Soy Protein Isolate Reduces Hepatosteatosis in Yellow A^{vy}/a Mice Without Altering Coat Color Phenotype

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Agouti (A^{vy}/a) mice fed an AIN-93G diet containing the soy isoflavone genistein (GEN) prior to and during pregnancy were reported to shift coat color and body composition phenotypes from obese-yellow towards lean pseudoagouti, suggesting epigenetic programming. Human consumption of purified GEN is rare and soy protein is the primary source of GEN. Virgin a/a female and A^{vy}/a male mice were fed AIN-93G diets made with casein (CAS) or soy protein isolate (SPI) (the same approximate GEN levels as in the above mentioned study) for 2 wks prior to mating. A^{vy}/a offspring were weaned to the same diets and studied at age 75 d. Coat color distribution did not differ among diets, but SPI-fed, obese A^{vy}/a offspring had lower hepatosteatosis ($P < 0.05$) and increased ($P < 0.05$) expression of CYP4a 14, a PPARα-regulated gene compared to CAS controls. Similarly, weanling male Sprague-Dawley (SD) rats fed SPI had elevated hepatic Acyl Co-A Oxidase (ACO) mRNA levels and increased *in vitro* binding of PPARα to the PPRE promoter response element. In another hepatosteatosis model, adult SD rats fed a high fat/cholesterol diet, SPI reduced ($P < 0.05$) steatosis. Thus, 1) consumption of diets made with SPI partially protected against hepatosteatosis in yellow mice and in SD rats, and this may involve induction of PPARα-regulated genes; and 2) the lifetime (*in utero*, neonatal and adult) exposure to dietary soy protein did not result in a shift in coat color phenotype of A^{vy}/a mice. These findings, when compared with those of previously published studies of A^{vy}/a mice, lead us to conclude that: 1) the effects of purified GEN differ from those of SPI when GEN equivalents are closely matched; 2) SPI does not epigenetically regulate the agouti locus to shift the coat color phenotype in the same fashion as GEN alone; and 3) SPI may be beneficial in management of non-alcoholic fatty liver disease. Exp Biol Med 233:1242–1254, 2008

Key words: soy; epigenetics; steatosis; PPAR-alpha

Introduction

Body fat mass and distribution are not necessarily static conditions, but are always subject to change depending upon many variables, such as total caloric intake, diet composition (including alcohol), endocrine status, metabolism, physical activity, use of medications and general health. Energy storage as fat is part of a dynamic process necessary to capture food energy to power cells and organs between meals. However, chronic and excessive accumulation of fat stores in the liver (hepatosteatosis) can be a precursor to serious liver disease, and preventing or otherwise managing this condition is problematic in overweight or obese individuals.

Soybeans and foods derived from soy have been consumed by human populations and animals for centuries, and a substantial number of published reports make claims of beneficial or adverse effects of consuming soybeans or components of soybeans, such as the proteins, oils, or phytochemicals. Among the beneficial effects attributed to consumption of foods prepared from soybeans are: reduced risk for breast cancer in women who regularly consumed soy in their youth; improved cardiovascular health; and leaner body composition (1). Soybeans contain more than one hundred phytochemicals, some of which have been studied for their bioactivity in animals and human subjects (2–9). Genistein (GEN), an isoflavone phytoestrogen metabolite of the soybean phytochemical genistin, is one of the most widely studied soy components. Recent studies in a yellow agouti mouse model have suggested that exposure to dietary GEN *in utero* and during early neonatal

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development can modulate epigenetic regulation of coat color and body composition (10).

The coat color pattern and other phenotypes of A^{vy}/a mice, such as obesity, diabetes and cancer susceptibility, are determined by the transcriptional regulation of the *agouti* locus encoding the agouti signaling protein (ASIP). The yellow color (pheomelanin) found in the subapical band in the hair is formed when ASIP is expressed during the first 4–7 days of hair growth. This determines the characteristic dorsal agouti coat color pattern of the wild-type (A^{w}/- mice). In contrast, most A^{vy}/a mice synthesize ASIP continuously in all tissues, resulting in a mostly yellow coat color and an obese phenotype as the result of altered melanocortin signaling in the CNS and hyperphagia. This is due to the insertion of a retrotransposon, intracisternal A particle (IAP), into exon 1A of the *agouti* gene (11). The cryptic IAP promoter usurps transcriptional regulation of the *agouti* gene from the gene's two hair-cycle-specific promoters on exon 1B and 1C resulting in continuous synthesis of ASIP. Interestingly, some A^{vy}/a mice (the pseudoagouti phenotype) are not yellow and obese, but more similar to the wild-type agouti (lean and brownish/black). In this phenotype, expression of the *agouti* gene reverts to the dorsal hair-cycle-specific promoter as a result of DNA methylation of CpG islands on the LTR of the cryptic promoter of the IAP retrotransposon. This results in non-obese mice and a yellow-banded black coat color pattern resembling the wild-type dorsal coat pattern. This phenotype is designated "pseudoagouti" (PAG). Thus, even though PAG mice (brownish/black) are genetically identical to yellow A^{vy}/a mice, they differ epigenetically. Furthermore, body composition phenotype of the PAG and yellow A^{vy}/a mice differ significantly, with the PAG mice being lean and having normal circulating glucose and insulin concentrations, like the wild-type mice; whereas, the yellow A^{vy}/a mice (referred to hereafter as yellow mice) develop adult-onset obesity and insulin resistance. This is an excellent model to study dietary factors for their epigenetic potential.

Recently, Dolinoy *et al* (10) reported that A^{vy}/a offspring of a/a mouse dams, mated by A^{vy}/a sires, had significant shifts in the coat color pattern towards PAG and were partially protected against obesity when compared to the control diet group if the dams consumed AIN-93G diets supplemented with GEN prior to conception and throughout pregnancy/lactation. The authors attributed these effects to GEN-induced epigenetic regulation via hypermethylation of CG sites on the promoter regions of specific genes. Although this is interesting, it should be noted that human populations are not typically exposed to pure GEN, but rather consume either soy foods (which are complex mixtures having isoflavone glycoside conjugates such as the genistein conjugate genistin and many other phytochemicals associated with soy proteins) or dietary supplements (mixtures of soy phytochemicals, many of which are also glycoside conjugates) extracted from soybeans. Several studies have demonstrated that the biological effects (i.e., gene expression profiles) in tissues following purified GEN consumption differ substantially from that of consuming soy protein that contains the same GEN equivalents, most likely because there are many other bioactive components in soy foods, soy protein and isoflavone supplements, and these either have their own actions or can modify GEN effects. Thus, the question arises as to whether the effects of diets made with soy protein that contain approximately the same GEN equivalents as studied by Dolinoy *et al* (10) would produce similar results on body composition and other biochemical markers of obesity.

In the present report, we describe the effects of feeding soy protein isolate (SPI), rather than GEN, on A^{vy}/a mice. We found that in contrast to GEN, SPI did not shift the coat color of A^{vy}/a mice, but did significantly reduce hepatosteatosis in yellow A^{vy}/a mice and up-regulated PPARα-regulated genes. We further studied the effects of SPI on body composition and hepatosteatosis in two rat models and confirmed that SPI provided significant reductions of obesity-associated hepatosteatosis and this protection may be linked to the nuclear receptor PPARα.

Methods

Reagents. All the chemicals, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO). Synthetic oligonucleotides were obtained from Integrated DNA Technologies Inc. (Coraville, IA). SYBR Green PCR mix was purchased from Applied Biosystems (Foster City, CA). TRI Reagent used for RNA extraction was obtained from Molecular Research Center, Inc. (Cincinnati, OH). Reagents for assessment of RNA quality using the Agilent Bioanalyzer were acquired from Agilent Technologies (Foster City, CA).

Experimental Animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal maintenance and experimental treatments were conducted in accordance with ethical guidelines for animal research and were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Yellow A^{vy}/a Mice. A breeding colony was derived from a highly inbred VY/WffC3Hf/Nctr-A^{vy} colony at the National Center for Toxicological Research/FDA, Jefferson, AR. Black a/a dams were bred to mottled yellow and pseudoagouti A^{vy}/a sires to produce A^{vy}/a and a/a offspring for this study. Mice were housed in filtered polycarbonate cages in the AAALAC approved facility at the Arkansas Children's Nutrition Center. Cages were changed weekly with Tek-Fresh bedding (Harlan Teklad), mice had *ad libitum* access to food and water, the lights were off between 6 PM and 6 AM and the temperature and humidity were automatically maintained at ~22°C and ~48%, respectively.

Experimental diets were made by Harlan Teklad

Industries (Indianapolis, IN) using the published AIN-93G formula (12) except that soy oil was replaced with corn oil. There were two diets, one having 100% of the protein as casein (CAS, Harlan Teklad Industries, Indianapolis, IN) and the other had 100% of the protein as soy protein isolate (SPI, Solae, St. Louis, MO). Essential amino acids levels in the SPI diet were equalized to the CAS diet by amino acid supplementation. Breeding pairs were fed either CAS or SPI diets two weeks prior to mating and throughout pregnancy and lactation. At age 4 wk, offspring were implanted with transponder chips, separated by sex, and weaned to the same diets as their dams. Weanling mice were visually inspected by an investigator experienced in agouti mouse phenotyping (author GW) and categorized according their coat color: black, pseudoagouti (PAG), almost pseudoagouti (APAG), heavily mottled yellow (HMY), mottled yellow (MY), slightly mottled yellow (SMY), and clear yellow (CY). Offspring were sacrificed at age 75 days, and samples collected (blood, liver, fat pads and the entire skin). The skins of the A^{vy}/a mice were also removed, digitally photographed, the photo scanned by a computerized imaging system and analyzed using the MCID5+ software program (Imaging Research Inc., St. Catherine's, Ontario, Canada). Pixel analysis was used to determine the percentage of mottling on each coat. By doing this, we were able to obtain objective and blinded results of coat patterns. The photos were also categorized by our experienced investigator (author GW), providing another opportunity to analyze the coat pattern at the end of the experiment and to compare results of visual assignment to coat pattern groups and computer assignment. Livers sections were stained with Oil-Red-O and quantified by MCID imaging analysis as described previously (13).

Hepatic gene expression profiles from male CAS- or SPI-fed A^{vy}/a MY/SMY/CY mice ($n = 3$ per group) were assessed using Affymetrix MOE430A GeneChip microarrays (Affymetrix, Santa Clara, CA) containing 22,690 transcripts. Total RNA was isolated from livers using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction, and cleaned using RNeasy kit (QIAGEN, Valencia, CA). RNA was quantified and analyzed for integrity using RNA 6000 Nano Chip on Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). First- and second-strand cDNA synthesis, biotin-labeled cRNA synthesis, fragmentation of cRNA and hybridization reactions were performed using one cycle cDNA synthesis kit (Affymetrix, Santa Clara, CA). Briefly, 8 μg of purified RNA was used to synthesize cDNA. Labeled cRNA was synthesized from double stranded cDNA using the GeneChip IVT labeling kit (Affymetrix) according to the manufacturer's instructions.

Microarray data analyses were carried out using GeneSpring version 7.2X software (Agilent Technologies, Santa Clara, CA). The data files (CEL files) containing the probe level intensities were processed using the robust multiarray analysis (RMA) algorithm (GeneSpring), for background adjustment, normalization and $\log_2$-transformation of perfect match values (14). Subsequently, the data were subjected to normalization by setting measurements less than 0.01 to 0.01 and by per-chip and per-gene normalization using GeneSpring normalization algorithms. For comparison analysis, genes were filtered based on minimum 1.5-fold change (SPI vs. CAS) and P value $\leq$ 0.05 using Student's t test. Data analyses were performed using Microsoft Excel and GeneSpring GX v7.2. Correlation-based hierarchical clustering between treatment groups and visualization of data were performed using GeneSpring GX v7.2. Known biological functions of genes were queried and acquired from Affymetrix online data analysis resource NetAffx and gene ontology (GO) analyses performed using Affymetrix GO Browser (14).

SPI Feeding Induces PPARα Signaling. Time impregnated Sprague-Dawley rats (Harlan, Indianapolis, IN) arrived at our facility on gestational day 4. They had *ad libitum* access to the AIN-93G diet described above that was made with either casein (CAS) or soy protein isolate (SPI) as the sole source of protein. The offspring were weaned onto the same diet and sacrificed prior to the beginning of puberty on PND34. Liver samples were frozen in liquid nitrogen and stored at –80°C. Hepatic Acyl Co-A Oxidase (ACO) levels were determined by real-time reverse-transcription PCR analysis of mRNA and binding of PPARα to the ACO promoter assessed by electrophoretic mobility shift assay (EMSA) as described below (13, 15).

Overfeeding-Induced Hepatosteatosis in Adult Rats Is Partially Prevented by SPI. Adult (250 g) male Sprague-Dawley rats (Harlan, Indianapolis, IN) were surgically cannulated with an intragastric tube, allowed to recover two weeks and thereafter fed by total enteral nutrition as previously described (16). These rats were fed 115% of National Research Council (NRC) recommended caloric needs of adult rats, which produces obesity. The diets contained 16% protein (CAS or SPI), 39% carbohydrate (maltodextrin), 45% fat (corn oil), and 0.5% cholesterol. Diets were supplemented with minerals, vitamins and amino acids to meet NRC recommendations; isocaloric (890 kcal/L); isonitrogenous and fed at 220 kcal/$kg^{.75}$/d. Diet details are given in Table 1. Following 21 d of TEN, rats were anesthetized by isoflurane and decapitated. Hepatic steatosis was determined by Oil-Red-O staining (13). Total lipids were extracted from liver homogenates according to the method of Folch *et al* (17) and triglycerides and cholesterol concentrations assayed using commercially available reagents (Synermed, Westfield, IN).

Real-Time Reverse-Transcription PCR Analysis of ACO mRNA Expression. Total RNA was isolated from the liver using the Tri Reagent according to manufacturer's protocol (Molecular Research Center, Cincinnati, OH). RNA was treated with RNase-free DNase (Ambion, Inc., Austin, TX) to remove contaminating genomic DNA and was further purified with RNeasy mini kits (QIAGEN Inc., Valencia, CA). cDNA for ACO was

Table 1. Total Enteral Nutrition Diet[a,b]

	Per liter	Total kcal
Protein[c]	37.05 g	16%
Carbohydrate[d]	86.72 g	39%
Fat[e]	46.71 g	45%

[a] The caloric density was 890 kcal/L and diets were fed at 220 kcal/kg$^{.75}$/d.
[b] Vitamins and minerals added to meet or exceed National Research Council recommended levels.
[c] Protein (casein) was supplemented with an amino acid mix to assure that all essential amino acids were present at levels to meet or exceed the National Research Council recommended levels.
[d] 25% maltodextrin and 75% dextrose.
[e] Corn oil (8.57 kcal/g).

synthesized from 1 μg of total RNA in 20 μL using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI). One twentieth of the reverse transcription mixture was mixed with 2 X SYBR Green PCR master mixture and ACO gene specific primers (Primer sequence [5′-3′], F = GAGACCTTGTGCATGGCTGA; R = GAATCGGACCTCTGCCTCCT) in a final volume of 50 μL. The primers were designed with Primer express software (Applied Biosystems). The primer pairs were selected to yield a single amplicon based on dissociation curves and analysis on agarose gel. RT-PCR was performed in an ABI Prism Model 7000 sequence detection system. Real time RT-PCR was quantitated within the linear range of product amplification. GAPDH amplicons were used as endogenous internal standards to normalize the values, and the relative expression of each gene was expressed as the gene expression/GAPDH expression ratio relative to the mean ratio for the male CAS control group = 1 (GAPDH [F = TGAGGTGACCGCATCTTCTTG; R = TGGTAACCAGGCGTCCGATA]) (14).

EMSA of Protein Binding to the PPRE Element of the ACO Promoter. EMSA analysis of protein binding to the PPARα response element (PPRE) in the promoter of the ACO gene was performed as described previously for other genes (18). The probe was labeled by using Roche T-4 polynucleotide kinase kit, (PerkinElmer Life Science), ^{32}P-ATP, and the complimentary annealed PPRE sequence (gatcCTCCCGAACGTGACCTTTGTCCTGGTCCAgatc). A 4% acrylamide gel made with low ionic strength Tris borate/EDTA buffer was prerun for an hour. The binding buffer was 12 mM HEPES, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 15% Glycerol, and 1 μg poly[dI-dC]. Using 12.5 μg nuclear protein per sample, 5 μL of binding buffer was added. This mixture was incubated on ice for 15 min. The samples were mixed with 2 μL of labeled probe and incubated 20 min at room temperature. 10X loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue and 40% glycerol) was added. The samples were run onto the gel at 200 V for 2 h. The gel was applied to filter paper and dried at 72°C for 110 min. The image was acquired by phosphoimaging and quantitated by densitometric scanning using a GS700 molecular imager (Bio-Rad Laboratories, Hercules, CA). Specificity of the shifted band was determined by competition with increasing concentrations of cold probe.

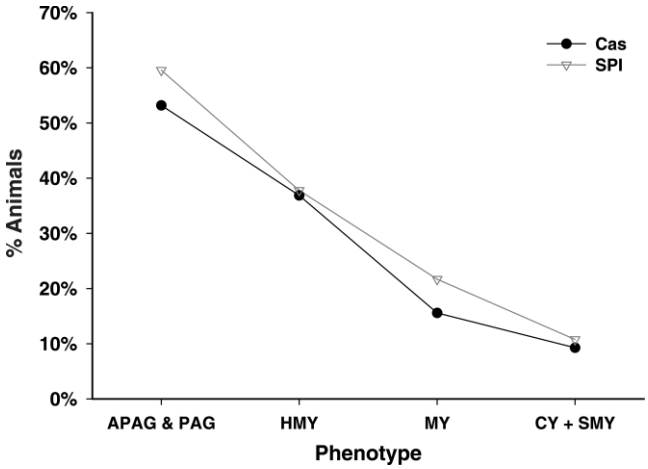

Figure 1. Color coat distribution of Agouti (A^{vy}/a) mice fed AIN-93G diets made with either casein (CAS) or soy protein isolate (SPI). Pseudoagouti (PAG), almost pseudoagouti (APAG), heavily mottled yellow (HMY), mottled yellow (MY), slightly mottled yellow (SMY), and clear yellow (CY).

Statistical Analysis. Data are presented as mean ± SEM. All data were analyzed using Sigma Stat for Windows (version 3.5, Systat Software, Inc., San Jose, CA). Student's *t* test was used to compare data from 2 groups. For multiple group comparisons, data were analyzed by 1-way or 2-way ANOVA with post hoc Student-Neuman-Keuls tests. Differences were considered significant if $P < 0.05$. Linear regression was used to quantify the relationship between body composition variables and the coat color (% black).

Results

Yellow A^{vy}/a Mice. Based upon a previous report by Dolinoy *et al.* (10), we predicted that the coat color distribution of A^{vy}/a mice fed SPI-containing diets would differ from that of A^{vy}/a mice fed CAS-containing diets. However, no differences in coat color distribution were observed between diet groups. Figure 1 depicts the coat color distribution in all the mice (male + female). Although the body weights and fat pad weight in male SPI-fed mice were slightly lower than CAS-fed mice, these effects did not reach statistical significance when analyzed by linear regression (Fig. 2A, B). Similarly, diet did not affect body or fat pad weights in female mice (Fig. 2C, D). Polynomial (cubic or quadratic) analysis also did not reveal significant diet effects, but there was a tendency for differences in the CY groups. SPI-fed CY female A^{vy}/a offspring had lower ($P < 0.05$) abdominal fat pad mass if these groups were analyzed separately by Student's *t* test. A^{vy}/a mice with the MY/SMY/CY phenotypes had hepatosteatosis; whereas, the lean APAG and PAG phenotypes had no obvious ectopic fat

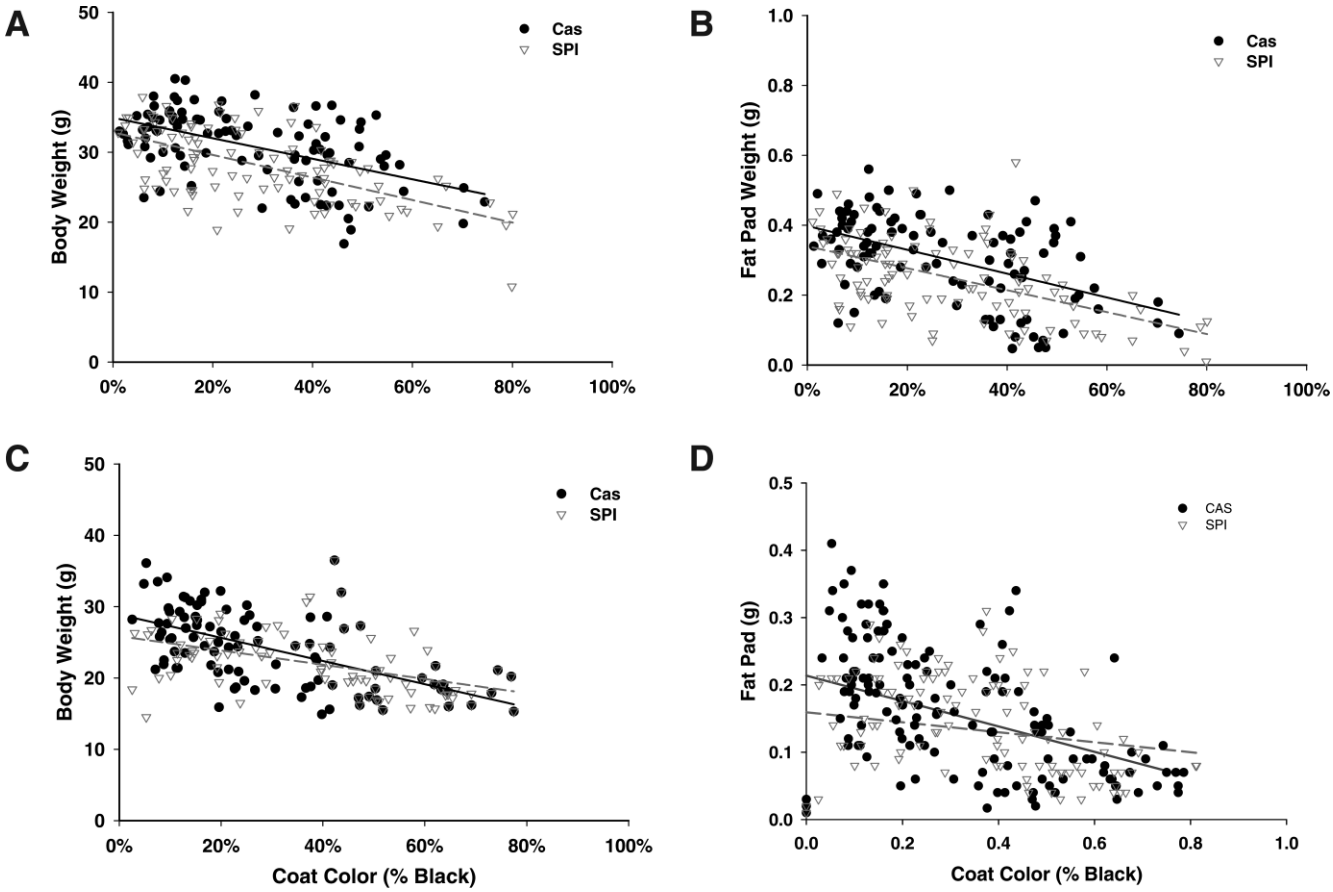

Figure 2. Effects of AIN-93G diets made with either casein (CAS) or soy protein isolate (SPI) on body weights and abdominal fat pad weights of mice at age 8 weeks. Coat color was determined by a standardized imaging system described in Methods and presented as the percent of black. Lines represent best-fit regression analyses and no significant differences were detected. However, polynomial analysis revealed significantly ($P < 0.05$) lower fat pad weight for CY female mice. A and B are males, and C and D are females.

deposition. A^{vy}/a SMY and CY mice fed SPI-containing diets had reduced ($P < 0.05$) hepatosteatosis as compared with CAS-fed, SMY and CY offspring (Figs. 3 and 4).

To examine the underlying mechanisms associated with decreased liver steatosis we performed microarray-based expression profiling. Forty-nine gene transcripts were altered ($\pm$ 1.5-fold, $P \leq 0.05$) by SPI feeding in livers of male A^{vy}/a SMY/CY mice, which are listed in Table 2. Two-way hierarchical clustering of altered genes is represented in Figure 5A, B. Each column represents a single animal. Genes involved in cellular metabolic processes such as electron transport, fatty acid oxidation and steroid biosynthesis clustered together (Fig. 5A and Table 2). Furthermore, gene ontology analyses revealed that the majority of genes affected by consumption of SPI had important catalytic activities or gene binding properties (Fig. 6A). Approximately 10% of the genes (5 out of 49) also had a known role in regulating gene transcription. Most importantly, we observed a 4.19-fold increase in gene expression of CYP4a14, an important PPAR-α regulated P450 enzyme, mediating ω-hydroxylation of fatty acids. This was confirmed by real-time PCR normalized to Rps20, Srp14, Rps13, OAZ1, β-actin and 18S as house keeping genes (19). A representative real-time PCR quantitation using Rps13 is shown in Figure 7. Also, we have measured expression of Cpt1a, another PPARα responsive gene, in the mouse and this was increased (0.43 ± 0.05 to 0.63 ± 0.05, $P < 0.05$) using OAZ1 as a housekeeping gene. A similar result was observed using Rps13 as a house keeping gene. Activation of PPARα and CYP4A family of enzymes has been shown to ameliorate liver steatosis (20). Finally, using Ingenuity Pathway Analyses, we examined whether coordinated changes in gene expression were occurring following SPI feeding (Fig. 6B). Our analyses suggest that expression of 4 genes regulated by SREB-1c were increased ($P < 0.05$): cysteine sulfinic acid decarboxylase; FABP5; isopentenyl-diphosphate delta isomerase; and 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1. However, neither SREB-1c mRNA nor the classical lipogenic targets such as fatty acid synthase or malic enzyme 1 were increased. Hence, at least in the mouse model of agouti-driven hepatosteatosis, SPI associated decreased fatty liver was associated with PPARα-mediated fatty acid degradation, but was not associated with decreased SREBP-1c signaling.

PPARα-Regulated Genes in Young Rats. Acyl Co-A oxidase (ACO) is a peroxisomal enzyme responsible

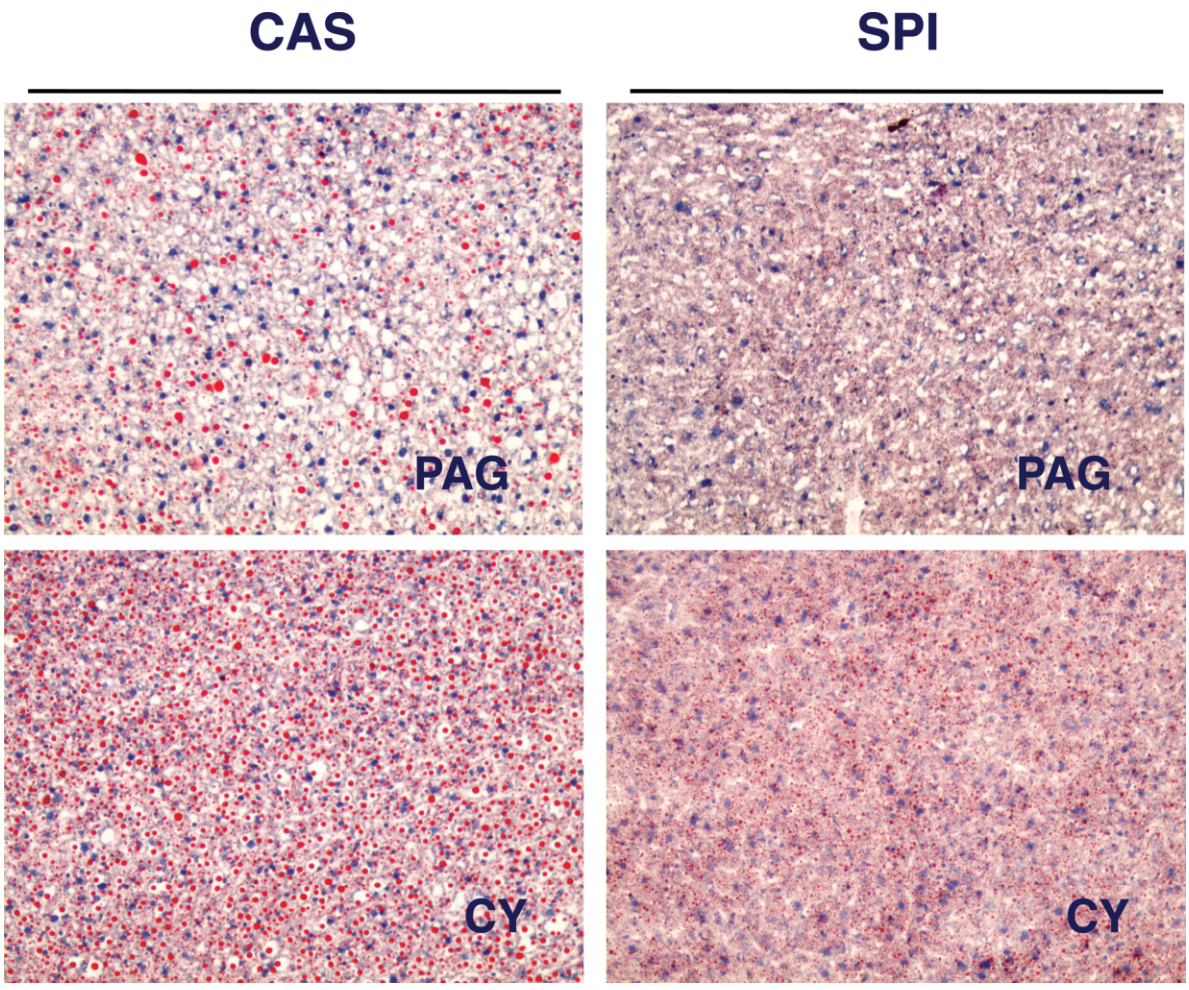

Figure 3. Effects of AIN-93G diets made with either casein (CAS) or soy protein isolate (SPI) on hepatosteatosis in agouti mice. Each micrograph shows fat droplets stained with Oil-Red-O for one representative liver section from pseudoagouti (PAG) mice, or clear yellow (CY) mice at 8 wk of age. See Figure 4 for quantitation.

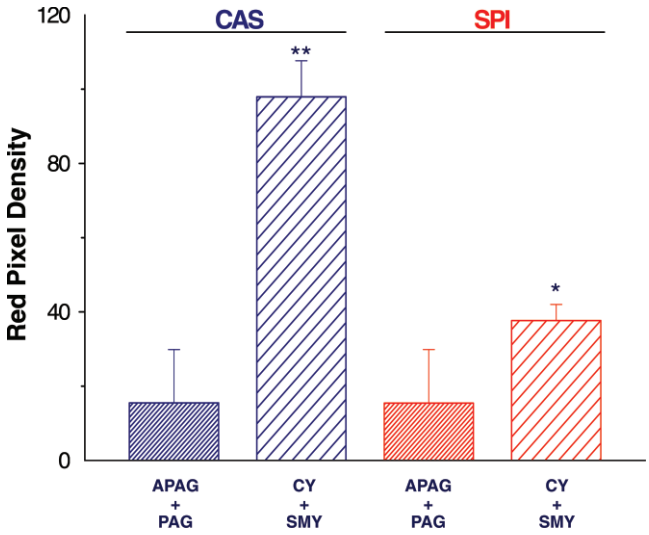

Figure 4. Histograms are the quantitation of Oil-Red-O staining in Figure 3. Data are the means (± SEM) of PAG ($n = 8$) plus almost pseudoagouti (APAG, $n = 8$), or CY ($n = 8$) plus slightly mottled yellow (SMY, $n = 8$). ** $P < 0.01$ compared to APAG + PAG control; * $P < 0.05$ compared to APAG + PAG control.

for fatty acid β-oxidation. Expression of the ACO gene is positively regulated by the nuclear transcription factor PPARα (21, 22). Real time RT-PCR analysis of liver from CAS- or SPI-fed rats demonstrated increases in ACO mRNA (1.00 ± 0.06 vs. 1.86 ± 0.08) ($P < 0.05$). We performed EMSA analysis of protein binding to the PPRE element in the ACO promoter using nuclear extracts from rats fed either CAS- or SPI-containing diets. As seen in Figures 8 and 9, hepatic nuclear extracts from rats fed the SPI diet had greater binding to the PPRE ($P < 0.05$). Furthermore, real time RT-PCR analysis demonstrated increased ($P < 0.05$) expression of mRNA encoding for PPARα itself in the SPI-fed rats compared with CAS-fed rats (1.00 ± 0.08 vs. 1.46 ± 0.06).

Overfeeding-Induced Hepatosteatosis in Adult Rats Is Partially Prevented by SPI. To test the hypothesis that dietary SPI could protect against hepatosteatosis in an animal model other than the A^{vy}/a mouse, male Sprague-Dawley rats (250 g) were fed high fat diets (45% corn oil + 0.5% cholesterol) at a caloric intake (220 kcal/$kg^{.75}$/d) that exceeded the National Research Council

Table 2. Effects of SPI on Hepatic Gene Expression in A^{vy}/a SMY/CY[a]

Gene identifier	Gene description	Gene symbol	Fold change
Electron transport			
1419523_at	cytochrome P450 3a13	Cyp3a13	−1.52
1423599_a_at	phosducin-like	Pdcl	−1.64
1418780_at	cytochrome P450 39a1	Cyp39a1	−2.02
1423257_at	cytochrome P450 4a14	Cyp4a14	4.19
1416809_at	cytochrome P450 3a11	Cyp3a11	1.55
1424273_at	cytochrome P450 2c70	Cyp2c70	−1.55
Cellular metabolic processes			
1416372_at	phosphatidylserine synthase 1	Ptdss1	−1.56
1417339_a_at	Dynein light chain 1	Dnclc1	1.66
1419093_at	tryptophan 2,3-dioxygenase	Tdo2	−1.51
1427981_a_at	cysteine sulfinic acid decarboxylase	Csad	1.67
1451348_at	DEP domain containing 6	Depdc6	−1.55
1416022_at	fatty acid binding protein 5	Fabp5	1.63
1418601_at	aldehyde dehydrogenase family 1A7	Aldh1a7	1.83
1433443_a_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Hmgcs1	1.62
1427960_at	UDP glucuronosyltransferase 2B34	Ugt2b34	−1.56
1418138_at	sulfotransferase family 1D1	Sult1d1	−2.09
1423804_a_at	isopentenyl-diphosphate delta isomerase	Idi1	1.85
1433691_at	protein phosphatase 13C	Ppp1r3c	−1.60
Regulators of transcription			
1417130_s_at	angiopoietin-like 4	Angptl4	2.46
1434620_s_at	RIKEN cDNA 2610024E20 gene	2610024E20Rik	−1.51
1425281_a_at	TSC22 domain family 3	Tsc22d3	−2.17
1433733_a_at	cryptochrome 1 (photolyase-like)	Cry1	−1.92
1417065_at	early growth response 1	Egr1	2.38
Cellular transport and cytoskeleton			
1427866_x_at	hemoglobin beta chain complex	Hbb	1.96
1428361_x_at	hemoglobin alpha, adult chain 1	Hba-a1	1.77
1423090_x_at	SEC61, gamma subunit	Sec61g	1.55
1424826_s_at	metastasis suppressor 1	Mtss1	−1.56
1437185_s_at	thymosin, beta 10	Tmsb10	1.59
Apoptosis, angiogenesis or stress response			
1428942_at	metallothionein 2	Mt2	4.17
1422557_s_at	metallothionein 1	Mt1	2.77
1416855_at	growth arrest specific 1	Gas1	−1.59
1418835_at	pleckstrin homology-like domain, A1	Phlda1	1.68
1416050_a_at	scavenger receptor class B1	Scarb1	−1.53
1422603_at	ribonuclease, RNase A4	Rnase4	−1.71
1450717_at	angiogenin, ribonuclease A1	Ang1	−1.91
1428306_at	DNA-damage-inducible transcript 4	Ddit4	−1.68
Miscellaneous or unknown functions			
1435725_x_at	ribosomal protein L12	Rpl12	1.54
1449375_at	carboxylesterase 6	Ces6	1.66
1436058_at	radical S-adenosyl methionine domain containing 2	Rsad2	1.61
1419510_at	esterase 22	Es22	1.58
1426840_at	YTH domain family 3	Ythdf3	−1.51
1427422_at	Mus musculus, clone IMAGE:5050186		−1.70
1450060_at	polymeric immunoglobulin receptor	Pigr	−1.71
1418604_at	arginine vasopressin receptor 1A	Avpr1a	−1.51
1448986_x_at	deoxyribonuclease II alpha	Dnase2a	2.08

[a] Data were collected from male A^{vy} SMY/CY ($n = 3$) mice fed diets as described under Materials and Methods. Hepatic gene expression profiles were assessed using Affymetrix MOE430A GeneChip microarrays according to manufacturer's instructions. Fold change column represents ratios of mean gene expression of SPI group compared to CAS group. All fold changes showing ± 1.5-fold change are also significantly different ($P \leq 0.05$) using a Student's t test.

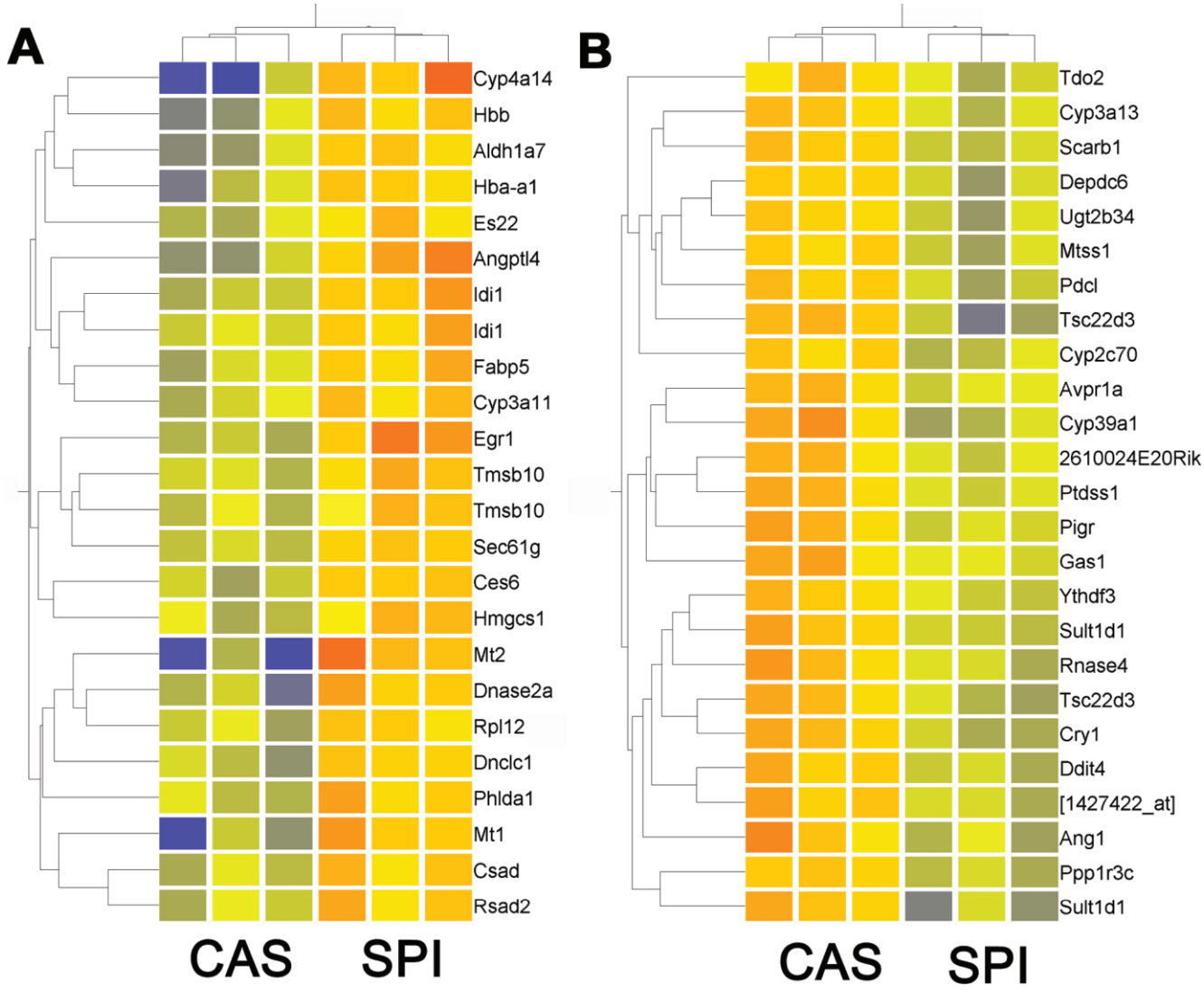

Figure 5. Microarray-based expression profiling of (A^{vy}/a) mice fed AIN-93G diets made with either casein (CAS) or soy protein isolate (SPI). Two-way hierarchical clustering of altered genes is represented in panels A and B. Each column represents a single animal.

recommended level by 15%. Rats were fed by TEN for 21 d. The diets were prepared with either CAS or SPI as the sole protein source. While neither body weight gain nor body composition differed significantly between diet groups (Table 3), hepatosteatosis developed in the high fat-casein-fed group and levels of hepatosteatosis was 30% lower ($P < 0.05$) in SPI-fed rats as determined by Oil-Red-O staining (Fig. 10). Liver triglyceride and cholesterol content was also reduced ($P < 0.05$) by SPI feeding (Table 3).

Discussion

The offspring from mating virgin *a*/*a* female mice with A^{vy}/a males represent an example of epigenetic imprinting that results in differing phenotypes ranging from the pseudoagouti mouse (brown/black and lean) at one extreme to the obese yellow mouse at the other. The difference in phenotype is dependent on methylation status of the cryptic promoter in the retrotransposon, intracisternal A particle (IAP), inserted into exon 1A of the *agouti* locus. In a recently published study by Dolinoy *et al*, the A^{vy}/a offspring fed AIN-93G diets supplemented with genistein (GEN) had a coat color distribution shift from yellow towards PAG associated with hypermethylation at CpG sites on the IAP promoter and decreased adult obesity (10). We conducted a study very similar to that of Dolinoy *et al* (10), except throughout the study (starting from before mating) mice were fed AIN-93G diets made with 100% protein as either casein (CAS) or soy protein isolate (SPI), rather than the CAS supplemented with purified GEN diet. Our data suggest that the *in utero* effects on the fetal epigenome and resultant shift in phenotype previously reported (10) in A^{vy}/a mice fed diets with purified GEN does not occur when GEN is supplied as SPI, demonstrating that the effects of purified GEN differ from those of SPI containing the same aglycone equivalents of this phytoestrogen. In retrospect, these findings are not surprising, because the effects of purified

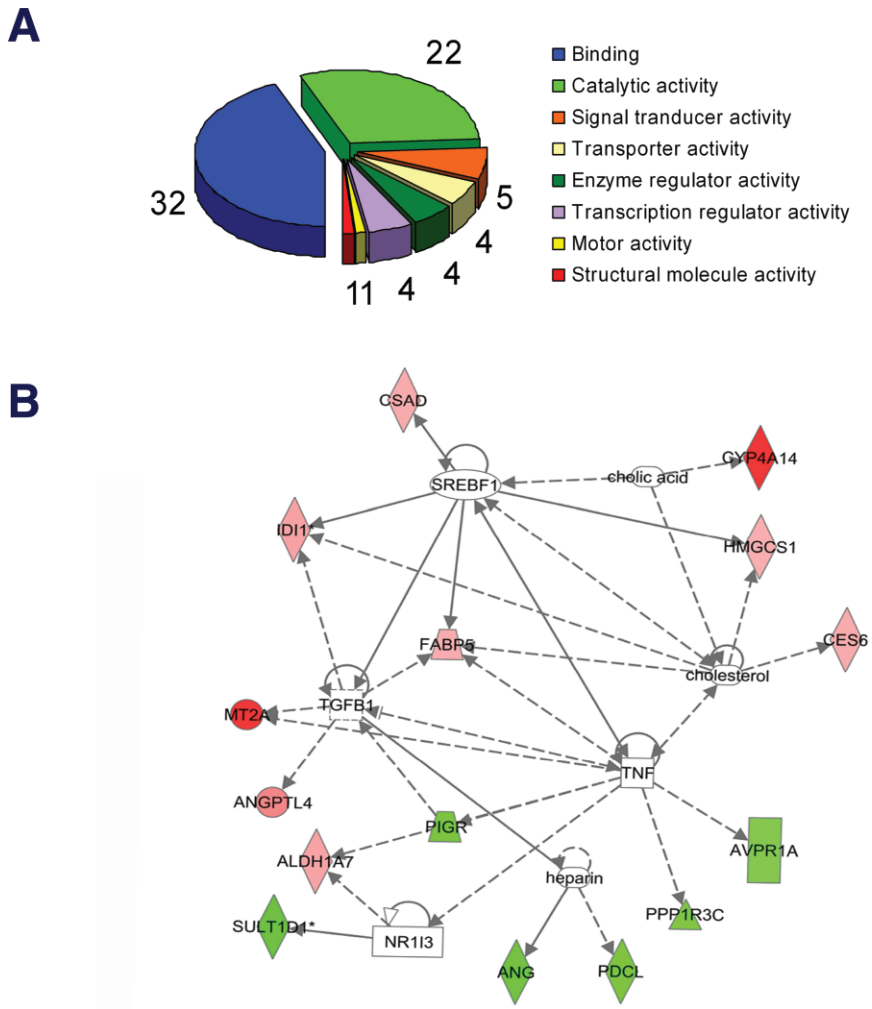

Figure 6. Catalytic activities or gene binding properties of genes displayed in Figure 5 and that are affected by SPI consumption are shown in panel A. Ingenuity Pathway Analyses demonstrated coordinated changes in gene expression occurred following SPI feeding (panel B).

GEN are well known to differ from the effects of soy foods that have the same GEN aglycone equivalents (23–26). For example, the gene expression profile in the mammary gland of female rats differs between rats fed AIN-93G diets made with SPI and rats fed diets made with CAS to which purified GEN is added to the same level of the SPI (aglycone equivalents) (26). The present microarray studies have revealed a significantly different gene expression profile in livers of mice fed CAS diets when compared with those fed SPI diets.

It has been suggested that exposure to genistein during development can reduce adult obesity as a result of estrogenic actions on genes regulating lipogenesis, lipolysis and on adipocyte differentiation via ERβ (27) and also via epigenetic effects when exposure occurs *in utero* (10). Our data from experiments where rats are fed AIN-93G diets made with SPI rather than CAS throughout development support the actions of soy to maintain a leaner body composition (28). However, effects of soy feeding during early development may differ from those in adults as a result of differences in: expression of estrogen receptors; levels of endogenous estrogens; estrogen-independent actions of isoflavones; and other soy components including phytochemical metabolites generated by gut bacteria or soy proteins and bioactive peptides generated during digestion of SPI (e.g., equol and beta conglycinin, respectively).

It should be pointed out that feeding SPI to mice in the present study not only achieved similar serum genistein concentrations, but also produced high levels of equol. While we have no evidence that differences between results reported herein from those of Dolinoy *et al* are related to equol, it is possible. However, it is just as likely that one of the more than one hundred soy phytochemicals in the SPI, or a soy peptide could be responsible. If the mechanism underlying the SPI effects involved equol, this might not occur in most human subjects, because only about 25% of adult people are equol producers and virtually no infants produce equol.

There was a tendency towards a lower level of abdominal fat pad weights in yellow female A^{vy}/a mice

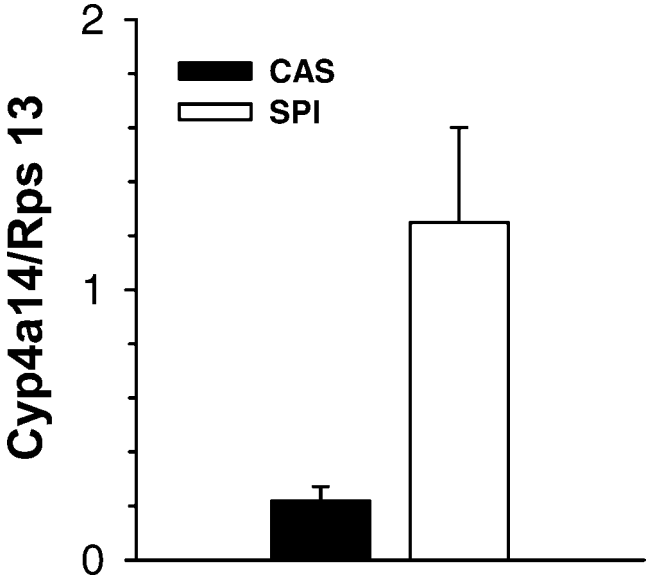

Figure 7. Real-time PRC results showing >4-fold increase ($P < 0.05$) in gene expression of CYP4a14, an important PPAR-α regulated P450 enzyme, mediating ω-hydroxylation of fatty acids. Data are means (± SEM) of $n = 4$/group.

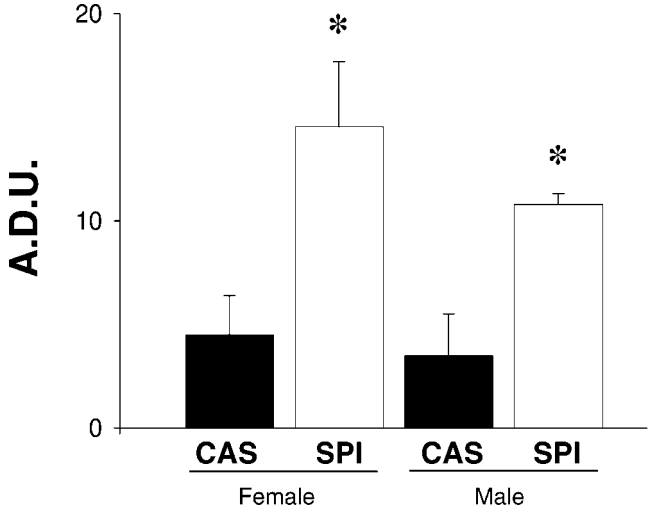

Figure 9. Quantitation of EMSA shown in Figure 8. The bars represent means (± SEM) of $n = 6$ mice per group. * $P < 0.05$ compared to CAS control.

(CY) that were fed SPI diets during early development. While this was not a robust effect, it is interesting because we have previously reported that adult rats fed standard (pellets) AIN-93G diets made with SPI as the sole protein source during development were leaner than their CAS-fed controls (28). Furthermore, preliminary data from a longitudinal prospective study of infants fed soy formula suggest that 6-month-old infants who were fed soy formula have lower body fat (29). The lack of effect of SPI on body weight in TEN infused rats may reflect an effect of either age differences or the diet. The SPI-infusion study was conducted in adults as opposed to during early development. Taken together, these results suggest that SPI may have a

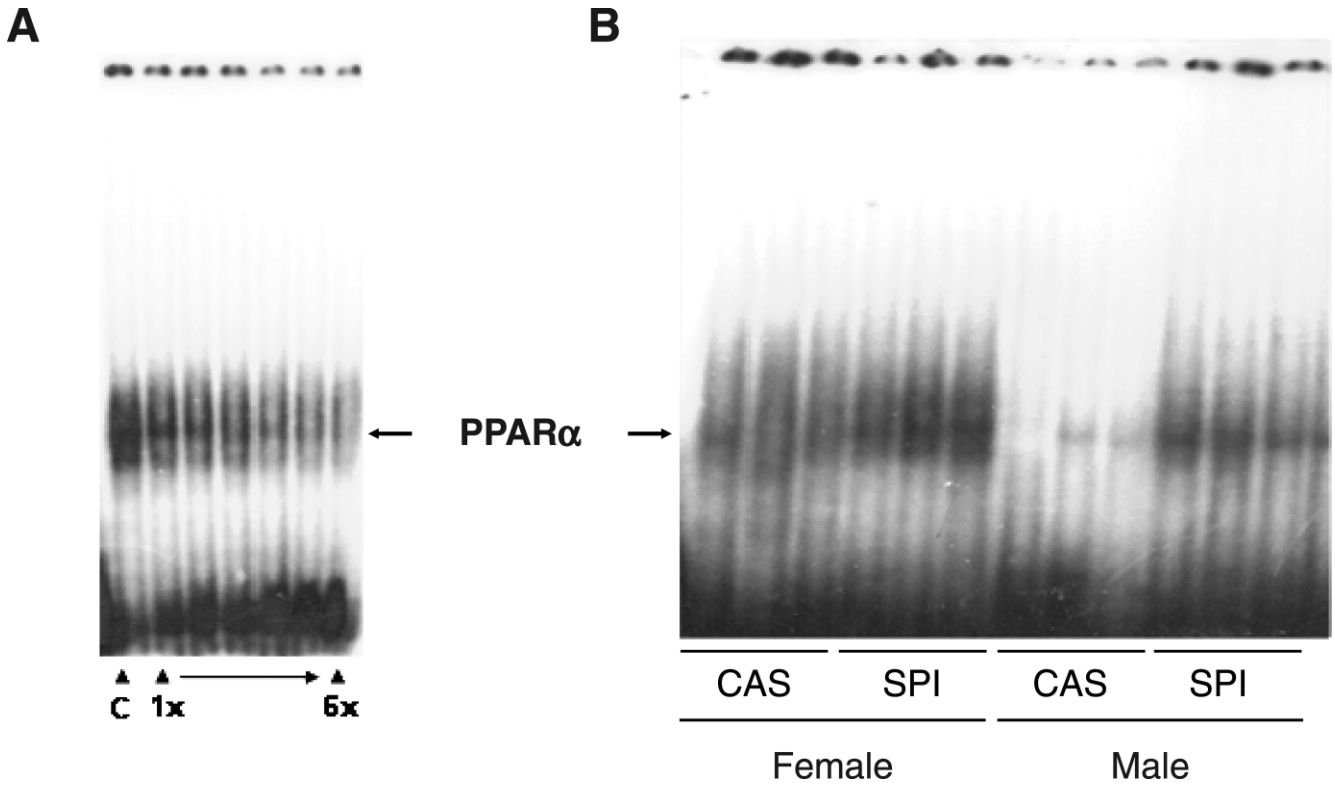

Figure 8. EMSA of nuclear protein binding to the PPARα response element (PPRE) in the promoter of the *ACO* gene. Each lane of panel B represents binding of nuclear protein from individual female or male rats that were fed AIN-93G diets made with casein (CAS) or soy protein isolate (SPI). Specificity of the shifted band was determined by competition with increasing concentrations of cold probe (panel A).

Table 3. Effects of SPI on Body and Liver Composition[1]

Diet group	Casein	Soy protein isolate
Final body weight (g)	320 ± 5.7	325 ± 6.7
Body weight gain (g)	108 ± 1.9	113 ± 2.0
Body fat mass (%)	15.2 ± 0.6	16.7 ± 0.4
Perirenal adipose tissue (g/100 g BW)	0.88 ± 0.09	0.95 ± 0.05
Liver weight (g/100 g BW)	4.12 ± 0.14	4.02 ± 0.09
Liver triglyceride (μmol/g wet weight)	67.6 ± 5.94[a]	42.3 ± 2.8[b]
Liver total cholesterol (μmol/g wet weight)	15.2 ± 1.5[a]	7.9 ± 0.98[b]

[1] Values are means + SEM, $n = 12$ CAS; $n = 11$ SPI. Means in a row with different superscripts differ ($P < 0.05$) where a > b.
[2] Analysis of body composition by NMR (See Materials and Methods for details).

role in modulating body composition of rodents, and perhaps human infants during early development, by reducing body fat storage.

Nonalcoholic fatty liver disease (NAFLD) is the most common liver pathology and is closely associated with obesity and the metabolic syndrome. Based on retrospective analysis of autopsies, NAFLD has been estimated to occur in up to 70% of obese patients (30). There is a wide spectrum of pathologies covered by NAFLD ranging from simple benign reversible steatosis to nonalcoholic steatohepatitis (NASH) in which steatosis is accompanied by inflammation, necrosis, apoptosis and fibrosis. NASH pathology in adults is characterized by evidence of hepatic oxidative stress including appearance of oxidized proteins,

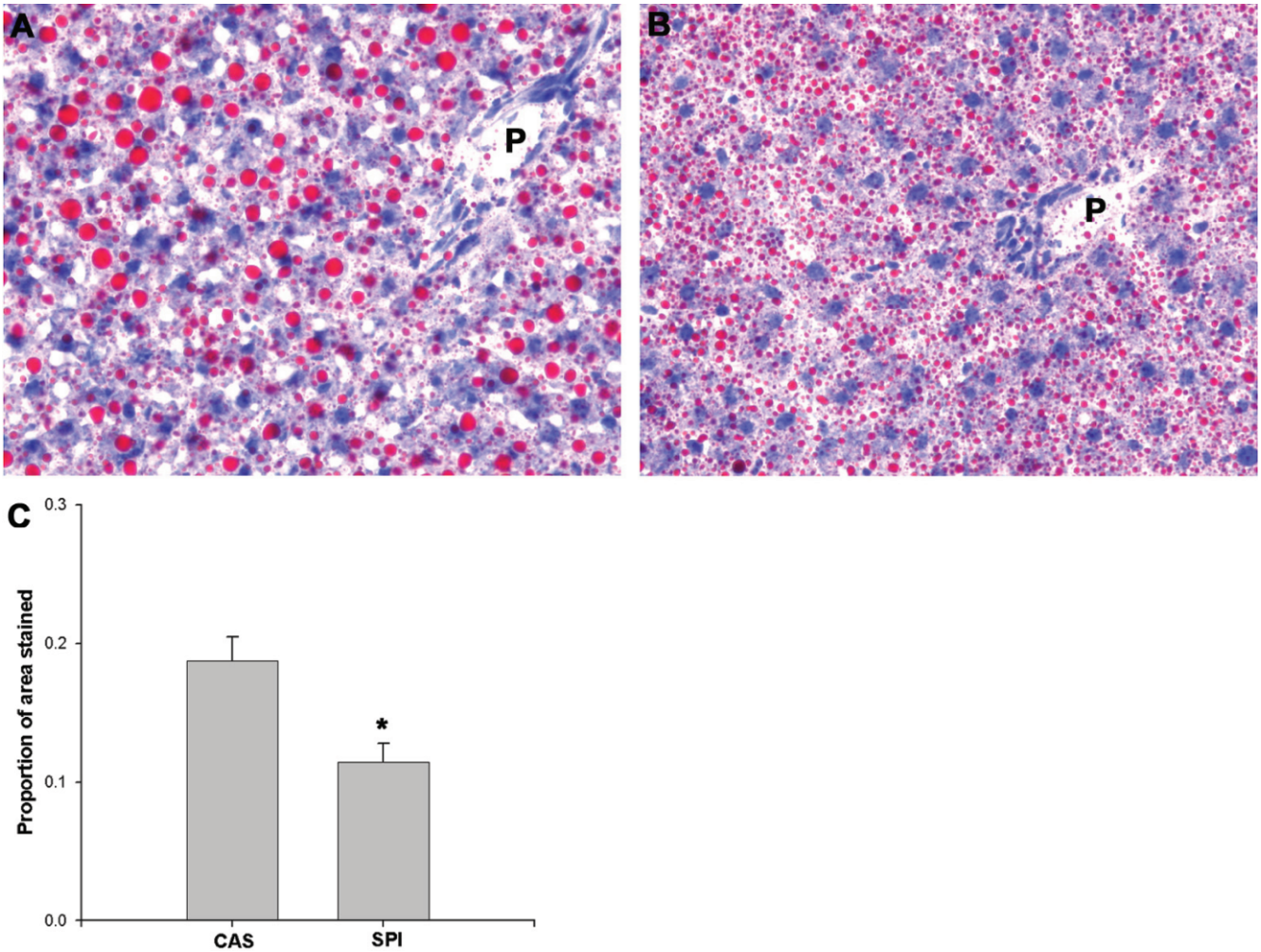

Figure 10. Effects of AIN-93G diets made with either casein (CAS) or soy protein isolate (SPI) on hepatosteatosis in Sprague-Dawley rats. Each micrograph shows fat droplets stained with Oil-Red-O for one representative liver section from rats fed AIN-93G diets made with CAS protein (left panel) or SPI (right panel). The bar graphs represent the proportion of area stained with Oil-Red-O (means ± SEM) of $n = 6$ rats each. * $P < 0.05$ compared to CAS control.

DNA and lipids (31–34), and the magnitude of oxidative stress has been shown to correlate with disease severity (33, 34). Recent studies have reported that insulin resistance is present in almost all patients with NAFLD (32) and that the presence of central obesity, type 2 diabetes or the severity of insulin resistance are predictors of NASH independent of the severity of steatosis (30).

Several diet-related conditions lead to hepatosteatosis. For example, it is well known that human subjects and animals that consume high fat diets and excessive alcohol develop hepatosteatosis that can lead to necrosis, fibrosis and cirrhosis of the liver (35, 36). Hepatosteatosis is a common feature of obesity and even in people with BMIs < 30. Identification of dietary factors with the potential to protect against hepatosteatosis could be important in reducing morbidity associated with overweight, obesity, or other conditions, such as excessive alcohol intake. Results of the current study in A^{vy}/a mice and Sprague-Dawley rats suggest that SPI may be a dietary factor that can at least partially protect against hepatosteatosis. Although further studies are required to completely define the mechanisms responsible for these effects, induction of PPARα-regulated genes encoding for proteins responsible for fatty acid metabolism appear to be involved. These results are similar to those in obese Zucker fa/fa rats that were fed diets containing soy protein (35). In that model, soy protein diet ameliorated fatty liver and hepatic cholesterol and triglyceride levels, even in the presence of hyperinsulinemia.

Our data are consistent with previous studies suggesting that soy feeding can activate hepatic PPARα-signaling *in vivo* and suggest that SPI feeding may increase hepatic fatty acid degradation. This might contribute to the reduction of hepatic triglycerides and reduced steatosis observed upon feeding SPI in a variety of animal models. SPI-fed rats and mice demonstrated up-regulation of several PPARα-regulated genes, increased protein binding to the PPRE on the ACO gene and increased expression of PPARα mRNA. Mezei *et al* (37) have reported similar increases in PPARα-regulated gene expression in 129/Sv mice fed SPI and a lack of effects of SPI feeding on these genes in PPARα knockout mice. In addition, Tovar *et al* (35) have reported increased expression of the PPARα-regulated CPT-1 gene in the liver of hyperinsulinemic obese Zucker rats fed SPI. It is unclear if the increased binding of PPARα to its promoter element reflects activation of the transcription factor by ligands appearing in plasma following consumption of SPI or increased expression of more transcription factor, since PPARα mRNA was significantly elevated following SPI consumption. Similar increases of hepatic PPARα mRNA expression were reported by Tovar *et al* (35) following SPI consumption in Zucker rats and increased receptor expression have also been observed following *in vitro* and *in vivo* treatment with the peroxisome proliferator clofibric acid (38).

In summary, while the major soy-containing isoflavone, genistein, has been reported to modulate epigenetic regulation of coat color and body composition in A^{vy}/a mice (10), we found that SPI fed at levels that provide the same approximate level of genistein failed to modulate coat color. These data further support the growing published data base demonstrating the differences between the effects of purified isoflavones and soy foods with soy protein or SPI, such as soy infant formula. SPI-containing diets did reduce hepatosteatosis in two different rodent models and there was a gender-specific effect (reduction) on ectopic fat pad mass in mice. These results suggest that soy foods may be beneficial in treatment or prevention of hepatosteatosis. Although more studies are needed to expand our understanding of the molecular mechanisms involved, it appears the SPI can act on PPAR to increase fatty acid metabolism and soy products may be of future use in treatment or prevention of NAFLD or NASH.

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