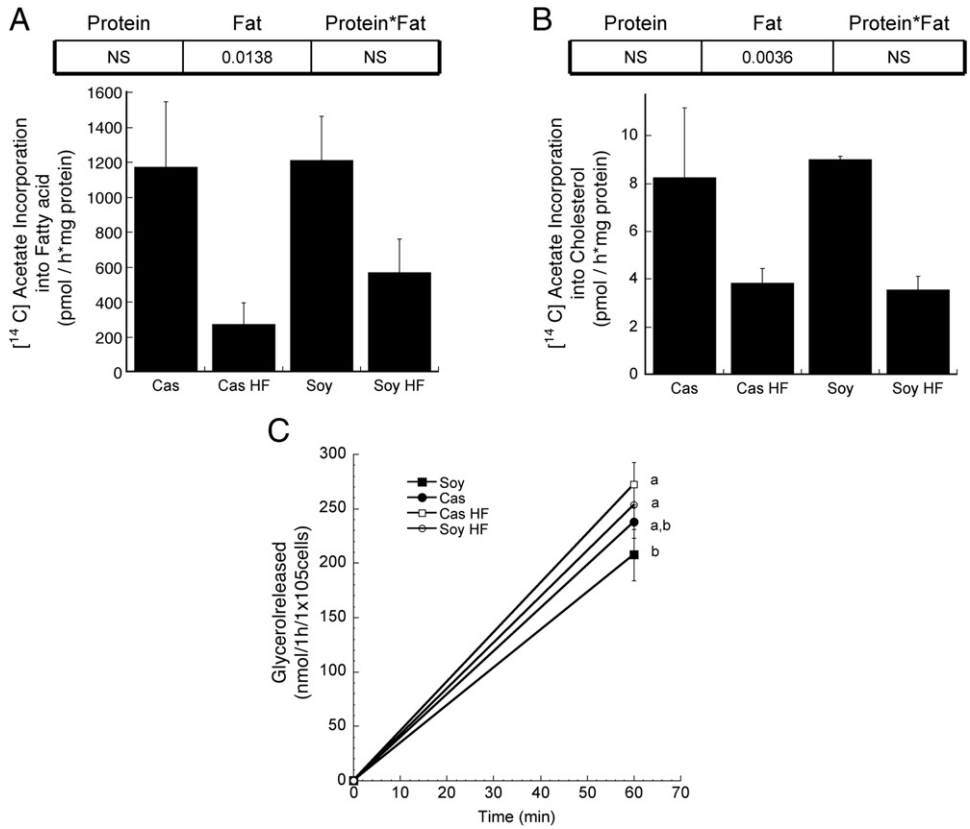

Fig. 3. High-fat diets promote decreased lipogenesis and increased lipolysis in vitro. (A) [^{14}C] acetate incorporation into fatty acids. (B) [^{14}C] acetate incorporation into cholesterol. (C) Glycerol release from retroperitoneal adipocytes of rats fed Cas, Cas HF, Soy and Soy HF for 160 days. The results represent the mean±S.E.M., n=5. Means without a common letter differ (P<.05).

70°C. For measurements, the tissue was thawed and homogenized in 10 mM Tris buffer, pH 8.0 containing 150 mM sodium chloride and 0.1% triton at 40–50°C with a polytron homogenizer. Fifty microliters of the homogenized solution was used for triglyceride determination as described above. One milliliter of the homogenized solution was incubated with a solution containing sodium dodecyl sulfate, proteinase K and EDTA at 37°C. DNA was extracted by the phenol-chloroform extraction method. The amount of DNA was measured by its absorbance at 260 nm.

2.10. Quantification of adipocyte size

Paraffin sections of fixed fat pads were stained with hematoxylin and eosin and analyzed with a Leica microscope equipped with a digital camera. For each sample, three areas and 10 cells in each area were evaluated. Cell areas were obtained using Leica software for digital imaging processing.

2.11. Statistical analysis

Results obtained in this study are presented as the means±S.E.M. and were evaluated with one- or two-way analysis of variance, followed by Fisher's protected least significant difference test when the interaction was significant, to determine statistical differences. Differences were considered statistically significant at $P<.05$ and are indicated by letters in the figures (a>b>c>d).

3. Results

3.1. Weight gain, energy intake and biochemical parameters

At the end of the study, rats fed Cas HF or Soy HF diet gained significantly more weight than the respective control groups. However, rats fed Cas HF diet had significantly more retroperitoneal adipose tissue weight than rats fed Soy HF. These results are in agreement with a previous study from our lab demonstrating that soy protein induces less weight gain and less total body fat (15). Differences in weight gain were not attributed to changes in energy intake since the amount of kcal/day consumed on average for each group was not significantly different. Rats fed Soy diets had lower serum triglyceride and free fatty acid concentrations than rats fed Cas diets. Serum cholesterol concentration did not show a significant difference among groups (Table 2). Animals fed Cas HF or Cas diets showed higher serum leptin concentrations than those fed Soy HF or Soy diets, respectively (Table 2). Rats fed Cas diets also had higher concentrations of serum adiponectin than groups fed Soy diets. However, the

Table 3
Differentially expressed transcripts of metabolically relevant genes in adipose tissue of rats fed Casein or Soy Protein and Cas HF or Soy HF diets for 160 days

Affymetrix ID	Gene	Cas vs. Soy (FC)	*P*	Cas HF vs. Soy HF (FC)	*P*
Renin-angiotensin system					
1387791_at	Angiotensin converting enzyme	0.84	.033		
1369439_at	Angiotensin receptor 1b	0.91	.045		
Adipokines					
1387748_at	Leptin	2.18	.003		
	Leptin receptor			0.92	.05
1388373_at	Adiponectin receptor	0.84	NS		
1371876_at	Tumor necrosis factor 5			1.17	.006
1376327_at	Tumor necrosis factor receptor 14	1.51	.003		
Lipid metabolism					
1386880_at	Acetyl CoA acyltransferase 2			1.44	.002
1370893_at	Acetyl CoA carboxylase alpha			0.69	.027
	Acyl CoA dehydrogenase, very long chain	1.15	.042	1.18	.0214
1367708_a_at	Fatty acid synthase	1.34	.008	0.74	.0077
1387132_at	Hormone sensitive lipase			1.52	.0067
1375852_at	Hydroxy-methylglutaryl-CoA reductase	0.87	.021		
1375209_at	Oxyesterol binding protein-like 11	0.81	.016	1.23	.0165
1369098_at	Very low density lipoprotein receptor	1.14	.013	1.24	.0055
1390383_at	Adipose differentiation related protein	0.75	.038		
1371038_at	CCAAT enhancer binding protein gamma	1.18	.007	1.11	.046
1367660_at	Fatty acid binding protein 3	1.5	.002		
1368271_a_at	Fatty acid binding protein 4			1.15	.035
1368941_at	Prostaglandin E receptor	1.16	.016	0.9	.016
1368989_at	Tissue inhibitor of metalloproteinase3	1.4	.017	0.41	.0002
Cytokines					
1376895_at	Interleukin 16	1.3	.0007		
1369665_ a_at	Interleukin 18	1.19	.007		
1370728_at	Interleukin 13 receptor, alpha 1			1.15	.0085
Signaling					
1370196_at	Signal transducer and activator of transcription 3	0.91	.038		
1377131_at	Suppressor of cytokine signaling 7			1.13	.044
1372879_at	AKT 1 substrate 1	0.78	.015	1.18	.048
1368486_at	Insulin receptor substrate 3	0.72	.022		
1370114_a_at	PI3K, regulatory subunit 1	1.14	.009	1.35	.028
Carbohydrate metabolism					
1369794_a_at	6-phosphofructo-2-kinase	1.29	.0096	1.91	3.2 E-05
1369967_at	Citrate synthase	1.45	.0002	1.21	.0083
1369560_at	Glycerol-3-phosphate dehydrogenase 1	2.99	1.29E-05	1.59	.0029
AFFX_Rat_Hexokinase_M_at	Hexokinase 1	1.85	.00097	1.36	.034
1368674_at	Liver glycogen phosphorylase	1.16	.008		
1383698_at	Piruvate dehydrogenase E1 alpha 1	1.7	8.1E-05		
1386917_at	Piruvate carboxylase			1.28	.036
1371311_at	Succinate dehydrogenase, subunit C	1.16	.023		

adiponectin/leptin ratio was significantly higher in control rats than those fed high-fat diets, indicating that high dietary fat content reduces serum adiponectin concentration as adipose tissue mass increased. In vitro studies demonstrated that isolated adipocytes from rats fed Soy released more adiponectin than those from the other groups (data not shown). On the other hand, there were no significant differences in serum concentrations of IL-6 and TNFα among the groups. Thus, soy protein consumption has a beneficial effect on biochemical and hormonal parameters despite the consumption of a high-fat diet.

3.2. Histological analysis of adipose tissue

Adipose tissue from rats fed the Cas HF diet (Fig. 1B) contained bigger adipocytes than tissue from rats fed Cas (Fig. 1A), Soy (Fig. 1C), or Soy HF (Fig. 1D). Quantitative analysis of adipocyte size showed that animals fed high-fat diets had bigger adipocytes than the respective control groups. Adipocytes from rats fed Soy HF or Cas HF diets were 72% and 80% larger, respectively, than those of the control groups. Moreover, adipocytes of rats fed the Soy HF diet were 49% smaller than those of rats fed the Cas HF diet (Fig. 2A).

3.3. Triglyceride and DNA content in adipose tissue

Adipose tissue from rats fed Cas or Cas HF diets contained higher levels of triglyceride per gram of tissue than rats fed Soy or Soy HF diets (Fig. 2B). Interestingly, the amount of DNA content per gram of tissue was significantly higher in rats fed Cas or Soy diets than in rats fed Cas HF or Soy HF diets (Fig. 2C). As a result, the triglyceride/DNA ratio was significantly higher in rats fed the Cas HF diet than in the other groups, indicating a large extent of adipocyte hypertrophy. Statistical analysis indicated that adipose tissue from rats fed Soy protein diets maintained a low triglyceride/DNA ratio, suggesting an active adipogenic process that avoids adipocyte hypertrophy (Fig. 2D). Thus, the difference in size observed in the histological studies between adipocytes from rats fed Soy HF and Cas HF diets was not only due to a difference in the amount of triglyceride stored, but also to an important difference in the number of adipocytes. Our results are in agreement with a previous study, which used histological studies of a different fat pad (epididymal) to demonstrate that rats fed Soy protein produced smaller adipocytes [15]. Thus, soy protein maintains high adipose tissue cellularity, preventing hypertrophy.

3.4. Fatty acid and cholesterol synthesis in isolated adipocytes

To determine the capacity of adipocytes of rats fed Soy HF or Cas HF diets to synthesize lipids, we measured the incorporation of [^{14}C] acetate into fatty acids and cholesterol. As shown in Fig. 3A and B, rats fed high-fat diets showed a significant decrease in the rate of incorporation of radiolabeled acetate into fatty acids and cholesterol as compared to the control groups fed Soy or Cas. [^{14}C] Acetate was mainly incorporated into fatty acids, indicating that adipocytes actively synthesize fatty acids; however, only a small fraction was incorporated into cholesterol. Addition of high fat content to the Soy

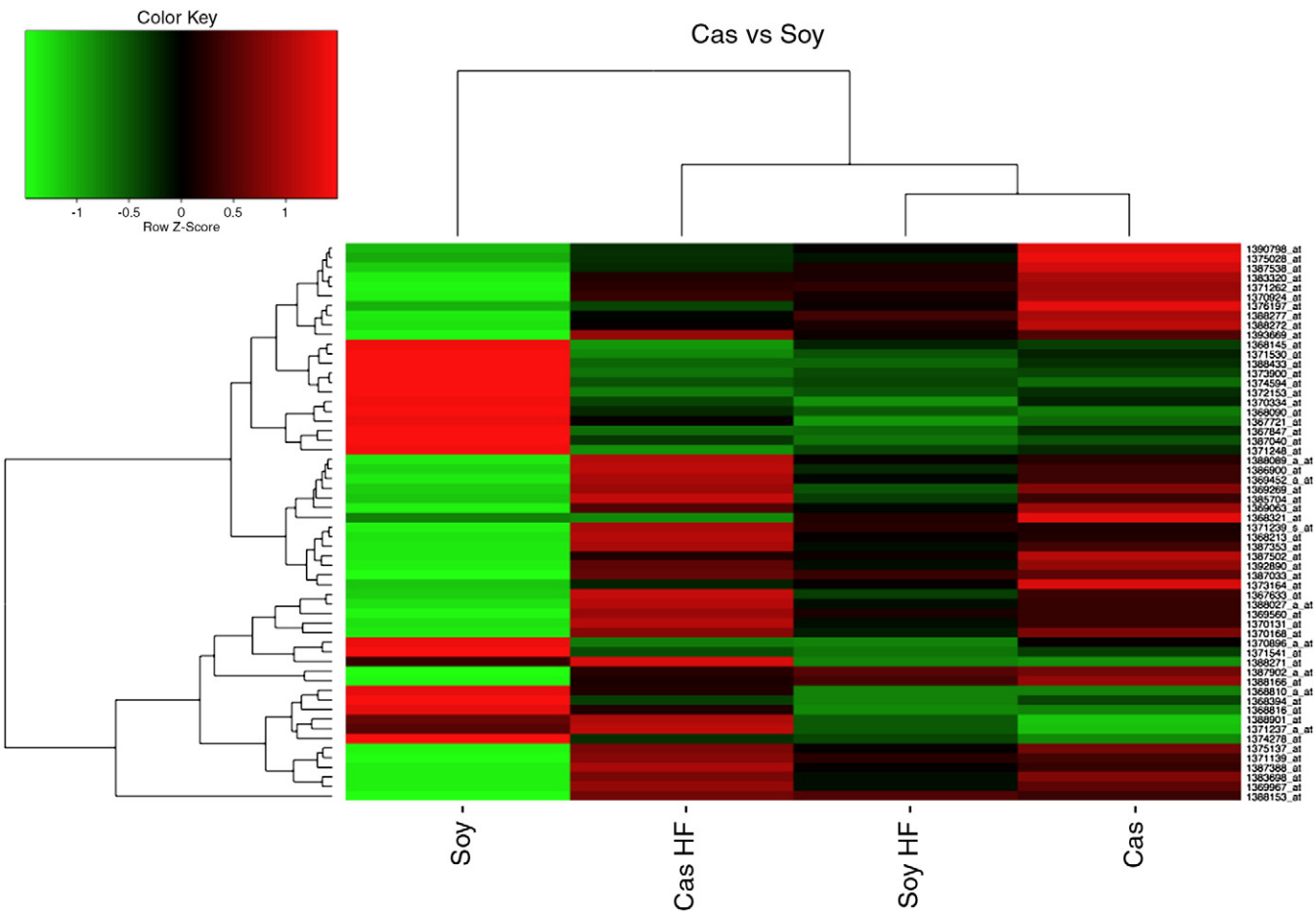

Fig. 4. Hierarchical clustering of soy protein modified genes. A hierarchical search of genes was based on log-fold changes. All of these expressed genes in the RAE 230A chip were clustered based on their expression patterns across the 12 samples using R software.

or Cas diets reduced the rate of [^{14}C] incorporation into fatty acids by 53 and 77%, respectively, as compared to the control groups.

3.5. Glycerol release from isolated adipocytes

To establish if there was a difference in the lipolytic capacity of the adipocytes of the groups studied, we measured the amount of glycerol released into the medium from incubated adipocytes. The data showed that adipocytes from rats fed the Soy diet released significantly less glycerol than cells from the groups fed HF diets. Adipocytes from rats fed Cas diets tended to release more glycerol than those from rats fed Soy diets, although the difference did not reach statistical significance (Fig. 3C).

3.6. Transcriptome analysis of adipose tissue

To study the effect of dietary protein type on the adipose tissue transcriptome of rats fed adequate or HF diets, we measured changes in global gene expression using Affymetrix RAE 230A arrays. Data on significantly regulated genes are summarized in Supplemental Fig. 1A. After adequate fat consumption, expression levels of 219 genes (out of 14,200 genes analyzed) were significantly different between rats fed Soy and Cas (fold change >1.4). After high fat consumption, expression levels 121 genes were significantly different between rats fed Soy and Cas. Analysis of genes differentially expressed in rats fed Soy or Cas diets and those fed Soy HF or Cas HF diets showed that 90 genes were differentially expressed as a result of the type of the dietary protein consumed (Supplemental Figure 1A). Fifteen of these genes were down-regulated, and 75 were up-regulated by soy protein consumption. A list of regulated genes involved in adipose tissue metabolism is shown in Table 3. These genes fall into several different categories of biological function, including the renin-angiotensin system, adipokines, lipid metabolism, cytokines, signaling and carbohydrate metabolism. qRT PCR analysis was performed for selected genes, which confirmed the array results (Supplemental Figure 1B). A hierarchical clustering of genes that were differentially regulated in adipose tissue of rats fed Soy versus Cas for 160 days is shown in Fig. 4. In addition to lipid and carbohydrate metabolism genes, this analysis showed that genes involved in the immune system, cytoskeleton regulation and signal transduction were overrepresented. Genes that were highly over- or under-expressed in adipose tissue of rats fed Cas compared to Soy are shown in Table 4, and Cas HF versus Soy HF are shown in Table 5. Interestingly, the function of many of the latter genes in adipose tissue remains unknown. We also searched the potential biological interaction networks that are activated in the adipose tissue of rats fed Soy, Cas, Soy HF and Cas HF diets using the Ingenuity Pathways Analysis. For the comparison of genes in the adipose tissue of rats fed Soy or Cas with adequate fat content, 341 genes were considered for the network analysis, obtaining a score of 14 (P<.05) (Fig. 5A). Subsequently, we analyzed these interactions in adipose tissue between the groups fed Soy HF and Cas HF diets, using 303 genes for this purpose, and the maximal score was 11 (P<.05) (Fig. 5B). For the comparison between the Soy versus Soy HF diet and the Cas versus Cas HF diet, 711 and 225 genes were considered for network analysis, with scores of 9 and 22, respectively. In the last two comparisons, the leptin gene was greatly induced and appeared as a main node. Additionally, the networks showed that soy protein consumption reduced leptin expression as compared to casein consumption. In contrast, consumption of Soy HF or Cas HF diets increased leptin expression compared to consumption of Soy or Cas diets, as observed in the network analysis of Fig. 5C and D.

Table 4
Transcripts of genes highly over or under expressed in adipose tissue of rats fed Casein vs. Soy Protein diet for 160 days

Affymetrix ID	Gene	Fold change	*P* value
Overexpressed			
1387902_a_at	Similar to IG kappa chain V-V region K2 precursor	8.47	9.37E-07
1388166_at	Similar to immunoglobulin heavy chain 6 (Igh-6)	5.39	1.17E-06
1368337_at	Glycosylation dependent cell adhesion molecule 1	3.03	.0014
1369560_at	Glycerol-3-phosphate dehydrogenase 1	2.99	1.29E-05
1369063_at	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A		2.47E-06
1370967_at	Claudin 10	2.37	.0098
1368551_at	Phosphoribosyl pyrophosphate synthetase 2	2.23	.0069
1387748_at	Leptin	2.18	.0029
1368321_at	Early growth response	2.15	2.58E-05
1370131_at	Caveolin	2.10	6.22E-05
Underexpressed			
1370863_at	Keratin complex 2, basic, gene	0.62	2.70E-05
1367721_at	Syndecan 4	0.61	.0007
1371725_at	Myosin, heavy polypeptide 9	0.61	.0291
1371237_a_at	Metallothionein 1a	0.61	.0002
1368145_at	Purkinje cell protein 4	0.57	5.11E-06
1368810_a_at	Myelin basic protein	0.56	.0004
1372153_at	Type I keratin KA15	0.55	1.93E-06
1371541_at	Myosin, light polypeptide kinase	0.52	.0004
1388433_at	Keratin complex 1, acidic, gene 19	0.46	6.67E-06
1371248_at	Similar to cornifin A (small proline-rich protein 1A)	0.38	9.25E-06

Table 5
Transcripts of genes highly over or under expressed in adipose tissue of rats fed Cas HF diet in comparison with those fed Soy Protein High-Fat diet for 160 days

Affymetrix ID	Gene	Fold change	*P*
Overexpressed			
1387748_at	Leptin	2.24	.0024
1367605_at	Profilin 1	2.20	.044
1387630_at	ELOVL family member 5, elongation of long chain fatty acids (yeast)	2.05	.0037
1388271_at	Similar to metallothionein-2	1.97	1.94E-05
1368821_at	Follistatin-like 1	1.92	.0077
1369794_a_at	6-phosphofructo-2-kinase	1.91	3.17E-05
1370135_at	Caveolin 2	1.88	.00043
1369736_at	Epithelial membrane protein 1	1.87	.00183
1367660_at	Fatty acid binding protein 3	1.81	.00019
1387769_a_at	Inhibitor of DNA binding 3	1.78	.0038
Underexpressed			
1371248_at	Similar to Cornifin A (small proline-rich protein 1A)	0.78	.0260
1368806_at	Selenoprotein P, plasma, 1	0.77	.0257
1367896_at	Carbonic anhydrase	0.76	.0188
1367708_a_at	Fatty acid synthase	0.74	.0077
1398364_at	Similar to chromosome 1 open reading frame 63	0.74	.000998
1367555_at	Albumin	0.73	.0394
1387189_at	Solute carrier family 22, member 3	0.71	.0036
1398390_at	Similar to small inducible cytokine B13 precursor (CXCL13) (B lymphocyte chemoattractant)	0.70	.0055
1368321_at	Early growth response 1	0.70	.0030
1370893_at	Acetyl-coenzyme A carboxylase alpha	0.69	.0268

3.7. Leptin mRNA expression in adipose tissue

Since leptin was found to be a central node in the network analysis, we measured by qRT-PCR the expression of leptin mRNA in adipose tissue from rats fed each of the experimental diets. There was an agreement between the network analysis and leptin mRNA expression because adipose tissue from rats fed Soy HF or Soy expressed significantly less leptin mRNA than the respective groups

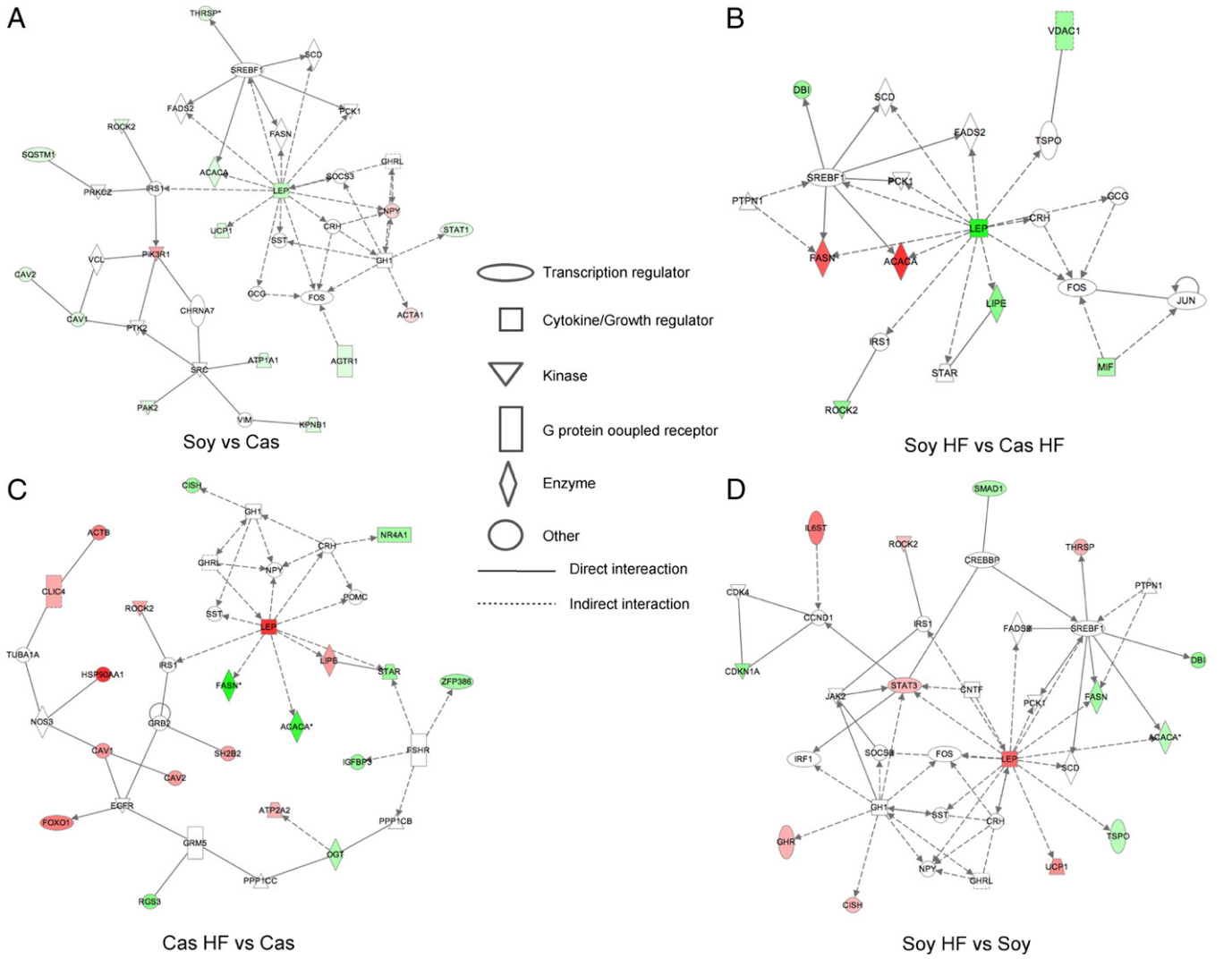

Fig. 5. Generation of biological interaction networks identifies central genes. Ingenuity Pathway Analysis was used to search for biological interaction networks. Four networks scored best. (A) Soy versus Cas. (B) Soy HF versus Cas HF. (C) Cas HF versus Cas. (D) Soy HF versus Soy. Color-coding: red, up-regulated genes; green, down-regulated genes. The intensity of the colors indicates the degree of up- or down-regulation, respectively; a greater intensity represents a higher degree of regulation.

fed Cas HF or Cas (Fig. 6A). Secretion of leptin from cultured adipocytes (Fig. 6B) from rats fed Cas or Soy diets followed a similar trend in leptin mRNA abundance, particularly in the groups fed high-fat diets. Therefore, it seems that the type of dietary protein can influence adipocyte leptin expression, thus modulating the cellular metabolic response.

3.8. Regulation of gene expression in adipose tissue by soy protein

Expression of PPARγ and PPARα (Fig. 7A and D) was significantly higher in the Soy group than the Cas group, indicating a more active adipogenic and oxidative capacity in rats fed Soy Protein. CPT-1 expression (Fig. 7F) in rats fed Soy or Soy HF increased 1.25- and 0.9-fold in comparison with rats fed Cas or Cas HF, respectively. We did not observe significant differences in the expression of SREBP-1, FAS, or UCP-1 between the Soy and Cas groups (Fig. 7B, C, E). The addition of high fat content to the diet reduced the expression of PPARγ, PPARα, SREBP-1, CPT-1 and FAS but increased UCP-1 mRNA concentration. Expression of glucose transporter GLUT4 tended to increase in adipose tissue of rats fed Soy in comparison with those fed Cas (Supplemental Figure 2).

4. Discussion

Obesity has been associated with the consumption of high-carbohydrate or high-fat diets [11,24,25]. However, few studies have focused on the effect of dietary protein on the development of obesity. Our results show that the type of dietary protein has a significant influence on adipose tissue phenotype. This is the first study to analyze in depth the genomic and metabolic changes that occur in adipose tissue as a result of the consumption of dietary soy protein. Soy diets reduced adipocyte size due to a reduction in the triglyceride/DNA ratio, indicating an increase in fat cell number and thus preventing hypertrophy. In contrast, Cas diets produced the opposite effect. We believe that these changes are in part the result of hormonal modifications, as the findings of this study and evidence from previous studies [26,27] both demonstrate that soy protein prevents excessive elevation of serum insulin and leptin concentrations. We have previously demonstrated that consumption of soy protein was able to stimulate insulin secretion to a lower extent [27]. In addition, we found in this study that soy protein reduced leptin mRNA expression in adipose tissue, resulting in low levels of circulating serum leptin.

The specific mechanism by which soy protein reduces leptin mRNA expression is not known. A possible explanation is that the

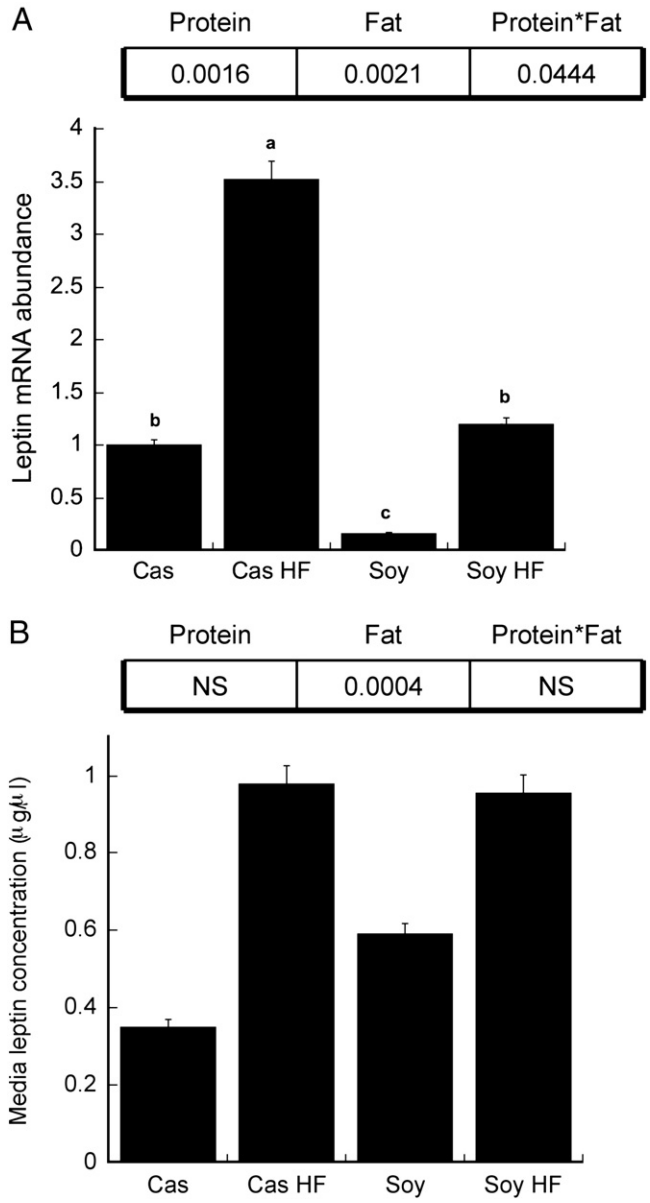

Fig. 6. Leptin. mRNA abundance in adipose tissue (A), its secretion from isolated adipocytes (B), and serum concentration (C), from rats fed Cas or Soy, and Cas HF or Soy HF diets. Results represent the mean±S.E.M., $n=5$. Means without a common letter differ, $P<.05$.

prevention of hyperinsulinemia by soy protein reduces the abundance of leptin mRNA. It has been postulated that a feedback loop between adipose tissue and the pancreas regulates the secretion of leptin and insulin, respectively. It is known that leptin regulates insulin secretion by several mechanisms: by increasing β-oxidation and decreasing lipogenesis, thus preventing β-cell lipotoxicity [28]; by activating K_{ATP} channels in the β-cell [29]; and by reducing insulin mRNA levels [30]. On the other hand, insulin modulates leptin synthesis and secretion via both transcriptional and translational mechanisms [31,32]. Thus, soy protein, by maintaining normal serum insulin and leptin concentrations, leads to an improved sensitive response to these hormones in target organs and tissues, including adipocytes. These hormonal changes are also responsible for maintaining low retroperitoneal adipose tissue weight. In fact, it has been demonstrated that serum leptin concentration is associated with the amount of adipose tissue [33,34].

Several studies have shown that serum leptin not only regulates hypothalamic and peripheral tissues but also regulates adipocyte metabolism by a paracrine mechanism [35]. Our microarray analysis showed increased STAT3 and decreased SOCS7 expression in adipose tissue of rats fed the Soy diet, suggesting that soy protein contributes to leptin sensitivity. Although SOCS7 has been shown to enhance insulin sensitivity [36], its function in adipocytes is unknown. Furthermore, it has been established that PPARα is a target gene of leptin in non-adipose tissues such as liver [4]. Our results suggest that leptin may also stimulate PPARα expression in adipose tissue; however, more studies are needed to understand this interaction. Nevertheless, the increase of PPARα by soy protein can promote adipocyte fatty acid oxidation, preventing triglyceride over-accumulation in adipocytes and thus reducing hypertrophy, as observed in this study.

Soy protein also seems to stimulate insulin sensitivity in adipose tissue. We have previously demonstrated that consumption of soy protein increases peripheral insulin sensitivity. Our results extend this finding to adipose tissue, where microarray analysis showed an increased expression of IRS-3 and Akt, and qRT-PCR analysis showed a tendency to increase GLUT4 expression. We were expecting a significant increase in lipogenesis mediated by insulin in rats fed the Soy Protein diet. However, the rate of incorporation of [^{14}C] acetate into fatty acids between rats fed Soy and Cas was unchanged. In accordance, there was no significant change in the expression of SREBP-1 and FAS between these groups. Addition of high fat to the diets reduced adipocyte lipogenesis for both the Soy and Cas diets. It is important to determine the role of SREBP-1 in controlling lipogenesis in adipose tissue because there is controversial evidence in the literature indicating that SREBP-1 may [37] or may not [38] contribute to adipocyte lipogenesis. Interestingly, despite the reduction in adipocyte lipogenesis in groups fed Soy HF or Cas HF diets, it was not associated with the size of adipocytes. Thus, the oxidative capacity of the adipocyte to use fatty acids as fuel is enhanced by the consumption of soy protein in the control and high fat diets, as demonstrated with the increase in mRNA abundance of PPARα and its target gene CPT-1. These results, again suggest that the smaller adipocyte size in adipose tissue of rats fed soy protein is a consequence of an accelerated fatty acid oxidation process.

The lower serum leptin concentration in rats fed soy protein was associated with an increase in PPARγ mRNA in adipose tissue. An increase in PPARγ by soy protein also reduced the expression of inflammatory genes such as interleukin 16, 18, a-1 interleukin 13 receptor and TNF (Table 3). It has been demonstrated that during the development of metabolic syndrome and obesity these cytokines are elevated in serum in humans and mice [39,40]. The increase in interleukin 18 and TNF is known to reduce insulin sensitivity [41,42]. High expression of PPARγ, an essential transcription factor for preadipocyte differentiation, has been associated with a decrease in systemic inflammation accompanied by a decrease in adipocyte expression of proinflammatory cytokines [43]. Furthermore, the increase in PPARγ after the consumption of soy protein was associated with an increase in the expression of the adiponectin receptor. It has been reported that adiponectin receptor expression in white adipose tissue is controlled by PPARγ [44]. Thus, the effect of adiponectin on adipocytes is mediated by the amount of adiponectin receptor, as well as the concentration of serum adiponectin. Nonetheless, in the present study, serum adiponectin was reduced in the Soy groups, in contrast to a previous study showing that consumption of this protein increases serum adiponectin concentration [45]. However, it is unclear whether the secretion of this hormone is different in short- versus long-term dietary treatments, but this could explain the ambiguity between results.

Other potential sites of regulation of lipogenesis and lipolysis in adipocytes by the type of dietary protein were explored through biological interaction networks among differentially regulated genes

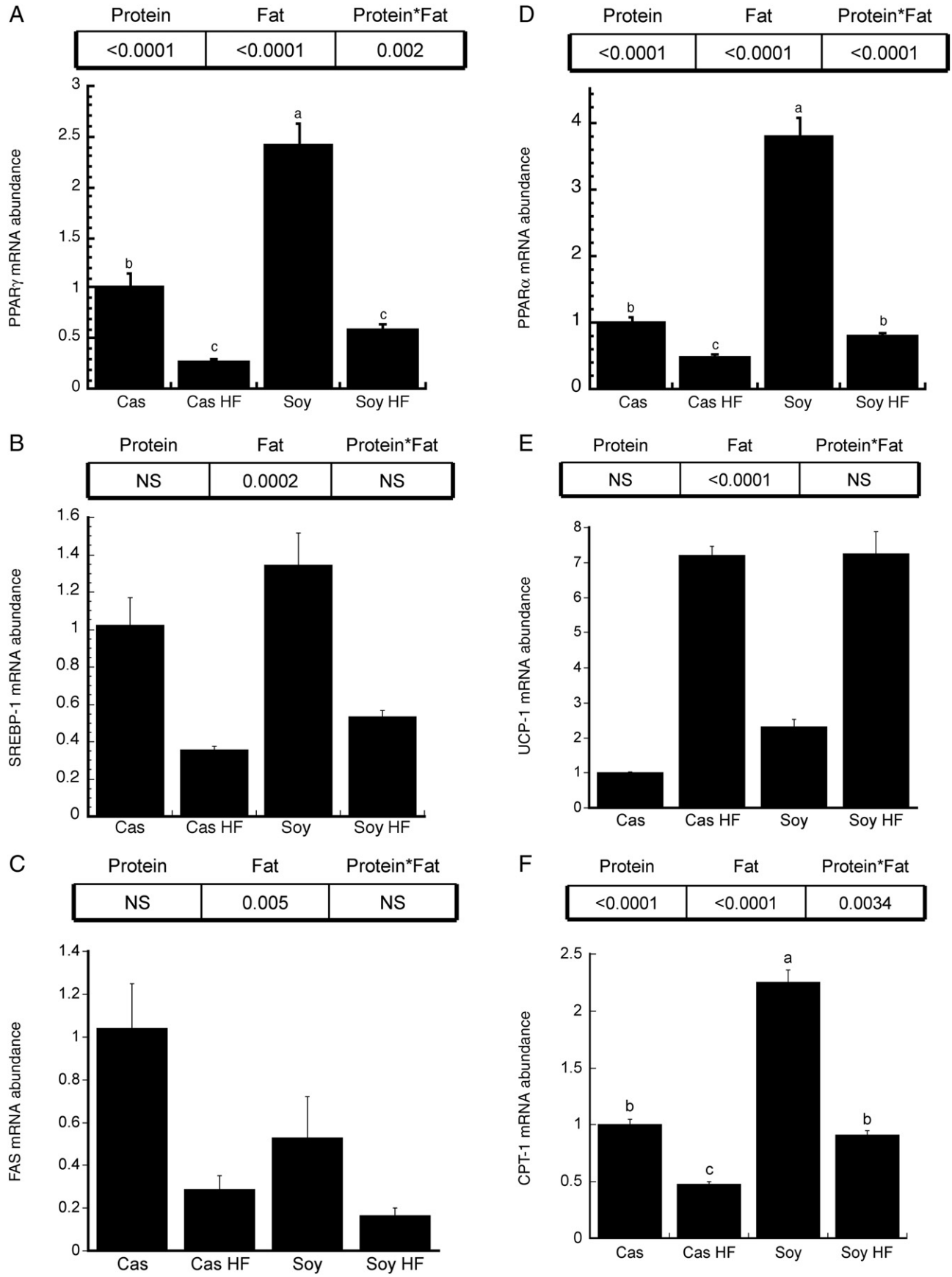

Fig. 7. Expression of lipogenic and fatty acid oxidation genes. Relative mRNA abundance of PPARγ (A), SREBP-1 (B), and FAS (C), PPARα (D), UCP-1 (E) and CPT-1 (F) was measured by qRT-PCR and normalized to HPRT from rats fed Cas or Soy, and Cas HF or Soy HF diets. Results represent the mean±S.E.M., $n=5$. Means without a common letter differ ($P<.05$).

using Ingenuity Pathways analysis. This analysis revealed an important finding, that one of the central modified genes (or nodes) was leptin. Leptin expression was down-regulated in the adipose tissue of rats fed Soy or Soy HF diets as compared to those fed Cas or Cas HF. Addition of HF to diet caused up-regulation of leptin in adipose tissue and decreased FAS and acetyl-CoA carboxylase (ACC) expression. Furthermore, down-regulation of leptin expression by soy protein was accompanied by a decrease in the expression of the hormone-sensitive lipase (HSL or LIPE in the pathway analysis), an important enzyme in the regulation of adipocyte lipolysis [46] with no significant change in the expression of uncoupling protein 1, which is involved in the thermogenic process [47]. It has been recently described that central action of adipokines such as leptin has a direct communication with white adipose tissue to regulate lipolysis [48].

Our results also showed that the dietary protein type significantly modified a large set of genes involved in different biological functions, including the renin-angiotensin system, adipokines, lipid metabolism, cytokines, signaling genes, carbohydrate metabolism, immune response, cytoskeleton and certain unknown genes. Some of these genes, such as fatty acid binding protein, leptin and its receptor, Na^+/K^+-ATPase, phosphofructokinase, pyruvate dehydrogenase, heat shock proteins and adenylate cyclase play metabolic roles. In addition to changes in the expression of metabolic genes, we found a set of genes that were the most overexpressed, including those similar to immunoglobulin heavy chain 6, glycosylation-dependent cell adhesion molecule 1, acidic nuclear phosphoprotein 32 family member A, claudin 10, phosphoribosyl pyrophosphate synthetase 2 and profilin 1, among others, or underexpressed, such as those similar to cornifin A, keratin complex 1 acidic gen 19 and myosin-like polypeptide kinase. However, the biological roles in adipose tissue of some of these genes remains unknown.

The addition of high fat content to the diets increased the serum free fatty acid concentration in the Cas HF group more than in the Soy HF group. This pattern was also seen for the release of glycerol from adipocytes in these groups. Excessive release of free fatty acids is related to an increase in adipocyte size, elevated macrophage infiltration and a limited capacity of adipocytes to accommodate excess energy surplus; these factors, together with altered adipokine secretion, leads to ectopic lipid deposition [49]. Therefore, there is an increase in the flux of free fatty acids from the adipocyte to non-adipose tissues. When lipids over-accumulate in non-adipose tissues during overnutrition, fatty acids enter deleterious pathways. One such pathway is ceramide production, which, through increased nitric oxide formation, causes apoptosis of β cells, myocardium and skeletal muscle, leading to type 2 diabetes, cardiomyopathy and insulin resistance, respectively [50]. In the liver, ceramide overproduction is associated with hepatic steatosis, which under certain circumstances may lead to fibrosis and cirrhosis. Our group has demonstrated that soy protein has a protective role against lipid accumulation and lipotoxicity in the pancreas and liver of different animal models of obesity, such as diet-induced obesity rats and Zucker fa/fa rats. The findings of these previous studies suggest that lipid accumulation in non-adipose tissues is influenced by a reduced rate of influx of fatty acids from adipose tissue into the bloodstream [15,27,51].

In summary, the type of dietary protein modifies the gene expression profile of adipose tissue, resulting in biochemical changes that affect its metabolic functionality. The type of protein consumed also influences adipocyte size and function, which is associated with the development of obesity and its complications. Our results indicate that soy protein prevents obesity and adipocyte hypertrophy, by an increase in adipocyte fatty acid oxidation, through changes in gene expression patterns, and a reduction in the expression of inflammatory genes. Therefore, soy protein could be used as a dietary strategy for the prevention or treatment of the metabolic abnormalities of obesity.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2009.12.006.

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