

Lipogenesis Is Decreased by Grape Seed Proanthocyanidins According to Liver Proteomics of Rats Fed a High Fat Diet*[§]

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Bioactive proanthocyanidins have been reported to have several beneficial effects on health in relation to metabolic syndrome, type 2 diabetes, and cardiovascular disease. We studied the effect of grape seed proanthocyanidin extract (GSPE) in rats fed a high fat diet (HFD). This is the first study of the effects of flavonoids on the liver proteome of rats suffering from metabolic syndrome. Three groups of rats were fed over a period of 13 weeks either a chow diet (control), an HFD, or a high fat diet supplemented for the last 10 days with GSPE (HFD + GSPE). The liver proteome was fractionated, using a Triton X-114-based two-phase separation, into soluble and membrane protein fractions so that total proteome coverage was considerably improved. The data from isobaric tag for relative and absolute quantitation (iTRAQ)-based nano-LC-MS/MS analysis revealed 90 proteins with a significant ($p < 0.05$) minimal expression difference of 20% due to metabolic syndrome (HFD *versus* control) and 75 proteins due to GSPE treatment (HFD + GSPE *versus* HFD). The same animals have previously been studied (Quesada, H., del Bas, J. M., Pajuelo, D., Díaz, S., Fernandez-Larrea, J., Pinent, M., Arola, L., Salvadó, M. J., and Bladé, C. (2009) Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obes.* 33, 1007–1012), and GSPE was shown to correct dyslipidemia observed in HFD-fed rats probably through the repression of hepatic lipogenesis. Our data corroborate those findings with an extensive list of proteins describing the induction of hepatic glycogenesis, glycolysis, and fatty acid and triglyceride synthesis in HFD, whereas the opposite pattern was observed to a large extent in GSPE-treated animals. GSPE was shown to have a wider effect than previously thought, and putative targets of GSPE involved in the reversal of the symptoms of metabolic syndrome were revealed. Some of these novel candidate proteins such as GFPT1, CD36, PLAA (phospholipase A₂-activating protein), METTL7B,

SLC30A1, several G signaling proteins, and the sulfide-metabolizing ETHE1 and SQRDL (sulfide-quinone reductase-like) might be considered as drug targets for the treatment of metabolic syndrome. *Molecular & Cellular Proteomics* 9:1499–1513, 2010.

An increase in high calorie diets and a sedentary lifestyle are considered the key factors in explaining the epidemic rise in obesity in developed countries (1). Obese patients, especially those with abdominal obesity due to visceral adipose tissue accumulation, run a higher risk of impaired glucose tolerance, which frequently evolves into insulin resistance (2). Obesity and insulin resistance are frequently associated with hypertension, proatherogenic dyslipidemia, chronic inflammation, a prothrombotic state, and recently also fatty liver (3), conditions that together make up what is known as metabolic syndrome and lead to an increased risk of developing cardiovascular disease (CVD)¹ and type 2 diabetes (4). Conversely,

¹ The abbreviations used are: CVD, cardiovascular disease; ACACA, acetyl-CoA carboxylase α ; ACADL, long chain acyl-CoA dehydrogenase; ACADS, short chain acyl-CoA dehydrogenase; ACLY, ATP-citrate lyase isoform 1; ACOT2, mitochondrial acyl-CoA thioesterase 2; ACOX1, palmitoyl acyl-CoA oxidase 1; BUCS1, butyryl-CoA synthetase 1 protein; C, control; CD36, CD36 antigen; CPT-I, carnitine palmitoyltransferase 1; CPT-II, carnitine palmitoyltransferase 2; DECR1, 2,4-dienoyl-CoA reductase 1, mitochondrial; DGAT2, diglyceride acyltransferase 2; ETHE1, ethylmalonic encephalopathy 1; FA, fatty acid; FAM82A, regulator of microtubule dynamics protein 2; FASN, fatty-acid synthase; FXR, farnesoid X receptor; G6PD, glucose-6-phosphate dehydrogenase; GBE1, glucan (1,4- α -), branching enzyme 1; GFPT1, glutamine fructose-6-phosphate transaminase 1; GO, gene ontology; GPD1L, glycerol-3-phosphate dehydrogenase 1; GSPE, grape seed proanthocyanidin extract; H₂S, hydrogen sulfide; HCCS, cytochrome c-type heme-lyase; HFD, high fat diet; iTRAQ, isobaric tag for relative and absolute quantitation; ME1, malic enzyme 1; METTL7B, methyltransferase-like 7B; M, membrane protein(s); MT-CO3, cytochrome c oxidase subunit II; MYL12B, myosin light chain regulatory B; NCBI, National Center for Biotechnology Information; PCK2, phosphoenolpyruvate carboxykinase 2; PGD, 6-phosphogluconate dehydrogenase, decarboxylating; PKLR, pyruvate kinase; PLAA, phospholipase A₂-activating protein; S, soluble protein(s); SHP, small heterodimer partner; SLC30A1, solute carrier family 30 member 1; SQRDL, sulfide-quinone reductase-like; SREBP1c, sterol regulatory element-binding protein 1; TALDO1, transaldolase 1; TG, triglyceride; UGP2, UDP-glucose pyrophosphorylase 2; VLDL, very low density lipoprotein; LTQ, linear trap quadrupole; START, steroidogenic acute regulatory protein-related lipid transfer.

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some dietary patterns and specific food components have been associated with a lower prevalence of obesity, type 2 diabetes, and CVD. In this sense, the traditional Mediterranean diet (characterized by a high fiber content, low glycemic index carbohydrates, unsaturated fats, vitamins, and antioxidant polyphenols) has been linked to a lower incidence of CVD, obesity, and type 2 diabetes (5–8). Moreover, the French population presents a very low prevalence of death due to CVD despite consuming a diet rich in saturated fats and cholesterol. This phenomenon, known as “the French paradox” (9), has been ascribed to the moderate consumption of red wine and specifically to its content of polyphenols (10–12).

Polyphenols include flavonoids of which flavan-3-ols and their oligomeric forms (proanthocyanidins) have been reported to exhibit several beneficial health effects by acting as antioxidant, anticarcinogen, cardioprotective, antimicrobial, antiviral, and neuroprotective agents (for a review, see Ref. 13). Specifically, grape and wine proanthocyanidins have a cardioprotective effect through increasing plasma high density lipoprotein cholesterol, decreasing low density lipoprotein-derived atherosclerotic foam cell lesions, attenuating oxidant formation by quenching harmful radicals, increasing endothelium-dependent vasorelaxation, etc. (13). In this context, our group has been working for years on the effect of a grape seed proanthocyanidin extract (GSPE) (containing monomers and oligomers of flavan-3-ols) in relation to metabolic syndrome. In previous works, we have found that GSPE prevents oxidative injury (14), has an insulinomimetic effect on adipocytes and adipose tissue (15), modulates glucose homeostasis (16), decreases plasma levels of triglycerides (TGs) and apolipoprotein B in normolipidemic rats (17), and acts as an *in vitro* (18, 19) and *in vivo* (20) anti-inflammatory. We have also shown that GSPE decreases postprandial plasma TG and apolipoprotein B in mice through a hepatic induction of a farnesoid X receptor (FXR) and the small heterodimer partner (SHP) that in turn down-regulates SREBP1c and other lipogenic genes in the liver (21, 22). Furthermore, we have demonstrated that the molecules responsible for the reduced TG synthesis in HepG2 cells treated with GSPE are the sum of a proanthocyanidins trimer and a dimer gallate because they reproduce the GSPE effect (23).

The effect of GSPE on metabolic syndrome has been studied in our laboratory by feeding rats a “cafeteria diet.” This diet is an experimental model of a western high sugar and high fat diet extensively used to produce obesity in rats because its palatability induces the animals to increase their energy intake (24). In a recent study conducted by our group (25) as well as this study, the rats were fed a high fat diet (HFD) (cafeteria diet) for 13 weeks, and one group of the animals was treated with a daily dose of GSPE (25 mg/kg of body weight) for the last 10 days (HFD + GSPE). In that study, HFD was shown to cause the animals to be overweight and to suffer from fatty liver, dyslipidemia, and hepatic overexpression of key genes

involved in lipogenesis and VLDL assembly, whereas GSPE treatment corrected dyslipidemia and down-regulated some of the genes up-regulated by HFD (25).

To better investigate the mechanism behind the changes observed in HFD- and HFD + GSPE-fed rats, we analyzed protein expression in the liver. Because GSPE treatment and obesity have multiple effects, a proteome-wide approach is needed to map proteins from different pathways. Proteomics studies related to obesity, metabolic syndrome, fatty liver, or insulin resistance have previously been performed on the liver (26–32). Two such studies looked into the effects of flavonoids in mouse livers (33, 34), but to our knowledge, this is the first hepatic proteome analysis of the effect of flavonoids in rats suffering from metabolic syndrome. To improve the proteome coverage of the complex liver samples, we performed a proteome fractionation according to protein solubility using a two-phase detergent protocol (35). This strategy was advantageous because it captured membrane proteins that otherwise would have been difficult to detect. The resulting soluble and membrane protein fractions were digested, iTRAQ-labeled, fractionated according to isoelectric point, and analyzed by nano-LC-MS/MS. The proteomics study presented here reports a differential expression due to HFD or HFD + GSPE for approximately 140 proteins, indicating that both conditions were potent modifiers of the liver proteome. We have focused on the sugar and lipid metabolism data, which confirmed the repression of hepatic lipogenesis in HFD + GSPE rats. Additionally, new proteins have been revealed as putative GSPE targets.

EXPERIMENTAL PROCEDURES

Animal Treatment—Wistar female rats weighing about 150 g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22 °C with a 12-h light/dark cycle. All rats were fed a standard chow diet (Panlab A03, Cornellà, Spain) for 1 week and then separated into three groups. Six control (C) rats continued to receive a standard chow diet and water, whereas 12 animals also had *ad libitum* access to fresh daily renewed cafeteria diet as an HFD model. The cafeteria diet had 13.6% fat, 21% carbohydrates, 9% protein, 51.3% water, and 5.1% others (36) and consisted of bacon, sweets, biscuits with foie gras, cheese, muffins, carrots, and milk with sugar. After 13 weeks of metabolic syndrome induction, the 12 rats on the HFD were divided into two groups of six rats. One group (HFD + GSPE) received an oral daily dose of 25 mg of GSPE/kg of body weight dissolved in condensed milk as a vehicle, whereas the other group (HFD) received only the equivalent amount of vehicle. GSPE contains monomers and oligomers of flavan-3-ols. After 10 days of treatment, C, HFD, and HFD + GSPE rats were sacrificed. On the day of sacrifice, the rats received the daily dose of the vehicle (HFD group) or GSPE (HFD + GSPE group) and had access to fresh cafeteria diet for 2 h. Afterward, food was withdrawn from all three groups, and after 3 h of fasting, the animals were sacrificed by beheading. The livers were excised, frozen immediately in liquid nitrogen, and stored at –80 °C until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University.

Proteome Fractionation—A new protocol based on Moebius *et al.* (37) was tried with test rat liver samples to set up the proteome

fractionation procedure into soluble and membrane protein fractions. Once the protocol was established, each of the 18 experimental samples was processed separately. Rat liver tissue was homogenized with a Potter-Elvehjem grinder, sonicated three times for 10 s, and briefly centrifuged to remove tissue particles and cell debris. The supernatant was ultracentrifuged ($120,000 \times g$, 60 min, 4°C) to separate the membranes from the new supernatant (soluble proteins (S)). The membrane pellet was washed in sodium carbonate buffer according to Fujiki *et al.* (38) to remove contaminant soluble proteins and ultracentrifuged again. The new membrane pellet was further purified by dissolving it in Triton X-114 buffer and separating the proteins into two phases according to Bordier (35). The detergent phase mostly contained membrane proteins (M) and was further analyzed.

Protein Digestion—The proteins in S and M fractions were precipitated with trichloroacetic acid and acetone, respectively, to concentrate and purify the protein sample. The pellet was resuspended in dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5 and 0.1% SDS) or dissolution buffer plus 0.1% Triton X-100 for S and M pellets, respectively. Disulfide bridges in proteins were reduced, cysteine residues were blocked, and proteins were digested overnight with trypsin following the manufacturer's instructions included with the iTRAQ reagent kit (Applied Biosystems, Foster City, CA).

Peptide Labeling and Fractionation—The labeling procedure was performed three times corresponding to biological triplicates for each S and M fraction. Two samples of each group were pooled (50 μg of peptides each), and each of the resulting 100- μg peptide samples was labeled with a different iTRAQ reagent in accordance with the manufacturer's instructions (Applied Biosystems). The subsequent analytical steps were essentially performed as described previously (39). Briefly, labeled peptides were mixed and purified through a strong cation exchange chromatography column followed by elution with a volatile buffer containing 5% ammonia and 30% methanol. Pure peptides were dried and resuspended in denaturing buffer. The peptides were separated on an Immobiline DryStrip gel (GE Healthcare) using isoelectric focusing on a Multiphor II unit (GE Healthcare). The pH range was 3.5–4.5 for S peptides and 3–10 for M peptides because initial experiments showed that these ranges give somewhat increased numbers of detected proteins in the respective samples. The gel strip was cut into 12 pieces, and the peptides were extracted from the gel in two steps with 0.5% TFA in 5% ACN. Peptides were vacuum-dried and redissolved in 0.5% TFA in 5% ACN. Peptides were purified on PepClean C_{18} spin columns (Pierce) according to the manufacturer's protocol prior to nano-LC analysis.

Nano-LC and MS Analysis—The peptide mixtures were analyzed by liquid chromatography (Easy nLC from Proxeon, Odense, Denmark) coupled to mass spectrometry (LTQ-Orbitrap, Thermo Fisher Scientific, Waltham, MA) through a nanoelectrospray source with a stainless steel emitter (Proxeon). The peptides were separated on a 75- μm reverse phase column packed with 3.5- μm Kromasil C_{18} particles (Eka Chemicals, Bohus, Sweden) using a 100-min gradient of ACN in 0.4% acetic acid, starting with 5% and ending with 35% ACN. The mass spectrometry detection constituted a full scan (m/z 400–2000) with Orbitrap detection at resolution $R = 60,000$ (at m/z 400) followed by up to four data-dependent MS/MS scans with LTQ detection of the most intense ions. Dynamic exclusion of 25 s was used as well as rejection of charge state 1+. Pulsed Q dissociation fragmentation was performed with an ion accumulation time of 140 ms, a target value of automatic gain control of 40,000, one microscan, an activation time of 0.1 s, an activation Q of 0.7, and a normalized collision energy of 33.

Database Searches and Statistics—The raw data files containing the MS spectra were processed using extract_msn.exe from February 15, 2005 (Thermo Fischer Scientific) to generate peak lists of the

tandem spectra. The processed data were searched with Mascot, version 2.2.04 (Matrix Science, London, UK), which was used for protein identification and iTRAQ reporter quantification. The raw files in each study from the 12 different fractions of peptides analyzed in duplicate by nano-LC-MS/MS were merged together and searched against the NCBI non-redundant database for the taxonomy *Rattus* containing 68,505 sequences (released September 11, 2008) using the Mascot multidimensional protein identification technology (MudPIT) scoring algorithm. Full-scan tolerance was 5 ppm, MS/MS tolerance was 0.75 Da, and up to two missed trypsin cleavages were accepted. Protein modifications were taken from the iTRAQ protocol of the manufacturer: 1) fixed modifications were iTRAQ label on lysine and the N terminus and methylthio modification of cysteines, and 2) variable modifications were oxidation of methionine and iTRAQ label on tyrosine. The threshold of protein identification significance was set to 0.001, which resulted in a false discovery frequency of 0.0009 when searched against a decoy database generated in Mascot. iTRAQ values were reported for proteins with four or more measured iTRAQ values where each peptide should have an expectation value of 0.02 or below. When identification of a protein in an analysis yielded several possible protein isoforms, all of them were considered for quantification. In the quantitative calculations, only protein isoforms with iTRAQ values in all three analyses were included. For each protein, the relative expression levels were calculated as a ratio between the sample and the average of all three samples in the same analysis. Mean and S.E. values of the three biological replicates were calculated. A two-tailed student's *t* test for equal variance data was applied with the criterion of $p < 0.05$. In addition, a threshold test was applied, requiring at least 20% difference (corresponding to $\sim 2\times$ median global standard error), to filter out alterations with a putative low biological impact and to further reduce the risk of false positives.

Mapping of gene name and Swiss-Prot codes from NCBI gi identifiers was done through the protein cross-reference file for International Protein Index rat release 3.61 and the protein knowledgebase (UniProtKB) ID mapping tool. The classification of differentially expressed proteins was performed manually according to the protein information in the protein knowledgebase (UniProtKB) and literature searches. Gene ontology (GO) data were retrieved from the downloaded Gene Association File for Rat (released October 8, 2009).

Hierarchical clustering of protein expression data to investigate overall similarities of the proteome samples was performed in Cluster 3.0 (40). Proteins that were statistically significant in at least one of the pairwise comparisons, HFD versus HFD + GSPE, HFD versus C, or C versus HFD + GSPE, were included. The data on a logarithmic scale were clustered using the similarity metric "correlation uncentered" and the clustering method "average linkage." The clusters were viewed in Java Treeview (40, 41).

RESULTS

Analysis of Rat Liver Proteome Fractionated According to Hydrophobicity—The aim of this study was to gain insight into the proteome in relation to the liver, including sugar and lipid metabolism as well as various membrane-associated transport and signaling pathways. Protein extracts from rat liver cells were therefore fractionated into two fractions: the M fraction containing hydrophobic proteins associated with the various membranes in the cell and the S fraction containing water-soluble proteins. This sample fractionation was used to prevent low abundance proteins from being masked by high abundance proteins. The fractionation strategy was performed using a combination of ultracentrifugation of membranes and two-phase separation based on the detergent

Triton X-114. The effect of fractionation on the number of proteins detected was assessed in an initial study. The sum of the number of proteins from the two fractionated samples was more than 2 times higher than the number of proteins from the unfractionated sample (data not shown). Of the additional proteins detected as a result of fractionation, ~35% originated from the S fraction, and 65% originated from the M fraction, emphasizing the value of isolating the M fraction in particular. Furthermore, the solubility problem of membrane proteins was minimized by cleaving the proteins into peptides, which were subsequently analyzed by nano-LC-MS/MS for identification and relative quantification. Using this proteomics approach, more than 1850 protein hits, with high quality quantification data, were obtained. All the quantified proteins are listed in supplemental Table S1, whereas proteomics data, including peptide data, from the two fractions can be found at the Proteomics Identifications database, PRIDE (42) (<http://www.ebi.ac.uk/pride/>, accession numbers 11674 and 11675). To further assess the fractionation efficiency, we looked at the overlap of the S and M fractions. When not taking protein isoforms into account, a total of 1131 gene products were detected: 607 from S and 449 from M fractions and only 75 detected in both fractions. That means that only 11% of proteins in the S fraction and 14% of proteins in the M fraction were also present in the other fraction, showing that the fractionation method used was efficient.

To further evaluate the fractionation of membrane proteins, we compared the percentage of known membrane proteins in the two protein fractions. Proteins assigned with the GO term “integral membrane protein” (GO:0016021) were found to be highly enriched in the membrane fraction with 34% of all annotated proteins compared with the soluble fraction with less than 1%. Thus, the applied strategy using ultracentrifugation, washing of the pellet, and two-phase separation followed by peptide-based proteomics proved to be suitable for the isolation and detection of membrane proteins, which due to low abundance and solubility problems are otherwise hard to detect.

Differential Protein Expression—A high fat diet and treatment by GSPE strongly influence obesity-related changes of the liver function. To study their effects on the liver proteome, we applied proteomics on three groups of rats: a C group fed a standard chow diet, rats fed an HFD, and rats fed a high fat diet combined with GSPE treatment (HFD + GSPE). Protein abundance data from the two proteome fractions were analyzed to filter out proteins with statistically significant ($p < 0.05$) altered levels as a function of metabolic syndrome (HFD *versus* C) and GSPE treatment (HFD *versus* HFD + GSPE). More than 140 of these proteins exhibited differences in abundance exceeding 20% in at least one of the comparisons. These proteins were manually classified according to similar involvement in metabolism or biological process and are presented in supplemental Table S2 under the following categories: sugar metabolism, lipid metabolism, nitrogen metabo-

lism, detoxification and oxidative stress protection, cytoskeletal proteins, G protein signaling and cell dynamics, transporters, nucleic acid binding, protein binding or modification, miscellaneous, and uncharacterized proteins. Table I contains proteins classified into the categories of sugar and lipid metabolisms, which are of particular interest in this study on metabolic syndrome in rats, and Fig. 1 graphically shows the expression changes of most of these proteins. Table II contains proteins from other categories exhibiting statistical alterations of a higher magnitude ($>50\%$). These proteins contribute to providing the full spectrum of physiological effects, and some of them are novel putative hallmarks of the effects of obesity and GSPE. The effects of HFD and GSPE treatment are described in the following sections.

Effects of High Fat Diet on Rat Liver Proteome—A high fat diet changed the expression of 90 proteins, with at least 20% change and $p < 0.05$, *versus* the control group (supplemental Table S2). As shown in Table I and Fig. 1A, several proteins involved in sugar metabolism were altered in HFD-fed rats. Two proteins involved in glycogen synthesis were up-regulated, UDP-glucose pyrophosphorylase 2 (UGP2) and glucan (1,4- α -), branching enzyme 1 (GBE1), which along with the increase in one of the glycolysis regulatory enzymes, pyruvate kinase (PKLR), point to surplus sugar metabolism. Glycolysis induction in HFD-fed rats was consistent with the observed down-regulation, although not significant, of one of the gluconeogenesis-regulating proteins, phosphoenolpyruvate carboxykinase 2 (PCK2). In support of this repression, the gluconeogenic enzyme responsible for the transformation of alanine into pyruvate, glutamic-pyruvate transaminase, was repressed more than 2-fold in HFD-fed rats. Two proteins related to the respiratory chain were induced in HFD-fed rats: the component of the respiratory chain that catalyzes the reduction of oxygen to water, cytochrome c oxidase subunit II (MT-CO3), and the protein that catalyzes the covalent attachment of heme to apocytochrome c and c_1 , cytochrome c-type heme-lyase (HCCS). Taken together, these results suggest that HFD induces glycogenesis, glycolysis, and the respiratory chain in the liver (Fig. 1A).

As expected, the levels of proteins involved in lipid metabolism were found to be altered in HFD-fed rats (Table I and Fig. 1A). Two of the enzymes involved in *de novo* FA synthesis, ATP-citrate lyase isoform 1 (ACLY) and fatty-acid synthase (FASN), increased more than 40% together with a 53% increase in one enzyme of the pentose phosphate pathway that generates NADPH for lipogenesis, transaldolase 1 (TALDO1). Furthermore, glycerol-3-phosphate dehydrogenase 1 (GPD1L), which, in addition to transferring reducing equivalents from cytosolic NADH to mitochondrial FADH₂, provides activated glycerol backbone for TG synthesis, was up-regulated by 51%. We also detected a 34% significant induction in HFD-fed rats of two enzymes involved in the control of acyl-CoAs in the cell: mitochondrial acyl-CoA thioesterase 2 (ACOT2) and butyryl-CoA synthetase 1 protein

TABLE I
Proteins of sugar and lipid metabolism with altered expression due to HFD and/or HFD + GSPE

Shown is the list of proteins involved in sugar and lipid metabolism with a significant (t test; $p < 0.05$) 20% or more difference in relative abundance between C rats and rats that consumed an HFD and/or between HFD rats and treated rats (HFD + GSPE) that consumed a high fat diet plus 25 mg of GSPE/kg of body weight for 10 days. The protein relative abundance ratios, HFD/C and HFD/(HFD + GSPE), were calculated from the averages of three biological replicates (each from a pool of two animals) from each sample group. Only proteins with quantitative signal from four of more MS scans were included. HFD/C and HFD/(HFD + GSPE) ratios are shown for a better comparison of the correction effect of GSPE; i.e. similar values in the two columns indicate similarity between C and GSPE-treated samples.

	gi number	UniProt code	Gene name	S/M fraction	Function or biological process	HFD/C	HFD/HFD + GSPE
Sugar metabolism							
UDP-glucose pyrophosphorylase 2	gi 67078526	Q4V8I9	<i>Ugp2</i>	S	Glycogen synthesis	1.28 ^a	1.25
Glucan (1,4- α -), branching enzyme 1	gi 109492774	ENSRNOP00000039448	<i>Gbe1</i>	S	Glycogen synthesis	1.21 ^a	0.95
Galactokinase 1	gi 56605662	Q5RKH2	<i>Galk1</i>	S	Sugar metabolism	1.57 ^a	0.98
Glucokinase regulatory protein	gi 6978884	Q07071	<i>Gckr</i>	S	Sugar metabolism	1.29 ^a	0.99
Pyruvate kinase	gi 185134818	B1WB99	<i>Pklr</i>	S	Glycolysis	1.42 ^a	1.34 ^a
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	gi 189163483	B2RYG2	<i>Pck2</i>	S	Gluconeogenesis	0.66	0.64 ^a
Glutamic-pyruvate transaminase	gi 13591961	P25409	<i>Gpt</i>	S	Gluconeogenesis, amino acid degradation	0.47 ^a	0.99
Lactate dehydrogenase A	gi 8393706	P04642	<i>Ldha</i>	S	Anaerobic glycolysis	1.26 ^a	1.05
Glutamine fructose-6-phosphate transaminase 1	gi 54400724	P82808	<i>Gfpt1</i>	S	Hexosamine pathway and protein glycosylation	0.51 ^a	0.63 ^a
Mitochondrial protein 18 kDa	gi 55741522	Q5XIG9	<i>Mtp18</i>	M	Carbon utilization	0.93	1.61 ^a
Cytochrome c oxidase subunit II	gi 110189718	Q8SEZ5	<i>mt-Co3</i>	M	Respiratory chain	1.26 ^a	1.03
Cytochrome c-type heme-lyase	gi 109510612	ENSRNOP00000034552	<i>Hccs</i>	M	Respiratory chain	2.15 ^a	2.54
Lipid metabolism							
ATP-citrate lyase isoform 1	gi 162287306	P16638	<i>Acly</i>	S	Lipid synthesis	1.41 ^a	1.74 ^a
Acetyl-coenzyme A carboxylase α	gi 11559962	P11497	<i>Acaca</i>	S	Lipid synthesis	1.15	1.35 ^a
Fatty-acid synthase	gi 2506136	P12785	<i>Fasn</i>	S	Lipid synthesis	1.50 ^a	1.75 ^a
Glycerol-3-phosphate dehydrogenase 1	gi 109484025		<i>Gpd1l</i>	S	Lipid synthesis	1.51 ^a	1.48 ^a
Malic enzyme 1	gi 158341689	ENSRNOP00000013244	<i>Me1</i>	S	Lipid synthesis	1.19	1.67 ^a
Glucose-6-phosphate dehydrogenase	gi 8393381	P05370	<i>G6pd</i>	S	Pentose phosphate pathway	1.07	1.44 ^a
Transaldolase 1	gi 42476292	Q9EQS0	<i>Taldo1</i>	S	Pentose phosphate pathway	1.53 ^a	1.37
6-Phosphogluconate dehydrogenase, decarboxylating	gi 149024656	P85968	<i>Pgd</i>	S	Pentose phosphate pathway	1.08	1.45 ^a
Butyryl-coenzyme A synthetase 1 protein	gi 197245828	B5DFA3	<i>Bucc1</i>	S	Fatty acid activation	1.34 ^a	1.22 ^a
Acyl-coenzyme A thioesterase 2, mitochondrial	gi 6166586	O55171	<i>Aco2</i>	S	Acyl-CoA metabolic process	1.34 ^a	1.39 ^a
Carnitine palmitoyltransferase 2	gi 134104110		<i>Cpt2</i>	S	FA oxidation	1.38	1.68 ^a
Acyl-coenzyme A dehydrogenase, short chain	gi 11968090	P15651	<i>Acads</i>	S	Mitochondrial FA β -oxidation	1.17	1.28 ^a
Acyl-coenzyme A dehydrogenase, long chain	gi 6978431	P15650	<i>Acadl</i>	S	Mitochondrial FA β -oxidation	0.91	0.75 ^a
2,4-Dienoyl-CoA reductase 1, mitochondrial	gi 17105350	Q64591	<i>Decr1</i>	M	Mitochondrial FA β -oxidation	1.16	1.36 ^a

TABLE I—continued

gi number	UniProt code	Gene name	S/M fraction	Function or biological process	HFD/C	HFD/HFD + GSPE
gi 8394149	P07872-1	Acox1	S	Peroxisomal FA β -oxidation.	0.45 ^a	0.77
gi 158341628	Q4QQW8	Pld2	S	Lipid degradation	0.68 ^a	0.56
gi 146345388	Q07969	Cd36	M	Multiple functions including FA oxidation and transport	0.87	1.21 ^a
gi 149068738	Q5BJN1	Stard10	S	Lipid metabolism	1.05	1.62 ^a
gi 404382			M	Intracellular lipid transport	0.71	0.68 ^a
gi 5732982	P16303	Ces3	S	Lipid metabolism	1.95 ^a	1.24
gi 66730429	Q562C4	Mettl7b	M	Found in fatty liver	1.05	1.53 ^a
gi 61556857	Q5BK21	Tm7sf2	M	Cholesterol synthesis	0.80	1.66 ^a

^a A value with a statistically significant difference ($p < 0.05$), of at least a factor of 1.2, between HFD and C abundance or between HFD and HFD + GSPE abundance values.

(BUCS1). ACOT2 catalyzes the hydrolysis of acyl-CoAs to the free FA and CoA (43), whereas BUCS1 is a mitochondrial medium chain acyl-CoA synthetase that activates 4–12-carbon atom FAs, adding CoA to them (for a review, see Ref. 44). On the other hand, two proteins related to lipid degradation were down-regulated in HFD-fed rats: the enzyme that desaturates very long chain acyl-CoAs in peroxisomal FA β -oxidation, palmitoyl acyl-CoA oxidase 1 (ACOX1), and a putative phospholipase B involved in lysosomal lipid degradation, mannose 6-phosphate protein p76 (PLBD2), showed a clear repression due to HFD (more than a 2- and 1.5-fold change, respectively). Carboxylesterase (CES3), which is involved in the metabolism of xenobiotics but also catalyzes several lipid metabolism reactions such as monoacylglycerol and triglycerol hydrolysis (45, 46), showed an almost doubled induction in HFD. These results suggest an increased lipogenesis and decreased lipid catabolism in HFD-fed rats (Fig. 1A).

In addition to proteins related to sugar and lipid metabolisms, other processes were also clearly altered by HFD (Table II). Some cytoskeletal proteins such as myosin light chain regulatory B (MYL12B), destrin, and regulator of microtubule dynamics protein 2 (FAM82A) were strongly up-regulated by HFD (between a 1.5- and 2-fold increase). Interestingly, two enzymes involved in hydrogen sulfide (H_2S) detoxification, sulfide-quinone reductase-like (SQRDL) and ethylmalonic encephalopathy 1 (ETHE1) (47, 48), were down-regulated more than 50% in HFD-fed rats with possible effects on cardiovascular health due to H_2S properties as an antioxidant and vasodilative (for a review, see Ref. 49). An enzyme related to inflammation (50), phospholipase A_2 -activating protein (PLAA), was also affected by HFD, showing a slight induction (29%). Moreover, the expression of two other proteins with reported involvement in cardiovascular disease changed slightly: cytochrome P450 family 2 subfamily e polypeptide 1 (CYP2E1) (51) was up-regulated by 21%, and betaine-homocysteine S-methyltransferase 1 (BHMT) (52) was down-regulated by 24% (supplemental Table S2).

Effects of GSPE Treatment on Liver Proteome of HFD-fed Rats—In this study, we detected 75 proteins whose expression was altered by GSPE in the livers of HFD-fed rats (supplemental Table S2). Of the proteins with a significant differential abundance of over 20% in HFD *versus* C or in HFD *versus* HFD + GSPE, Fig. 2 shows the 21 proteins changed in both comparisons. Curiously, these proteins exhibited similar expression in the HFD + GSPE and C groups, meaning that GSPE was able to correct HFD effects on rat livers and did not in any case worsen the situation, which would suggest some putative targets for the treatment of metabolic syndrome. Some of these proteins are related to lipid metabolism and were already mentioned in the previous section (PKLR, ACLY, FASN, GPD1L, ACOT2, and BUCS1). Additionally, other proteins were altered by the GSPE treatment although not significantly changed by HFD (Table I), but when looking at other members of the pathway, it seems as though the pathway

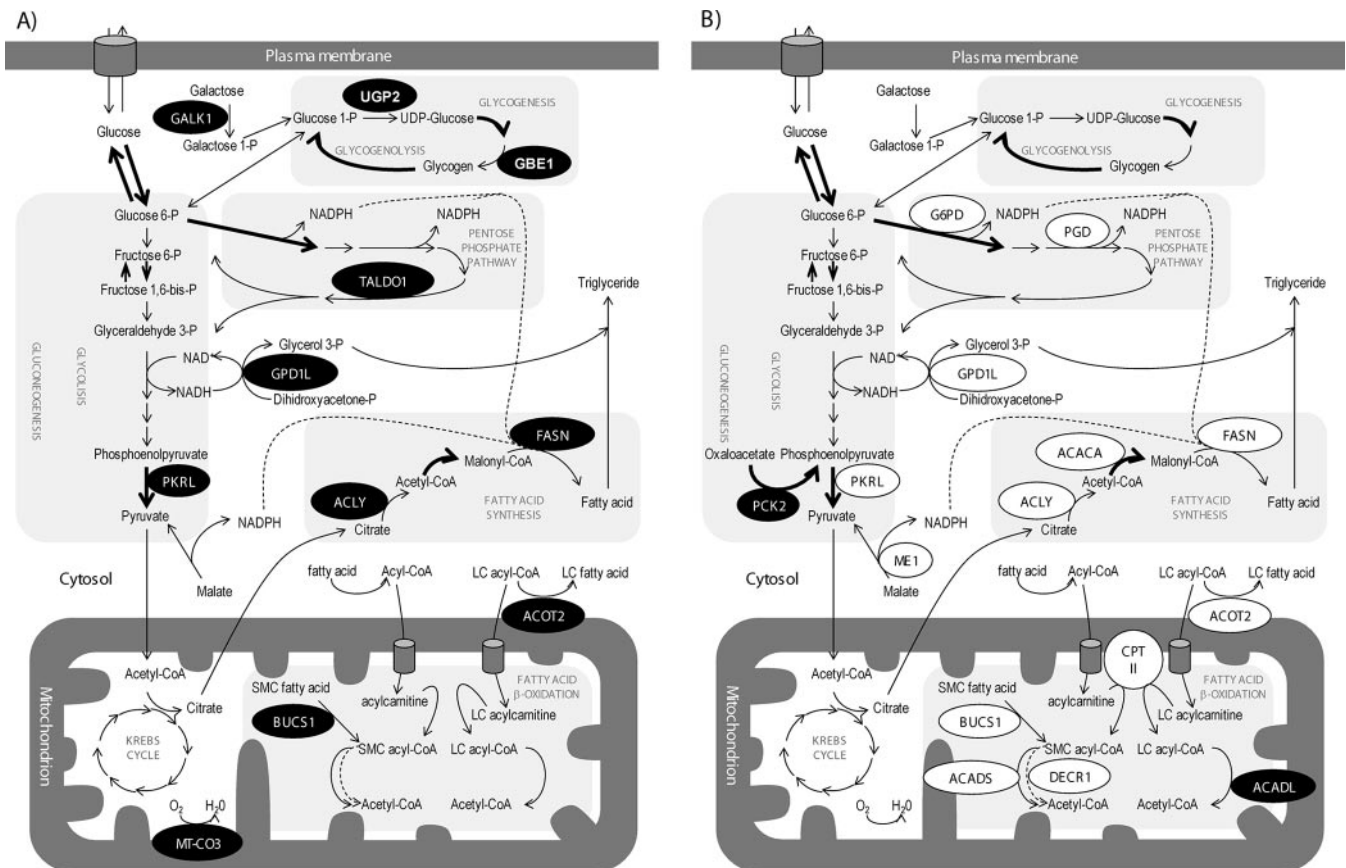


FIG. 1. Altered expression of proteins related to sugar and lipid metabolism in liver of HFD- and HFD + GSPE-fed rats. A shows changes in the expression of proteins involved in sugar and lipid metabolisms in the livers of rats fed an HFD versus control rats. B shows changes in rats fed HFD + GSPE (10 days of 25 mg of GSPE/kg of body weight) versus HFD-fed rats. Black circles mean up-regulation and white circles mean down-regulation of protein expression. GALK1, galactokinase 1; LC, long chain; SMC, short and medium chain; P, phosphate.

was indeed affected by HFD and corrected by GSPE (Fig. 1). For example, the lower abundance of acetyl-CoA carboxylase α (ACACA) in HFD + GSPE-fed rats fits the higher expression of the other FA synthesis-involved enzymes, ACLY and FASN, in HFD-fed rats. Likewise, PCK2 induction due to GSPE is consistent with PKLR induction in HFD-fed rats. Moreover, three proteins involved in supplying NADPH for FA synthesis, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase, decarboxylating (PGD), and malic enzyme 1 (ME1), were also corrected (down-regulated) by GSPE, whereas TALDO1 of the same pathway was up-regulated in HFD-fed rats (Fig. 1). Two proteins of fatty acid β -oxidation also seemed to support each other, long chain acyl-CoA dehydrogenase (ACADL) and the peroxisomal ACOX1, taking part in the β -oxidation of long and very long chain acyl-CoAs, respectively. Together, they indicate an increase in long chain fatty acid β -oxidation due to GSPE treatment (Table I). However, other parts of β -oxidation seemed to decrease as a result of GSPE treatment as demonstrated by three proteins: the inner mitochondrial membrane protein of the carnitine shuttle, carnitine palmitoyltrans-

ferase 2 (CPT-II); the enzyme catalyzing the first step of FA β -oxidation, short chain acyl-CoA dehydrogenase (ACADS); and the enzyme participating in the mitochondrial β -oxidation of unsaturated FA, 2,4-dienoyl-CoA reductase 1 mitochondrial (DECR1). Other proteins involved in lipid metabolism whose expression was only significantly altered in HFD + GSPE-fed rats (Table I) were the fatty-acid translocase CD36 antigen (CD36) (53); a protein associated with lipid droplets in fatty liver, methyltransferase-like 7B (METTL7B) (54); a lipid transfer protein capable of shuttling phosphatidylcholine and phosphatidylethanolamine between membranes, START domain-containing 10, found to be overexpressed in breast cancer but also present in the liver (55); and transmembrane 7 superfamily member 2, an endoplasmic reticulum 3β -hydroxysterol Δ^{14} -reductase acting on Δ^{14} -unsaturated sterol intermediates during the conversion of lanosterol to cholesterol (56). GSPE also affected the fatty acid-binding protein (FABP-II), increasing its expression by 50%. Taken together, these results show the clear impact of GSPE on proteins related to lipid metabolism.

GSPE also corrected (down-regulated) the expression of other proteins involved in various other processes (Fig. 2):

TABLE II
Proteins with at least 50% altered expression due to HFD and/or HFD + GSPE

Shown is the list of proteins with a significant (*t* test; *p* < 0.05) and at least 50% altered abundance in the comparison of HFD versus C and/or of HFD versus HFD + GSPE. For sample descriptions, see Table I. Proteins have been grouped according to their involvement in similar metabolism or biological process. Sugar and lipid metabolism-related proteins are not included. MHC, major histocompatibility complex.

	gi number	UniProt code	Gene name	S/M fraction	Function or biological process	HFD/C	HFD/HFD + GSPE
Nitrogen metabolism							
Solute carrier family 38, member 3	gi 21955257	Q9JHZ9	<i>Slc38a3</i>	M	Sodium-dependent amino acid/proton antiport	0.56 ^a	0.68 ^a
Detoxification and oxidative stress protection							
Sulfide-quinone reductase-like (yeast)	gi 149023155	B0BMT9	<i>Sqrdl</i>	M	Sulfur metabolism	0.65 ^a	0.53
Ethylmalonic encephalopathy 1	gi 157819563	B0BNJ4	<i>Ethe1</i>	S	Sulfur metabolism	0.62 ^a	0.83
S-Glutathiolated carbonic anhydrase III	gi 157875870	P14141	<i>Ca3</i>	S	Hydration of carbon dioxide, response to oxidative stress	0.94	0.62 ^a
Cytoskeletal proteins							
Myosin light chain, regulatory B	gi 8393781	P18666	<i>My12b</i>	S	Motor protein	1.63 ^a	1.52 ^a
Desrin	gi 75991707	Q7M0E3	<i>Dstn</i>	S	Actin depolymerization	1.51 ^a	1.10
Coronin, actin-binding protein, 1B	gi 149061951	O89046	<i>Coro1b</i>	S	Actin binding	1.08	1.57 ^a
Regulator of microtubule dynamics protein 2	gi 81295355	Q498D5	<i>Fam82a</i>	M	Interaction with microtubules	1.92 ^a	1.32 ^a
Bifunctional and Golgi-associated formiminotransferase cyclodeaminase octamer	gi 71041625			M	Cytoskeleton	1.10	1.61 ^a
Integrin α 1	gi 149059384	P18614	<i>Itga1</i>	M	Receptor for laminin and collagen	0.82	0.65 ^a
G protein signaling and cell dynamics							
RAB2B	gi 83415090	Q3B7V5	<i>Rab2b</i>	M	Signaling, membrane traffic, protein transport	1.89 ^a	1.29
RhoA	gi 2225894	O35791	<i>Rhoa</i>	S	Signaling, cytoskeleton	1.40	2.49 ^a
Ras homolog gene family, member B (<i>Homo sapiens</i>)	gi 4757764	P62745 or B2R692	<i>RHOB</i>	S	Angiogenesis, apoptosis, cell adhesion, differentiation, protein transport	1.38	2.56 ^a
Synembryn-A	gi 97181289	Q80ZG1	<i>Ric8a</i>	S	Signaling, mitosis	1.45	1.67 ^a
RAB21	gi 51948448	Q6AXT5	<i>Rab21</i>	M	Cell adhesion and migration	0.73	0.66 ^a
Transporters							
Solute carrier organic anion transporter family, member 1a4	gi 18777755	O35913	<i>Slco1a4</i>	M	Organic anion transport	0.56 ^a	0.92
Solute carrier family 30, member 1	gi 12408302	Q62720	<i>Slc30a1</i>	M	Zinc transport	1.06	1.54 ^a
Translocase of inner mitochondrial membrane 44	gi 8394449	O35094	<i>Timm44</i>	S	Mitochondrial protein translocation	0.69	0.64 ^a

TABLE II—continued

	gi number	UniProt code	Gene name	S/M fraction	Function or biological process	HFD/C	HFD/HFD + GSPE
Ribosome-binding protein 1	gi 109468922	XP_230637	<i>Rrbp1</i>	S	Protein translocation	0.66	0.65 ^a
Nucleic acid binding							
Heterogeneous nuclear ribonucleoprotein K isoform a (<i>H. sapiens</i>)	gi 14165437	P61978 or Q61BN1	<i>HNRNPK</i> or <i>HNRPK</i>	S	Host-virus interaction, mRNA processing	1.53 ^a	1.12
Heterogeneous nuclear ribonucleoprotein A2	gi 157059859	A7VJC2	<i>Hnrnpa2b1</i>	S	mRNA processing	0.58 ^a	0.70 ^a
Ribosomal protein L32 (<i>H. sapiens</i>)	gi 45066635	P62910 or B2R4Q3	<i>RPL32</i>	M	Translation	1.87 ^a	1.24
Ribosomal protein L10	gi 62664113			M	Translation, transcription regulation	1.62 ^a	1.24
3-Hydroxyacyl-CoA dehydrogenase type 2	gi 7387724	O70351	<i>Hsd17b10</i>	M	Mitochondrial tRNA maturation	1.43 ^a	1.85 ^a
Carbamyl-phosphatate synthetase 2	gi 149050765		<i>cad</i>	S	Pyrimidine biosynthesis	0.52 ^a	0.76
Protein binding or modification							
Protein kinase C substrate 80K-H	gi 157818781	B1WC34_RAT	<i>PrkcsH</i>	S	Protein binding	1.62 ^a	0.94
Protein phosphatase 2	gi 149041647	Q4QQT4	<i>Ppp2r1b</i>	S	Protein phosphatase	1.51 ^a	0.99
Alanyl (membrane) aminopeptidase	gi 149057276	P15684	<i>Anpep</i>	M	Proteolysis, angiogenesis, differentiation	0.73 ^a	0.63
Miscellaneous							
Ab2-079	gi 33086530	Q7TP70		M	Carotene metabolic process	1.16	1.62 ^a
Haloacid dehalogenase-like hydrolase	gi 157824168	B2RYT7	<i>Hdhc3</i>	S		0.58 ^a	0.72
Dihydroxyacetone kinase 2 homolog	gi 84781664	Q4KLZ6	<i>Dak</i>	S	Glycerol metabolic process, splitting of ribonucleoside diphosphate-X compounds	1.56 ^a	1.41 ^a
β -Globin minor	gi 164448680	P11517	<i>Hbb2</i>	S	Oxygen transport	0.59 ^a	0.86
Hemoglobin subunit β -2	gi 55825	P11517	<i>Hbb2</i>	S	Oxygen transport	0.56 ^a	0.82
Zero β -globin	gi 802111	Q63011	MGC72973	S	Oxygen transport	0.51 ^a	0.68
Hemoglobin α 1 chain	gi 6981010	P01946	<i>Hba1</i>	M	Oxygen transport	0.65 ^a	0.60 ^a
Hemoglobin α 2 chain	gi 60678292	P01946	<i>Hba1</i>	M	Oxygen transport	0.67 ^a	0.55 ^a
MHC class Ib antigen	gi 27805185	Q861J1	<i>RT1-CE5</i>	M	Antigen presentation	1.19	1.65 ^a
Complement component 9	gi 16924006	Q62930	C9	S	Complement pathway	1.51 ^a	0.82
Uncharacterized proteins							
rCG62519	gi 149048094			S	Predicted from sequence, "trans-isoprenyl diphosphate synthases"	0.65 ^a	0.94
rCG59263	gi 149017560			M	Predicted from sequence, "synthesis of cytochrome c oxidase family"	0.58 ^a	0.69

^a A value with a statistically significant difference ($p < 0.05$), of at least a factor of 1.2, between HFD and C abundance or between HFD and HFD + GSPE abundance values.

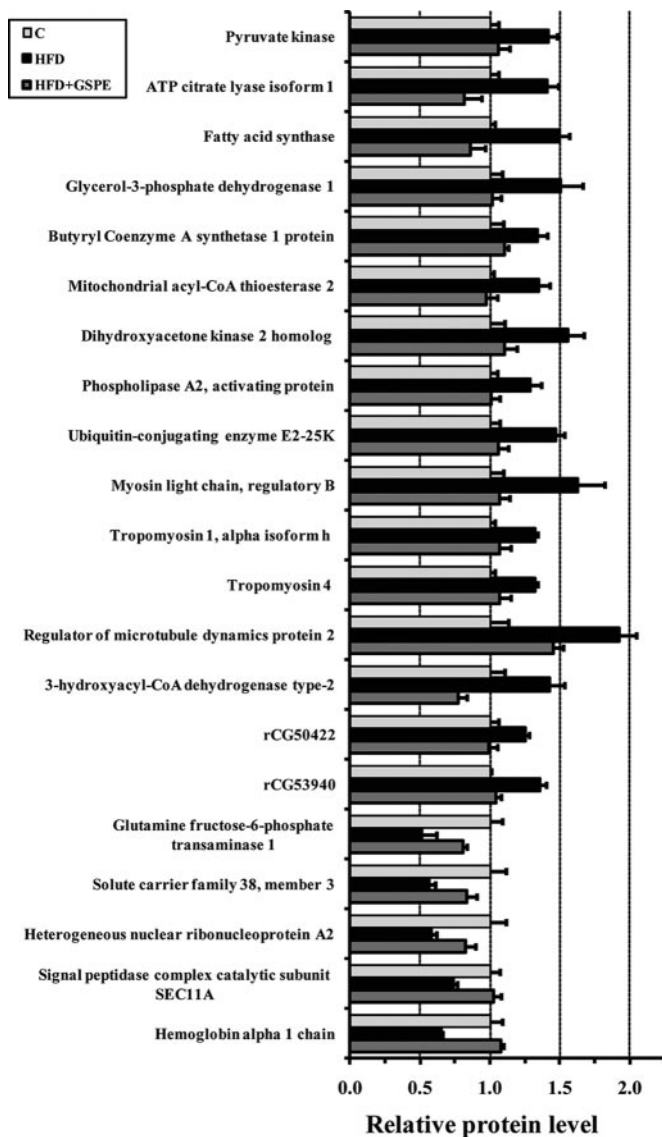


FIG. 2. **Correction of HFD-altered protein expression by GSPE.** Relative abundance of proteins in rats fed for 13 weeks with a chow diet (C), HFD, or high fat diet plus an oral dose of 25 mg of GSPE/kg of body weight for the last 10 days (HFD + GSPE) is shown. C, light gray; HFD, black; and HFD + GSPE, dark gray. The values are the average of three biological replicates and error bars represent Standard Error of the Mean. For each protein, the three values were normalized to get a C value of 1.0. All proteins showed a significant ($p < 0.05$) difference above 20% between C and HFD and between HFD and HFD + GSPE groups, indicating proteins for which the GSPE treatment reversed the effect of HFD back to the C level.

dihydroxyacetone kinase, an enzyme that catalyzes both the phosphorylation of dihydroxyacetone and the splitting of ribonucleoside diphosphate-X compounds; ubiquitin-conjugating enzyme E2-25K, which may mediate foam cell formation by the suppression of apoptosis of lipid-bearing macrophages through ubiquitination and subsequent degradation of p53; PLAA, which is related to inflammation; MYL12B, which plays an important role in the regulation of cell con-

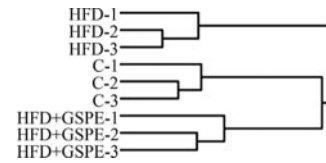


FIG. 3. **Clustering of experimental replicates according to protein expression.** Hierarchical clustering of protein quantity values of the various replicates shows the degree of similarity between the replicates as well as between the experimental groups.

tractile activity and is implicated in cytokinesis, receptor coupling, and cell locomotion; several tropomyosins that bind actin filaments and have been recently found in the wall of blood vessels in a portal area of liver (57); a protein that interacts with microtubules (FAM82A); and 3-hydroxyacyl-CoA dehydrogenase type 2, a mitochondrial multifunctional enzyme implicated in steroid and isoleucine metabolism (58) among other processes. Two more proteins, rCG50422 and rCG53940, were also up-regulated in HFD-fed rats and corrected in HFD + GSPE-fed rats. For these two proteins, no protein annotation is accessible yet, but sequence motifs (from NCBI data) indicate endonuclease and lactate dehydrogenase activities, respectively. Of interest, a number of cytoskeletal, G protein signaling, and cell dynamics proteins showed the same tendency; that is, HFD up-regulated their expression, and GSPE treatment acted in the opposite sense with more than 2-fold change in some cases (Table II and supplemental Table S2). Moreover, the zinc transporter solute carrier family 30 member 1 (SLC30A1), that has a putative effect on signaling and other zinc mediated processes in the liver, was also decreased by GSPE.

GSPE also significantly relieved the suppressed level of several proteins in HFD-fed rats (Fig. 2): glutamine fructose-6-phosphate transaminase 1 (GFPT1), which controls the flux of glucose into the hexosamine pathway; the sodium-dependent amino acid/proton antiporter solute carrier family 38 member 3, which may play a role in nitrogen metabolism and synaptic transmission; the heterogeneous nuclear ribonucleoprotein A2, which is involved with pre-mRNA processing; the signal peptidase complex catalytic subunit SEC11A, which removes signal peptides from nascent proteins, and finally hemoglobin.

The potential of GSPE to correct HFD-induced changes was further demonstrated by the comparison of the overall protein profiles between the three groups of rats. Hierarchical clustering was carried out on proteins that were statistically significant in at least one of the pairwise comparisons: HFD versus HFD + GSPE, HFD versus C, or C versus HFD + GSPE. As shown in Fig. 3, the C and HFD + GSPE samples resembled each other to a high degree, indicating the potential of GSPE treatment to normalize protein levels. Notably, this effect of the treatment was observed even if the diet was the same in the HFD + GSPE and HFD group and despite the short duration of the treatment. This led us to expect that the

GSPE treatment of metabolic diseases would produce a general beneficial effect on the liver.

DISCUSSION

In this study, we detected proteins modulated by GSPE treatment in the liver of a rat model of metabolic syndrome. More than 1000 proteins were detected, aided by fractionation of the proteome into soluble and membrane proteins, enabling mapping of the major metabolic pathways of the liver. Metabolic syndrome was induced by offering the animals a very tasty HFD that they could eat *ad libitum*. Other studies by our research group on the same animals, whose liver proteome has been studied here, have shown that HFD-fed rats suffer from metabolic syndrome symptoms such as obesity, fatty liver due to hepatic TG and cholesterol accumulation, dyslipidemia caused by high plasma TG and low density lipoprotein cholesterol (25), hyperinsulinemia (16), and inflammation (88). All these symptoms were slightly reduced in the group of HFD-fed rats treated with GSPE for 10 days (HFD + GSPE group) except dyslipidemia, which was completely corrected. Our results show that sugar and lipid metabolisms were particularly altered, describing the extensive effects of metabolic syndrome and its reversal by treatment with GSPE.

The animals were sacrificed 3 h after their respective diets were removed. This must be considered a postprandial state in which insulin promotes glucose transformation into glycogen and FA in the liver. Because HFD is also a sugar-rich diet, a higher sugar concentration is expected to reach the liver through the portal vein. In this study, we found two enzymes involved in glycogen synthesis (UGP2 and GBE1) up-regulated in HFD-fed rats (Fig. 1A). The expression of gluconeogenesis- and glycolysis-controlling enzymes (PCK2 and PKLR, respectively) indicates induction of gluconeogenesis in rats treated with GSPE and high glycolytic activity in HFD-fed rats. During glycolysis, NADH is produced, and we found an increase of GPD1L, an enzyme of one of the two shuttles that transfer the cytosolic reducing equivalents of NADH to the mitochondrion where it enters the respiratory chain to produce ATP, in response to HFD. Pyruvate, another product of glycolysis, can enter mitochondria and be converted to citrate, which in turn either 1) is oxidized in the Krebs cycle or 2) serves as an acetyl-CoA precursor necessary for *de novo* FA synthesis outside mitochondria. Actually, our results indicate that both pathways were activated in HFD-fed rats. First, MT-CO3 and HCCS, two enzymes related to the respiratory chain, were up-regulated in HFD-fed rats and tended to decrease with GSPE treatment. Second, ACLY, which converts citrate into acetyl-CoA, was found to be both induced in HFD- and repressed in HFD + GSPE-fed rats. Acetyl-CoA is one of the substrates of the FA synthesis regulatory enzyme ACACA, which has been shown to have higher gene expression as a result of a cafeteria diet (59). According to our results, ACACA expression was reduced in response to GSPE, suggesting a

reduction of FA synthesis, but not altered by HFD. The last enzyme in the FA biosynthetic pathway, FASN, was found to be 1.5-fold induced in HFD-fed rats. This cytosolic enzyme catalyzes the formation of long chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH. Other authors also found that high carbohydrate (60) and cafeteria diets (high fat and high sucrose diet) (59) induced FASN expression at the mRNA level in rats. Patients suffering from non-alcoholic fatty liver disease also show an increase of expression of FASN (61) consistent with the increased FASN expression and fatty liver found in HFD-fed rats. FASN is believed to be a determinant of the maximal capacity of a tissue to synthesize long chain fatty acids by *de novo* lipogenesis, and FASN inhibitors have been studied as a treatment for obesity because they mediate profound weight loss in animals (62). The results of this study suggest that GSPE may be a good candidate for FASN regulation because FASN expression was greatly down-regulated (even below control levels) in HFD + GSPE-fed rats. Similarly, other studies have shown a decreased expression of FASN due to different flavonoids in HFD-fed animals (63, 64). In addition, several other studies also describe flavonoids that inhibit FASN activity (65–72). Specifically, Kweon *et al.* (73) also found that the activity of FASN in rat liver was lowered by half by treatment with a grape seed extract.

NADPH is the other substrate needed for FA synthesis, and it is provided either in a reaction catalyzed by malic enzyme or via the pentose phosphate pathway. TALDO1, an enzyme of the pentose phosphate pathway, was induced in HFD-fed rats. The opposite phenomenon was observed in the HFD + GSPE group: a decrease in the expression of ME1, PGD, and G6PD. The latter, a key enzyme in the pathway, has previously been reported to be decreased by other flavonoids, hesperidin and naringin, in type 2 diabetic mice (72).

The normal fate of *de novo* synthesized FA is incorporation into triglycerides. In fact, HFD-fed rat livers had a significantly higher amount of triglycerides (25). The increase of GPD1L in HFD-fed rats would be consistent with the need for glycerol 3-phosphate for triglyceride synthesis, whereas the opposite would be true for HFD + GSPE-fed rats. This is in accordance with the finding, in the same rats, of low mRNA levels of diglyceride acyltransferase 2 (DGAT2) (25) because DGAT2 is a key enzyme in the free FA reesterification process that delivers TG to the nascent VLDL (74). Decreases in the microsomal transfer protein mRNA (key controller of VLDL assembly) have also been reported in HFD + GSPE-fed rats (25) that could diminish VLDL assembly. In this study, conclusions about VLDL assembly cannot be drawn because microsomal transfer protein expression only was changed with a low magnitude.

Taken together, these results indicate that GSPE decreased TG synthesis in obese rats despite the HFD they received. In fact, our group previously demonstrated that the hypotriglyceridemic effect of GSPE *in vivo* acts through an FXR- and SHP-dependent mechanism (21, 22). GSPE-enhanced FXR

activity up-regulates the expression of SHP, which in turn represses SREBP1c, the transcription factor that controls the expression of hepatic genes involved in FA and TG synthesis (75). Now, we have reported a decrease of lipogenesis in HFD + GSPE rats through the expression of an extended number of proteins. This would further confirm the effect of GSPE at an upstream transcriptional regulatory level, although additional direct effects on the expression or functionality of individual proteins cannot be ruled out. Similarly, other flavonoids decrease SREBP1c, ACACA, and FASN liver expression (63, 64) and activity (71) in rats fed an HFD.

According to Quesada *et al.* (25), both HFD- and HFD + GSPE-fed rats suffered from fatty liver, but GSPE showed a tendency to ameliorate this condition. Fatty liver consists of hepatic lipid accumulation and can be caused by, in addition to a higher uptake of lipids of external origin, increased lipogenesis or decreased lipid oxidation in the liver or lipoprotein secretion (for a review, see Ref. 76). It is generally thought that an activation of FA synthesis reduces β -oxidation through the allosteric inhibition of carnitine palmitoyltransferase 1 (CPT-I), the rate-determining step in mitochondrial FA β -oxidation. However, CPT-I was not detected in this study, and other enzymes involved in mitochondrial FA β -oxidation such as CPT-II, ACADS, ACADL, and DECR1 were not significantly changed in HFD-fed rats (Table I). In the present study, GSPE effects on mitochondrial FA β -oxidation seem to be dependent on FA chain length because CPT-II, ACADS, and DECR1 were repressed due to GSPE treatment, whereas ACADL was overexpressed (Fig. 1B). The repression of ACADS and induction of ACADL appear to be controversial, and further investigation would be needed to elucidate their impact on the pathway fluxes. Changes in protein levels are not always consistent with changes in activities because of posttranslational modifications, subcellular location, allosteric regulation, etc. In fact, activities are also not conclusive, which means that pathway fluxes should be measured to find out how the different parts of FA β -oxidation are influenced by HFD and GSPE.

Acyl-CoAs are substrates in FA β -oxidation, and BUCS1 and ACOT2 are two mitochondrial enzymes that control acyl-CoA levels. In this study, we found that both BUCS1 and ACOT2 were up-regulated in HFD- (Fig. 1A) and down-regulated in HFD + GSPE-fed rats (Fig. 1B). BUCS1 adds CoA to 4–11-carbon FAs, whereas ACOT2 removes CoA preferably from 14–20-carbon acyl-CoAs. According to their preferred substrate, down-regulation of BUCS1 and ACOT2 in mitochondria of HFD + GSPE rats would result in the presence of deactivated short and medium chain FAs and activated long chain FAs. The consequence could be a decrease in β -oxidation of short and medium chain acyl-CoAs and an increase in long chain FA β -oxidation in HFD + GSPE-fed rats, whereas the opposite would occur in HFD-fed rats. This correlates with the observed levels of ACADS and ACADL in both groups and with the already observed down-regulation of short and medium chain acyl-CoA synthetases in GSPE-

treated mice (21, 22). ACOX1, the first enzyme in peroxisomal FA β -oxidation of straight very long chain FAs, was reduced more than 2-fold in HFD-fed rats, indicating a lower β -oxidation of very long chain FAs, and the opposite seemed to be the case for HFD + GSPE-fed rats. Moreover, we found higher levels of catalase in HFD + GSPE-fed rats that could be due to a higher need for depletion of hydrogen peroxide produced by a higher ACOX1 activity in peroxisomes, reinforcing that FA β -oxidation is probably activated in HFD + GSPE-fed rats. Taken together, our results suggest that mitochondrial β -oxidation of FAs of different chain lengths could be controlled by BUCS1 and ACOT2 activities. In fact, this controlling role of BUCS1 would be consistent with the observation that polymorphisms in some human medium chain acyl-CoA synthetases are correlated with hypertriglyceridemia, visceral obesity (77), and cholesterol high density lipoprotein (78).

PLAA is another protein related to cardiovascular disease that was corrected by GSPE treatment in this study. PLAA activates a regulator of eicosanoid biosynthesis (phospholipase A₂) (79) and is induced in several inflammation situations (50). HFD-fed rats have been shown to have high tumor necrosis factor α and C-reactive protein levels in plasma, whereas GSPE treatment reduced high tumor necrosis factor α (88), indicating a decrease in inflammation. This might explain the decreased PLAA expression in HFD + GSPE-fed rats found in the present study. In fact, other flavonoids are known to inhibit PLA2A activity (80–82).

In addition to counteracting the effects produced by HFD, GSPE also modified the expression of some proteins not affected by diet and revealed them to be proteins with possible positive effects on cardiovascular disease. These include CD36 and METTL7B (Table I) among other putative GSPE targets such as SLC30A1, G protein signaling proteins, etc. (Table II and supplemental Table S2). We report a more than 50% decrease in response to GSPE of SLC30A1, the transporter that exports zinc ions out of cells. Previous results in our group showed a decrease in the mRNA level of this zinc transporter and an increase in labile zinc cytosolic levels in HepG2 hepatocytes treated with GSPE, and we hypothesized that through these changes in labile zinc levels GSPE would affect signaling processes and/or metabolic pathways modulated by zinc (83).

HFD and GSPE also strongly influenced the expression of G protein signaling proteins. Among them, the Ras-like protein Ras-related protein 1b (RAP1B) showed increased expression due to HFD, and GSPE treatment tended to decrease its expression. RAP1B has previously been found to be upregulated during a hyperglycemic state (84). Other flavonoids have been reported to have an effect on G protein signaling. For example, cocoa procyanidins inhibit the activity of the mitogen-activated protein kinase (85), and isoflavones attenuate vascular contraction, at least in part, through inhibition of the RhoA/Rho kinase signaling pathway (86).

We also found a lower expression of ETHE1 and SQRDL in HFD-fed rats (Table II), and GSPE tended to normalize their expression. ETHE1 and SQRDL are two enzymes involved in sulfur metabolism that take part in the pathway in which H₂S is oxidized to thiosulfate in three steps (48). H₂S oxidation has previously been found in the liver (87), and although H₂S has toxic properties (e.g. inhibiting the respiratory chain), the basal endogenous concentration of H₂S has been found to have a cardioprotective effect mainly due to its antioxidant and vasorelaxation properties (49). The decreased levels of these two enzymes in HFD-fed rats may allow the presence of higher concentrations of H₂S, which may balance out some of the risks of high blood pressure and oxidative stress associated with obesity. On the other hand, H₂S seems to be implicated in several biological processes (49), and we cannot exclude the possibility of other regulatory properties of H₂S in relation to obesity.

In summary, the increased expression of some enzymes involved in glycogenesis (UGP2 and GBE1), glycolysis (PKLR), FA synthesis (ACLY and FASN), NADPH synthesis (TALDO1), and glycerol synthesis (GPD1L) reinforces each other and demonstrates that glycogen synthesis and *de novo* lipogenesis were induced in the livers of rats fed an HFD. GSPE treatment of this metabolic syndrome animal model seems to reverse this effect, modifying the expression of PKLR, PCK2, ACLY, ACACA, FASN, G6PD, PGD, ME1, and GPD1L in the opposite direction. Decreased lipid synthesis due to GSPE is consistent with the corrected dyslipidemia reported previously for these animals (25) and extends the already demonstrated effect of GSPE in SREBP1c repression (21, 22, 25). Similarly, an effect of GSPE on the peroxisome proliferator-activated receptor α transcription factor may also be possible and needs to be further investigated as our results show that GSPE also modulates FA β -oxidation. Specifically, we found that β -oxidation would be induced in HFD-fed rats according to BUCS1 expression and reduced by GSPE for short and medium chain FAs (in view of ACADS repression), whereas long and very long chain FAs would be more actively oxidized in HFD + GSPE-fed rats (in view of ACADL induction). We hypothesize that this action could be controlled by acyl-CoA synthetases (BUCS1) and thioesterases (ACOT2), which would regulate the acyl-CoA levels in mitochondria. Some putative drug targets were also revealed during the course of this study that could ameliorate the deleterious effects of metabolic diseases on the liver such as GFPT1, CD36, PLAA, METTL7B, SLC30A1, ETHE1, and SQRDL and several proteins of G protein signaling.

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