Dietary soy protein inhibits DNA damage and cell survival of colon epithelial cells through attenuated expression of fatty acid synthase

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Xiao R, Su Y, Simmen RC, Simmen FA. Dietary soy protein inhibits DNA damage and cell survival of colon epithelial cells through attenuated expression of fatty acid synthase. *Am J Physiol Gastrointest Liver Physiol* 294: G868–G876, 2008. First published January 31, 2008; doi:10.1152/ajpgi.00515.2007.—Dietary intake of soy protein decreases tumor incidence in rat models of chemically induced colon cancer. We hypothesized that decreased expression of fatty acid synthase (FASN) underlies, in part, the tumor-preventive effects of soy protein, since FASN overexpression characterizes early tumorigenesis. Here, we show that colonic FASN levels are reduced with dietary intake of soy protein isolate (SPI), compared with a control casein diet, in male Sprague-Dawley rats administered the colon carcinogen azoxymethane. SPI consumption resulted in decreased serum insulin levels and decreased azoxymethane-induced tumor suppressor p53 phosphorylation in colon crypt epithelium. To evaluate potential links between insulin and FASN leading to DNA damage and FASN-mediated anti-apoptosis during carcinogen-mediated apoptosis of C2BBe1, as well as intestinal epithelial cell apoptosis, these sites was decreased with FASN knockdown. By contrast, NMU-induced apoptosis of C2BBe1, as well as intestinal epithelial cell (IEC)-6, was enhanced by transfection FASN siRNA. Increased FASN expression in IEC-6 cells by addition of liver X receptor agonist T0901397 did not affect apurinic/apyrimidinic site number, but enhanced cell killing by cerulin, a FASN inhibitor. Moreover, insulin rescued NMU-treated cells from apoptosis in an FASN-dependent manner. Results suggest that dietary SPI, by decreasing circulating insulin levels and colon FASN expression, attenuates insulin-induced DNA damage and FASN-mediated anti-apoptosis during carcinogenesis, resulting in an overall reduced tumorigenic state.

**FATTY ACID SYNTHASE (FASN)** catalyzes the complex sequence of reaction steps underlying the conversion of acetyl-CoA and malonyl-CoA to long-chain saturated fatty acids (9). FASN expression and activity are subject to multifactorial and tissue-specific regulation, with insulin being a major hormonal inducer of synthesis of this protein (17, 20). The frequent upregulation of FASN gene and protein expression in human tumors and the association of FASN overexpression with poor clinical outcome of cancer patients have implicated this enzyme in the initiation and progression of malignancy (16, 20, 26, 43). Treatment of cancer cells with natural product (cerulenin) or chemical (C75) inhibitors of FASN activity, as well as RNA interference (RNAi) knockdown of FASN mRNA, induces growth arrest and apoptosis (2, 13, 21, 25). Because of the presumed functional linkages of FASN overexpression with tumorigenesis, this enzyme has become an attractive target for new chemotherapeutic intervention strategies (25). Mechanistically, however, it remains unclear how upregulated FASN expression promotes tumorigenesis and, conversely, why chemical or RNAi inhibition of FASN selectively kills cancerous, but not normal, cells (8, 13, 25).

The loss of DNA integrity elicited by endogenous or exogenous genotoxic agents is the initial transforming event for many pre-neoplastic lesions (4). In response to DNA damage, a series of cellular pathways are activated to induce either growth arrest and DNA repair, or, when irreparable DNA damage occurs, apoptosis (44). Under certain conditions, however, the DNA repair machinery fails to correct the DNA lesion or to initiate apoptosis, thereby leading to acquisition of mutation(s) by cells that clonally expand, acquire further mutations, and give rise to tumors. The ability of mutated cells to survive (i.e., escape apoptosis) has been associated with FASN knockdown. In pre-neoplastic cells, however, the potential relationships of FASN expression with DNA damage and apoptosis remain less clear, due to the generally low basal expression of this gene. Colon is one of only a few tissues that express FASN in significant levels before malignancy (43); interestingly, the incidence of tumors in this tissue is relatively high and affected by diet.

Soybeans have been an important dietary protein source for humans for ~5,000 years (40). Many epidemiological and animal studies suggest that regular soy protein consumption may have beneficial effects on lipid metabolism (including reduced hepatic FASN expression), diabetes, obesity, cancer, and renal dysfunction (11, 40). Dietary soy also may offer a reduced risk of colorectal cancer in humans (35). Using the classic rat model of colon cancer, we and others have found that consumption of soy protein isolates (SPI) inhibited azoxymethane (AOM)-induced colon tumor formation, as reflected by the reduced occurrence of aberrant crypt foci and reduced tumor incidence (14, 22, 27, 38, 46). The cancer-inhibitory mechanism(s) underlying dietary SPI action remains undefined. Among the several possibilities are diet-induced changes in J cell proliferation, 2 apoptosis, 3 cell survival, 4 endocrine milieu, 5 DNA repair, and 6 immune system gene expression (5, 45).

In the present study, we used male Sprague-Dawley rats to investigate the consequence of dietary SPI, relative to the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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control protein casein (CAS), on circulating insulin, colonic FASN expression, and DNA damage during initiation of colon carcinogenesis. Furthermore, using C2BBe1 and intestinal epithelial cell (IEC)-6 cell lines, we show that FASN mediates insulin-induced DNA damage and inhibits DNA damage-induced apoptosis. The results suggest that reduction in insulin levels leading to decreased expression of FASN may underlie, in part, the protective effects of dietary SPI against colon tumorigenesis in rats exposed to chemical carcinogens.

MATERIALS AND METHODS

Chemicals and antibodies. Reagents were obtained from the following sources: AOM from Midwest Research Institute (Kansas City, MO); N-nitroso-N-methylurea (NMU) from Ash Stevens (Detroit, MI); T090137 (T09) from Cayman Chemical (Ann Arbor, MI); insulin (human) and cerulenin from Sigma-Aldrich (St. Louis, MO); anti-proliferating cell nuclear antigen (PCNA) antibody from Dako (Carpinteria, CA); anti-FASN antibody from Abcam (Cambridge, MA); antibodies against total and phosphorylated p53 (P-p53ser15) from Cell Signaling (Danvers, MA); and anti-sterol regulatory element binding factor 1 (SREBF1) and anti-α-tubulin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal experiments. Animal use protocols were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Isocaloric, isonitrogenous AIN93G diets containing CAS or SPI as the sole protein source (200 g/kg diet) were formulated by Harlan Teklad (Madison, WI), as previously described (14, 22, 46). Pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were received at gestation day 4 and randomly assigned to one of the two diet groups. At postnatal day (PND) 2, each litter was culled to 5 males and 5 females. Beginning at PND 21, males were weaned to the same diet as their dams for the duration of the study. Females were used in unrelated studies of carcinogen-induced mammary cancers. At PND 50, male rats from multiple randomly selected litters were injected subcutaneously with AOM in saline at a dose of 15 mg/kg body wt. Serum and colon tissues were collected at D1 and D4 post-AOM. Animals were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a daily photoperiod (D4) post-AOM. Animals were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a daily photoperiod of 12 h light and 12 h dark and were allowed food and water ad libitum. Serum and colon tissues were frozen at −70°C; colon tissues also were fixed in formalin for immunohistochemistry (IHC). Whole colon rather than scraped mucosa tissues were collected for purposes of IHC analysis.

Cell culture. C2BBe1 cells [subclone of parental human colon adenocarcinoma-derived Caco2 cells (29)] were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM containing 10% (vol/vol) fetal bovine serum (FBS; Gibco, Carlsbad, CA), 0.01 mg/ml human transferrin (Gibco), and 1% antibiotic/antimycotic solution (Gibco) in an atmosphere of 5% CO2-95% air at 37°C. IEC-6 cells (derived from rat small intestine crypt epithelium; American Type Culture Collection) were cultured in DMEM containing 10% FBS, 0.1 U/ml bovine insulin (Sigma-Aldrich), and 1% antibiotic/antimycotic solution.

Hormone measurements. Serum insulin was measured by ELISA (Linco Research, Billerica, MA). Samples were analyzed in duplicate; assay detection limit was 0.2 ng/ml (35 pm). Serum C-peptide (Linco Research) was quantified by RIA; assay sensitivity was 25 pM.

IHC. General IHC procedures have been described in previous publications from our laboratories (10, 36, 45). Slides were incubated overnight at 4°C with anti-FASN rabbit polyclonal antibody or anti-P-p53ser15 rabbit polyclonal antibody, each at 1:200 dilutions. PCNA and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) staining of tissue sections was as previously described (10).

TUNEL assay of cells. The fluorescein FragEL DNA fragment detection kit (Calbiochem, San Diego, CA) was used to monitor in situ apoptosis of cultured cells. IEC-6 cells were seeded at a density of 5 × 10^4 cells/well on immunofluorescence chamber slides. After 12 h in 10% FBS/DMEM media, cells were washed with Hanks’
balanced salt solution, treated with liver X receptor (LXR) agonist T09 (10 μM) for 24 h, and incubated in medium containing the FASN inhibitor cerulenin (2.5 μg/ml) for an additional 24 h. After rinsing with HBBS, cells were fixed in 4% paraformaldehyde for 30 min and sequentially treated with 0.1% Triton X-100/PBS for 15 min and proteinase K (20 μg/ml) for 5 min. Cells were incubated with terminal deoxynucleotidyl transferase reaction mix in a humidified chamber at 37°C for 1 h. Labeled nuclei were counted in five separate fields (×200 magnification) containing 300–400 cells each, using an Olympus IX71 microscope with a standard fluorescence filter.

RNAi (small interfering RNA)-mediated gene silencing. Human FASN small interfering RNA (siRNA) (5′-GGA UAA CUG GGU GAC CUU C-3′, siRNA ID no. 107315), human SREBF1 siRNA (5′-GGC AAA GCU GAA UAA AUC UTT-3′, siRNA ID no. 5140), and scrambled siRNA (silencer negative control no. 1 siRNA, AM4611) were from Ambion (Austin, TX). FASN siRNA, SREBF1 siRNA, the combination, or negative control (scrambled) siRNAs (all 50 nM) were transfected into C2BBe1 cells, using siPORT NeoFX transfection agent (Ambion), in Opti-MEM medium (Invitrogen, Carlsbad, CA), following published procedures (10). Cells were placed in normal medium at 8 h after transfection.

Flow cytometry. siRNA-transfected C2BBe1 cells were incubated for 24 h in normal medium, followed by treatment with insulin, NMU, or the combination for 24 h. Cells were harvested, rinsed, suspended in PBS, and stained using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences). Cells were subjected to flow cytometry in a BD FACSCalibur Flow Cytometer (BD Biosciences).

Quantification of DNA apurinic/apyrimidinic sites. Cells treated with insulin, NMU, or the combination were harvested, and DNA was isolated with the QiAamp DNA Mini Kit (Qiagen). DNA concentrations for each sample were adjusted to 0.1 μg/ml, and numbers of apurinic [apurinic/apyrimidinic (AP)] sites were determined (DNA Damage Quantification Assay, BioVision, Mountain View, CA). Western blot analysis. C2BBe1 cells, treated with siRNAs and/or NMU (0 or 10 μM) for 24 h, were solubilized in RIPA buffer (Santa Cruz Biotechnology). IEC-6 cells pretreated with 10 μM T09 (24 h) were further incubated in medium containing 10 μM NMU for 24 h, harvested, and solubilized in RIPA buffer. Immediately before use, phenylmethylsulfonyl fluoride (1 mM final concentration), sodium orthovanadate (100 mM final concentration), and protease inhibitor cocktail (Santa Cruz Biotechnology) were added to RIPA buffer. Thirty micrograms of lysate protein were subjected to 8% (for FASN) and 10% (for SREBF1, P-p53ser15, p53, and α-tubulin) SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against FASN (1 μg/ml), SREBF1 (1 μg/ml), P-p53ser15 (0.5 μg/ml), p53 (0.5 μg/ml), and α-tubulin (0.1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized using the Chemiluminescence Reagent Plus system (Perkin Elmer Life Sciences, Waltham, MA). Blots were stripped with Restore Western Blot stripping buffer (Pierce Biotechnology, Rockford, IL) before reprobing with other antibodies. Whole cell lysates (RIPA solubilized) of colons from AOM-treated rats were analyzed for FASN and α-tubulin, as above.

Real-time reverse transcription-PCR. Total RNA extracted from distal colons (DCs) or from cells using TRizol reagent (Invitrogen) was reverse transcribed with iScript reagents (Bio-Rad Laboratories, Hercules, CA). A SYBRgreen-based protocol and the MyiQ single-color real-time PCR detection system (Bio-Rad Laboratories) were used for real-time PCR (45). Primers and corresponding rat genes (gene, forward primer, and reverse primer, respectively) were as follows: Srebf1 (5′-CAG AGG GAC TAC AGG CTG AGA AAG-3′, 5′-CAG GTA GAT CTC TGC CAG TGT TG-3′); Fasn (5′-CCG CGA CCA GTA TAA ACC CAA-3′, 5′-TCA CCC TCA ATG TGC ACA-3′); cytochrome P-450 2e1 (Cyp2e1) (5′-GAA GGA TGT GCC GAG GTT TTC-3′, 5′-GCT GCC CTG TTT TTC TGA-3′); alcohol dehydrogenase 4 (Adh4) (5′-CTT GCC CGA GTG GAT

Fig. 2. SPI diet decreases circulating insulin levels and suppresses expression of fatty acid synthase (FASN) in rat DC. A: SPI decreases circulating insulin and C-peptide concentrations at D1 post-AOM. B: SPI suppresses sterol regulatory element binding factor 1 (Srebf1) and Fasn mRNA expression in DC. For A and B, values are means ± SE (n = 10 animals/group). *Significant difference compared with CAS (P < 0.05); †0.05 < P < 0.1. C: immunohistochemistry (IHC) of FASN in DC. Boxed areas are expanded in corresponding lower areas. D: Western blots of FASN and α-tubulin in DC at D1 post-AOM. Each lane is lysate from an individual animal.
GAT GA-3', 5'-AAA GAC AGC ACA GAG GGA ACC-3'); 8-oxo-guanine DNA glycosylase (Ogg1) (5'-AAG ACC CCA CTG AAT GCC TTT T-3', 5'-TCA TCG TCT CTC CTA CAG CAC-3'). Cyclophilin-A (Ppia) mRNA was used to normalize real-time RT-PCR results (forward primer, 5'-AAG CAT ACA GGT CCT GGC ATC T-3'; reverse primer, 5'-TGC CAT CCA GCC ACT CAG T-3'). Human gene primers (for C2BBe1 cells) were as follows: SREBF1 (5'-AGG CCA TCG ACT ACA TTC GCT-3', 5'-TCC ACC TCA GTC TTC ACG CC-3'); FASN (5'-TGA ACT CCT TGG CGG AAG AGA-3', 5'-AAG ATA GCC ATG CCG AGC G-3'). 18S RNA was used as reference RNA for C2 BBe1 transcript normalization (5'-TCT TAG CTG AGT GTC CCG CG-3', 5'-ATC ATG GCC TCA GTT CGG AA-3').

Statistical analysis. Statistical computations were performed using SigmaStat for Windows, version 3.11 (SSI, Richmond, CA). For comparisons of means between treatments, t-test or one-way ANOVA with the Holm-Sidak method was used. Statistical significance was set at P < 0.05; a tendency for an effect was indicated by 0.05 < P ≤ 0.10.

RESULTS

Dietary SPI inhibits colonic p53ser15 phosphorylation in response to AOM. To determine effects of dietary proteins on carcinogen-induced proliferation, apoptosis, and DNA damage, male Sprague-Dawley rats lifetime-fed CAS or SPI were administered AOM. Colons and sera were collected at D1 and D4 post-AOM. The cellular expression of PCNA and tumor suppressor p53 phosphorylation on serine 15 (P-p53ser15) were evaluated by IHC, whereas apoptosis was evaluated in situ by TUNEL. Phosphorylation of p53 on serine 15 is a well-recognized response to DNA damage. As shown in Fig. 1A, the type of dietary protein had no effect on the number of PCNA-positive cells in colon crypts at D1 or D4 post-AOM. The number of crypt cells undergoing apoptosis was significantly increased by AOM treatment (data not shown). However, no significant differences were observed between diet groups at D1 or D4 post-AOM (Fig. 1B). Total number of apoptosing cells declined by D4 relative to D1. After AOM treatment,
and repair of 8-oxo-7,8-dihydroguanine (8-oxoG; Ogg1) or products are key to metabolic activation of AOM (Cyp2e1, Adh4) shown). Colonic expression of mRNAs, whose protein products declined by D4 relative to D1 in DC. Immunostaining of consecutive sections demonstrated that some, but not all, P-p53ser15-positive cells also were TUNEL positive (data not shown). At D1 post-AOM, animals fed SPI had fewer numbers of P-p53ser15-positive cells (per crypt) (P < 0.05) than corresponding animals fed CAS (Fig. 1C). At this same time point, DC manifested more TUNEL- and P-p53ser15-positive cells than the proximal colon. The total number of P-p53ser15-positive cells declined by D4 relative to D1 in DC. Immunostaining of consecutive sections demonstrated that some, but not all, P-p53ser15-positive cells also were TUNEL positive (data not shown). Colonic expression of mRNAs, whose protein products are key to metabolic activation of AOM (Cyp2e1, Adh4) and repair of 8-oxo-7,8-dihydroguanine (8-oxoG; Ogg1) or O'-methyl-deoxyguanosine (O'-MeG; Mgmt), were unaffected by diet (Fig. 1D).

Circulating insulin and colon Fasn gene expression are downregulated by dietary SPI. SPI consumption reduced circulating insulin levels in rats at D1 post-AOM (P < 0.05) (Fig. 2A). Serum C-peptide, a biomarker of insulin synthesis and secretion, was similarly suppressed by SPI at D1 post-AOM (Fig. 2A). The DC of the rat is the most responsive to AOM with respect to subsequent tumor formation (15) and indeed manifested a greater genotoxic response to AOM by D1 (Fig. 1); hence we focused on this region. Since insulin stimulates lipogenesis, the expression levels of SREBF1 (insulin-induced transcriptional activator of FASN) and FASN were next evaluated. The relative abundance of Srebf1 and Fasn mRNAs in the DC at D1 post-AOM was suppressed by SPI (Fig. 2B). FASN protein was undetectable, by IHC, in DCs of SPI-fed animals at D1 post-AOM, whereas DCs from CAS-fed rats had detectable FASN staining in the lower regions of crypts (Fig. 2C). In agreement with these results, FASN protein was undetectable, by Western blot, in colon lysates of SPI-fed animals at D1 post-AOM, whereas colon lysates from CAS-fed rats had detectable FASN (Fig. 2D).

Insulin-stimulated DNA damage in C2BBe1 cells is diminished by SREBF1 and/or FASN knockdown. We investigated presumptive functional linkages among insulin, its downstream effectors, SREBF1 and FASN, and endogenous DNA damage. C2BBe1 cells, a model of transformed colon epithelium derived from the parental Caco-2 human colon tumor cell line and with high FASN expression, were transfected with SREBF1 and FASN siRNAs before insulin treatment. For FASN, we initially tested two distinct siRNAs, and all results shown below were obtained using the more efficient of the two siRNAs. As expected, transfection with specific siRNAs to FASN and SREBF1 reduced the levels of the corresponding mRNAs and proteins for each (Fig. 3, A and B). Moreover, knockdown of FASN resulted in an induction of SREBF1 mRNA but not SREBF1 protein, confirming a previously recognized regulatory link of SREBF1 and FASN gene (20). Cells were treated with insulin to examine effects on DNA damage (measured by frequency of AP sites; Ref. 28) and the roles of FASN and SREBF1 in this process. DNA damage was increased by insulin in a dose-dependent manner (Fig. 3C); this effect was lost after transfection with FASN siRNA, alone and in combination with SREBF1 siRNA (Fig. 3D). Thus insulin-stimulated DNA damage in C2BBe1 cells, directly or indirectly, involves FASN.

FASN mediates insulin promotion of anti-apoptosis. We next examined associations of FASN and SREBF1 with apoptotic status. C2BBe1 cells were analyzed for number of apoptosing cells after transfection with FASN and/or SREBF1 siRNAs. Reduction in levels of FASN or SREBF1 by RNA knockdown increased cell death (Fig. 4). To further examine associations of FASN and chemically induced carcinogenesis, nontransformed rat IEC-6 were treated with the LXR agonist T09 to induce FASN from undetectable basal levels, followed by addition of the DNA alkylating and colon cancer-causing agent cerulenin. 

![Fig. 4](image-url) Knockdown of FASN and/or SREBF1 stimulates apoptosis of C2BBe1 cells. C2BBe1 cells were transfected with scrambled (control) siRNA, FASN siRNA, SREBF1 siRNA, or the combination and, after 48 h, were subjected to flow cytometry to monitor apoptosis (MATERIALS AND METHODS). Values are means ± SE of two independent experiments. *Differences relative to control siRNA (P < 0.05).

![Fig. 5](image-url) Treatment with liver X receptor agonist T0901317 (T09) sensitizes intestinal epithelial cell (IEC)-6 cells to cerULEIN-induced apoptosis. A: T09 induces IEC-6 FASN expression. Thirty micrograms of cell lysate (from duplicate cell cultures) were subjected to Western blot with FASN or α-tubulin antibodies. B: IEC-6 cells were incubated in medium containing T09 (0, 10 μM) for 24 h, followed by treatment with cerulenin (0, 2.5 μg/ml) for 48 h. Cells were fixed in 4% paraformaldehyde and subjected to TUNEL assay. Values are means ± SE (n = 5 fields of view per treatment). *Effect of T09 (P < 0.05). #Effects of cerulenin (P < 0.05).
NMU. NMU was used for in vitro studies since it is a direct-acting alkylating agent that does not require prior metabolic activation by liver or colon tissues, as is the case for AOM. Increased FASN expression with T09 treatment was observed as expected (Fig. 5, A and B); however, this did not result in a corresponding increase in the number of AP sites (data not shown). NMU alone induced a modest increase in Fasn mRNA levels and a significant rise in the number of AP sites (data not shown). However, the latter occurred independently of changes in FASN levels. An anti-apoptotic role for FASN was demonstrated by use of cerulenin, an inhibitor of FASN enzyme activity, in control and T09-treated IEC-6 cells. Apoptotic cell number was increased by cerulenin (Fig. 5B). Interestingly, pretreatment with T09 (to induce FASN synthesis) followed by cerulenin elicited a robust enhancement (by nearly 10-fold) of cell apoptosis over those of control cells (Fig. 5B).

To examine the functional association of FASN with carcinogen-induced apoptosis, C2BBe1 cells were treated with FASN or control siRNAs, followed by NMU. Under these experimental conditions, the FASN siRNA led to significant reduction in steady-state FASN protein level, with no changes in that for SREBF1, P-p53ser15, total p53, and α-tubulin (Fig. 6A). NMU alone had no effect on apoptotic cell number (Fig. 6B). FASN siRNA alone increased apoptotic cell numbers by nearly twofold, and this was further enhanced by exposure to NMU under this condition, nearly 50% of cells were apoptosing (Fig. 6B). However, NMU-elicited DNA damage was independent of FASN levels (Fig. 6C), similar to results shown for IEC-6 cells.

To confirm that insulin suppresses carcinogen-induced apoptosis, in part, via induction of FASN, C2BBe1 cells were treated with NMU in the presence and absence of added insulin. Insulin and NMU had nearly additive effects on DNA damage and were not attenuated by FASN siRNA transfection (Fig. 7A). Insulin was anti-apoptotic under basal conditions and after NMU addition, and the latter effect was abolished with FASN knockdown (Fig. 7B).

**DISCUSSION**

This study evaluated the biochemical pathway involving insulin and the lipogenic enzyme FASN within the framework of NMU-induced colon carcinogenesis. The results suggest that insulin suppresses NMU-induced DNA damage and apoptosis in part via induction of FASN. FASN knockdown in the presence of NMU resulted in a reduction in DNA damage and apoptosis, indicating a possible role for FASN in the anti-apoptotic effects of insulin.

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**Fig. 6.** FASN inhibits N-nitroso-N-methylurea (NMU)-induced apoptosis. For A and B, C2BBe1 cells were treated with control or FASN siRNAs for 24 h, followed by NMU (or vehicle) for 48 h. A: Western blots of SREBF1, FASN, P-p53ser15, total p53, and α-tubulin in transfected C2BBe1 cells. B: apoptosis is reduced in FASN-expressing cells. Treated cells were used in flow cytometry. *Effects of FASN siRNA (P < 0.05). #Effect of NMU (P < 0.05) within FASN siRNA-treated groups. C: FASN knockdown does not affect number of NMU-induced AP sites in DNA. P < 0.05 indicates effects of NMU compared with vehicle. Values are means ± SE (n = 3 replicates per group).
of dietary SPI inhibition of colon cancer in AOM-treated rats, the latter finding previously demonstrated by this and other laboratories (14, 38, 45, 46). Using P-pS3\textsuperscript{ser15} and cell apoptosis as functional read-outs for colonic epithelial cell procarcinogenic status, we report a potential link between the reduced circulating insulin levels in carcinogen-treated male rats elicited by dietary SPI and the previously described decreased incidence of colon tumors with SPI (46). The present data demonstrate that, in AOM-exposed rats fed SPI compared with counterparts that consumed CAS: 1) colonic expression of FASN and its major insulin-induced transactivator SREBF1 was suppressed; 2) reduced expression of FASN occurred coincident with (and likely as a consequence of) decreased systemic levels of insulin; and 3) DNA damage after AOM treatment, measured by numbers of P-pS3\textsuperscript{ser15}-positive cells (3), was lower in colonic crypt epithelium. Given the reported positive associations of circulating insulin and colonic FASN with colon tumorigenesis (20, 24, 31, 41, 43) and the present localization of FASN to lower crypt epithelium, the cellular sites of AOM-induced DNA damage (15) and of AOM-induced P-pS3\textsuperscript{ser15} immunostaining (this study), results suggest that decreased colonic FASN expression in SPI-fed animals contributes to the previously documented inhibitory effects of this dietary protein on chemically induced colon cancers (14, 27, 38, 46).

The observation that rat colonic mRNA abundance of the AOM-activating enzymes, ADH4 and CYP2E1, was unchanged with the type of dietary protein did not support a role for these enzymes/gens in mediating the suppressive effect of SPI on AOM-induced DNA damage. Similarly, we observed no diet differences in colonic expression of \textit{Ogg1} and \textit{Mgmt} mRNAs, suggesting that rates of colonic DNA adduct (O\textsuperscript{6}-MeG and 8-oxo-G) removal, as mediated by the protein products from these mRNAs, are similar with the two dietary proteins. O\textsuperscript{6}-MeG, in particular, is a prevalent procarcinogenic colon DNA adduct generated in vivo by AOM and other alkylating agents (30). Others have suggested that dietary soy protein favors decreased tissue concentrations of activated carcinogens as a consequence of increased expression of hepatic phase I and II detoxification enzymes (1, 33). The present study did not examine the many candidate hepatic and colon carcinogen-detoxifying enzymes as a function of diet; hence, this pathway(s) remains a possible contributor to the differences in AOM-induced DNA damage with diet. Most interestingly, TUNEL assay revealed no significant dietary effect on the numbers of cells undergoing apoptosis after AOM treatment. Apoptosis, a critical pathway in cancer control (32), is inhibited by insulin. Thus the elevated serum insulin levels may have countered the apoptosis-inducing effects of greater DNA damage in the CAS-fed rats, such that overall apoptosis rates after carcinogen treatment did not differ.

As mentioned above, reduced circulating levels of insulin in SPI-fed animals (this study, Ref. 19) can account for lowered colon \textit{Srebf1} and \textit{Fasn} mRNA and protein expression. Higher systemic insulin levels in CAS-fed animals may directly impact tumor development by two distinct, albeit not mutually exclusive, mechanisms. The first relates to the contribution of elevated insulin in increasing DNA damage, whereas the second may be a function of insulin’s ability to induce expression of SREBF1 and FASN (20, 42) and thus greater FASN-mediated anti-apoptotic activity. We examined these possibilities in vitro using \textit{C2BBe1} cells treated with insulin and showed that insulin dose-dependently induced DNA damage in these cells, as indicated by the appearance of AP sites in genomic DNA. Most interestingly, the number of insulin-induced AP sites was reduced when FASN was suppressed by RNAi, thereby indicating a possible direct connection between insulin, FASN, and genomic DNA damage in this cellular context. The increased DNA damage may be associated with excessive production of free fatty acids and lipids, which can create a

![Fig. 7. Rescue of C2\textsubscript{mm},1 cells from NMU-induced apoptosis by insulin involves FASN. A: C2\textsubscript{mm},1 cells were placed in low (0.5%) serum-containing medium for 24 h and then treated with insulin (100 nM), NMU (10 \textmu M), or the combination for 48 h. Parallel cell cultures were treated with control or FASN siRNAs for 24 h, followed by 100 nM insulin and 10 \textmu M NMU for 48 h. B: cells were harvested and used for AP site determination and apoptosis assay (flow cytometry). Values are means ± SE [n = 4 replicates/group (A); n = 3 replicates/group (B)]. Significant difference (P < 0.05) *from each other and #from controls.](G875)

![Fig. 8. Model for insulin and FASN actions during colon carcinogenesis in AOM-treated rats.](G876)
proinflammatory oxidative environment with attendant DNA damage (12). The observation that T09 induction of FASN in IEC-6 cells did not increase AP site number implies a requirement for both insulin and FASN in insulin-induced DNA damage. Alternatively, upregulated FASN protein levels may have led to an inhibition of basal apoptosis in response to insulin-induced DNA damage, thereby causing an accumulation of cells containing DNA adducts, which would be reflected by an increased number of AP sites.

The potential importance of FASN (and de novo lipogenesis) in tumor cell growth and survival was supported by results from the present study, since knockdown of FASN or SREBF1 mRNAs triggered apoptosis of C2BBe1 cells. Knockdown of FASN led to a compensatory increase in gene expression of SREBF1, consistent with results from Li et al. (20), who showed that cerulenin inhibition of FASN led to an induction of SREBF1 mRNA in HCT116 colon cancer cells. Taken together, the data indicate the presence of a feedback loop operative in at least two human colon tumor cell lines, in which deficiencies in FASN expression and activity cause upregulation of its cognate transcriptional activator gene. Such a mechanism would favor increased lipogenesis in support of proliferation.

The mechanism(s) by which inhibitors or RNAi of FASN trigger apoptosis of tumor cells is the subject of intense study. Excessive accumulation of the FASN substrate malonyl-CoA (2, 6, 21, 39), as well as depletion of intracellular palmitic and other end-product fatty acids (8), are likely to be involved. Additionally, FASN inhibition has been reported to induce endoplasmic reticulum stress in tumor cells (23), to impair the synthesis of phospholipids that contribute to membrane lipid rafts, wherein signaling complexes for autocrine/paracrine cell growth are localized (37), to induce the synthesis of the pro-apoptotic molecule ceramide (18), and to upregulate cell cycle arrest- and apoptosis-related genes (18). To our knowledge, the present study is the first to report that the induced elevation of FASN expression in nontransformed (i.e., IEC-6) intestinal cells results in their increased sensitivity to the pro-apoptotic effects of cerulenin; this mimics the response of human tumor cells to this agent. The LXR agonist T09 induces SREBF1 and FASN genes, as well as the full program of lipogenesis in liver cells (7, 17, 34). Thus the present results suggest that the overall elevated lipogenic state of tumor cells results in their increased susceptibility to apoptosis by inhibitors of FASN activity or expression.

Intriguingly, IEC-6 cells treated with the carcinogen NMU exhibited a small induction of Fasn mRNA levels. Moreover, NMU-induced apoptosis of C2BBe1 cells was enhanced when cells were pretreated with FASN siRNA. To our knowledge, these data are the first to implicate FASN in anti-apoptosis during NMU carcinogenesis of any target cell type. Following exposure to DNA-damaging agents and cell stressors, p53 is rapidly induced and activated, after which cells undergo apoptosis or G1 arrest (and DNA repair). NMU caused a comparable level of p53 phosphorylation in FASN siRNA-treated and nontreated cells (data not shown); whereas cell death was significantly lower in the non-FASN siRNA-treated group. Therefore, elevated FASN expression appears to inhibit NMU-induced apoptosis, independent of changes in P-p53ser15.

Based on the present results, we propose a model for how FASN may contribute to the cancer-inhibitory effects of a putative SPI protein in AOM-treated rats and with broader implications for insulin, aging, and cancer risk (Fig. 8). We suggest that the overall decreased circulating insulin levels elicited with SPI diet result in reduced colonic SREBF1 and FASN expression, a lowering of insulin-mediated colonic DNA damage, and a reduction in FASN-repressed apoptosis in response to carcinogen (Fig. 8). In this model, DNA damage is the sum of that elicited by insulin and AOM (or NMU) and is inferred to be greater in CAS- than SPI-fed rats (from p53ser15 immunostaining). The net result is that colon epithelial cells exhibit less genomic DNA damage due to increased apoptosis with SPI diet, resulting in inhibition of cell transformation and carcinogenesis. A testable prediction of this model is that hyperinsulinemia, such as in obesity or during insulin-resistance states, will lead to chronic elevations in DNA damage in colonic epithelial stem/transit cells and will accumulate with age, due to FASN anti-apoptosis. This prediction is in keeping with the well-known increased incidence of colon cancer with aging and its positive links with hyperinsulinemia and obesity (24, 41). Given that SPI diets are tumor inhibitory in certain other contexts, including NMU-elicited rat mammary carcinogenesis (10, 36), and that many human tumors, including those of the breast, typically overexpress FASN (16, 18, 25), it is tempting to speculate that the mechanisms described above may have relevance beyond chemically induced rat colon carcinogenesis.

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