Soy protein reduces hepatic lipotoxicity in hyperinsulinemic obese Zucker fa/fa rats

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Abstract Hepatic steatosis is commonly present during the development of insulin resistance, and it is a clear sign of lipotoxicity attributable in part to an accelerated lipogenesis. There is evidence that a soy protein diet prevents the overexpression of hepatic sterol-regulatory element binding protein-1 (SREBP-1), decreasing lipid accumulation. Therefore, the aim of the present work was to study whether a soy protein diet may prevent the development of fatty liver through the regulation of transcription factors involved in lipid metabolism in hyperinsulinemic and hyperleptinemic Zucker obese fa/fa rats. Serum and hepatic cholesterol and triglyceride levels, as well as VLDL-triglyceride and LDL-cholesterol, were significantly lower in rats fed soy protein than in rats fed a casein diet for 160 days. The reduction in hepatic cholesterol was associated with a low expression of liver X receptor-α and its target genes, 7-α hydroxylase and ABCA1. Soy protein also decreased the expression of SREBP-1 and several of its target genes, FAS, stearoyl-CoA desaturase-1, and Δ5 and Δ6 desaturases, decreasing lipogenesis even in the presence of hyperinsulinemia. Reduction in SREBP-1 was not associated with the presence of soy isoflavones. Finally, soy protein reduced SREBP-1 expression in adipocytes, preventing hypertrophy, which also helps prevent the development of hepatic lipotoxicity.—Tovar, A. R., I. Torre-Villalvazo, M. Ochoa, A. L. Elías, V. Ortíz, C. A. Aguilar-Salinas, and N. Torres. **Soy protein reduces hepatic lipotoxicity in hyperinsulinemic obese Zucker fa/fa rats. *J. Lipid Res.* 2005.** 46: **1823–1832.**

Supplementary key words steatosis • liver • liver X receptor-α • sterol-regulatory element binding protein-1

Obesity and associated diabetes are epidemic throughout the world. Obesity is the leading cause of insulin resistance and hyperinsulinemia, which predispose to glucose intolerance, diabetes, and cardiovascular diseases. As many as 40% of type 2 diabetics develop evidence of hepatic steatosis or fatty liver (1, 2).

Several lines of evidence indicate that fatty liver in insulin-resistant states is caused by the activation of the sterol-regulatory element binding protein-1c (SREBP-1c), which is increased in response to high insulin levels even in resistant states (3). SREBP-1c is a member of the family of SREBP membrane-bound transcription factors that activates mainly the transcription of genes involved in fatty acid synthesis (4). Thus, an increase in SREBP-1c expression increases the rate of lipogenesis in the liver (5). Analysis of the SREBP-1c promoter has revealed an insulin-responsive region that maps to a binding site for liver X receptors (LXRs), suggesting a common pathway of action (6, 7).

LXRs are nuclear hormone receptors that form active heterodimers with retinoid X receptors. They are activated by oxysterols and serve as key sensors of intracellular sterol levels by regulating the expression of genes that control cholesterol absorption, storage, transport, and elimination (8). Studies have demonstrated that the addition of the synthetic LXR ligand T0901317 increased the expression of SREBP-1c (9), indicating that these nuclear receptors provide interregulatory control of the cholesterol and fatty acid metabolism.

Several studies in humans and experimental animals have demonstrated that the ingestion of soy protein reduces serum cholesterol and triglycerides (10–12). Furthermore, it has been shown that soy protein consumption also reduces the accumulation of cholesterol and triglycerides in the liver, preventing the development of fatty liver (13). There is evidence that soy protein regulates the concentration of serum and hepatic lipids by different mechanisms. We have demonstrated that rats fed a soy protein diet can control serum and hepatic lipid concentration by modulating serum insulin concentration (14). In addition, short-term ingestion of soy protein leads to lower serum insulin concentration compared with rats fed casein, and this response is accompanied by a small increase in hepatic SREBP-1 mRNA (13). Also, long-term consump-

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tion of soy protein prevents hyperinsulinemia compared with rats fed casein, which in turn reduces the expression of SREBP-1 mRNA and its target genes FAS and malic enzyme, leading to low lipid hepatic depots of triglycerides and cholesterol (13).

However, it is not known whether soy protein has the same effect on hepatic steatosis in animal models that develop hyperinsulinemia. The obese male Zucker diabetic rat (ZDF fa/fa) develops hyperglycemia, hyperinsulinemia, hyperlipidemia, mild hypertension, and kidney disease (15). Because of a mutation in the intracellular domain of the leptin receptor, ZDF fa/fa rats are completely insensitive to the antilipogenic action of leptin (16). In addition, ZDF fa/fa rats also develop hepatic steatosis, and they have significant increases in triglyceride-rich lipoproteins and LDL-cholesterol. Therefore, the purposes of the present study were to assess whether the consumption of a soy protein diet may prevent the appearance of hepatic lipid abnormalities in the ZDF fa/fa rat and to investigate the mechanisms by which soy protein may regulate the different transcription factors and enzymes involved in lipid metabolism.

EXPERIMENTAL PROCEDURES

Materials and methods

All chemicals used were from Sigma. Nylon membranes (Hybond-XL), the Rediprime DNA labeling kit, and Redivue [α-^{32}P] dCTP (110 Terabecquerel/mmol) were purchased from Amersham Pharmacia. The vitamin-free test casein and the rest of the ingredients were obtained from Teklad (Madison, WI). Isolated soy protein (Supro 710) was kindly donated by Solae de México.

Diets

Diets were administered in dry form and contained 20% casein or soy protein, 5% corn oil, 5% mineral mix, 10% vitamin mix, and 0.0165% choline citrate. Cornstarch and sucrose in a 1:1 proportion were added to complete 100% of diet.

Animals

Five week old male obese homozygous (fa/fa) Zucker diabetic rats from Harlan (Indianapolis, IN) were kept in a room with a 12 h light/dark cycle at 22°C. The day after arrival, rats were placed in individual cages and randomly assigned to the casein or soy protein diet (n = 33). Rats had free access to their experimental diet and were studied in the fasting state. Animals had free access to water and were weighed every other day. Rats were deprived of food overnight before killing on day 0, 30, 60, 90, 120, and 160 by decapitation after being anesthetized with CO_2. Blood was collected in tubes with gel and clot activator (BD, Franklin Lakes, NJ) and centrifuged at 1,000 *g* for 10 min, and fasting serum glucose, cholesterol, triglycerides, and insulin were determined. Liver and adipose tissue samples were removed rapidly and immediately frozen and stored at −80°C for extraction of total RNA or histological studies. An adipose tissue sample was taken for hematoxylin and eosin staining. The animal protocol was approved by the Animal Committee of the National Institute of Medical Sciences and Nutrition, Mexico City.

Serum measurements

Serum insulin was measured with monoclonal anti-rat insulin radioimmunoassay using the Rat Insulin RIA kit (Linco Research, Inc., St. Charles, MO). Immune complexes were counted with a Cobra II γ counter (Packard Instruments, Meriden, CT). Serum glucose was measured by the glucose oxidase method with the Glucose Analyzer II (Beckman Instruments, Fullerton, CA). Serum triglycerides and cholesterol concentrations were assayed using an enzymatic/colorimetric assay kit (SERAPAK; Bayer).

Liver lipids

Total lipids were extracted from 100 mg of tissue according to the method of Folch, Lees, and Sloane-Stanley (17). Briefly, total lipids from tissues were extracted and homogenized in chloroform-methanol (2:1). The extraction solvent was evaporated, lipids were resuspended in isopropanol and 10% Triton, and triglycerides and cholesterol concentrations were assayed as described above.

Density gradient ultracentrifugation

To characterize the density distributions and lipid composition of the apolipoprotein B-containing lipoproteins, 3 ml of pooled serum (five rats per group) was fractionated by isopycnic density gradient ultracentrifugation using a Beckman SW 40 Ti rotor at 202,000 *g* for 40 h at 15°C (18). Briefly, serum density was increased to 1.063 g/ml by the addition of dry, solid KBr. A 0.5 ml cushion of 1.21 g/ml solution was placed at the bottom of the tube followed in order by 2 ml of the density-adjusted serum sample, 1 ml of 1.0464 g/ml solution, 1 ml of 1.0336 g/ml solution, 2 ml of 1.0271 g/ml solution, 2 ml of 1.0197 g/ml solution, 2 ml of 1.0117 g/ml solution, and 2 ml of 1.006 g/ml solution. After centrifugation, the gradient was eluted from the top using a peristaltic pump operating at a flow rate of 0.5 ml/min. Cholesterol and triglycerides were measured in each 0.5 ml fraction as described previously. VLDL-, intermediate density lipoprotein-, and LDL-cholesterol were measured by ultracentrifugation using a sequential procedure.

Histological analysis and Oil Red O staining

Adipose tissue was fixed by immersion in ethanol and embedded in paraffin, and 3 μm sections were stained with hematoxylin and eosin. For Oil Red O staining, frozen liver samples were sliced (4 μm) and stained in 60% Oil Red O stock solution (0.5 g of Oil Red O in 100 ml of isopropanol) for 5 min. Tissues were washed briefly in 60% polyethylene glycol and then rinsed in distilled water for microscopic observation and photography. Morphological analysis of adipose tissue was performed using the Leica Qwin image-analyzer system on a Leica DMLS microscope.

Isolation of total RNA and Northern blot analysis

Total RNA was isolated from tissues according to Chomczynski and Sacchi (19). For Northern blot analysis, equal aliquots of total RNA made from each rat liver were pooled (15 μg total) and electrophoresed on a 1% agarose gel containing 37% formaldehyde, transferred onto a nylon membrane (Hybond-XL; Amersham Pharmacia) by capillary blotting, and cross-linked with an ultraviolet cross-linker (Amersham). cDNA probes for the rat SREBP-1, peroxisome proliferator-activated receptor α (PPARα) and PPARγ, microsomal triglyceride transfer protein (MTP), stearoyl-CoA desaturase (SCD-1), FAS, 7-α hydroxylase (CYP7A1), ABCA1, carnitine palmitoyltransferase-1 (CPT-1), and Δ5 and Δ6 desaturases were prepared by reverse transcriptase-polymerase chain reaction with the primers shown in **Table 1**. Hybridization conditions and cDNA probe preparations were carried out as described (13). Digitization of the images and quantitation of the radioactivity of the bands were done using the Instant Imager (Packard Instruments). Membranes were also exposed to Extascan film (Kodak) at −70°C with an intensifying screen.

TABLE 1. Primers used for RT-PCR and real-time PCR

Gene	Primers (5′ to 3′)	mRNA	Predicted Size	Species	GenBank Accession Number
			bp		
Northern blot analysis					
ABCA1					
Sense	AAG GCA TCG GGG TCC AAT	6,801	1,287	Rat	NM_178095
Antisense	GGC GGT CAT CAA TCT CGT G				
7-α hydroxylase					
Sense	AAA GCG GGA AAG CAA AG	3,545	846	Rat	NM_012942
Antisense	AAC AAT TTT ATA CAG CGT AA				
Δ5 desaturase					
Sense	TCT TGC CCA CGA TGC CAC GAC	3,413	688	Rat	AF320509
Antisense	CTT TGC CCC GCC TGC TTC TGA				
Δ6 desaturase					
Sense	TGC CTT CCG TGC CTT CCA C	1,546	925	Rat	AB021980
Antisense	GTG CCC GCT GAA CCA GTC ATT				
FAS					
Sense	GCT TTG CTG CCG TGT CCT TCT	9,143	793	Rat	X62888
Antisense	GTG TCT GCT GGG GTC CTC CTC GTT				
Microsomal triglyceride transfer protein					
Sense	TCA TTC AGC ACC TCC GCA CTT	3,519	1,636	Rat	LOC310900
Antisense	ACC ACA GCC ACC CGA TTT TTC				
Peroxisome proliferator-activated receptor α					
Sense	CCC CAC CAG TAC AGA TGA GTC	2,022	1,022	Rat	NM_013196
Antisense	GGA GTT TTG GGA AGA GAA AGG				
Peroxisome proliferator-activated receptor γ					
Sense	GTT GAC ACA GAG ATG CCA TTC	1,838	1,445	Rat	AF246458
Antisense	CAG CGA CTG GGA CTT TTC T				
Stearoyl-CoA desaturase-1					
Sense	GCT GAG TTC TGG GCT TCT G	4,689	1,020	Rat	NM_139192
Antisense	CAT GTG CGG ATT TTG CTT A				
Sterol-regulatory element binding protein-1					
Sense	TCC CAG AGT AGC CCC TTG TCC	2,972	1,008	Rat	AF286470
Antisense	CCA GTC CCC ATC CAC GAA				
Carnitine palmitoyltransferase-1					
Sense	TAT GTG AGG ATG CTG CTT CC	4,377	629	Rat	NM_031559
Antisense	CTC GGA GAG CTA AGC TTG TC				
Real-time RT-PCR					
Liver X receptor-α	Rn 00581185_m1				

Multiplex RT-PCR

For quantitative real-time PCR, total RNA was extracted from liver according to Chomczynski and Sacchi (19) and pooled. The first-strand cDNA was synthesized from 100 ng of total RNA with the Multiscribe Master Mix (PE Applied Biosystems). Samples were subjected to quantitative real-time PCR using the TaqMan probe and primer sets for rat LXR-α (Rn 00581185-M1). The ABI PRISM 7000 system was used for the reaction and detection (Applied Biosystems). The probe was labeled with FAM at the 5′ end and with TAMRA at the 3′ end. PCR amplification was performed in a total volume of 25 μl containing 30 ng of cDNA sample, 200 nM of each primer, 100 nM LXR probe, and 12.5 μl of TaqMan Universal PCR Master Mix. For each reaction, the polymerase was activated by preincubation at 95°C for 10 min. Amplification was then performed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The LXR-α cDNA quantity in each sample was normalized to the housekeeping gene for 18 S rRNA. The probes and primers for rat LXR-α and 18 S rRNA were obtained from PE Applied Biosystems (Pre-Developed TaqMan Assay Reagents Control Kits). Real-time PCR was carried out in triplicate for each sample.

Preparation and culture of primary rat hepatocytes

Hepatocytes were isolated from rat liver prepared by the collagenase perfusion technique and separated from nonparenchymal liver cells and debris by centrifugation (20). Cell viability was assessed by the Trypan Blue exclusion test and was always >90%. Cells (65,000/cm^2) were plated on treated culture dishes (100 mm diameter; Corning, Corning, NY) and maintained in DMEM (Gibco BRL) supplemented with glucose, L-glutamine, pyridoxine hydrochloride, and sodium pyruvate. After 2 h, cells were washed and culture was continued in DMEM containing 10% heat-inactivated fetal bovine serum and 100 mg/ml streptomycin.

Statistical analysis

The results are presented as means ± SEM. Statistical analysis was done by unpaired *t*-test or by one-way ANOVA followed by Fisher's protected least-square difference test to determine significant differences among groups. Differences were considered significant at $P < 0.05$ (Statview statistical analysis program, version 4.5; Abacus Concepts, Berkeley, CA).

RESULTS

To examine the effect of soy protein on hepatic and adipose tissue lipid metabolism in an animal model with hyperinsulinemia and hyperleptinemia, ZDF fa/fa rats were fed a soy protein or casein diet for 30, 60, 90, 120, and 160 d. The average intake of ZDF fa/fa rats fed the casein or soy protein diet was similar during the 160 days of dietary treatment (24.6 ± 2.2 and 24.9 ± 2.4 g/day, respectively). Weight gain was the same in both groups until day 140; however, from day 150 on, rats fed the casein diet began to lose weight

compared with rats fed the soy protein diet ($P = 0.004$). By day 160, rats fed the casein diet weighed 592 ± 48 g, whereas those fed the soy protein diet weighed 687 ± 22 g ($P < 0.0004$). The difference in weight gain between groups can be explained by a physical deterioration observed in rats fed the casein diet in the last days of the study. Rats showed moderate hyperglycemia during the experimental period, and no significant difference in serum glucose concentration was observed between rats fed casein (9.3 ± 1.1 mmol/l) and rats fed soy protein (7.5 ± 0.6 mmol/l) (**Fig. 1A**). Fur-

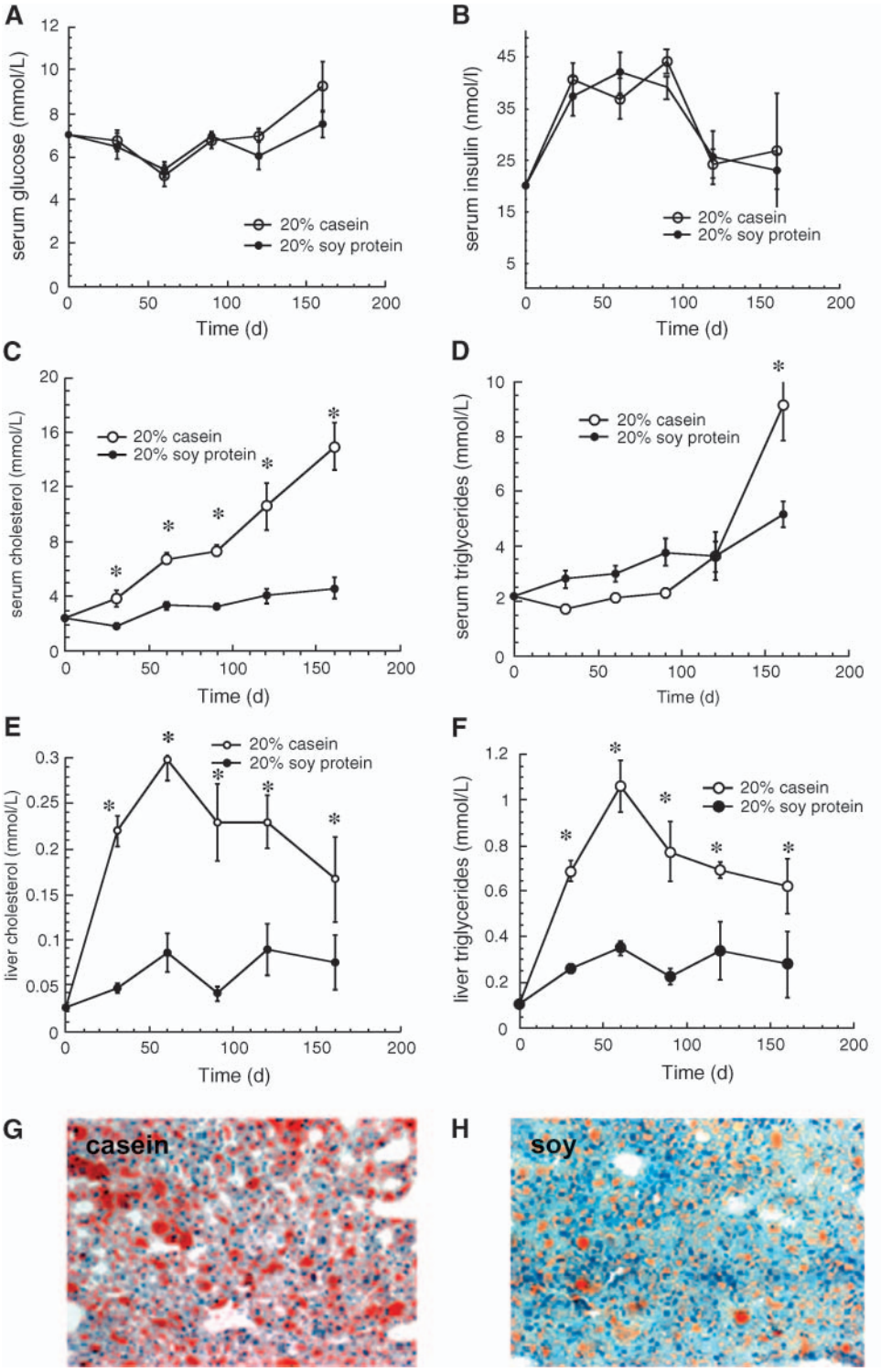

Fig. 1. Serum glucose (A), insulin (B), cholesterol (C), and triglycerides (D), hepatic cholesterol (E) and triglycerides (F), and Oil Red O staining of liver sections (G, H) were determined after overnight fasting from Zucker diabetic rats (ZDF fa/fa) fed a 20% casein or soy protein diet for 30, 60, 90, 120, and 160 days. Values are means ± SEM (n = 5). * Different from the soy protein group at that time ($P < 0.05$).

thermore, both groups showed hyperinsulinemia from the beginning of the dietary treatment (Fig. 1B). Nonetheless, serum glucose concentration tended to increase at the end of the study, whereas serum insulin levels decreased. The ZDF fa/fa rats fed both diets showed the highest insulin levels (~40 nmol/l) between days 30 and 90, and no difference was observed between the two groups. By day 120, insulin levels decreased to ~24 nmol/l. This pattern is probably associated with the progress from the prediabetic/insulin-resistant state to overt diabetes (21), perhaps indicating a decrease in the capacity of the pancreas to secrete insulin.

Previous studies in our laboratory demonstrated that soy protein decreases serum and liver lipids. These changes are in part associated with the capacity of soy protein to maintain insulin levels in the normal range. However, it is not known whether soy protein has the same effect on lipids in rats with hyperinsulinemia. Our results showed that soy protein intake maintained the capacity to control blood lipids even during diabetes. Thus, serum cholesterol concentrations in ZDF fa/fa rats fed the casein diet increased over time, reaching the highest concentration at day 160 (14.9 mmol/l), whereas ZDF fa/fa rats fed the soy protein diet showed 69% less serum cholesterol concentration (4.6 mmol/l) (Fig. 1C). In addition, serum triglyceride concentration in rats fed the soy protein diet was significantly higher than that in rats fed the casein diet until day 90. By day 120, there was no difference between the groups, although by day 160, rats fed the soy protein diet showed 43.8% lower serum triglyceride concentration (5.2 ± 0.5 mmol/l) than rats fed the casein diet (9.2 ± 1.3 mmol/l) (Fig. 1D). Furthermore, liver cholesterol concentration in rats fed the casein diet was significantly higher than that in rats fed the soy protein diet ($P < 0.001$). By day 160, ZDF fa/fa rats fed the soy protein diet showed a 68% lower concentration of liver cholesterol (0.075 mmol/l) than rats fed the casein diet (0.22 mmol/l) (Fig. 1E). Similarly, the liver triglyceride concentration of ZDF fa/fa rats fed the soy protein diet was 55% lower than that in rats fed the casein diet at the end of the study (Fig. 1F). The difference in hepatic lipid accumulation between rats fed soy protein and casein was clearly observed with Oil Red O staining, where liver from ZDF fa/fa rats fed soy protein showed lower lipid accumulation than that in rats fed casein (Fig. 1G, H).

To study whether the hypolipidemic effect of the soy protein diet in liver, despite the presence of hyperinsulinemia, was related to the expression of different transcription factors and enzymes involved in lipid metabolism, Northern blot analysis was performed to quantitate the concentration of SREBP-1 mRNA by measuring the radioactivity emitted by the bands in an Instant Imager (Packard Instruments). It has been reported that SREBP-1 is regulated by insulin; unexpectedly, however, in the present study, SREBP-1 mRNA abundance in the liver was significantly lower in rats fed soy protein than in rats fed casein, despite the high insulin concentration observed in both groups. On day 160, rats fed soy protein had 43% lower expression of SREBP-1c than rats

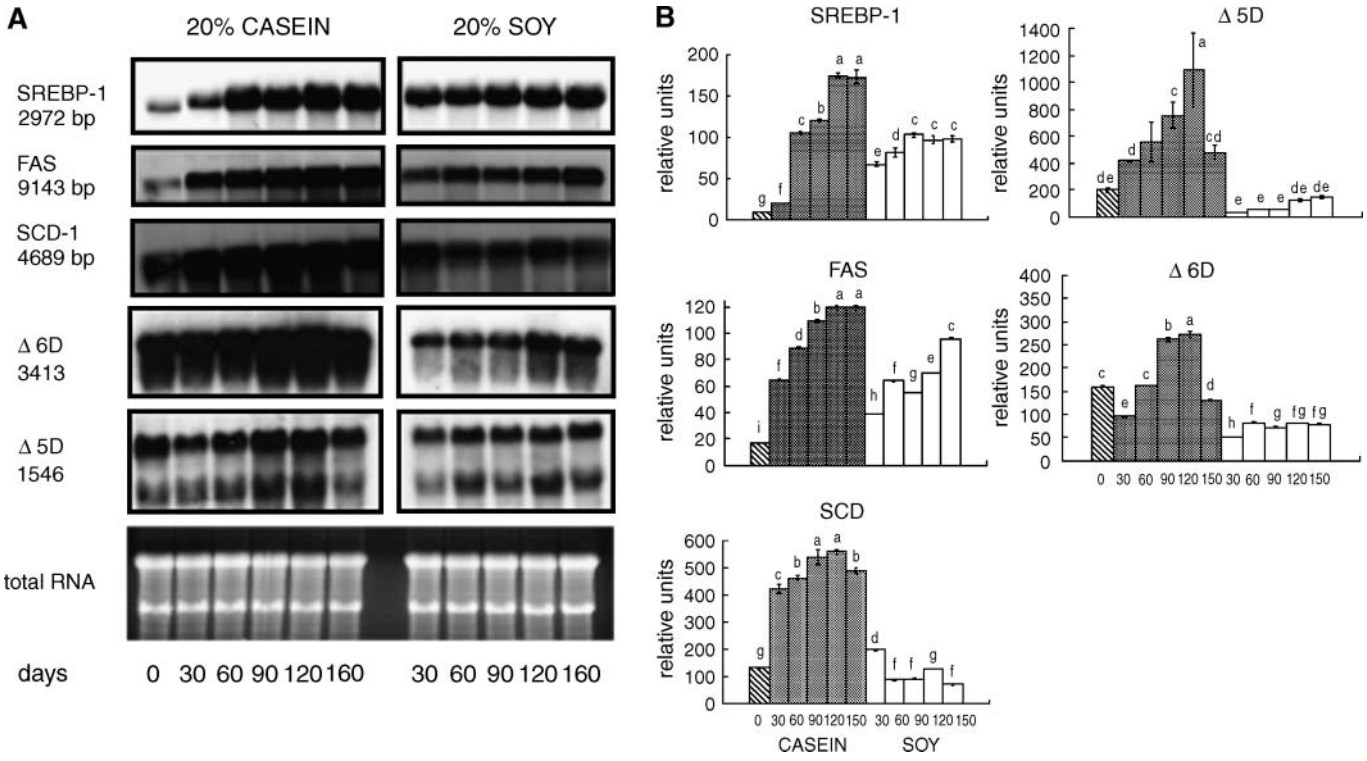

Fig. 2. A: Northern blot analysis of hepatic sterol-regulatory element binding protein-1 (SREBP-1), FAS, stearoyl-CoA desaturase-1 (SCD-1), and Δ6 and Δ5 desaturase genes involved in fatty acid biosynthesis from ZDF fa/fa rats fed a 20% casein or soy protein diet for 30, 60, 90, 120, and 160 days. The bottom row represents ethidium bromide gel staining. B: Densitometric analysis of hepatic SREBP-1, FAS, SCD-1, and Δ6 and Δ5 desaturases mRNA of ZDF fa/fa rats fed a 20% casein or soy protein diet for 30, 60, 90, 120, and 160 days. Values are means ± SEM (n = 5). Different letters indicate significant differences among groups ($P < 0.05$).

fed casein (**Fig. 2A**). SREBP-1 regulates the transcription of lipogenic genes that contain sterol-regulatory elements in their promoter regions. Among other genes regulated by this transcription factor are FAS, SCD-1, and Δ5 and Δ6 desaturases (22–24). As a result of the low SREBP-1 expression in rats fed soy protein, there was a concomitant significant reduction in the expression of FAS, SCD-1, and Δ5 and Δ6 desaturases. Reduction in the expression of these genes was evident beginning on day 30, and by the end of the experimental period, rats fed soy protein had 18, 85, 69, and 40% lower expression of FAS, SCD-1, and Δ5 and Δ6 desaturases, respectively, than rats fed casein ($P < 0.005$) (Fig. 2B). These results indicate that soy protein reduces the biosynthesis of fatty acids in the liver of ZDF fa/fa rats.

Nonetheless, the reduction of hepatic lipids by soy protein could also be the result of an increase in fatty acid oxidation. Therefore, we investigated the expression of PPARα, a transcription factor involved in regulating the expression of genes of fatty acid oxidation (25). The expression of this transcription factor in rats fed the casein diet decreased over time compared with that in rats fed the soy protein diet (**Fig. 3A**). Rats fed soy protein showed, at most time points, a significantly higher PPARα mRNA concentration than rats fed casein, indicating higher fatty acid oxidation capacity, which could in part contribute to the lesser accumulation of lipids in the liver. This observation is in agreement with recent work that established, using a low-density cDNA array, that the expression of hepatic PPARα increased in ZDF fa/fa rats fed a soy protein diet (26). In addition, CPT-1 expression increased significantly over time in rats fed the soy protein diet, whereas rats fed casein showed the opposite pattern (Fig. 3A, B). On the other hand, the expression of MTP, which is involved in the assembly and secretion of VLDL particles from the liver (27), showed a decreasing pattern with time in rats fed casein, whereas rats fed soy protein showed the opposite trend (Fig. 3A). At the end of the study, rats fed soy protein showed a 45% increase in MTP expression compared with those fed casein ($P < 0.05$) (Fig. 3B). The low expression of MTP observed in rats fed casein occurred despite the high serum and hepatic triglyceride concentrations as well as high VLDL-triglyceride and LDL-cholesterol concentrations (**Fig. 4A**, **B**) and probably represents a negative feedback mechanism to reduce the formation of hepatic de novo lipoproteins. Furthermore, animals fed soy protein had lower cholesterol and triglycerides in the VLDL particles (Fig. 4A, B), especially in the VLDL-1 and -2 subclasses (28) (Fig. 4C, D), and lower cholesterol content of all LDL subclasses compared with rats fed casein (Fig. 4A). This observation demonstrated that soy protein has hypolipidemic actions in insulin-resistant states.

As mentioned above, SREBP-1 expression is regulated by insulin; however, ZDF fa/fa rats fed soy protein had lower expression of this transcription factor than rats fed casein. This indicates that SREBP-1 expression during diabetes could be modulated by other mechanisms that do not involve insulin. To probe this hypothesis, we first studied whether the changes in SREBP-1 mRNA, attributable to the consumption of the soy protein or casein diet, were associated with changes in the expression of LXR-α. The expression of this transcription factor was measured by real-time PCR. As observed in **Fig. 5**, rats fed soy protein showed a trend toward lower LXR-α expression than rats

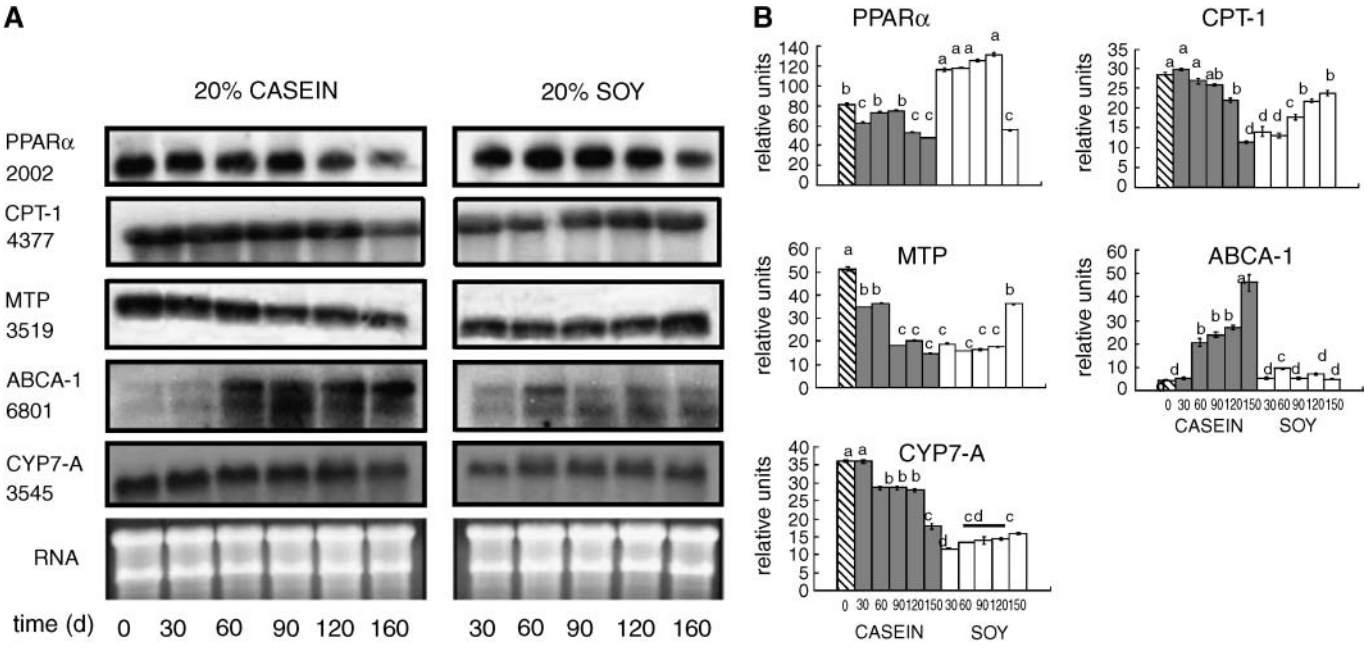

Fig. 3. A: Northern blot analysis of hepatic peroxisome proliferator-activated receptor α (PPARα), carnitine palmitoyltransferase-1 (CPT-1), microsomal triglyceride transfer protein (MTP), ABCA1, and 7-α hydroxylase (CYP7A1) mRNA from ZDF fa/fa rats fed a 20% casein or soy protein diet for 30, 60, 90, 120, and 160 days. B: Densitometric analysis of hepatic PPARα, CPT-1, MTP, ABCA1, and CYP7A1. Values are means ± SEM (n = 5). Different letters indicate significant differences among groups ($P < 0.05$).

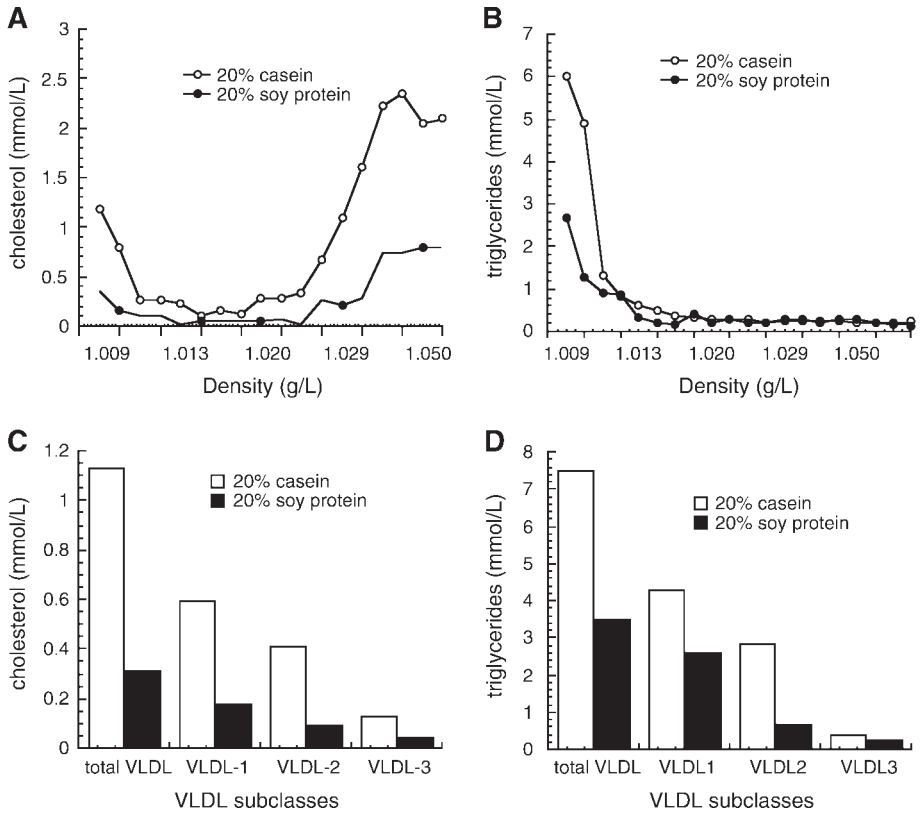

Fig. 4. Density gradient ultracentrifugation of pooled serum lipoprotein cholesterol (A) and triglycerides (B) from ZDF fa/fa rats after 160 days of consuming a 20% casein or soy protein diet. Cholesterol (C) and triglyceride (D) concentrations in the VLDL subclasses were measured in the serum samples.

fed casein during the experimental period, and this was significantly different on days 90 and 120 of the study. This suggests that LXR-α is partially involved in the regulation of SREBP-1 expression by soy protein in ZDF fa/fa rats. It has been demonstrated that LXR-α promotes cholesterol excretion by increasing bile acid synthesis and cholesterol secretion to bile through the induction of CYP7A1 (29) and ABCA1 transporter (30), respectively. As can be seen in Fig. 3A, rats fed the soy protein diet showed lower expression of hepatic ABCA1 and CYP7A1 than rats fed the casein diet. In addition, the low hepatic cholesterol concentrations in rats fed soy protein may reduce the formation of oxysterols, downregulating LXR-α activity and decreasing the hepatic expression of genes involved in the reverse transport and elimination of cholesterol.

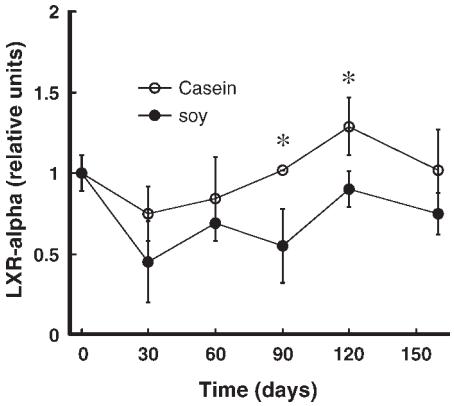

Fig. 5. Liver X receptor-α (LXR-α) gene expression in the livers of ZDF fa/fa rats fed 20% soy protein or casein for 30, 60, 90, 120, and 160 days. mRNA abundance was measured by real-time PCR analysis using a TaqMan gene expression assay. LXR-α values were normalized using the TaqMan assay for ribosomal 18S in the same reaction (multiplex assay). Values are means ± SEM (n = 5). Asterisks represent a significant difference between casein and soy protein groups at each time point ($P < 0.05$).

On the other hand, the hypolipidemic effect of soy protein has been associated with the presence of its isoflavones. To assess whether SREBP-1 was repressed by soy isoflavones, isolated hepatocytes from rats were incubated in the presence of increasing concentrations of genistein and daidzein, the main phytoestrogens found in soy. **Figure 6** shows that there was no change in SREBP-1 expression by the addition of different concentrations of isoflavones to the incubation medium, whereas hepatocytes incubated in the presence of insulin increased SREBP-1 mRNA abundance independently of isoflavones.

Finally, SREBP-1 and PPARγ gene expression in adipose tissue were also regulated by soy protein consumption. As can be seen in **Fig. 7A**, SREBP-1 was lower in rats fed soy protein than in rats fed casein, and PPARγ was constantly higher in rats fed soy protein than in rats fed casein. Histological analysis of adipose tissue in rats fed the soy pro-

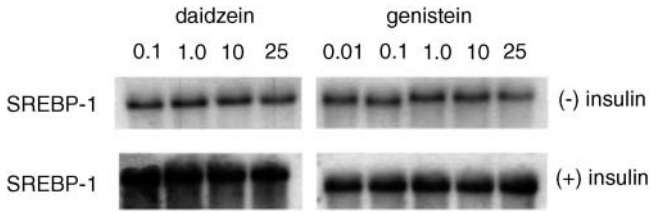

Fig. 6. Effect of different concentrations of soy isoflavones (daidzein or genistein) on SREBP-1 expression in cultured hepatocytes in the presence or absence of insulin. Hepatocytes were incubated for 18 h in DMEM with fetal bovine serum (10%) in the presence or absence of 20 mU/mol insulin and graded concentrations of isoflavones. RNA was isolated as described in Experimental Procedures.

tein diet showed that there were more adipocytes per area but they were smaller than those observed in rats fed the casein diet (Fig. 7B). These results suggest more hyperplasia in adipose tissue of rats fed soy protein, whereas adipose tissue of rats fed casein showed more hypertrophy attributable to an increased lipogenesis mediated by SREBP-1 (Fig. 7C, D).

DISCUSSION

As many as 40% of type 2 diabetics or ZDF obese fa/fa rats accumulate significant amounts of triglycerides not only in adipose tissue but also in liver. This leads to hepatic steatosis or "fatty liver," a condition that progresses to hepatic fibrosis and cirrhosis in a subset of individuals (2). Hepatic steatosis during obesity and insulin resistance occurs as a result of the increased uptake of free fatty acids released from adipose tissue but also, at least in rodents, through an increase in endogenous hepatic fatty acid biosynthesis (3, 31, 32). Interestingly, our results showed that in ZDF obese fa/fa rats, a soy protein diet ameliorates fatty liver and hepatic cholesterol and triglyceride levels to a greater extent than does a casein diet, despite the fact that the rats had high serum insulin concentrations. The consumption of soy protein reduced hepatic triglyceride levels by 50%, the same extent reported in obese mice with inactivation of SREBP-1c (33). In addition, dietary soy protein reduced serum cholesterol and triglycerides, accompanied by low LDL-cholesterol and VLDL-triglycerides.

The accumulation of lipids in the liver could be regulated by different transcription factors involved in the expression of genes for fatty acid biosynthesis. SREBP-1c has been established as the principal regulator of hepatic fatty acid biosynthesis (34). Interestingly, we found that this transcription factor is less abundant in the liver of rats fed soy protein than in rats fed casein. Several studies have demonstrated that insulin regulates the expression of SREBP-1 in liver and in rat primary hepatocytes (35). We demonstrated, in previous studies, that soy protein consumption prevented postprandial hyperinsulinemia and was associated with a decrease in SREBP-1 expression,

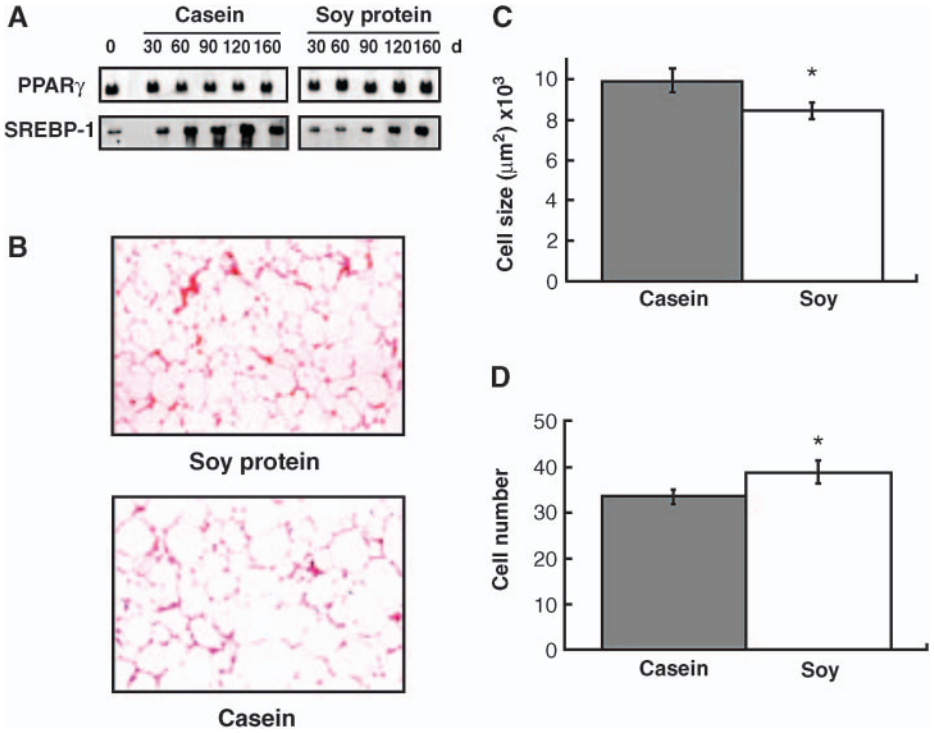

Fig. 7. Effect of the consumption of a casein or soy protein diet on adipose tissue from ZDF fa/fa rats. A: Northern blot analysis of PPARγ and SREBP-1 in adipocytes of rats fed soy protein or casein for 160 days. B: Histological analysis of adipose tissue of rats fed a soy or casein diet at day 160. C, D: Cell size (C) and cell count (D) in a representative population of white adipocytes from the epididymal depot. Data were analyzed on three slides for each animal. In each experiment, n = 5. Asterisks represent a significant difference between casein and soy protein group ($P < 0.05$). Data are mean ± SEM of 5 rats.

whereas the opposite occurred in rats fed casein (13). In the present study, despite high insulin concentrations in both groups, rats fed soy protein showed lower SREBP-1 expression than rats fed casein. These results clearly suggest that soy protein consumption regulates SREBP-1 expression through an insulin-independent mechanism even in the presence of profound insulin resistance. Evidence is emerging that dietary phytoestrogens play a beneficial role in decreasing serum lipid levels (36), although our results showed that SREBP-1 in cultured hepatocytes was not regulated by the presence of daidzein and genistein, the main isoflavones in soy. These results are in agreement with those described by Shay and colleagues (37), in which SREBP-1 expression was not affected by isoflavones in HepG2 cells, as well as studies in humans in which the consumption of soy-associated isoflavones was not related to changes in LDL- or HDL-cholesterol (38). However, there is evidence that SREBP-2 is increased in the presence of isoflavones (37).

As a result of low hepatic SREBP-1 mRNA concentration in rats fed soy protein, there was a concomitant reduction of FAS mRNA abundance, leading to a decrease in endogenous fatty acid biosynthesis. Additionally, low SREBP-1 expression also reduced the abundance of SCD-1 and Δ5 and Δ6 desaturase mRNAs. SCD-1 is one of the enzymes whose expression is increased in ob/ob mice and corrected by leptin administration (22). SCD-1 mRNA levels were highly increased in ZDF fa/fa rats fed the casein diet, whereas rats fed the soy protein diet showed an ~80% decrease, indicating that this enzyme can be regulated by SREBP-1 in spite of hyperleptinemia. The reduction in SCD-1 expression by soy protein could be associated with subsequent reductions in hepatic lipids and VLDL synthesis. In the liver, fatty acids are esterified with glycerol to form triglycerides or with cholesterol to form cholesteryl esters that can: *1*) accumulate, leading to increased hepatic lipid content; *2*) be packaged into VLDL for transport to other tissues; or *3*) be oxidized. Therefore, if SCD-1 is low, there will be low monosaturated fatty acids, the enzymatic products of SCD-1 required for phospholipid and cholesteryl ester synthesis, leading to low VLDL production.

Adipose tissue secretes several factors that may participate in metabolic cross-talk to other insulin-sensitive tissues (39). PPARγ and SREBP-1 participate in the development of insulin resistance in adipose tissue (33, 40). PPARγ, a member of the nuclear hormone receptor superfamily, is required for normal adipocyte differentiation, and SREBP-1 is responsible for lipogenesis. PPARγ mRNA expression in adipose tissue was constantly higher in ZDF fa/fa rats fed the soy protein diet than in rats fed the casein diet, and SREBP-1 was higher in rats fed casein than in rats fed soy protein. Also, histological analysis of adipose tissue of rats fed soy protein showed more and smaller adipocytes than those present in rats fed casein. These findings suggest that the consumption of a soy protein diet maintains a lower number of dysfunctional adipocytes than that found in rats fed casein, because the adipose tissue of rats fed soy protein develops less hypertrophy, possibly as a result of low lipogenesis.

Another possible mechanism by which soy protein may regulate lipogenesis and cholesterol homeostasis is through LXRs. One of the proposed target genes for LXR-α is CYP7A1, which encodes the enzyme cholesterol 7α-hydroxylase. Thus, a putative LXR response element was identified in the CYP7A1 promoter. CYP7A1 is the rate-limiting step in the classical pathway for the conversion of cholesterol to bile acids (41). Our results show that LXR-α and CYP7A1 genes were induced in response to hepatic cholesterol levels independent of the insulin concentration, because ZDF fa/fa rats fed the casein diet showed higher hepatic cholesterol and triglyceride levels than those fed the soy protein diet. It has been reported that the expression of the CYP7A1 gene is induced in response to dietary cholesterol, thereby accelerating the conversion of cholesterol to bile acids and promoting a net excretion of cholesterol (42).

LXRs also activate the transcription of genes that regulate reverse cholesterol transport, including ABCA1 (30). ABCA1 facilitates the efflux of cholesterol from extrahepatic tissues. ABC transporter family members in the liver also participate in bile formation and lipid metabolism (43). Hepatic ABCA1 transporter is also involved in the modulation of cholesterol in HDL particles, which in turn regulates hepatic cholesterol content (44). As shown in Fig. 3A, this transporter was induced in ZDF fa/fa rats fed the casein diet with time, whereas the expression of this gene was less induced and was constant over time in rats fed the soy protein diet. These results support the notion that soy protein regulates cholesterol excretion by modifying bile acid synthesis and cholesterol excretion via LXRs through the control of CYP7A1 and ABCA1 transporter gene expression, depending on hepatic oxysterol concentration.

Finally, we propose that the molecular mechanism for the regulation of SREBP-1 gene expression by soy protein is through the negative regulation of LXR-α, possibly mediated by hepatic cholesterol concentration in diabetic ZDF fa/fa rats independent of insulin or leptin concentration. This model excludes the direct repression of SREBP-1 by isoflavones. In addition, there is an increase in PPARα and CPT-1 that implies an increase in the oxidation of fatty acids. All of these changes in rats fed soy protein reduce the accumulation of triglycerides and cholesterol in the liver, leading to a reduction of hepatic steatosis.

REFERENCES

1. Browning, J. D., L. S. Szczepaniak, R. Dobbins, P. Nuremberg, J. D. Horton, J. Cohen, S. M. Grundy, and H. Hobbs. 2004. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology.* **40:** 1387–1395.
2. Marchesini, G., M. Brizi, G. Bianchi, S. Tomassetti, E. Bugianesi, M. Lenzi, A. J. McCullough, S. Natale, G. Forlani, and N. Melchionda. 2001. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes.* **50:** 1844–1850.
3. Browning, J. D., and J. D. Horton. 2004. Molecular mediators of hepatic steatosis. *J. Clin. Invest.* **114:** 147–152.
4. Horton, J. D., J. L. Goldstein, and M. S. Brown. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* **109:** 1125–1131.
5. Shimano, H., J. D. Horton, R. E. Hammer, I. Shimomura, M. S.

Brown, and J. L. Goldstein. 1996. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.* **98:** 1575–1584.
6. Chen, G., G. Liang, J. Ofu, J. L. Goldstein, and M. S. Brown. 2004. Central role for liver X receptor in insulin-mediated activation of SREBP-1c transcription and stimulation of fatty acid synthesis in liver. *Proc. Natl. Acad. Sci. USA.* **101:** 11245–11250.
7. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. A. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. *Genes Dev.* **14:** 2819–2830.
8. Zhang, Y., and D. J. Mangelsdorf. 2002. LuXuRies of lipid homeostasis. *Mol. Interv.* **2:** 78–87.
9. Russell, A. D., J. Ou, J. L. Goldstein, and M. S. Brown. 2001. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc. Natl. Acad. Sci. USA.* **98:** 1477–1482.
10. Anderson, J. W., B. M. Johnstone, and M. E. Cook-Newell. 1995. Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **333:** 276–282.
11. Tovar, A. R., F. Murguia, C. Cruz, R. Hernandez-Pando, C. A. Aguilar-Salinas, J. Pedraza-Chaverri, R. Correa-Rotter, and N. Torres. 2002. A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome. *J. Nutr.* **132:** 2562–2569.
12. Zhan, S., and S. C. Ho. 2005. Meta-analysis of the effects of soy protein containing isoflavones on the lipid profile. *Am. J. Clin. Nutr.* **81:** 397–408.
13. Ascencio, C., N. Torres, F. Isoard-Acosta, F. J. Gómez-Pérez, R. Hernández-Pando, and A. R. Tovar. 2004. Soy protein affects serum insulin and hepatic SREBP-1 mRNA and reduces fatty liver in rats. *J. Nutr.* **134:** 522–529.
14. Tovar, A. R., C. Ascencio, and N. Torres. 2002. Soy protein, casein, and zein regulate histidase gene expression by modulating serum glucagon. *Am. J. Physiol.* **283:** E1016–E1022.
15. Peterson, R. G., W. N. Shaw, M. A. Neel, L. A. Little, and J. Eichberg. 1990. Zucker diabetic fatty rat as a model for non-insulin-dependent diabetes mellitus. *Ilar News.* **32:** 16–19.
16. Phillips, M. S., Q. Liu, H. A. Hammond, V. Dugan, P. J. Hey, C. J. Caskey, and J. F. Hess. 1996. Leptin receptor missense mutation in the fatty Zucker rat. *Nat. Genet.* **13:** 18–19.
17. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
18. Aguilar-Salinas, C. A., P. H. Barrett, J. Kelber, J. Delmez, and G. Schonfeld. 1995. Physiologic mechanisms of action of lovastatin in nephrotic syndrome. *J. Lipid Res.* **36:** 188–199.
19. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
20. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J. Cell Biol.* **43:** 506–520.
21. Unger, R. H., and L. Orci. 2001. Diseases of liporegulation: new perspective on obesity and related disorders. *FASEB J.* **15:** 312–321.
22. Cohen, P., and J. M. Friedman. 2004. Leptin and the control of metabolism: role for stearoyl-CoA desaturase-1 (SCD-1). *J. Nutr.* **134 (Suppl.):** 2455–2463.
23. Kim, J. B., P. Sarraf, M. Wright, K. M. Yao, E. Mueller, G. Solanes, B. B. Lowell, and B. M. Spiegelman. 1998. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J. Clin. Invest.* **101:** 1–9.
24. Matsuzaka, T., H. Shimano, N. Yahagi, M. Amemiya-Kudo, T. Yoshikawa, A. H. Hasty, Y. Tamura, J. Osuga, H. Okazaki, Y. Iizuka, et al. 2002. Dual regulation of mouse Delta5- and Delta6-desturase gene expression by SREBP-1 and PPARα. *J. Lipid Res.* **43:** 107–114
25. Jump, D. B., and S. D. Clarke. 1999. Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* **19:** 63–90.
26. Banz, W. J., J. Davis, R. Peterson, and M. J. Iqbal. 2004. Gene expression and adipocity are modified by soy protein in male Zucker diabetic fatty rat. *Obes. Res.* **12:** 1907–1913.
27. Hussain, M. M., J. Shi, and P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* **44:** 22–32.
28. Adiels, M., C. Packard, M. J. Caslake, P. Stewart, A. Soto, J. Westerbacka, B. Wennberg, S. Olofsson, M. Taskinen, and J. Borén. 2005. A new combined multicompartmental model for apolipoprotein B-100 and triglyceride metabolism in VLDL subfractions. *J. Lipid Res.* **46:** 58–67.
29. Chiang, J., Y. Kimmel, and G. Stroup. 2001. Regulation of cholesterol 7 alpha-hydroxylase gene (CYP 7 A1) transcription by the liver orphan receptor (LXRalpha). *Gene.* **262:** 257–267.
30. Repa, J. J., S. D. Turley, J. A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R. A. Heyman, J. M. Dietschy, and D. J. Mangelsdorf. 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science.* **289:** 1524–1529.
31. Takaishi, K., L. Duplomb, M. Wang, J. Li, and R. H. Unger. 2004. Hepatic insig-1 or -2 overexpression reduces lipogenesis in obese Zucker diabetic fatty rats and in fasted/refed normal rats. *Proc. Natl. Acad. Sci. USA.* **101:** 7106–7111.
32. Weigman, C. H., R. H. Bandsma, M. Ouwens, F. van der Sluijs, R. Havinga, T. Boer, D. Reijngoud, J. A. Romijn, and F. Kuipers. 2003. Hepatic VLDL production in ob/ob mice is not stimulated by massive de novo lipogenesis but is less sensitive to the suppressive effects of insulin. *Diabetes.* **52:** 1081–1089.
33. Yahagi, N., H. Shimano, A. Hasty, T. Matzuzaka, T. Ide, T. Yoshikawa, M. Amemiya-Kudo, S. Tomita, H. Okasaki, Y. Tamura, et al. 2002. Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lepob/Lepob mice. *J. Biol. Chem.* **277:** 19353–19357.
34. Shimano, H., J. D. Horton, I. Shimomura, R. E. Hammer, M. S. Brown, and J. L. Goldstein. 1997. Isoform 1c of sterol regulatory element binding protein is less active than isoform 1a in livers of transgenic mice in cultured cells. *J. Clin. Invest.* **99:** 846–854.
35. Shimomura, I., Y. Bashmakov, and J. D. Horton. 1999. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J. Biol. Chem.* **274:** 30028–30032.
36. Potter, S. M., J. A. Baum, H. Teng, R. J. Stillman, N. F. Shay, and J. W. Erdman. 1998. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.* **68 (Suppl.):** 1375–1379.
37. Mullen, E., R. Brown, T. Osborne, and N. Shay. 2004. Soy isoflavones affect sterol regulatory element binding proteins (SREBPs) and SREBP-regulated genes in HepG2 cells. *J. Nutr.* **134:** 2942–2947.
38. Weggemans, R. M., and E. A. Trautwein. 2003. Relation between soy-associated isoflavones and LDL and HDL cholesterol concentrations in humans: a meta-analysis. *Eur. J. Clin. Nutr.* **57:** 40–46.
39. Pittas, A. G., N. A. Joseph, and A. S. Greenberg. 2004. Adipocytokines and insulin resistance. *J. Clin. Endocrinol. Metab.* **89:** 447–452.
40. Rangwala, S. M., and M. A. Lazar. 2004. Peroxisome proliferator-activated receptor gamma in diabetes and metabolism. *Trends Pharmacol. Sci.* **25:** 331–336.
41. Lu, T. T., M. Makishima, J. J. Repa, K. Schoonjans, T. A. Kerr, J. Auwerx, and D. J. Mangelsdorf. 2000. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol. Cell.* **6:** 507–515.
42. Horton, J. D., J. A. Cuthbert, and D. K. Spady. 1995. Regulation of hepatic 7 α-hydroxylase expression and response to dietary cholesterol in the rat and hamster. *J. Biol. Chem.* **270:** 5381–5387.
43. Repa, J. J., K. E. Berge, C. Pomajzl, J. A. Richardson, H. Hobbs, and D. J. Mangelsdorf. 2002. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the X receptors α and β. *J. Biol. Chem.* **277:** 18793–18800.
44. Basso F., L. Freeman, C. L. Knapper, A. Remaley, J. Stonik, E. B. Neufeld, T. Tansey, M. J. Amar, J. Fruchart-Najib, N. Duverger, et al. 2003. Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *J. Lipid Res.* **44:** 296–302.