Original Research

Soy protein isolate inhibits hepatic tumor promotion in mice fed a high-fat liquid diet

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Impact statement

The impact of dietary components on cancer is a topic of great interest for both the general public and the scientific community. Liver cancer is currently the second leading form of cancer deaths worldwide. Our study has addressed the effect of the protein source on hepatic tumor promotion in a mouse model reflecting aspects of non-alcoholic fatty liver disease (NAFLD). A high-fat liquid diet with casein as the protein source promotes hepatic injury and tumor promotion in diethylnitrosamine-treated mice. Replacing casein with a soy protein isolate led to a pronounced diminishment of tumor promotion and associated hepatic injury and inflammation. The study thus demonstrates that a dietary protein source can have beneficial, preventative effects on hepatic tumor promotion.

Abstract

Alcoholic and nonalcoholic fatty liver diseases are risk factors for development of hepatocellular carcinoma, but the underlying mechanisms are poorly understood. On the other hand, ingestion of soy-containing diets may oppose the development of certain cancers. We previously reported that replacing casein with a soy protein isolate reduced tumor promotion in the livers of mice with alcoholic liver disease after feeding a high fat ethanol liquid diet following initiation with diethylnitrosamine. Feeding soy protein isolate inhibited processes that may contribute to tumor promotion including inflammation, sphingolipid signaling, and Wnt/β-catenin signaling. We have extended these studies to characterize liver tumor promotion in a model of nonalcoholic fatty liver disease produced by chronic feeding of high-fat liquid diets in the absence of ethanol. Mice treated with diethylnitrosamine on postnatal day 14 were fed a high-fat liquid diet made with casein or SPI as the sole protein source for 16 weeks in adulthood. Relative to mice fed normal chow, a high fat/casein diet led to increased tumor promotion, hepatocyte proliferation, steatosis, and inflammation. Replacing casein with soy protein isolate counteracted these effects. The high fat diets also resulted in a general increase in transcripts for Wnt/β-catenin pathway components, which may be an important mechanism, whereby hepatic tumorigenesis is promoted. However, soy protein isolate did not block Wnt signaling in this nonalcoholic fatty liver disease model. We conclude that replacing casein with soy protein isolate blocks development of steatosis, inflammation, and tumor promotion in diethylnitrosamine-treated mice fed high fat diets.

Keywords: Tumor promotion, soy protein, casein, non-alcoholic fatty liver disease, high fat feeding, liver cancer

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Introduction

According to the International Agency for Research on Cancer of the World Health Organization, the second-most leading cause of cancer deaths in the world is liver cancer.[1] Infections with hepatitis B, hepatitis C, and alcohol consumption are well-established risk factors for liver cancer.[2] Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) occurring as a consequence of a typical modern high fat (HF) Western diet may also increase the risk of developing hepatocarcinomas.[3–5] The mechanisms whereby alcohol and HF diets promote tumor formation remain poorly understood. Dietary components may also exert protection from carcinogenesis. Soy-containing diets have been reported to reduce incidence of various cancers.[6–9]

We have developed a mouse model for studying the tumor-promoting properties of alcohol ingestion.[10,11] In this model, tumorigenesis is initiated by developmental exposure to diethylnitrosamine (DEN) that results in DNA adducts in a cytochrome 2E1-dependent manner.[12,13] Adult mice received alcohol as a part of HF liquid diet for 16 weeks. Compared to animals eating regular chow, the alcohol-fed animals had increased content of liver lesions in the form of preneoplastic foci and adenomas.[10,11] Alcohol diet consumption was further associated with hepatocyte

proliferation, activation of the Wnt/β-catenin pathway, fibrosis, inflammation, and activation of ceramide/sphingosine metabolism – all processes that may promote carcinogenesis.[10,11] Interestingly, animals given a HF liquid diet in the absence of ethanol, also exhibited increased inflammation and fibrosis as well as elevated level of liver injury compared to animals on regular chow.[11] These findings suggest that the liquid HF diet by itself has tumor-promoting properties. We further observed that replacing casein with a soy protein isolate (SPI) in alcohol-fed animals reduced hepatic carcinogenesis, liver injury, Wnt/β-catenin signaling, inflammation, and sphingolipid signaling.[10]

We hypothesize that a HF diet will promote hepatic carcinogenesis and associated mechanisms and that replacing casein with SPI will counteract these processes. We assessed the mechanisms by quantifying components thereof in DEN-treated mice fed HF liquid diets.

Materials and methods

Experimental animals

Male C57Bl/6 mice receiving a DEN injection at postnatal day 14 were subjected, starting on postnatal day 60, to 16 weeks of feeding with standard rodent chow (n = 10) or were fed liquid HF diets containing 18% protein; 35% fat and 47% carbohydrate adjusted daily to the same caloric intake as similar ethanol-fed animals.[10,11] The liquid diets were purchased from Dyets Inc., Bethlehem, PA. The protein source in the liquid diets was either casein (CAS) (n = 22) or SPI (n = 23). The composition was 41.4 g/l casein (100 Mesh) or SPI, 0.5 g/l L-cystine, 0.3 g/l DL-methionine, 8.5 g/l corn oil, 28.4 g/l olive oil, 2.7 g/l safflower oil, 115.2 g/l maltose dextrin, 10 g/l cellulose, 8.75 g/l mineral mix #210,011, 2.5 g/l vitamin mix #310,011, 0.53 g/l choline bitartrate and 0.3 g/l xanthan gum. The SPI was isolated soy protein provided by DuPont Nutrition and Health, St. Louis, MO containing 87% protein and a total isoflavone content of 3.2 mg/g, including daidzein-containing (1.2 mg/g), genistein-containing (1.9 mg/g), and glycitein-containing (0.1 mg/g) compounds. The standard chow was Teklad 22/5 rodent diet 8640 from Harlan Laboratories, Madison, WI. Further groups included control mice that instead of DEN were injected with saline followed by being fed chow (n = 4), HF/CAS (n = 6), or HF/SPI (n = 5). The data for several parameters for the DEN-treated, chow-fed group have been previously reported.[10] Mice were housed two per cage. They were euthanized by CO_2 asphyxiation early in their day cycle. The experiments were conducted in an AAALAC accredited animal facility and approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Soy isoflavones

The serum concentrations of soy isoflavones were determined as previously described.[14]

Liver pathology

Liver tissue collected at sacrifice were formalin fixed and evaluated for hepatic lesions as previously described.[10] Steatosis and inflammation were scored as reported earlier.[15] The pathologists were blinded to the treatment groups. Activities of alanine transaminase (ALT) in serum collected at sacrifice were determined by an InfinityTM ALT(GTP) kit (Cat. no. TR1121, Fisher Scientific, Middletown, VA) according to the guidelines of the manufacturer.

Immunohistochemistry and Western blotting

Proliferating cell nuclear antigen (PCNA) was assessed by immunohistochemistry using an anti-PCNA antibody (PC-10 from Abcam, Cambridge, MA) as previously described.[11] Hepatic mitochondria were isolated using a mitochondrial isolation kit for tissue (ThermoFisher, Waltham MA; cat. no. 89801) and mitochondrial oxidative phosphorylation (OXPHOS) complexes were determined using MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413 from Abcam) which allows simultaneous detection of key components of the complexes. β-catenin in membrane fractions, cytosolic extracts, and nuclear extracts were determined by Western blotting using antibody 05-665 from EMD Millipore, Billerica, MA. Nuclear phosphorylated NF-κB were determined by fractionation and Western blotting as previously described.[10] The primary antibody was 3033 from Cell Signaling Technology, Beverly, MA, and the secondary antibodies were goat anti-rabbit IgG-HRP sc-2004 and goat anti-mouse IgG1-HRP sc-2969 from Santa Cruz Biotechnology, Dallas, TX. Band densities are normalized relative to loading quantified by staining membranes with amido black.

Gene expression

RNA was isolated from aliquots of whole liver, including any tumor lesions that may be present. The RNA integrity number (RIN) was assessed on a 2200 TapeStation (Agilent Technologies, Santa Clara, CA) with average and standard deviation of 8.73 and 0.57. Gene expression of components of the Wnt/β-catenin signaling pathway was assessed by a 96-well RT^2 Profiler PCR Array (Qiagen, Germantown, MD; cat. no. 330231 PAMM-043ZA) using pools of hepatic RNA (three mice per pool) from each diet (n = three pools per diet). Δ C_T for each target gene was calculated as C_T for the target gene minus the average C_T for the five normalizer genes included in the arrays. Gene expression for individual genes was assessed by two-step quantitative RT-PCR as described[11] or by one-step quantitative RT-PCR using a Power SYBR Green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA). Expression was expressed relative to the expression of OAZ1, 36B4, or GADPH. Assays were conducted on an ABI 7500 Sequence Detection System (Applied Biosystems), or a LightCycler® 480 II (Roche, Indianapolis, IN). Primer sequences and assay efficiencies are listed in Supplementary Table 1. The efficiency of each assay was calculated from the slope of standard curves of C_T values vs. the logarithms of standard

concentrations, where the standards were dilutions of a certain cDNA or RNA sample.

Data and statistical analysis

Incidences of basophilic foci and adenomas are indicated as percentages. Statistical differences between groups are evaluated by Fisher's exact test. Other data are presented as means +/− standard error. For data that are assessed to be normally distributed with equal variances, data are analyzed by Student's *t*-test or by one-way analysis of variance (ANOVA) with post hoc comparisons of means with Student–Newman Keul adjustment for multiple comparisons. Parameters with unequal variance between groups (body weight, liver weight, PCNA staining, ALT value and steatosis score) were analyzed with non-parametric statistics (Kruskal–Wallis analysis with Dunn's post hoc analysis or Mann–Whitney U rank-sum test). qRT-PCR data were consistently analyzed by ANOVA of ΔC_T values or logarithm-transformed values. Statistics were calculated with SigmaPlot software package 13.0 or by entering formulas directly in Microsoft Excel. Differences were considered significant at test probabilities $P < 0.05$.

Results

SPI diminishes weight gain and hepatic steatosis mediated by the HF diet

SIPs contains many phytochemicals in addition to protein, of which several, including isoflavone phytoestrogens, are absorbed from the gastrointestinal tract and enter the circulation.[6,14,16] From mice that fed the HF/SPI diets ($n = 6$), we could thus detect the soy isoflavones equol, daidzein, and genistein in serum at concentrations of 0.31 ± 0.08, 0.07 ± 0.02, and $0.05 \pm 0.02\,\mu M$, respectively. Starting body weights for DEN-treated mice at the start of feeding them chow, liquid HF diet with casein (HF/CAS), and liquid HF diet with SPI (HF/SPI) were 22.8 ± 0.5 g, 23.0 ± 0.3 g, and 22.7 ± 0.2 g, respectively, which were not significantly different. At sacrifice, the body weights were 28.1 ± 1.1 g, 41.4 ± 1.2 g, and 33.0 ± 0.8 g, respectively. As shown in Figure 1(a), there were significant differences between the

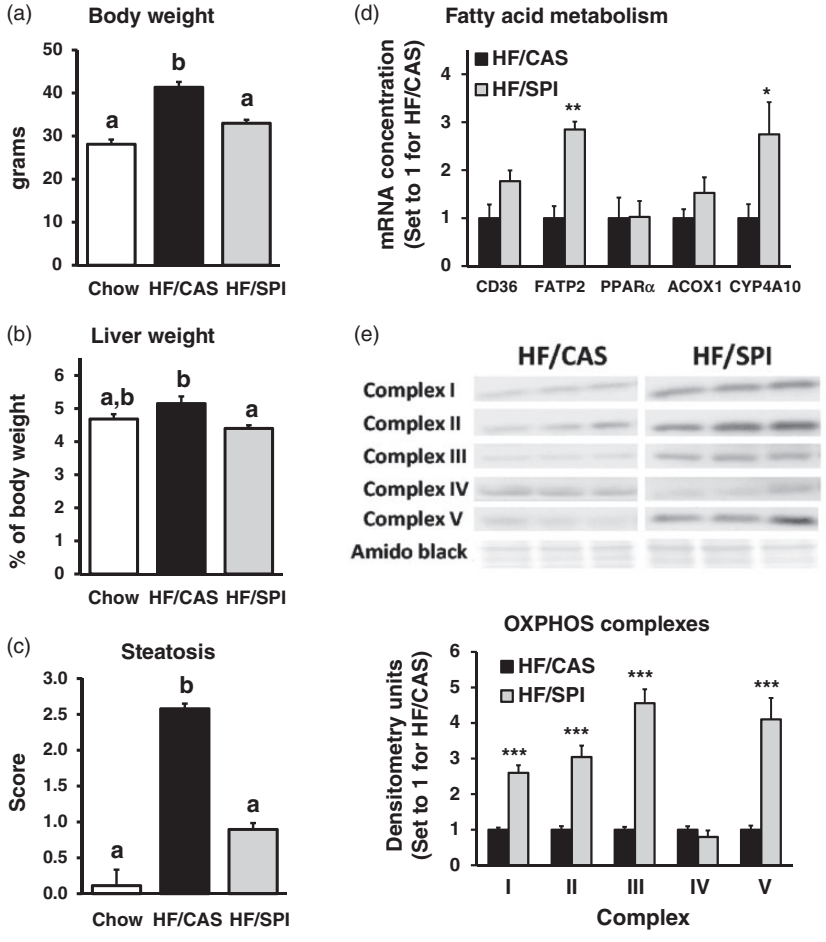

Figure 1 SPI reduces weight gain and hepatic steatosis in high fat-fed mice. (a) Body weight was measured in DEN-treated mice fed chow, HF/CAS, and HF/SPI ($n = 10$, 22, and 23, respectively). (b) Fresh liver weight relative to body weight was determined in DEN-treated mice fed chow, HF/CAS, and HF/SPI ($n = 10$, 19, and 22, respectively). (c) Hepatic steatosis scores were recorded for DEN-treated mice fed chow (n = 10), HF/CAS (n = 22) and HF/SPI (n = 23). Columns without letters (a and b) in common indicate significant differences at $P < 0.05$. (d) Transcripts of genes involved in hepatic fatty acid metabolism were determined in liver RNA isolated from saline-treated mice fed HF/CAS (n = 6) and HF/SPI ($n = 4$–5). The normalizing gene was GAPDH. (e) Western blot of oxidative phosphorylation (OXPHOS) complexes was conducted with liver mitochondria isolates from saline-treated mice fed HF/CAS ($n = 6$) and HF/SPI ($n = 4$). The panels show representative samples run in non-adjacent lanes on the same gel. 30 μg protein was loaded per well. *, **, ***; $P < 0.05$, $P < 0.01$, $P < 0.001$ vs. HF/CAS

three groups with the median body weight for the HF/CAS-fed mice significantly higher than for chow- and HF/SPI-fed mice ($P < 0.001$). The mean weight of the liver at sacrifice was also significantly higher for the HF/CAS group (2.19 ± 0.15 g) than for the chow (1.32 ± 0.06 g) and HF/SPI (1.45 ± 0.06 g) groups ($P < 0.001$). Furthermore, the relative liver weight was significantly lower in the HF/SPI group than the HF/CAS group (Figure 1(b)). Mice on the HF/SPI diet further showed significantly lower degree of hepatic steatosis than mice on the HF/CAS diet (Figure 1(c)). The SPI thus counteracts whole body weight gain, liver enlargement, and hepatic steatosis in the context of the HF liquid diet. This occurred despite no significant difference in the overall food intake observed in mice fed HF/CAS or HF/SPI diets (14.7 ± 0.1 ml/day/mouse and 14.5 ± 0.1 ml/day/mouse, respectively).

In rats, protective effects of dietary soy protein on the metabolic syndrome were mediated in part by activation of PPARα- and PPARγ signaling affecting genes involved in fatty acid metabolism.[17] For saline-treated mice on the HF diets, we measured the expression of genes involved in fatty acid uptake (CD36 and FATP2) and fatty acid degradation (PPARα, ACOX1, and CYP4A10). SPI led to a significant increase in both FATP2 and CYP4A10 mRNA (Figure 1(d)). In addition, the level of complexes I, II, III, and V of the mitochondrial electron transport chain and OXPHOS, which are also known PPARα targets[17] were all higher in HF/SPI-fed mice than HF/CAS-fed mice (Figure 1(e)). The SPI-mediated reduction of hepatic steatosis may therefore involve increased oxidative metabolism of fatty acids.

SPI opposes tumor promotion induced by the HF diet

In all dietary groups, lesions were observed, of which the predominant type was preneoplastic basophilic foci (Figure 2(a)). The incidences of total lesions and basophilic foci were higher in the HF-fed groups than in chow-fed mice. However, the incidence after feeding SPI was significantly lower than after feeding casein-based diets ($P = 0.023$). The multiplicity of total lesions and of basophilic foci (Figure 2(b)) was likewise significantly lower ($P = 0.004$ and $P = 0.005$, respectively) with SPI than with CAS. The incidence of adenomas (Figure 2(a)) was significantly higher ($P = 0.026$) in HF/CAS-fed mice than in chow-fed mice. There was also a trend towards lower incidence of adenomas in the HF/SPI group compared to the HF/CAS group ($P = 0.071$). The small differences in adenoma multiplicities were statistically insignificant. Overall, replacing casein with SPI counteracts the development of preneoplastic liver lesions in mice given the HF diet. In the non-tumorigenic liver tissue, SPI in the HF diet resulted in lower cell proliferation than casein ($P < 0.001$) as estimated by cell nuclei stained by PCNA (Figure 2(c)).

Cancer stem cells (CSCs) are theorized to be a cell population capable of generating all the cell types in a particular cancer.[18] Suggested markers for the CSC population and for increased stem cell-like attributes also known as "stemness" in the liver include CD24, PROM1 (also known as CD133), EPCAM, α-fetoprotein (APF), and SOX4.[19–22] We assessed the dietary effects on expression of these CSC markers by determining the transcriptional levels. Compared to mice not subjected to tumor initiation, i.e. saline-treated, and given normal chow diet, the only

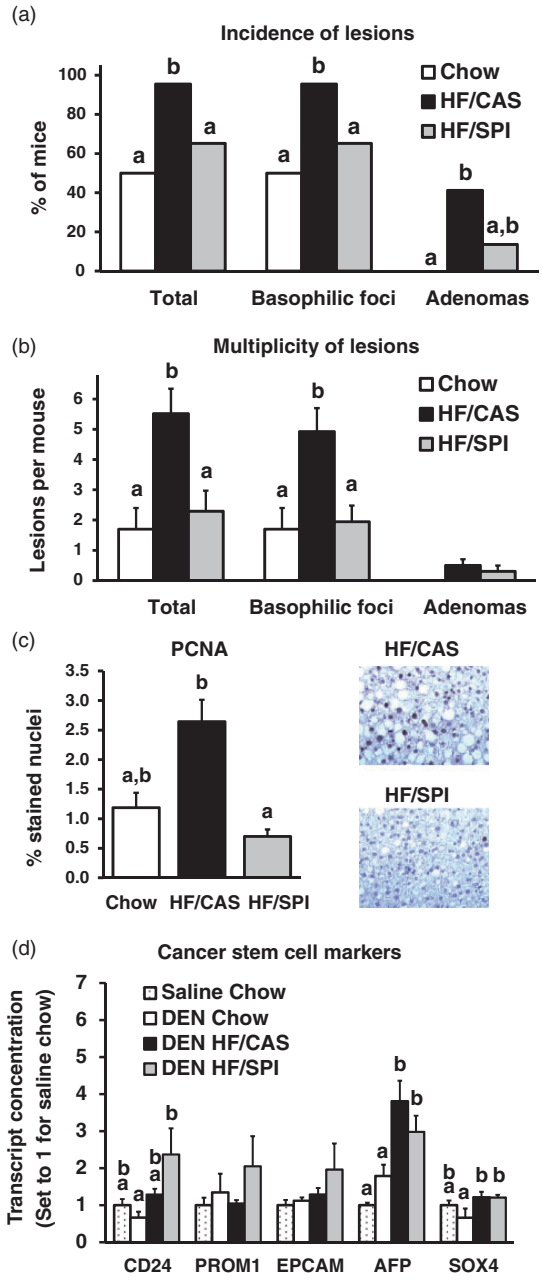

Figure 2 SPI opposes tumor promotion. (a) Incidences of total cancerous lesions, basophilic foci and adenomas were determined in mice fed chow, HF/CAS, and HF/SPI ($n = 10$, 21, and 23 for total cancerous lesions and foci and $n = 10$, 17, and 22 for adenomas, respectively). (b) Multiplicities of total cancerous lesions, basophilic foci and adenomas were calculated in mice fed chow, HF/CAS, and HF/SPI ($n = 10$, 22, and 23, respectively). (c) PCNA staining resulting in brown nuclei as shown in representative slides to the right was performed. PCNA quantification of stained nuclei in non-tumor-containing parts of the liver was done for 5, 12, and 8 mice, respectively, fed chow, HF/CAS, and HF/SPI. (d) Transcripts of cancer stem cell markers were determined in liver RNA isolated from saline-treated control mice fed chow ($n = 4$) and in DEN-treated mice fed chow ($n = 3$), HF/CAS ($n = 10$–21), and HF/SPI ($n = 12$–23). The normalizing gene was 36B4. Columns without letters (a and b) in common indicate significant differences at $P < 0.05$. (A color version of this figure is available in the online journal.)

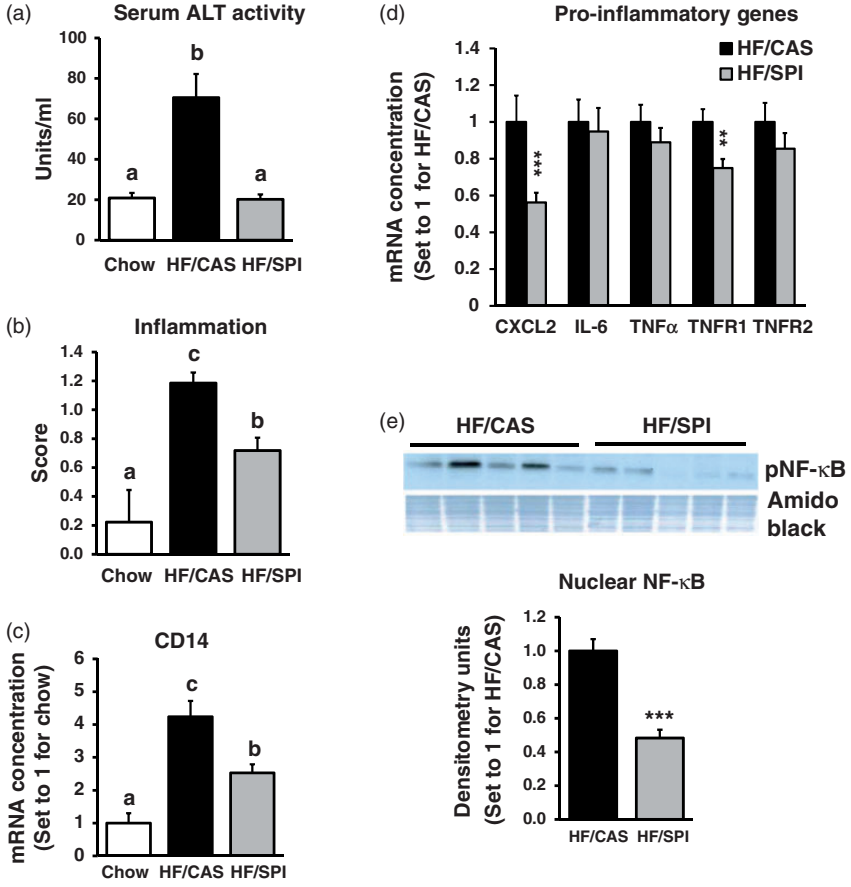

Figure 3 SPI relieves hepatic injury and inflammation. (a) Serum alanine transaminase activity was determined for mice fed chow ($n = 10$), HF/CAS ($n = 22$) and HF/SPI ($n = 23$). (b) Hepatic inflammation scores were recorded for mice fed chow ($n = 10$), HF/CAS ($n = 22$) and HF/SPI ($n = 23$). (c) The mRNA concentrations of the Kupffer cell marker CD14 were determined for DEN-treated mice fed chow ($n = 3$), HF/CAS ($n = 20$) and HF/SPI ($n = 21$). The normalizing gene was 36B4. (d) Transcriptional regulation of pro-inflammatory genes was determined in liver RNA from DEN-treated mice fed HF/CAS ($n = 19$–20) and HF/SPI ($n = 20$–21). The normalizing gene was 36B4. (e) NF-κB was determined in a Western blot of nuclear fractions from livers of five saline-treated mice fed HF/CAS and 5 mice fed HF/SPI. 40 μg protein was loaded per well. The NF-κB bands had a molecular weight of approximately 64 kDa. Columns without letters (a and b) in common indicate significant differences at $P < 0.05$. **, ***; $P < 0.01$, $P < 0.001$. (A color version of this figure is available in the online journal.)

significant difference was an increase in AFP for DEN-treated mice fed the HF diets (Figure 2(d)) relative to chow-fed controls.

SPI opposes liver pathology and inflammation induced by the HF diet

Liver damage was assessed by the activity of serum ALT. As illustrated in Figure 3(a), there was an increase in ALT activity in the HF/CAS-fed mice compared to the chow-fed mice ($P = 0.006$). This increase in HF-fed mice was completely reversed when casein was replaced with SPI ($P < 0.001$). Whereas liver fibrosis in this mouse model is mild, SPI may exert anti-fibrotic effects. DEN-treated mice fed the HF/SPI diet thus had a statistically significant ($P < 0.05$) 32% reduction in the hepatic content of mRNA for the collagen gene *Col1a1* compared to HF/CAS-fed mice (data not shown). In the context of the HF diets, SPI counteracted an index of hepatic inflammatory foci (Figure 3(b)). Transcripts for the Kupffer cell marker CD14 followed the same pattern with higher concentrations in HF/CAS-fed mice than in chow-fed mice and with SPI reducing the transcript concentrations relative to casein (Figure 3(c)). As illustrated in Figure 3(d), replacing casein with SPI also led to reductions in gene expression of the pro-inflammatory cytokine CXCL2 and tumor necrosis factor receptor 1 (TNFR1). Finally, nuclear content of NF-κB was highly significantly reduced ($P < 0.001$) for the HF/SPI diet compared to the HF/CAS diet (Figure 3(e)), suggesting reduced TNFα-signaling in the presence of SPI. We conclude that replacing casein with SPI in the HF diet opposes both liver damage and inflammation.

Ceramide/sphingosine metabolism is affected by the protein source in the HF diet

Previously, we have reported that alcohol exposure in DEN-treated mice significantly increased *de novo* ceramide synthesis, sphingosine kinase activity and sphingosine-1 P (S1P) accumulation, resulting in a proliferative environment conducive to tumorigenesis.[10] SPI feeding during alcohol exposure reduced ceramide/S1P generation, and tumor multiplicity, suggesting phospholipid signaling pathways are involved in tumor development and may represent a therapeutic target in alcoholic hepatocarcinogenesis.[10] In Figure 4(a), we determined the effect of the

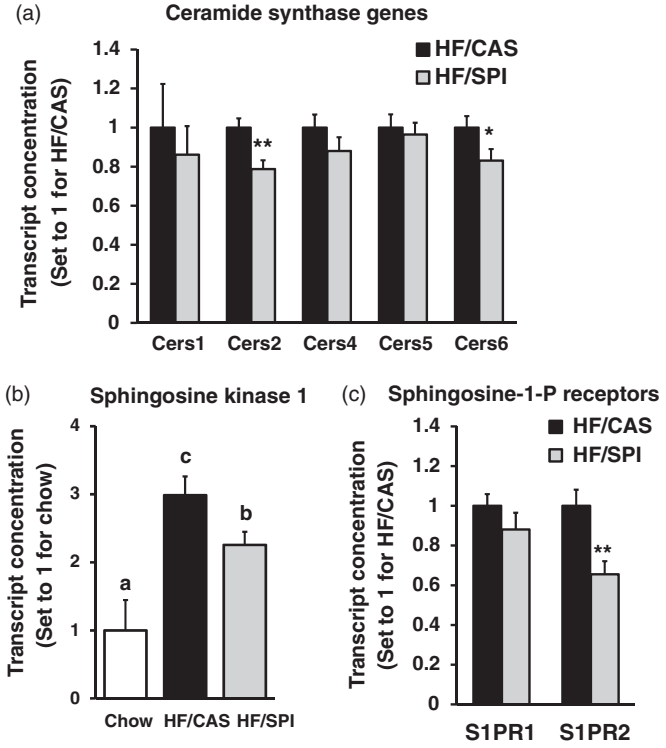

Figure 4 SPI attenuates sphingosine-1-phosphate signaling components. Transcripts of ceramide synthase genes (a) and components involved in sphingosine-1-phosphate signaling (b-c) were quantified in RNA from livers isolated from DEN-treated mice fed chow ($n = 3$), HF/CAS ($n = 19$–20), and HF/SPI ($n = 20$–21). The normalizing gene was 36B4. Columns without letters (a–c) in common indicate significant differences at $P < 0.05$. *, **: $P < 0.05$, $P < 0.01$

protein source on ceramide synthase genes in DEN-treated mice chronically fed HF liquid diet. Based on C_T values in qRT-PCR assays, the relative expression of the genes was Cers2 ≫ Cers4, Cers6, Cers5 ≫ Cers1. SPI had a small, but significant effect on Cers2 and Cers6 gene expression relative to casein. In response to HF/CAS feeding, sphingosine-1-phosphate kinase (SphK1) mRNA increased by 2-fold relative to chow ($P < 0.05$). Likewise, replacement of CAS with SPI opposed this effect ($P < 0.05$) (Figure 4(b)). S1P signals by binding to sphingosine-1-phosphate receptors. While the expression of sphingosine-1-phosphate receptor 1 (S1PR1) mRNA was unaffected by the protein source, expression of sphingosine-1-phosphate receptor 2 (S1PR2) mRNA was significantly decreased for the HF/SPI diet (Figure 4(c)). These data illustrate that similar to alcoholic tumorigenesis, in this HF feeding model, SPI feeding suppresses components of hepatic ceramide/sphingolipid signaling.

SPI's anti-tumor effects in the HF feeding model are not associated with inhibition of Wnt/β-catenin signaling

We have previously demonstrated that chronic ethanol consumption in rodent models is associated with activation of Wnt/β-catenin signaling, including an increase in phosphorylated GSK3β, an increase in active, unphosphorylated β-catenin, and increased expression of the β-catenin target genes glutamine synthase, Cyclin D1, and matrix metalloproteinase-7 (MMP7). In addition, SPI appeared to counteract Wnt/β-catenin signaling in ethanol-fed mice by opposing phosphorylation of GSK3β, nuclear accumulation of β-catenin, and transcription of target genes.[10,11] We tested whether dietary SPI could likewise reduce hepatic Wnt/β-catenin signaling relative to casein after chronic feeding of HF liquid diets in the absence of ethanol. A hall-mark of increased signaling is stabilization of β-catenin further leading to an increase in the content of nuclear β-catenin, where it functions as a transcriptional coactivator to stimulate activation of target genes.[23] We determined the content of hepatic β-catenin in saline-treated mice. While the content of β-catenin in cytoplasmic liver extracts was decreased by SPI relative to casein, there was, in fact, a significant increase in both membrane-associated and nuclear β-catenin in the HF model (Figure 5(a)).

We were further interested in assessing whether the diets given to DEN-treated mice could affect the Wnt/β-catenin pathway components at the transcriptional level. This was done using a commercial PCR array for mouse Wnt/β-catenin signaling components. The data for all 65 genes whose transcript were detected in all samples are listed in Supplementary Table 2. HF liquid diets affected transcript levels for many genes, but the differences between the CAS and SPI-containing HFD groups were quite small. For example, the expression of the gene encoding β-catenin, *Ctnnb1*, differed by less than 31% between groups. A subset of genes was re-examined by separate qRT-PCR, confirming the trends and small differences indicated by the arrays (Figure 5(b)).

Major trends in the array data set are illustrated in Figure 5(c). Compared to normal chow, the HF liquid diets lead to a general increase in transcript concentrations of pathway components, both components stimulating and inhibiting signaling. The average induction of components was +93% and +105%, respectively, in the presence of casein or SPI. An important exception was a strong down-regulation of PPARδ mRNA ($P < 0.001$) to 23% of the level in chow-fed mice (Figure 5(e)). The PCR arrays indicated that SPI led to a slight +21% overall increase in transcript concentrations compared to casein (Figure 5(c)). Seven Wnt genes were robustly quantified. Figure 5(d) shows the relative amount of transcripts for these genes as well as the dietary effects on expression. We also calculated the sum of expression of these genes (total Wnt). The expression of Wnt2, Wnt2b, Wnt4, Wnt9a, and total Wnt mRNA was significantly elevated in animals fed the HF diets compared to chow-fed animals. Eight Wnt/β-catenin target genes were robustly quantified by the PCR arrays: *Btrc*, *Ccnd1*, *Ccnd2*, *Dab2*, *Jun*, *Mmp7*, *Myc*, and *Ppard* (Figure 5(e) and Supplementary Table 2). *Btrc*, *Ccnd*1, *Ccnd*2, *Dab*2, and *Jun* were all significantly induced for the HF diets compared to chow. Exchanging casein with SPI led to no decrease in any target gene but rather a statistically significant increase in expression of the six target genes *Btrc*, *Ccnd1*, *Ccnd2*, *Dab2*, *Jun* and *Myc* (Figure 5(e)).

Discussion

Both alcohol consumption and the metabolic syndrome are risk factors for the development of fatty liver diseases with

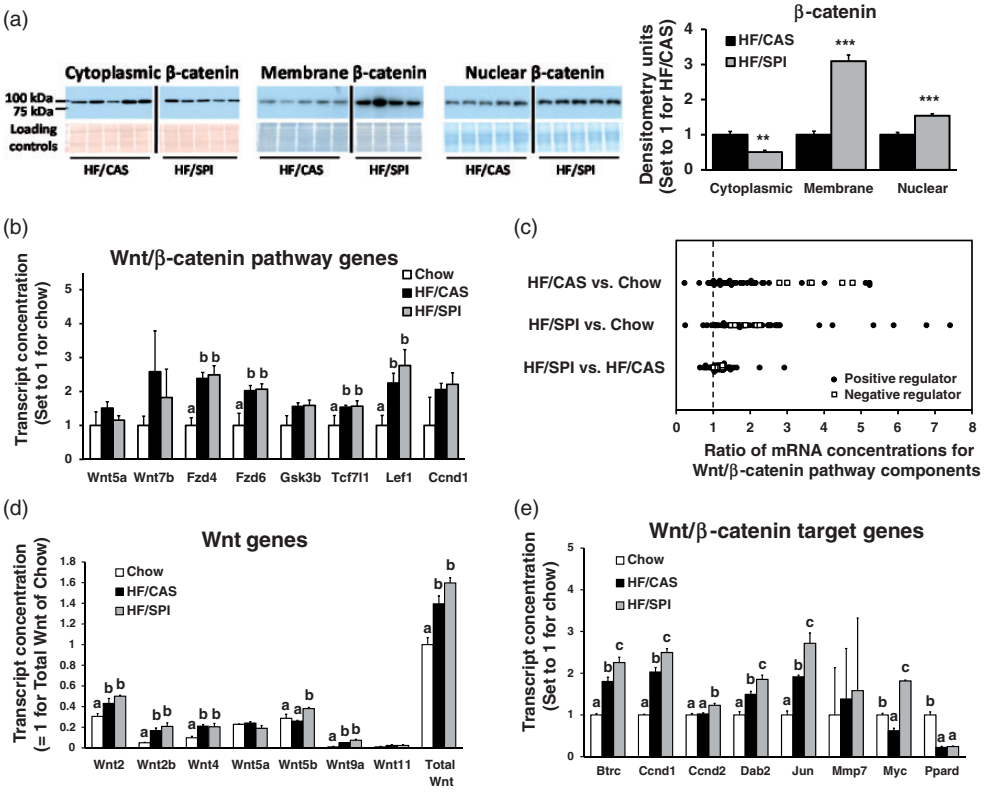

Figure 5 Effects of dietary components on the Wnt/β-catenin pathway. (a) Western blots of β-catenin in cytoplasmic extracts (60 μg protein per lane), membrane fractions (40 μg protein per lane), and nuclear extracts (40 μg protein per lane) from livers of DEN-untreated mice fed the HF/CAS and HF/SPI diets. The samples for the HF/CAS and HF/SPI diets were run on the same gel, but in non-adjacent lanes. Panels below the blots are images of amido black stained membranes which were used as loading controls. Densitometric quantification of the Western blots are illustrated on the right.**, ***; $P < 0.01$, $P < 0.001$, respectively. (b) Individual qRT-PCR assays for selected Wnt/β-catenin pathway genes were conducted with RNA from DEN-treated mice fed chow ($n = 3$), HF/CAS ($n = 9$–20), and HF/SPI ($n = 9$–21). The normalizing genes were OAZ1 for Wnt5a, Wnt7b, GSKβ, and CyclinD1 assays and 36B4 for the other assays. (c) Expression of Wnt/β-catenin pathway genes was measured with PCR arrays using liver RNA pooled from three mice on a particular diet. We tested three pools each from mice on each of the diets. From the PCR array data, the relative expression of each of 65 genes consistently detected in all samples is depicted when contrasting diets. The genes encoded proteins involved in promoting Wnt/β-catenin signaling (Positive regulators) and proteins inhibiting signaling (Negative Regulators). (d) The PCR array data are shown for all robustly quantified Wnt genes. Total Wnt is the calculated sum of transcripts from these Wnt genes. (e) The PCR array data are shown for all robustly quantified Wnt/β-catenin target genes. Columns without letters (a–c) in common indicate significant differences at $P < 0.05$. (A color version of this figure is available in the online journal.)

similar pathology which ultimately can result in the development of liver cancer.[3,4,24–27] Using a two-stage mouse model in which tumor initiation by DEN was followed by a 35% HF diet, we have previously reported increased tumor incidence, suggestive of a diet-related promotional effect.[11] Consistent with these findings, in the current study, we observed increased adenoma incidence and tumor multiplicity in the HF/CAS group relative to chow controls. More importantly, substituting SPI for casein in the diet produced a marked reduction in hepatic lipid accumulation, inflammation and cell proliferation, resulting in lower tumor incidence and multiplicity.

SPI feeding prevented liver weight increase and hepatic steatosis in HF-fed mice relative to mice fed casein (Figure 1). In rats, replacing casein with soy in various feeding regimes has also led to significant reductions in hepatic weight and steatosis.[17,28,29] This was found to be associated with hepatic activation of PPAR-signaling pathways and inhibition of SREBP signaling which might promote fatty acid utilization over fatty acid synthesis.[17] Similar mechanisms may be operating in our mouse model, as SPI induces genes involved in fatty acid uptake, fatty acid degradation, and OXPHOS.

The employed model demonstrates protective effects of SPI in early tumorigenesis. Whether SPI also has beneficial effects on later tumorigenesis stages involving invasion and angiogenesis will need to be investigated in additional studies. Still, our findings suggest a role for SPI dietary supplementation in reducing liver cancer risk in NAFLD patients. Support for this protective effect comes from a wealth of epidemiological and experimental animal studies associating soy protein or soy-derived phytochemicals with anti-cancer properties, including anti-proliferative effects associated with reduced Wnt/β-catenin signaling.[10,30,31] In this study, using PCR arrays, we observed an overall induction of Wnt/β-catenin pathway genes for HF liquid diets compared to chow (Supplementary Table 2 and Figure 5). Interestingly, the effects of SPI on Wnt/β-catenin signaling were found to be stimulatory rather than inhibitory. These findings suggest that the anti-proliferative effects observed in the HF/SPI group are not mediated through Wnt/β-catenin signaling.

Previously, we have reported that consumption of HF liquid diets containing ethanol also led to activation of the Wnt/β-catenin pathway in livers of mice and rats.[10,11] Moreover, reduction of tumorigenesis by SPI feeding

during alcohol exposure was associated with inhibition of Wnt/β-catenin signaling mechanisms.[10] PCR array data conducted on livers from animals fed ethanol diets (data not shown) further revealed only small differences between the ethanol-fed and HF-fed animals. While ethanol *per se* may induce a few Wnt/β-catenin pathway genes, it appears that a major factor causing increased Wnt/β-catenin signaling in these models is the lipotoxicity and necro-inflammatory injury associated with chronic feeding of HF liquid diets with or without ethanol. In human hepatic cancers, frequently mutated genes include the genes for β-catenin and Axin1 presumably leading to enhanced activation of Wnt/β-catenin signaling.[2,32] Yet, only 13% (3 of 23) cancers in human alcoholics had β-catenin mutations versus 100% in the RB1 pathway,[2] suggesting that β-catenin signaling is not universally required for alcohol-mediated tumorigenesis. However, ethanol consumption in the context of a HF liquid diet did result in a higher percentage of adenomas per animal (67%) than observed in the current study (41%), and a higher adenoma multiplicity (1.62 vs. 0.20 adenomas/animal, respectively), suggesting that ethanol has additional tumor-promoting properties relative to HF diets, which may include additional sphingolipid signaling mechanisms.[10] The observed induction of Wnt/β-catenin signaling by the HF diets relative to chow controls may be a crucial mechanism in tumor promotion. However, we recognize that our studies so far only describe an association between Wnt/β-catenin signaling and tumor promotion. To establish a causative link, it will be necessary in future studies to block signaling by genetic techniques or by pharmaceutical inhibitors that are currently available.[23]

Chronic liver injury results in certain pathophysiological changes, including the activation of specific CSC populations, which are known to enhance tumorigenesis.[19–22] Therefore, we screened for CSC population by real time RT-PCR in DEN-treated chow, HF/CAS, and HF/SOY groups. In response to the HF/CAS diet, AFP was the only marker for which HF diets increased the mRNA concentration relative to the chow diet; SPI feeding had no effect. It should also be noted that saline-treated chow-fed mice also had detectable levels of the markers used to characterize stemness. For these mice, the levels must represent normal physiological levels unrelated to carcinogenesis. While we can't exclude the possibility of a CSC population contributing to fat-mediated tumor promotion, it must be too small to detect at the level of liver lesions developed in the current model.

Presently, it is unclear which components of the SPI confer beneficial effects relative to casein on hepatosteatosis and tumorigenesis. SPI is not purely a protein preparation, as it also contains more than a 100 different phytochemicals including flavonoids.[10,16] Consistent with our findings, several phytochemicals are known to enter the circulation allowing them to exert physiological effects at the whole-body level. Since soy flavonoids have structural similarities to estrogen, they have been termed phytoestrogens.[33] However, estrogenic properties of SPI in rat liver and mammary gland are minimal,[34,35] suggesting that the anti-tumorigenic effects of SPI are not primarily estrogen receptor-mediated. Genistein, the major isoflavone phytoestrogen, has been reported to have anti-tumorigenic properties in several types of cancers.[6,30,36] However, in our DEN-mouse model, ethanol-containing diets supplemented with genistein in concentrations similar to that found in SPI actually increased hepatic tumorigenesis.[31] Alternative mechanisms may include bioactive peptides. Of note, supplementation of alcohol-containing diets with the flavonoid luteolin was reported to inhibit hepatic inflammation and precancerous lesions of DEN-treated mice.[37] While luteolin is not recognized as a soy component, it is possible that some of the soy flavonoids have a similar effect. Bioactive peptides from digestion or hydrolysis of soy protein products may also contribute to intestinal effects of soy protein diets such as protective effects on the intestinal mucosa and changes in the microbiome,[38,39] which conceivably could affect whole-animal and liver physiology. While attempts have been made to distinguish effects mediated by protein from those mediated by phytochemicals using SPI washed with ethanol to reduce the level of isoflavones,[17] it is difficult to interpret such results as those depletion treatments may not be complete and may contribute to altered ratios among the remaining phytochemicals. Obviously, it is the net effect of the phytochemicals and proteins contained in SPI that gives the protection from hepatosteatosis and tumor promotion that we empirically determined in this study.

As discussed in a recent review,[5] there is increasing epidemiological evidence for hepatocellular carcinoma risk in humans being associated with modern life-style morbidities such as obesity, diabetes, the metabolic syndrome, and non-alcoholic fatty liver disease. Our animal model employed here demonstrates that a HF diet can promote hepatic tumorigenesis. The energy density in a HF diet may itself promote obesity. But even with isocaloric intake, replacing carbohydrates with corn oil causes substantially more steatosis and liver injury in a rat model of nonalcoholic steatohepatitis.[40] The fatty acid composition is yet another dietary factor influencing hepatic steatosis.[41] The HF diet in our study contained olive oil, corn oil, and safflower oil in a 10:3:1 (w/w/w) ratio, resulting in a diet enriched with C18:1 and C18:2 fatty acids. Providing the HF diet as a liquid rather than a solid diet is also a factor that may affect outcomes, e.g. by lack of dietary fiber or through effects on intestinal morphology and microbiota.[42,43] Additional studies will be required to test whether hepatic tumor promotion by HF diets is a general phenomenon and to study how details of the tumorigenesis process depends on the composition of lipids in HF diets.

In summary, we have confirmed that a HF liquid diet can promote tumor promotion in DEN-treated mice in the absence of alcohol. Replacing casein with SPI in the HF diet leads to reduced content of hepatic preneoplastic lesions which is associated with reduced liver pathology and with lower levels of steatosis, inflammation, and hepatocyte proliferation.

Authors' contributions: KEM and MJJR designed the experiments. KEM, CFP, KBP, and LH conducted the experiments and generated the data. KEM, KBP, and MJJR wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTARY MATERIAL

Supplementary material for this paper can be found at http://ebm.sagepub.com/content/by/supplemental-data

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