Soy protein concentrate mitigates markers of colonic inflammation and loss of gut barrier function in vitro and in vivo☆

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Abstract

Whereas a number of studies have examined the effects of soy isoflavones and tocopherols on colonic inflammation, few have examined soy protein. We determined the radical scavenging and cytoprotective effects of soy protein concentrate (SPC) in vitro and its anti-inflammatory effects in dextran sulfate sodium (DSS)-treated mice. Cotreatment with SPC protected Caco-2 human colon cells from H_2O_2-induced cell death and mitigated intracellular oxidative stress. Treatment of differentiated Caco-2 cells with SPC blunted DSS-induced increases in monolayer permeability. Pepsin/pancreatin-digested SPC had reduced radical scavenging activity, but retained the monolayer protective effects of SPC. In vivo, 1.5% DSS caused body weight loss, colon shortening, and splenomegaly in *CF*-1 mice. Co-treatment with 12% SPC mitigated DSS-induced body weight loss and splenomegaly. DSS increased colonic interleukin (IL)-1β, IL-6, and monocyte chemotactic protein-1 expression. The levels of these markers were significantly lower in mice co-treated with SPC. SPC prevented DSS-mediated reductions in colonic glucagon-like peptide 2 levels, suggesting that SPC can prevent loss of gut barrier function, but no significant effect on claudin 1 and occludin mRNA levels of was observed. SPC-treated mice had lower colonic mRNA expression of toll-like receptor 4 and nucleotide-binding oligomerization domain-containing protein-like receptor family, pyrin domain containing protein 3 (NLRP3), and lower caspase-1 enzyme activity than DSS-treated mice. In summary, SPC exerted antioxidant and cytoprotective effects in vitro and moderated the severity of DSS-induced inflammation and loss of gut barrier function in vivo. These effects appear to be mediated in part through reduced NLRP3 expression and caspase 1 activity.
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1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are characterized by either continuous or periodic inflammation of the colon and represent a significant risk factor for colon cancer [1]. IBD affects nearly 4 million people worldwide with an economic impact of US$19–30 billion annually in the United States alone [2,3].

IBD is characterized by increased colonic oxidative stress, inflammation, and loss of gut barrier function [4]. Reactive oxygen species (ROS) and inflammatory cytokines, including interleukin (IL)-1β, are released by inflamed epithelial cells and resident immune cells. IL-1β is initially produced as an inactive precursor, which is enzymatically converted to its mature form by caspase-1. Caspase-1, itself, is activated by the proteolytic action of a multiprotein complex known as inflammasome [5]. The nucleotide-binding oligomerization

domain-containing protein-like receptor family, pyrin domain containing protein 3 (NLRP3) inflammasome is a complex composed of NLRP3 and apoptosis-associated speck-like (ASC) protein. The NLRP3 inflammasome forms as a result of ROS-mediated release of thioredoxin interacting protein (TXNIP) by thioredoxin, as well as through toll-like receptor (TLR)-4/nuclear factor (NF)κB-induced expression of NLRP3 [6]. Recent studies have demonstrated that blocking ROS generation leads to decreased NLRP3 inflammasome activity and subsequently to decreased IL-1β maturation and inflammation [7].

Colonic inflammation can lead to loss of gut barrier function and increased gut permeability by causing the reorganization and down-regulation of the tight junction proteins that join adjacent colonic epithelial cells [8,9]. This decrease in barrier function allows bulky or highly charged molecules, including pathogen-associated molecular patterns into the submucosa and systemic circulation perpetuating the inflammatory condition, increasing mucosal damage, and prolonging the colitic event. Interruption of this cycle can result in resolution of inflammation.

Soybeans (*Glycine max* L., Fabaceae) have been widely studied for their cancer preventive effects [10–14]. Epidemiological studies have suggested that soy consumption may reduce the risk of colon cancer; however, laboratory studies with purified soy components have yielded mixed results [15,16]. For example, Rao et al., have reported that dietary supplementation with genistein enhanced colonic oxidative stress and colon carcinogenesis in azoxymethane-treated F344 rats [17]. We have previously reported that genistein in combination with the green tea polyphenol, (−)-epigallocatechin-3-gallate, enhanced tumorigenesis in the $Apc^{Min/+}$ mouse [18].

Soy proteins and soy-derived peptides have been found to have antioxidant activity in cell-free models, and soy-derived peptides, such as lunasin, have also been found to have anti-inflammatory activity in macrophage cells in culture [19–22]. One study found that dietary supplementation of dextran sulfate sodium (DSS)-treated mice with 20% soy protein significantly reduced colon shortening, colonic inflammation scores, and reduced colonic mRNA expression of tumor necrosis factor α, however, the underlying mechanism of action was not explored [23]. More recently, studies in piglets and Balb/c mice with DSS-induced colitis found that soy derived di- and tripeptides, in particular the tripeptide, Val-Trp-Tyr, decreased inflammatory cytokine production as compared to the DSS-only control [24,25]. The relative contribution of this peptide to the overall activity of soy protein was not assessed.

Although recent studies on the effects of soy protein in animal models of IBD are promising, many questions remain including the identity of the underlying molecular mechanism of anti-inflammatory activity. In the current study, we examined the ability of an isoflavone-free soy protein concentrate (SPC) to prevent inflammation and loss of gut barrier function both in vitro and in vivo. We further investigated the impact of SPC on markers of the pathway for IL-1β expression and maturation.

2. Materials and methods

2.1. Materials

SPC (Arcon SJ #066–408) was a gift from the Archer Daniels Midland Company (Decatur, IL, USA). The composition of SPC is shown in Supplemental Table 1. 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), *N*-ethylmaleimide, fluorescein isothiocyanate-conjugated dextran (FITC-D, average MW 4000), pancreatin, and pepsin, were purchased from Sigma-Aldrich (St. Louis, MO). 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCDHFDA) was purchased from Invitrogen (Carlsbad, CA, USA). DSS (average MW=40,000) was purchased from MP Biomedical, LLC (Solon, OH, USA). All other chemicals used were of the highest grade commercially available.

2.2. Solubilization and fractionation of SPC

SPC was solubilized in water as follows for use in the oxygen radical absorbing capacity (ORAC) assay and cell culture assays. SPC was suspended in deionized water (1:20 w/v) and the pH was adjusted to 11 using NaOH (1 M) under constant agitation to increase solubility. After 2 h, the pH was slowly adjusted back to 7.4 using HCl (1 M). The solution was centrifuged at 3200×g for 20 min at 20°C. The resulting supernatant was lyophilized and stored at−80°C until use. To produce a high-molecular-weight (MW)-fraction (SPC-H, MW>10 kDa) and a low-molecular-weight fraction (SPC-L, MW≤10 kDa), solubilized SPC was dissolved in deionized water (1:20 w/v) and spun through a 10 kDa molecular weight cutoff centrifugal filter Millipore (Billerica, MA) according to the manufacturer's instructions. The flow-through was collected as SPC-L, lyophilized, and stored at −80°C until use. The retentate was collected as SPC-H, lyophilized, and stored at −80°C until use. Protein content was determined in SPC, SPC-L, and SPC-H using the Bradford assay.

2.3. Soy protein concentrate hydrolysis

The enzymatic hydrolysis of SPC was accomplished using a previously published method [26]. In brief, SPC was suspended in deionized water (1:20 w/v) and heated to 80°C for 5 min to reduce any potential bacteria population as well as to denature lipoxygenase. The pH was reduced to pH 2 using HCl, pepsin was added (1:100 w/w, enzyme/SPC), and the solution was incubated at 37°C for 3 h. The pH was then increased to 7.5 using NaOH, pancreatin was added (1:20 w/w, enzyme/SPC) and the mixture was then incubated at 37°C for 3 h. Hydrolysis was stopped by heating to 75°C for 20 min. The hydrolysate (HSPC) was centrifuged at 25,000×g for 15 min, and the resulting supernatant was lyophilized and stored at −80°C.

2.4. Thiol analysis, oxidation, and thiol blocking of SPC and HSPC

Free thiol groups in SPC were blocked using *N*-ethylmaleimide (NEM) as previously described. [27] In brief, SPC and SPH were dissolved in phosphate buffer (0.1 mM, pH 8) at final concentration of 25 mg/mL and reacted with NEM (3.45 mmol/g of protein) for 15 min at 25°C. SPC was oxidized in an analogous manner using H_2O_2 (10 mmol/g of protein). Excess NEM and H_2O_2 were removed by dialysis (500 Da cut-off membrane) against 10 mM sodium acetate and imidazole buffer (pH 7) at 5°C. The buffer was changed at 6, 12, 18, and 24 h [27]. Sulfhydryl groups were measured afterwards using Ellman's reagent (5,5′-dithio-bis-(2-nitrobenzoic acid)) [28]. Only samples where the levels of free thiols were decreased below the limit of detection were used for studies of radical scavenging activity (see Section 2.5). Samples were then lyophilized and stored at−80°C.

2.5. Antioxidant capacity of SPC preparations

The antioxidant capacity of the SPC preparations was determined using the oxygen radical absorbance capacity (ORAC) assay. In brief, SPC and modified SPC samples (see Section 2.4) were dissolved in phosphate buffer (10 mM, pH 7.4) to a final concentration of 100 μg/mL and combined with fluorescein (final concentration=8.6 nM) in a 96-well black opaque plate. After incubation for 30 min at 37°C, fluorescence was measured ($\lambda_{Ex.}$=485 nm, $\lambda_{Em.}$=520 nm). AAPH (final concentration=30 nM) or phosphate buffer was then added and fluorescence was measured every 90 s for 90 min. Fluorescence values were normalized to time 0 and the area under the curve determined for each treatment and converted to Trolox equivalents.

2.6. Cell culture and cell viability

Caco-2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in sub-confluence in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL) at 37°C under 5% CO_2 atmosphere. For cell viability assays, Caco-2 cells were seeded to a 96-well plate (5×10^3 cells/well) and allowed to attach for 24 h before treatment. For co-treatment experiments, cells were simultaneously exposed to 50 μM H_2O_2 and SPC (0–500 μg/mL) in phosphate buffered saline (PBS) for 60 min at 37°C. For the pre-treatment experiment, cells were incubated with SPC for 30 min at 37°C. The cells were then washed with PBS and treated with 50 μM H_2O_2 for 60 min at 37°C. After each experiment, cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and is expressed relative to control cells treated with neither H_2O_2 nor SPC.

2.7. Effect of SPC on intracellular ROS

Caco-2 cells were seeded in Petri dishes (1.5×10^4 cells/cm²). After 48 h, cells were exposed to 0 or 50 μM H_2O_2 in PBS for 60 min at 37°C in the presence or absence of 1.0 mg/mL SPC. Cells were washed with DMEM and incubated with 10 μM DCDHFDA at 37°C for 30 min, washed twice with PBS, and once with DMEM. Fluorescence was observed with an Olympus BX-51 Fluorescence Microscope ($\lambda_{Ex.}$=490 nm, $\lambda_{Em.}$=525 nm).

2.8. Caco-2 monolayer permeability

Caco-2 cells were seeded in polycarbonate transwell inserts (0.33 cm^2 area and 0.4 μm pore size, Corning Life Sciences, Tewksbury, MA, USA) and allowed to reach confluence and differentiate for 21 d. Based on previous studies, only monolayers with a transepithelial electrical resistance of 500–600 Ω-cm^2 were used [29]. The monolayers were treated with SPC samples (0–100 μg/mL) for 2 h prior to addition of 2% DSS to the media. After addition of DSS, cells were co-incubated for an additional 48 h. The paracellular permeability was observed by measuring the apical to basolateral flux of FITC-D as described previously using a Fluoroskan Ascent FL fluorescent plate reader (Thermo Scientific, Waltham, MA, USA) [30].

2.9. Animals and treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University (IACUC #29544). Male, *CF*-1 mice (Charles River Laboratory, Wilmington, MA, 5 weeks old) were randomized into 4 treatment groups (10 mice per treatment) and then housed 5 per cage in shoebox cages on corn cob bedding and maintained on a 12 h light/dark schedule with *ad libitum* access to food and water. Mice were allowed to acclimate for 7 d prior to the start of experiments. Experimental diets (Table 1) were prepared by Research Diets, Inc. (New Brunswick, NJ, USA). In order to maintain equivalency, macronutrient and micronutrient ingredient levels in the basal diet were reduced and replaced with the components found in the SPC powder (i.e., casein in AIN93G was replaced with increasing amounts of soy protein; cellulose in AIN93G was replaced with increasing amounts of soy fiber, etc.)

2.10. DSS-induced colitis

Mice were randomized based on weight into control (water and AIN93G diet), DSS (1.5% DSS in drinking fluid and AIN93G), DS6 (1.5% DSS and 6% dietary SPC), and DS12 (1.5% DSS and 12% dietary SPC). Mice were treated with DSS and experimental diets for 7 d, after which DSS was replaced with water and the mice were maintained for three additional days. Body weight was measured daily. Upon euthanasia, blood was collected by cardiac puncture. Spleens were removed, weighed, and fixed in formalin. Colons were resected and the length measured. They were then split longitudinally, washed with cold 0.9% NaCl solution, and frozen at −80°C.

Table 1
Composition of Experimental Mouse Diets*

	Control	6% SPC	12% SPC
Macronutrient Composition			
Protein *(% of energy)*	18	18	18
Carbohydrate *(% of energy)*	65	65	65
Fat *(% of energy)*	17	17	17
Energy *(kcal/g)*	3.9	3.9	3.9
Ingredient *(g)*			
Casein	200	158	116
L-Cystine	3	3	3
Corn Starch	397.5	396.3	395.2
Maltodextrin 10	132	132	132
Sucrose	100	100	100
Cellulose BW200	50	39.8	29.6
Soybean Oil	70	70	70
t-Butylhydroquinone	0.014	0.014	0.014
Mineral Mix (S10022G**)	35	0	0
Mineral Mix (S10042) (10X) No *Ca*, P, K, Na, Mg, Zn	0	3.5	3.5
Calcium Phosphate, Dibasic (29.5% *Ca*, 22.8% P)	0	6	5.2
Calcium Carbonate (40% *Ca*)	0	7.5	7.8
Sodium Chloride (39.3% Na)	0	2.4	2.2
Magnesium Oxide (60.3% Mg)	0	0.64	0.35
Ferric Citrate	0	0.18	0.145
Zinc Carbonate	0	0.058	0.058
Vitamin Mix (V10037***)	10	10	10
Choline Bitartrate	2.5	2.5	2.5
Soy Protein Concentrate	0	60	120
Total	1000	989.492	984.367

* Differences in cellulose and mineral mixes in the diets are a result of the mineral composition and ash content in the SPC received from the supplier.

** Composed of calcium phosphate, magnesium oxide, potassium citrate, potassium sulfate, sodium chloride, chromium potassium sulfate, cupric carbonate, potassium iodate, ferric citrate, manganous carbonate, sodium selenite, zinc carbonate and sucrose.

*** Composed of Vitamin A, Vitamin D3, Vitamin E acetate, menadione sodium bisulfate, biotin, cyanocobalamin, folic acid, nicotinic acid, calcium pantothenate, pyridoxine-HCl, riboflavin, thiamin HCl and sucrose.

2.11. Biochemical analysis

Colon tissues were homogenized in T-PER reagent (Thermo Scientific, Waltham, MA) supplemented with nordihydroguaiaretic acid (10 μM), indomethacin (10 μM), protease inhibitor (1:100), and phosphatase inhibitors (1:100) using a Bullet Blender (Next Advance, Averill Park, NY, USA) with 0.2 mm stainless steel beads. The resulting homogenate was centrifuged at 16,160 *g* at 4°C for 15 min and supernatant collected for analysis. Protein levels in the supernatant were determined using the Bradford reagent (Sigma-Aldrich Chemical, St. Louis, MO, USA) and the levels of IL-6, IL-1β, and monocyte chemotactic protein (MCP)-1 were determined using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN, USA). Levels of glucagon-like peptide 2 (GLP-2) were determined using an ELISA from MyBioSource, Inc. (San Diego, CA, USA). Caspase-1 activity was measured using a caspase-1 activity assay as per the manufacturer's protocol (Abcam, Cambridge, MA, USA).

2.12. Quantitative reverse-transcriptase (q)PCR

Total RNA was isolated and DNA contamination was removed from homogenized colon samples using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was assessed and quantified with a Nanodrop 2000 spectrophotometer. The RNA was reverse-transcribed to cDNA using the RT^2 HT First Strand Kit (SA Biosciences, Valencia, CA, USA). Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System (San Francisco, CA, USA) using primers (The Pennsylvania State University Genomics Core Facility, University Park, PA, USA) or TaqMan® hydrolysis probes (Life Technologies, Inc., Grand Island, NY, USA) (Supplemental Tables 2 and 3). The data was analyzed with Sequence Detector Software (Applied Biosystems). Relative gene expression was determined using the $2^{-\Delta CT}$ method, where $\Delta C_T = (C_{T,\ target} - C_{T,\ reference})$ and *GAPDH* was used as the reference gene. ΔC_T values were used for statistical analysis and $2^{-\Delta CT}$ values were used for graphical representation.

2.13. Data analysis

Statistical analysis was performed using SAS 9.4 (Cary, NC, USA) and graphs were prepared using GraphPad Prism (San Diego, CA, USA). All data are presented as the mean ± standard error of the mean (SEM). The Brown-Forsythe Test was used to determine if data from different treatment groups had equal variance. Cytoprotective effects in vitro were analyzed by two-way ANOVA with Bonferroni's post-test with treatment protocol (co-treatment *vs.* pre-treatment) and the concentrations of SPC as the independent variables. In vitro changes in permeability and radical scavenging activity were analyzed by one-way ANOVA with Tukey's post-test or Student's *t* test as appropriate. Changes in body weight were analyzed by two-way ANOVA with Bonferroni's post-test with time and treatment as independent variables. For data with equal variance, changes in in vivo markers of inflammation and gastrointestinal permeability were analyzed by one-way ANOVA with Tukey's post-test. For data with unequal variance, Welch's ANOVA with Dunnett's post-test was used for statistical analysis. Comparisons were made to the DSS-treated control mice.

3. Results

3.1. In vitro cytoprotective and antioxidant activity

The in vitro peroxyl radical scavenging activity of SPC was determined using the ORAC assay (Fig. 1). The activity of native SPC was compared to SPC that had been fractionated based on molecular weight (SPC-H and SPC-L), hydrolyzed by pepsin/pancreatin (HSPC), and pre-oxidized (SPC-H$_2$O$_2$ and HSPC-H$_2$O$_2$) or treated to block thiol groups (SPC-NEM and HSPC-NEM). We found that unmodified SPC had the greatest radical scavenging activity (Fig. 1). Fractionation revealed that the radical scavenging activity of the SPC-H fraction was significantly higher than that of the SPC-L fraction. There was no significant difference in free thiol levels in SPC and SPC-H, however the levels of thiols in the SPC-L were significantly lower (Fig. 1). Hydrolysis of SPC with pepsin/pancreatin significantly lowered both radical scavenging activity and free thiol levels (Fig. 1). Blocking free thiols by pre-treatment of SPC or HSPC with NEM or pre-oxidation of SPC or HSPC with H$_2$O$_2$ significantly reduced the radical scavenging activity of SPC but not HSPC (Fig. 1). Treating Caco-2 cells with 50 μM H$_2$O$_2$ reduced cell viability by 50% compared to untreated control cells (Fig. 1, dashed line indicates viability of untreated cells). Co-treatment, but not pre-treatment, with SPC significantly prevented H$_2$O$_2$-induced loss of cell viability (Fig. 1). Significant effects of SPC concentration ($P<.01$), treatment protocol ($P<.05$), and the interaction between the two factors ($P<.05$) on cell viability was observed. H$_2$O$_2$ treatment

Radical Scavenging Activity
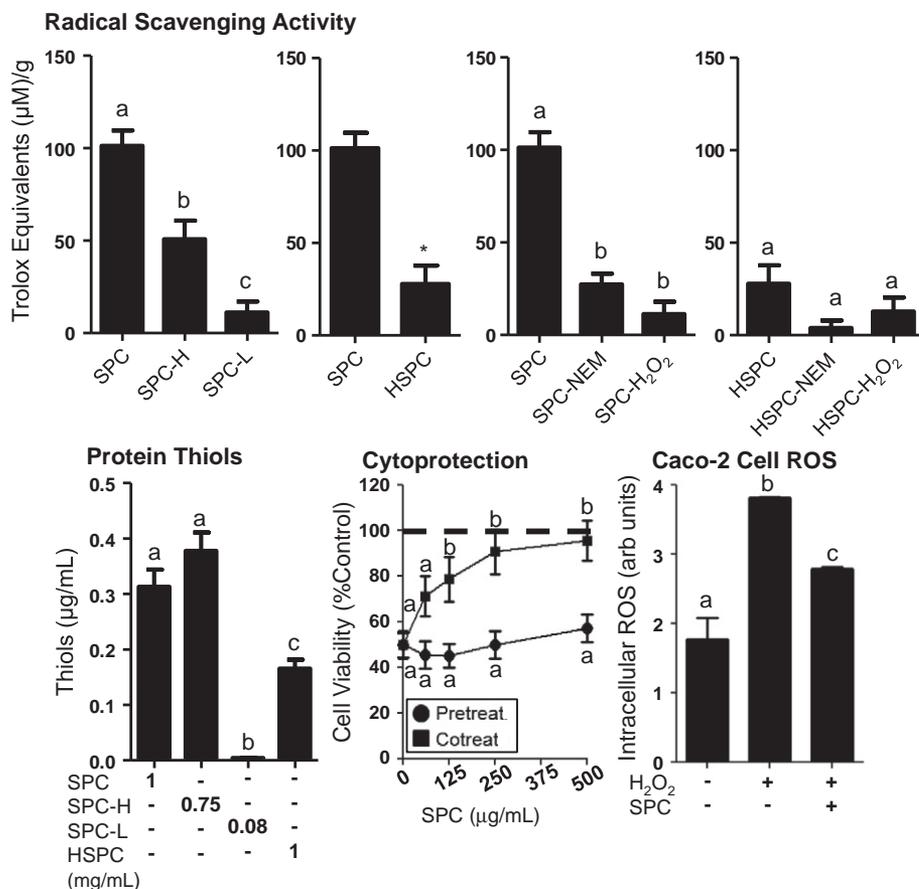
Fig. 1. Radical scavenging, cytoprotective, and permeability effects of SPC in vitro. The radical scavenging effects of SPC and its various modifications were determined chemically using the ORAC assay. Thiol groups as measured by Elman's reagent. The protective effects of pre-incubation and co-incubation with SPC on H_2O_2-induced loss of Caco-2 cell viability were assessed by the MTT assay. The dashed line at 100% viability indicates the viability of cells treated with neither H_2O_2 nor SPC. The effect of SPC on intracellular ROS was determined by measuring DCDHFDA fluorescence following treatment of Caco-2 cells with H_2O_2 alone or in combination with SPC. All values represent the means±S.E.M. and an $n=10$ for each group. Different letters denote $P<.05$ using one-way ANOVA with a Tukey's multiple comparison post-test. Asterisks indicate $P<.05$ by Student's t test. Cytoprotective data were analyzed by two-way ANOVA with Bonferroni's post-test. Different superscript letters indicate statistically significant differences between concentrations of SPC and between treatment schemes. "SPC-NEM", thiol blocked SPC; "HSPC-NEM", thiol blocked HSPC; "SPC-H_2O_2", pre-oxidized SPC; and "HSPC-H_2O_2", pre-oxidized HSPC.
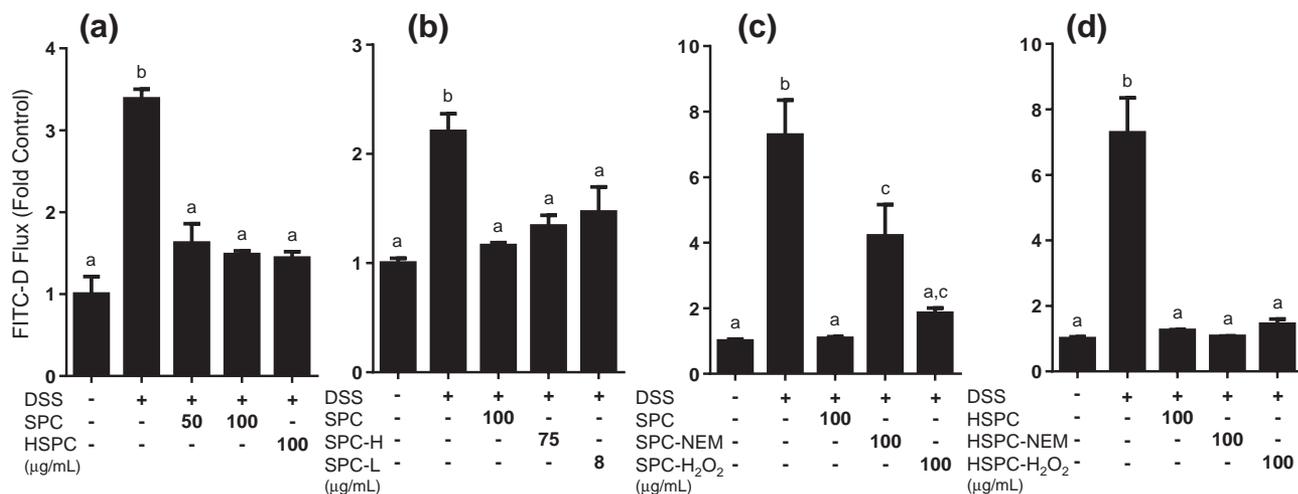
Fig. 2. Effect of SPC on DSS-induced permeability of Caco-2 human colonocyte monolayers. We examined the ability of (a) SPC and HSPC, (b) fractionated SPC, and oxidized or thiol blocked (c) SPC and (d) HSPC to protect differentiated Caco-2 cell monolayers from DSS-induced increases in permeability. Monolayer permeability was determined by measuring the apical to basolateral flux of FITC-D after 48 h incubation with 2% DSS and soy treatment. All experiments were repeated at least twice. All data represent the mean±S.E.M. Different letters denote $P<.05$ using one-way ANOVA with a Tukey's Multiple Comparison post-test. "SPC-NEM", thiol blocked SPC; "HSPC-NEM", thiol blocked HSPC; "SPC-H_2O_2", pre-oxidized SPC; and "HSPC-H_2O_2", pre-oxidized HSPC.

Fig. 4. The effect of dietary SPC on markers of colon inflammation and gut permeability in DSS-treated *CF*-1 mice. Protein levels of IL-1β, IL-6, and MCP-1 were determined in the colon homogenate using commercially-available ELISAs and normalized to the total protein of each sample. Relative mRNA expression of *Il1b, Emr1, Tlr4,* and *Nfkb* in colon homogenate was determined by qPCR and normalized to the expression of GAPDH expression. All values represent the means±S.E.M. and an *n*=10 for each group. Different letters denote *P*<.05 using one-way ANOVA with a Tukey's Multiple Comparison post-test. For IL-6 protein levels and *Il1b* and *Emr1* mRNA levels, the data had unequal variance and Welch's ANOVA with Dunnett's post-test was used for statistical analysis. Asterisk represents *P*<.05 compared to DSS-treated control mice.

inflammation and colon cancer; however, laboratory studies with purified soy components have yielded mixed results [15,16].

In the present study, we examined the radical scavenging and cytoprotective effects of SPC in vitro. Further, we examined the effect of dietary SPC on markers of colonic inflammation, gut barrier function, and the NLRP3 inflammasome in the DSS-treated mouse model of acute ulcerative colitis. Although previous studies have examined the anti-inflammatory effects of purified di- and tripeptides, as well as the Bowman-Birk protease inhibitor derived from soy, there

have been limited studies on whole soy protein preparations and the potential underlying mechanisms of action [24,25].

SPC exhibited significant radical scavenging activity in the ORAC assay, and antioxidant and cytoprotective effects in cultured Caco-2 human colon cells. The antioxidant activities of soy protein observed here are consistent with previous reports in various food systems, which correlated radical scavenging activity with free thiol content (reviewed in [19]). We found that pre-oxidation of SPC with H_2O_2 and blocking free thiols in SPC with NEM abolished the radical scavenging
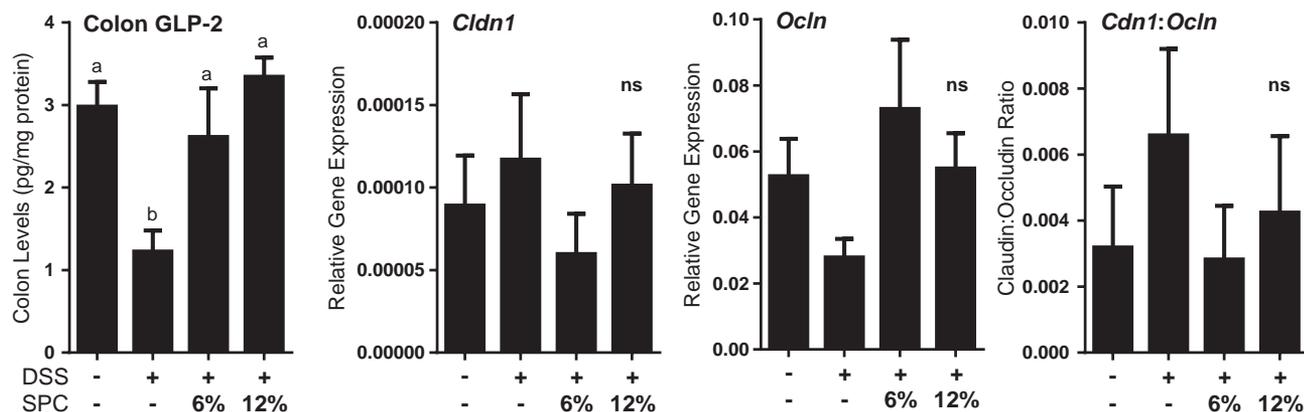
Fig. 5. Biochemical markers of intestinal permeability in DSS-treated *CF*-1 mice. GLP-2 protein levels in the colon homogenate as determined by ELISA and normalized to the total protein of each sample. Relative fold change of mRNA expression of claudin-1 (*Cldn1*) and occludin (*Ocln*) in colon homogenate was determined by qPCR and normalized to the expression of GAPDH. The ratio of *Cldn1* to *Ocln* was used as a marker of gut barrier function, with a decreased ratio representing compromised barrier function. All values represent the means±S.E.M. and an *n*=10 for each group. Different letters denote *P*<.05 using one-way ANOVA with a Tukey's Multiple Comparison post-test. "ns", not statistically significant.
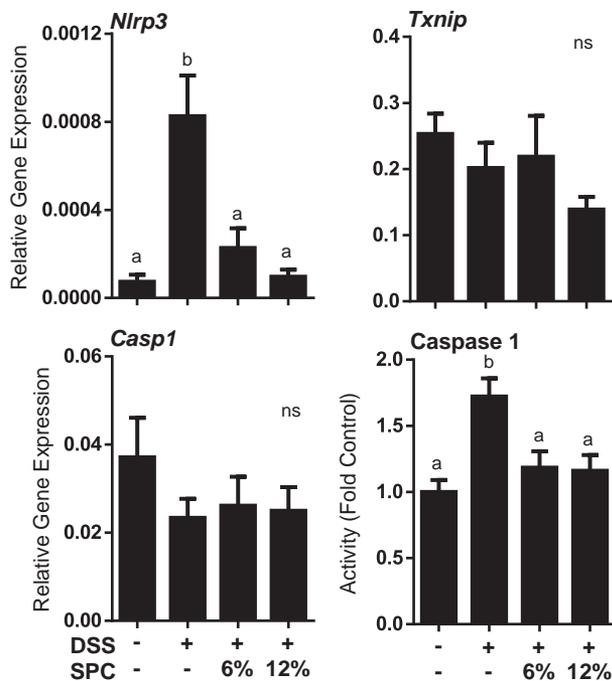
Fig. 6. Effects of dietary SPC on the inflammasome-related markers in DSS-treated *CF*-1 mice. Relative fold change of mRNA expression of *Nlrp3, Txnip*, and *Casp1* in colon homogenate was determined by qPCR and normalized to the expression of GAPDH expression. Caspase-1 enzyme activity was measured using a commercially available kit and normalized to the control. All values represent the means±S.E.M. and an n=10 for each group. Different letters denote P<.05 using one-way ANOVA with a Tukey's Multiple Comparison post-test. "ns", not significant.

activity in vitro. Similarly hydrolysis of SPC with pepsin and pancreatin (HSPC) significantly reduced its radical scavenging activity. This change was related to a decrease in the free thiol content of the final protein preparation. In addition to these antioxidant-related effects, we observed that both SPC and HSPC blunted DSS-induced increases in the permeability of Caco-2 cell monolayers. In contrast to the radical scavenging and antioxidant activity of SPC, the effects on gut barrier function were only partially-modulated by pre-oxidation of SPC with H_2O_2 or by blocking the free thiols in SPC with NEM. Treatment of HSPC with H_2O_2 and NEM had no effect on the protective activity of HSPC.

These in vitro results were unexpected since our initial hypothesis was that SPC exerted its monolayer protective effects *via* an antioxidant mechanism, and our results indicate that these two biological effects may result from different underlying mechanisms of action or from different components within soy protein. Previous studies have suggested that anti-inflammatory effects of specific peptides in soy protein [25]. The underlying mechanism of action of these peptides remains unclear, and we do not know the extent to which these peptides contribute to the anti-inflammatory effects of SPC, but future studies in this direction seem likely to be fruitful.

It is especially interesting and relevant that HSPC, which is prepared by proteolytic cleavage with pepsin and pancreatin, maintains its monolayer protective effects. Since this proteolytic digestion mimics what happens in vivo, we hypothesize that HSPC represents the material that reaches the colon more closely than SPC. Its in vitro biological activity is therefore more relevant to the in vivo situation. Further studies should focus on characterizing HSPC and exploring the anti-inflammatory effects of HSPC.

In vivo, we found that dietary SPC, at the 12% dose-level, ameliorated DSS-mediated body weight loss and splenomegaly. SPC also mitigated DSS-induced increases in protein markers of inflammation (IL-1β, IL-6, and MCP-1) and related gene expression (*Il1b* and

Tlr4). Colonic inflammation leads to reorganization of the tight junction proteins and loss of gut barrier function allowing intestinal microbiota and microbiota-derived components into the intestinal submucosa and into the systemic circulation. These effects have been linked to IL-1β-induced NFκB activation [8,9]. We observed here that SPC prevented DSS-induced decreases in colonic GLP-2 levels, indicating that SPC mitigated DSS-induced loss of gut barrier function [31–33]. By contrast, we observed no significant difference in *Cldn1, Ocln*, or the ratio of these two genes among the treatment groups. Although these results are inconsistent with what is expected based on the observed changes in GLP-2, it is worth noting that inflammation in the colon has been reported to lead to relocalization of claudin 1 and occludin protein from the plasma membrane to the interior of the cell [8,9]. This in turn leads to decreased tight-junction formation and compromised gut barrier function. We did not examine the protein expression or localization of claudin-1 and occludin, so it is possible that DSS and SPC treatment effects occur at the protein level. Further studies are needed to more directly examine changes in gut barrier function, including changes in the localization of tight junction proteins or the systemic availability of gut-derived bacterial endotoxin.

The underlying anti-inflammatory mechanisms of soy protein are unclear. IL-1β is an important cytokine in the IBD-associated inflammation, and in the present study we found that SPC supplementation blunted DSS-induced increases in both mRNA and protein expression of IL-1β. IL-1β is synthesized as an inactive protein precursor that is activated primarily by caspase-1-mediated proteolysis [34]. Here, we found that caspase-1 activity is elevated in DSS-treated mice, and that this increase was prevented by SPC supplementation. These results suggest that SPC may prevent proteolytic activation of pro-IL-1β.

Activation of caspase-1 occurs through the action of a multiprotein complex known as the NLRP3-inflammasome. This complex is formed by NLRP3 and ASC in response to ROS-induced association of TXNIP and NLRP3 [35]. TLR4-induced NFκB activation regulates the expression of both NLRP3 and pro-IL-1β expression, and plays a key role in innate immune response and is elevated in experimental models of IBD [36–38]. We found that mRNA levels of *Tlr4* were elevated in DSS-treated mice and that these increases were prevented in SPC-supplemented mice. Further, we found that SPC prevented DSS-induced increases in *Nlrp3* expression. By contrast, neither DSS treatment nor co-treatment with SPC had a significant effect on *Nfkb* mRNA expression. Although we were surprised by the lack of effect of DSS on *Nfkb* expression, we recognize that effects mediated by NFκB are due to nuclear translocation of the p65 subunit. Therefore, a lack of change in *Nfkb* expression may not indicate a lack of change in NFκB signaling. In the present study, we did not examine NFκB protein levels or subcellular location. While this represents a limitation to the present studies, we did observe changes in NFκB-responsive genes including IL-1β, IL-6, and MCP-1. This suggests that modulation of NFκB signaling may play a role in the observed effects of caspase-1 activity and the anti-inflammatory effects of SPC. Further studies are needed, however, to fully investigate the effects of SPC on the components of TLR4-NFκB-NLRP3 inflammasome pathway at the level of protein expression and activity.

The observed changes in inflammatory markers in vivo correlate well with what would be predicted by the in vitro monolayer protective effects of SPC and HSPC. Breakdown of the gut epithelial barrier results in the movement of bacterial components from the lumen of the colon to the basolateral space, allowing greater activation of TLR4/NFκB signaling, and increased expression of pro-inflammatory cytokines including IL-6 and IL-1β, as well as components of the NLRP3 inflammasome [39,40]. By protecting gut barrier integrity, SPC is able to prevent this increase in pro-inflammatory signaling and moderate the severity of colitis. Additional studies are needed to fully translate

the results of our in vitro studies to the in vivo situation, but overall these results indicate that SPC may exert its anti-inflammatory effects in part by targeting components of TLR4/NFκB signaling pathway and the NLRP3 inflammasome, and that these effects are related in part to the monolayer protective effects of SPC.

The present study has two limitations which must be highlighted. First, we group-housed mice and were not able to collect precise food consumption data, therefore, we are unable to comment on the impact of SPC on the palatability of the diet or on food intake by the mice. Second, we did not examine protein expression for all of the molecular markers that were examined at the mRNA level. As such, the changes observed in *Tlr4* and *Nlrp3* and the lack of change observed in *Nfkb* and *Txnip* may not correlate to similar effects at the protein level. In the present study we did not have sufficient tissue samples to examine these protein markers. Since we did observe changes in colonic caspase-1 activity (which is activated by NLRP3), and the expression of NFκB target proteins (e.g. IL-6, IL-1β) induced by DSS and SPC, we have some confidence that we will see changes in the expression of NLRP3 inflammasome-related proteins and the localization of NFκB.

Overall, the findings of our study support the efficacy of dietary SPC as a means of preventing colonic inflammation and loss of gut barrier function. These effects appear to result from mitigation of TLR4-mediated NLRP3 inflammasome formation and activity. Future studies will focus on identification of the active anti-inflammatory principles in soy protein, confirmation of the proposed mechanisms of action, and the potential efficacy of SPC as a means to prevent inflammation-associated colon cancer.

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References

[1] Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol 2004;287:G7–G17.

[2] Yu AP, Cabanilla LA, Wu EQ, Mulani PM, Chao J. The costs of Crohn's disease in the United States and other western countries: a systematic review. Curr Med Res Opin 2008;24:319–28.

[3] Cohen RD, Yu AP, Wu EQ, Xie J, Mulani PM, Chao J. Systematic review: the costs of ulcerative colitis in western countries. Aliment Pharmacol Ther 2010;31:693–707.

[4] Hashimoto K, Oshima T, Tomita T, Kim Y, Matsumoto T, Joh T, et al. Oxidative stress induces gastric epithelial permeability through claudin-3. Biochem Biophys Res Commun 2008;376:154–7.

[5] Davis BK, Philipson C, Hontecillas R, Eden K, Bassaganya-Riera J, Allen IC. Emerging significance of NLRs in inflammatory bowel disease. Inflamm Bowel Dis 2014.

[6] Davis BK, Ting JP. NLRP3 has a sweet tooth. Nat Immunol 2010;11:105–6.

[7] Schroder K, Tschopp J. The Inflammasomes. Cell 2010;140:821–32.

[8] Kucharzik T, Walsh SV, Chen J, Parkos CA, Nusrat A. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. Am J Pathol 2001;159:2001–9.

[9] Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol 2003;171:6164–72.

[10] Taylor CK, Levy RM, Elliott JC, Burnett BP. The effect of genistein aglycone on cancer and cancer risk: a review of in vitro, preclinical, and clinical studies. Nutr Rev 2009;67:398–415.

[11] Nagata C, Mizoue T, Tanaka K, Tsuji I, Tamakoshi A, Matsuo K, et al. Soy intake and breast cancer risk: an evaluation based on a systematic review of epidemiologic evidence among the Japanese population. Jpn J Clin Oncol 2014;44:282–95.

[12] Wu SH, Liu Z. Soy food consumption and lung cancer risk: a meta-analysis using a common measure across studies. Nutr Cancer 2013;65:625–32.

[13] Hsu A, Bray TM, Helferich WG, Doerge DR, Ho E. Differential effects of whole soy extract and soy isoflavones on apoptosis in prostate cancer cells. Exp Biol Med (Maywood) 2010;235:90–7.

[14] Guha N, Kwan ML, Quesenberry Jr CP, Weltzien EK, Castillo AL, Caan BJ. Soy isoflavones and risk of cancer recurrence in a cohort of breast cancer survivors: the life after cancer epidemiology study. Breast Cancer Res Treat 2009;118:395–405.

[15] Yan L, Spitznagel EL, Bosland MC. Soy consumption and colorectal cancer risk in humans: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2010;19:148–58.

[16] Yang G, Shu X-O, Li H, Chow W-H, Cai H, Zhang X, et al. Prospective cohort study of soy food intake and colorectal cancer risk in women. Am J Clin Nutr 2009;89:577–83.

[17] Koratkar R, Rao AV. Effect of soya bean saponins on azoxymethane-induced preneoplastic lesions in the colon of mice. Nutr Cancer 1997;27:206–9.

[18] Lambert JD, Kwon SJ, Ju J, Bose M, Lee MJ, Hong J, et al. Effect of genistein on the bioavailability and intestinal cancer chemopreventive activity of (−)-epigallo-catechin-3-gallate. Carcinogenesis 2008;29:2019–24.

[19] Elias RJ, Kellerby SS, Decker EA. Antioxidant activity of proteins and peptides. Crit Rev Food Sci Nutr 2008;48:430–41.

[20] Darmawan R, Bringe NA, de Mejia EG. Antioxidant capacity of alcalase hydrolysates and protein profiles of two conventional and seven low glycinin soybean cultivars. Plant Foods Hum Nutr 2010;65:233–40.

[21] de Mejia EG, Dia VP. Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-[kappa]B pathway in the macrophage. Peptides 2009;30:2388–98.

[22] Hernandez-Ledesma B, Hsieh CC, de Lumen BO. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem Biophys Res Commun 2009;390:803–8.

[23] Jiang H, Przybyszewski J, Mitra D, Becker C, Brehm-Stecher B, Tentinger A, et al. Soy protein diet, but not lactobacillus rhamnosus GG, decreases mucin-1, trefoil factor-3, and tumor necrosis factor-alpha in colon of dextran sodium sulfate-treated C57BL/6 mice. J Nutr 2011;141:1239–46.

[24] Young D, Ibuki M, Nakamori T, Fan M, Mine Y. Soy-derived di- and tripeptides alleviate Colon and Ileum inflammation in pigs with dextran sodium sulfate-induced colitis. J Nutr 2012;142:363–8.

[25] Kovacs-Nolan J, Zhang H, Ibuki M, Nakamori T, Yoshiura K, Turner PV, et al. The PepT1-transportable soy tripeptide VPY reduces intestinal inflammation. Biochim Biophys Acta 1820;2012:1753–63.

[26] Ju J, Hao X, Lee MJ, Lambert JD, Lu G, Xiao H, et al. A gamma-tocopherol-rich mixture of tocopherols inhibits colon inflammation and carcinogenesis in azoxymethane and dextran sulfate sodium-treated mice. Cancer Prev Res (Phila) 2009;2:143–52.

[27] Faraji H, McClements DJ, Decker EA. Role of continuous phase protein on the oxidative stability of fish oil-in-water emulsions. J Agric Food Chem 2004;52:4558–64.

[28] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–7.

[29] Ranaldi G, Consalvo R, Sambuy Y, Scarino ML. Permeability characteristics of parental and clonal human intestinal Caco-2 cell lines differentiated in serum-supplemented and serum-free media. Toxicol In Vitro 2003;17:761–7.

[30] Zhao H, Zhang H, Wu H, Li H, Liu L, Guo J, et al. Protective role of 1,25(OH)2vitamin D3 in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. BMC Gastroenterol 2012;12:57.

[31] Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091–103.

[32] Kissow H, Hartmann B, Holst JJ, Poulsen SS. Glucagon-like peptide-1 as a treatment for chemotherapy-induced mucositis. Gut 2013;62:1724–33.

[33] Poritz LS, Harris III LR, Kelly AA, Koltun WA. Increase in the tight junction protein claudin-1 in intestinal inflammation. Dig Dis Sci 2011;56:2802–9.

[34] Schönbeck U, Mach F, Libby P. Generation of biologically active IL-1β by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1β processing. J Immunol 1998;161:3340–6.

[35] Lane T, Flam B, Lockey R, Kolliputi N. TXNIP shuttling: missing link between oxidative stress and inflammasome activation. Front Physiol 2013;4:50.

[36] Qiao Y, Wang P, Qi J, Zhang L, Gao C. TLR-induced NF-kappaB activation regulates NLRP3 expression in murine macrophages. FEBS Lett 2012;586:1022–6.

[37] Østvik AE, Granlund AB, Torp SH, Flatberg A, Beisvåg V, Waldum HL, et al. Expression of toll-like receptor-3 is enhanced in active inflammatory bowel disease and mediates the excessive release of lipocalin 2. Clin Exper Immunol 2013;173:502–11.

[38] Igarashi H, Ohno K, Maeda S, Kanemoto H, Fukushima K, Uchida K, et al. Expression profiling of pattern recognition receptors and selected cytokines in miniature dachshunds with inflammatory colorectal polyps. Vet Immunol Immunopathol 2014;159:1–10.

[39] Vijay-Kumar M, Gewirtz AT. Guardians of the gut: newly appreciated role of epithelial toll-like receptors in protecting the intestine. Gastroenterology 2008;135:351–4.

[40] Wu XF, Ouyang ZJ, Feng LL, Chen G, Guo WJ, Shen Y, et al. Suppression of NF-kappaB signaling and NLRP3 inflammasome activation in macrophages is responsible for the amelioration of experimental murine colitis by the natural compound fraxinellone. Toxicol Appl Pharmacol 2014;281:146–56.