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Broccoli (*Brassica oleracea* var. *italica*) Sprouts and Extracts Rich in Glucosinolates and Isothiocyanates Affect Cholesterol Metabolism and Genes Involved in Lipid Homeostasis in Hamsters

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ABSTRACT: This study investigated the effects of broccoli sprouts (BS) on sterol and lipid homeostasis in Syrian hamsters with dietary-induced hypercholesterolemia. Treatments included freeze-dried BS containing 2 or 20 μmol of glucoraphanine (BSX, BS10X), glucoraphanine-rich BS extract (GRE), sulforaphane-rich BS extract (SFE), and simvastatin. Each experimental diet was offered to eight animals (male and female) for 7 weeks. Hepatic cholesterol was reduced by BS10X and SFE treatments in all animals. This correlated with a down-regulation of gene expression of sterol regulatory element-binding proteins (SREBP-1 and -2) and fatty acid synthase (FAS) caused by GRE and SFE diets. BS10X caused changes in gene expression in a gender-specific manner; additionally, it increased coprostanol excretion in females. With the same concentration of glucoraphanin, consumption of broccoli sprouts (BS10X) had more marked effects on cholesterol homeostasis than GRE; this finding reinforces the importance of the matrix effects on the bioactivity of functional ingredients.

KEYWORDS: Broccoli sprouts, glucosinolate, sulforaphane, cholesterol, SREBP, HMGR, FAS, LDLR, Apo B100

INTRODUCTION

Development of coronary heart disease due to high cholesterol levels is a major cause of death globally.[1] This problem has been targeted primarily by reducing low-density lipoprotein (LDL) concentrations with therapeutic treatments such as statin drugs.

Numerous food sources have been shown to lower blood cholesterol levels in rats, hamsters, and mice with very minimal side effects, in contrast to those frequently associated with pharmaceuticals.[2−4] It is unclear whether the observed positive effects are due to a single phytochemical or the synergistic effects of multiple compounds from within the food matrix. The cholesterol-lowering properties of most of these foods are due to absorption site competition[4,5] and reduced synthesis of endogenous cholesterol.[4]

Broccoli sprouts are very rich in glucosinolates, particularly glucoraphanin (GR), which is enzymatically converted to the chemoprotective isothiocyanate sulforaphane (SF) in the gastrointestinal system of mammals and when the raw vegetable is macerated or chewed. Predominance of either GR or SF in dried broccoli sprout preparations can be controlled by "predigesting" the extract with the enzyme myrosinase (EC 3.2.1.147). Broccoli sprouts have been shown to reduce cholesterol or lipid levels in addition to their well-known chemoprotective effects.[7,8] Consumption of a dose of 400 mg/kg (body weight) of ethanolic extracts from broccoli sprouts reduced body weight gain from 5.88 to 4.74 g/day and mesenteric adipose tissue weight from 4.97 to 2.91 g/100 g (body weight) in rats fed high-fat diets. The same dose of this extract also elicited a reduction in plasma triglyceride, LDL-cholesterol, and total cholesterol.[6]

In a separate animal study, several survival proteins were activated in addition to the redox cycling of thioredoxins in rats administered a broccoli sprout extract rich in SF, which is the main isothiocyanate. This was evidenced through improved postischemic ventricular function, reduced myocardial infarct size, and decreased cardiomyocyte apoptosis.[9]

A clinical study conducted with 12 healthy subjects that consumed 100 g/day of broccoli sprouts for 1 week produced a reduction in plasma total cholesterol (TC) and LDL in males and an increase in high-density lipoproteins (HDL) levels in females.[10]

Despite the potential hypocholesterolemic effect of sulforaphane-rich broccoli sprouts and the corresponding isothiocyanates, more research is needed to elucidate the mechanism that allows their cholesterol-lowering effect and regulation on lipid homeostasis. The genetic expression of some proteins such as sterol regulatory element binding protein (SREBP), HMG-CoA reductase (HMGR), fatty acid synthase (FAS), LDL receptors (LDLR), and apolipoprotein B (ApoB100) affected by food phytochemicals such as resveratrol, soy protein, or its isoflavones has been previously researched.[11−13] The phytochemicals associated with

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broccoli sprouts may have similar mechanisms and a significant anticholesterolemic effect.

Research aims in this work were focused on evaluating the dietary effects of GR or SF fractions obtained from broccoli sprouts as a whole or from their extracts on cholesterol balance, defined as the difference in cholesterol intake and fecal excretion of neutral sterols, and the cholesterol levels found in liver and plasma of hamsters that consumed hypercholesterolemic diets. Furthermore, gene expression of SREBP-1, SREBP-2, HMGR, FAS, Apo B100, and LDLR involved in the biosynthesis of lipids or their participation in cholesterol transport was studied.

This work sheds some light on possible mechanisms for hypocholesterolemic effects of broccoli sprouts, glucosinolates, and sulforaphane.

MATERIALS AND METHODS

Quantitation of GR, SF, and Their Dithiocarbamate (DTC) Metabolites. Analysis of GR in broccoli sprouts and broccoli sprout extracts was performed as described previously.[14] Briefly, BSE or broccoli sprouts were homogenized in water or solvent and assayed by HPLC utilizing a 150 × 4.6 mm, 5 μm particle size, 200 Å pore size hydrophilic interaction chromatography column (ZIC-HILIC; Sequant, Umeå, Sweeden), using an isocratic mobile phase consisting of 15 mM ammonium formate, pH 4.5, in 70:30 (v/v) acetonitrile/water, 0.5 mL/min flow rate, column temperature of 25 °C, and detection at 235 nm. A purified GR was used as a standard.

Analysis of SF in broccoli sprouts and broccoli sprout extracts was performed as described previously.[15] Briefly, BSE or broccoli sprouts were homogenized in water or solvent and assayed by HPLC utilizing a 250 × 4.6 mm, 10 μm particle size, Partisil 10, ODS-2 C18 column (Whatman, Clifton, NJ), which had been equilibrated with 1% acetonitrile/99% water and eluted at 1 mL/min with a linear gradient from 1% acetonitrile/99% water to 100% acetonitrile, in 60 min, initiated 2 min after sample injection. Column temperature was 25 °C, and elution was monitored at 240 nm. A SF standard was purchased from LKT Laboratories, St. Paul, MN.

DTC, the metabolites of SF, and other ITCs were measured by HPLC as described previously.[16] Briefly, 500 μL of urine (or urine diluted with water) was added to 500 μL of 500 mM sodium borate buffer, pH 9.25, and 1 mL of 20 mM 1,2-benzenedithiol in acetonitrile, incubated for 2 h at 65 °C, and centrifuged to sediment particulates, and a 200 μL of the supernatant was injected onto a Partisil 10 ODS-2 HPLC column (250 × 4.6 mm; Whatman) and eluted with 80% methanol/20% water (v/v). The reaction of all DTCs with 1,2-benzenedithiol forms 1, 3-benzodithiole-2-thione, which elutes at about 5 min, and is detected by monitoring at 365 nm.

Preliminary Glucosinolates and Isothiocyanates Metabolic Study. A preliminary study was conducted validating the ability of Syrian hamsters (*Mesocricetus auratus*) to metabolically convert glucosinolates to isothiocyanates and to absorb and excrete the dithiocarbamate metabolites of sulforaphane in a manner consistent with other mammals (e.g., rats, mice, and humans) to which broccoli sprout preparations have been administered to date. Broccoli sprout extracts were prepared as a freeze-dried powder as previously described,[8,15] and supplied to the animals. Glucoraphanin-rich broccoli sprout extract (GR-BSE) contained approximately 400 μmol of GR/g of powder, and thus the 1% GR-BSE diet contained 4 μmol of GR/g of diet. Sulforaphane-rich broccoli sprout extract (SF-BSE) contained about 70 μmol of SF/g of powder, and thus the 1% SF-BSE diet contained 0.7 μmol of SF/g of diet.

After 1 week for acclimatization in individual metabolic cages (Nalgene, Miami, FL), 4 16-week-old hamsters were fed ad libitum with a basal diet based on AIN-93 standard rodent formulation supplemented with 0.1 or 1% (w/w) powdered GR-BSE or SF-BSE for 3 consecutive days. Food intake was calculated daily through the collection of remaining food. Urine samples were collected daily from individual metabolic cages, mixed with 1.8% boric acid, and immediately frozen for further analysis. The samples of the 3 consecutive days were pooled for urinary DTC excretion of each hamster and quantified as previously described. All values were expressed on a per-day basis.

Animals and Dietary Groups for Cholesterol Metabolism and Lipid Homeostasis Study. Syrian hamsters were bred and raised at the School of Medicine animal facilities of Tecnologico de Monterrey. A total of 48 16-week-old animals with initial weights ranging from 100 to 120 g were blocked by weight and sex and thereafter randomly assigned to six experimental groups. Each group consisted of four animals of each sex. Hamsters were raised under standard environmental conditions (20 °C with diurnal cycles of 12 h light/12 h darkness).

After 4 weeks, animals were transferred to individual metabolic cages (Nalgene) for 1-week baseline collections of feces and urine. During the metabolic study, animals were provided with 15 g of diet each day, and leftovers were collected to calculate precise feed intake. Feces were collected daily and kept at −4 °C. At the end of the collection period, feces of each hamster were weighed and dried at 60 °C for 36 h (Imperial III incubator, Lab-line Instruments, Melrose Park, IL). Dry feces weights were recorded before grinding in a laboratory mortar. The ground fecal material was stored at −80 °C (Revco) until analyses. After the collection period, animals were returned to their cages for an additional 2 weeks.

Fresh broccoli sprouts were graciously donated by a commercial green-sprout producer (Alimentos Lee, Monterrey, NL, Mexico) and lyophilized prior to grinding and diet formulation. Sprouts contained approximately 6 μmol of GR/g fresh weight or about 75 μmol of GR/g dry weight. All diets were formulated to be isocaloric, isoproteic, isolipidic, and isofibrous. Hamsters were fed a basal hypercholesterolemic diet consisting of 20% casein (MP Biomedicals Inc., Solon, OH), 0.2% L-methionine (Sigma-Aldrich Co., St. Louis, MO), 5% commercial corn oil, 3.5% cellulose (AIN Alphacel-Nutritive Bulk, MP Biomedicals Inc.), 10% commercial sucrose, 46.7% corn starch (Maizena, Mexico), 10% commercial pork lard, 1% AIN-93 vitamin mix (MP Biomedicals Inc.), 3.5% AIN-93 mineral mix (MP Biomedicals, Inc.), and 0.1% cholesterol (Amresco, Solon, OH).

Six different diets were compared: a nonamended (negative) control, a positive control diet containing simvastatin (Zocor; 15 μmol/kg body weight), and four experimental diets. Two of the experimental diets contained freeze-dried broccoli sprouts calculated to provide 2 or 20 μmol/day of GR (BSX and BS10X, respectively), whereas the remaining two diets contained GR-BSE or SF-BSE each calculated to deliver 20 μmol/day of either SF or GR, hereinafter referred to as GRE and SFE, respectively.

After 7 weeks of feeding, and following 12 h of food deprivation, the animals were first anesthetized for 45 s in an ether-rich atmosphere to obtain blood through intracardiac puncture. Then, animals were immediately euthanized in a >70%-rich CO_2 atmosphere. Blood obtained through intracardiac puncture was placed in EDTA K3 tubes and centrifuged at 3500 rpm (Eppendorf 5804C Centrifuge, Hamburg, Germany) for 7 min at 4 °C. Plasma samples were stored at −80 °C for further analyses. Livers were surgically removed, weighed, divided into four lobes, individually placed in sterile bags (Nasco, Salida, CA), and immediately frozen using liquid N_2, and then samples were stored at −80 °C for analysis of metabolites and gene expression of each experimental unit.

Extraction of Hepatic and Plasma Cholesterol. The lipids of hepatic tissue samples (200 mg) were extracted twice with chloroform/methanol (2:1, v/v) and then treated with KCl (0.88%).[17] Resulting extracts were centrifuged at 2400 rpm for 10 min at 4 °C, and the chloroform layer was evaporated at 35 °C in a Speed Vac SC250EXP concentrator (Thermo Savant). Samples were redissolved in 1 mL of chloroform/methanol (2:1, v/v) containing 5α-cholestane (Sigma-Aldrich Co.),

Table 1. Sequences of Real-Time RT-PCR Primers Designed for Expression Studies in *Mesocricetus aureatus*[a]

gene		primer (5′−3′)[b]	amplified fragment size (bp)	organism	Genbank accession no.
SREBP-1[c]	S	AGTGCCTCTTGCCATGCAGT	68	*Cricetulus griseus*	U09103
	A	cgtttTCCCCATCCACGAAGAAAcG			
SREBP-2	S	cggtcCTGGAGAAGGTGGGTGACcG	66	*Cricetulus griseus*	U12330
	A	CCCAGCTTGACAATCATCTGCT			
HMGR	S	TCCTCGTGCTTGTGATTCTGC	84	*Mesocricetus auratus*	X00494
	A	cggtgGAAGGCGTCCTTTATCACaG			
FAS	S	cacttgGTTGTGAAGCCCCTCAAGtG	79	*Cricetulus griseus*	AF356086
	A	CCTGAGCCATGTAGCGGAAG			
LDLR	S	CCAAACTCCACTCTATCTCCAGCA	78	*Cricetulus griseus*	M94387
	A	gaccaTGCTTCTCATCTTCCAAAATGGtC			
ApoB100	S	GCATGTGGGTTCCAGCATTCTA	98	*Mesocricetus auratus*	M35187
	A	cgagcTTCAAGTCAGCATTGTGCTcG			

[a] Abbreviations: SREBP, sterol regulatory element binding protein; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; FAS, fatty acid synthase; LDLR, low-density lipoprotein receptor; ApoB100, apolipoprotein B100; S, sense; A, antisense. [b] Lower case letters at the 5′ end denote bases added to form hairpin. Lower case base at the 3′ end indicates the base to which the fluorophore was attached. [c] SREBP-1 gene generates two isoforms by alternative transcription start, 1a and 1c. SREBP-1c is the predominant isoform expressed in mammalian liver.[35] These primers amplify a region present in both transcripts; hence we refer to them as SREBP-1.

which was used as an internal standard. Thereafter, 1 M KOH/ethanol was added for saponification and allowed to react for 1 h at 80 °C. Distilled water was added to each tube, and this sample was then extracted twice with hexane. The hexane layer was transferred to glass vials and evaporated under N_2 before derivatization with BSTFA (99%) plus TMCS (1%) (Sigma-Aldrich Co.) in preparation for gas chromatography with detection by flame ionization (GC-FID).

TC in plasma was extracted using 10 μL of sample following the saponification, extraction, and derivatization steps previously described in preparation for quantification by GC-FID. High-density lipoproteins were measured according to the same method after LDL and very low density lipoproteins (VLDL) were precipitated with phosphotungstic acid in the presence of magnesium ions according to the procedure of Lopes-Virella et al.[18] LDL was calculated by subtracting HDL and 1/5 of plasma triglycerides values from TC.

Extraction of Total Fecal Neutral Sterols for Cholesterol Balance Determination. Dried feces (100 mg) of each hamster were acidified with 0.5 M HCl and neutral sterols extracted with chloroform/methanol (2:1, v/v), [17] and centrifuged (3000 rpm), and the resulting supernatants were pooled. KCl (0.88%) was added to each extract, and the mixture was vigorously shaken. The resulting chloroform layer was concentrated in a Speed Vac SC250EXP concentrator (Thermo Savant, Holbrook, NY). Fecal neutral sterols were redissolved in 1 mL of chloroform/methanol (2;1, v/v), saponified, extracted with hexane, and derivatized as previously described in preparation for GC-FID. Total fecal neutral sterols were calculated by the sum of cholesterol and coprostanol excretion. Cholesterol balance was calculated as the difference in cholesterol intake and total fecal neutral sterols excretion.[3] Daily dietary cholesterol intake was calculated by multiplying food intake by the percentage of cholesterol in the diet and was expressed as micromoles per day.

Analysis of Sterols by GC. Samples were analyzed using a gas chromatograph (Hewlett-Packard HP-6890) equipped with a FID detector (Agilent Technologies Inc., Palo Alto, CA) and a capillary BPX5 column (30 m × 0.25 mm i.d.) (SGE Inc., Austin, TX). The method recommended by the column maker was used with slight modifications according to Du and Ahn.[19] Sample volume was brought to 100 μL with hexane, and 2 μL aliquots were injected with a split mode of 10:1. Helium was used as a carrier at constant pressure of 137 kPa or 20 psi. The injector and detector temperatures were 270 and 360 °C, respectively. Oven temperature was programmed at 240 °C for 1 min and ramped to 300 °C at a rate of 7.5 °C/min and held at this maximum temperature for an additional 4 min. Analytes were identified and quantified in comparison to commercial standards of cholesterol and coprostan-3-ol (Sigma-Aldrich Co.). All data were corrected according to internal standards used in each sample.

Determination of Plasma Triglycerides. Plasma triglyceride concentration was analyzed using enzymatic hydrolysis and detection of glycerol produced by a colorimetric assay (Randox Laboratories Ltd., Antrim, U.K.).

RT-PCR Analysis. Total RNA was isolated from hepatic tissue of each hamster using a Qiagen RNeasy Mini Kit (Hilden, Germany) according to the manufacturer's instructions and quantified on a NanoDrop spectrophotometer (NanoDrop Technologies, Houston, TX). Reverse transcription of RNA and real time PCR were performed using a SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen Corp., Carlsbad, CA). Table 1 outlines specific primers designed in this work using the D-LUX Designer program (Invitrogen Corp.) for amplification of genes coding for HMG-CoA reductase (HMGR), SREBP-1, SREBP-2, FAS, LDLR, and Apo B100. RNA serial dilutions were used to calculate amplification efficiencies for each primer. In cases when sequences from *M. aureatus* were not available, primer design was based on highly conserved mRNA regions among *Cricetulus griseus*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* transcript sequences. Real-time RT-PCR was conducted in 25 μL reaction volume containing 25 ng of total RNA on a RotorGene 3000 (Corbett Research, Sydney, Australia) with the following run conditions: one cycle of cDNA synthesis at 50 °C for 15 min, one denaturing cycle at 95 °C for 2 min, 40 cycles of denaturing at 95 °C for 15 s, annealing at 55 °C for 10 s, and extension at 72 °C for 10 s. A melting curve was the final step, which consisted of a temperature ramp from 60 to 95 °C, rising by 1 °C in a span of 3.4 h. Relative expression values were normalized using the housekeeping gene coding for β-actin (Invitrogen Corp.). We confirmed that β-actin gene expression was not

Table 2. Effect of Oral Administration of Glucosinolate and Sulforaphane-Rich Extracts (GR-BSE and SF-BSE) on Urinary Excretion of Dithiocarbamates (DTC) by Syrian Hamsters[a]

type of extract	total daily GR/SF consumption (μmol/day)	DTC excretion (μmol/day)	DTC excretion (% of consumption)
1.0% GR-BSE	22.00 ± 1.89	0.42 ± 0.05	1.91 ± 0.27
0.1% GR-BSE	1.47 ± 0.57	0.06 ± 0.00	4.28 ± 1.28
1.0% SF-BSE	2.80 ± 0.49	0.65 ± 0.06	23.70 ± 4.38
0.1% SF-BSE	0.25 ± 0.05	0.10 ± 0.04	41.69 ± 5.66

[a] Values expressed as means of four hamsters in each diet ± SEM.

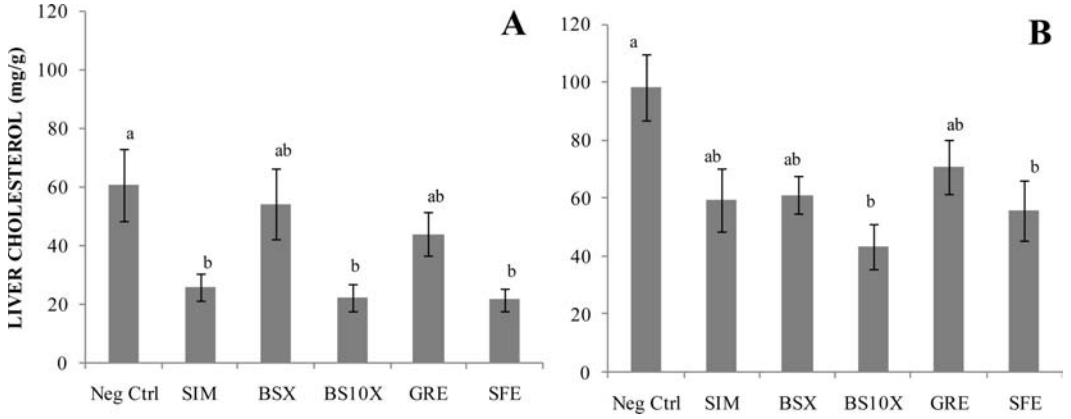

Figure 1. Liver cholesterol in female (A) and male (B) hamsters treated with basal hypercholesterolemic diet as negative control (Neg Ctrl), simvastatin (SIM), broccoli sprouts (BSX or BS10X), and broccoli sprout extracts rich in GR (GRE) or SF (SFE). Values are mean ± SEM for four hamsters in each diet. Values with the same letter indicate they are not significantly different ($p < 0.05$).

affected by the variable groups in our experiments (ANOVA, $\alpha = 0-0.5$, $p = 0.6096$). Threshold cycle values were calculated using Rotor-Gene 6 data analysis software (Corbett Research). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.[30] Representative amplicons were sequenced to confirm target amplification. PCR efficiencies were optimized for each primer pair, and efficiency values ranged from 91.37 to 102.35% with median slopes of the standard curves ranging from −3.54 to −3.26.

Statistical Analysis. Data is reported as mean and standard error. One-way ANOVA was used to detect differences among treatments and the Tukey test to compare means at $p < 0.05$. Statistical data analysis was performed using JMP version 4.0.4 (SAS Institute Inc.).

RESULTS AND DISCUSSION

Preliminary Glucosinolate and Isothiocyanate Study. Catabolism of GR or SF to their DTC congeners has never been reported in Syrian hamsters. A preliminary study was therefore required to ensure that, as with other mammals studied, conversion of GR to SF and uptake and metabolism of SF occurred. Our data indicated that sulforaphane was bioavailable in this model (Table 2). Conversion efficiencies were low compared to reported data for mice, rats, and humans,[20−22] but all treated animals excreted DTC metabolites, whereas untreated control animals did not.

DTC detected in the urine of hamsters that consumed GR-BSE confirmed that GR was metabolized to its cognate isothiocyanate (SF). This conversion is mediated by the activity of the enzyme myrosinase produced by microbes found in the gastrointestinal tract, and it is thought to vary in accordance with gut colonization and perhaps with transit time variation as well.[21,22]

DTC excretion levels of hamsters fed the SF-supplemented diet were substantially higher compared to counterparts fed the GR-supplemented diet (Table 2). Consumption of SF led to a higher DTC recovery (41.69%) that was almost 10 times the amount obtained in hamsters that consumed GR-supplemented diet (4.28%). Previous studies in humans also demonstrated (a) that peak DTC excretion was higher in individuals who consumed isothiocyanates when compared to those who consumed equimolar quantities of glucosinolates; (b) that this excretion was more rapid, peaking in 1−2 h, versus about 8 h with GR; and (c) that cumulative excretion was much higher, all indicating that isothiocyanates are approximately 6 times more bioavailable than glucosinolates.[21−23] This is all readily explained by the incomplete action of the enzyme myrosinase in hydrolyzing GR to SF (or glucosinolates, in general, to isothiocyanates). Experiments in which myrosinase associated with the gut microflora was reduced or eliminated support this observation.[22] Many animal experiments with mice and rats have also demonstrated that conversion of GR to SF occurs via gut microflora, but at levels on the order of 10%.[24] Thus, it appears that gut-microflora-mediated conversion of GR to SF is the rate-limiting step controlling bioavailability of SF from GR in humans and in animal models.

Hepatic and Plasma Lipids. Liver weight of female and male hamsters (6.00 ± 0.57 g) was not significantly different among treatments. Hepatic cholesterol levels of hamsters fed BS10X and SFE diets were significantly different from negative controls both in females (Figure 1A) and in males (Figure 1B). In females, these treatments and the simvastatin (SIM) positive control reduced hepatic cholesterol by approximately 60% compared to negative controls, which is equivalent to the in vitro and in vivo percentages reported for the pharmaceutical SIM.[25,26] BS10X and SFE diets were calculated to deliver 20 μmol/day of SF per animal, but considering that the excretion of DTC after consumption of GR was significantly lower than with SF, having the same hypocholesterolemic effects suggested that sulforaphane was not the only

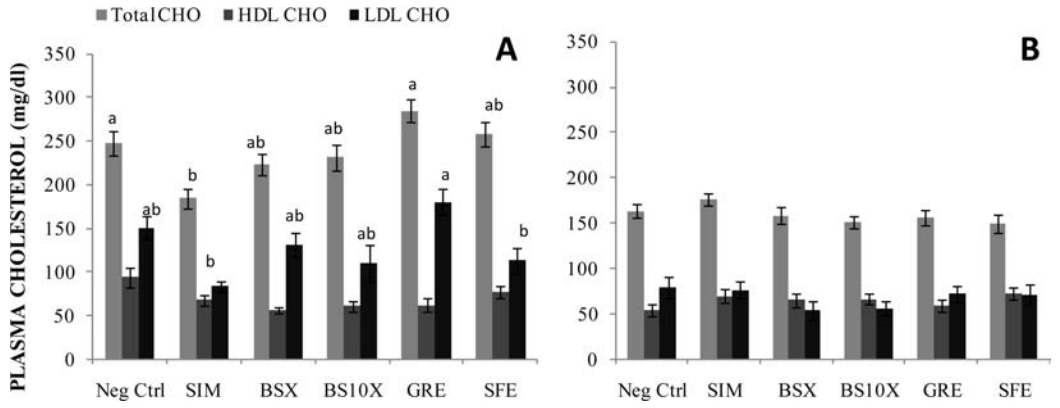

Figure 2. Plasma cholesterol in female (A) and male (B) hamsters treated with basal hypercholesterolemic diet as negative control (Neg Ctrl), simvastatin (SIM), broccoli sprouts (BSX or BS10X), and broccoli sprout extracts rich in GR (GRE) or SF (SFE). Values are mean ± SEM for four hamsters in each diet. Values with the same letter indicate they are not significantly different ($p < 0.05$).

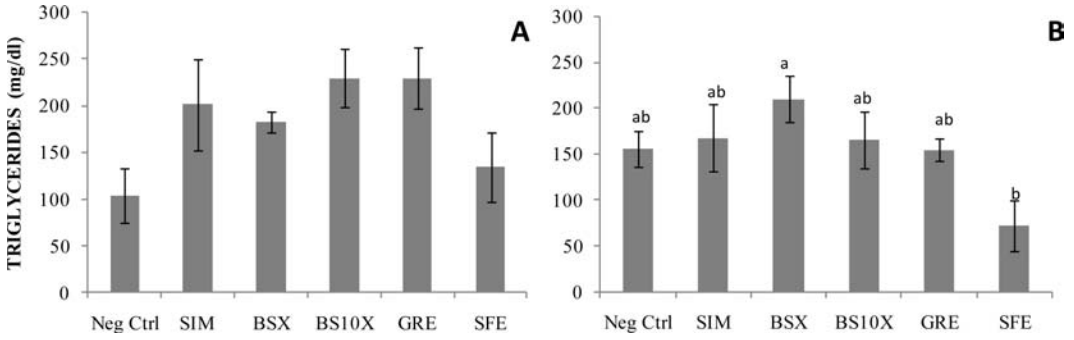

Figure 3. Plasma triglycerides in female (A) and male (B) hamsters treated with basal hypercholesterolemic diet as negative control (Neg Ctrl), simvastatin (SIM), broccoli sprouts (BSX or BS10X), and broccoli sprout extracts rich in GR (GRE) or SF (SFE). Values are mean ± SEM for four hamsters in each diet. Values with the same letter indicate they are not significantly different ($p < 0.05$).

factor responsible for the reduction of hepatic cholesterol. The consumption of glucosinolates from BS10X reinforced this idea because hamsters fed this diet had lower hepatic cholesterol than counterparts fed GRE even if both deliver the same amount of glucosinolates (Figure 1A).

In male hamsters, BS10X and SFE also promoted a significant reduction on hepatic cholesterol (Figure 1B). Previous papers indicated that the consumption of broccoli sprout extracts in rats fed a high-fat diet reduced hepatic cholesterol to levels observed in the normal diet.[6] Interestingly, the BS10X diet had a more robust cholesterol-lowering effect compared to the SFE diet, indicating that broccoli sprouts may be more effective than the individual phytochemicals (e.g., SF).

TC levels in plasma were higher in females (Figure 2A) than in males (Figure 2B), contrary to what was observed in hepatic cholesterol (Figure 1). The difference in the levels of plasma cholesterol between males and females was probably due to a higher sensitivity of female hamsters to high concentrations of cholesterol and saturated fatty acids in their diets and a subsequently higher synthesis of LDL that was transported to plasma.[27] In our study, only simvastatin reduced plasma total cholesterol levels in female hamsters. Simvastatin treatment reduced TC, by affecting both LDL and HDL cholesterol, to levels that were not significantly different from the negative control when analyzed separately.

In males, no significant differences were observed in plasma cholesterol levels of hamsters ingesting different treatments (Figure 2B) contrary to what could be expected according to the results in liver cholesterol. This lack of correlation between hepatic and plasma cholesterol levels could be due to a difference in cholesterol excretion through the bile that was not considered in this experiment. However, some compounds such as indole-3-carbinol fed to male CD-1 mice at 500 and 750 mg/kg/day lowered hepatic cholesterol levels without affecting serum cholesterol,[28] indicating a similar regulatory mechanism for BSX10 and SFE treatment.

In males, plasma triglyceride levels were also similar among treatments, and the only difference detected was between treatments BSX and SFE (Figure 3B). Male hamsters fed SFE had less than half the triglyceride levels of those fed BSX. In females, plasma triglycerides were similar among treatments (Figure 3A).

Cholesterol Balance and Coprostanol Excretion. The only statistically significant reduction in cholesterol balance was observed for BS10X fed to female hamsters (Figure 4A) due to a conversion of cholesterol into coprostanol of twice the amount observed in hamsters that consumed the control diet (Figure 5A). Nicolle et al.[3] reported a negative cholesterol balance when carrots rich in fiber and antioxidant activity were fed to mice. Another study[29] indicated that consumption of insoluble fiber-rich fractions extracted from carrots diminished plasma and hepatic cholesterol, which was associated with increased fecal cholesterol, lipids, and bile acids. Female hamsters fed BS10X also had lower hepatic cholesterol levels (Figure 1A). No significant effects were observed in cholesterol balance or coprostanol excreted by males (Figures 4B and 5B).

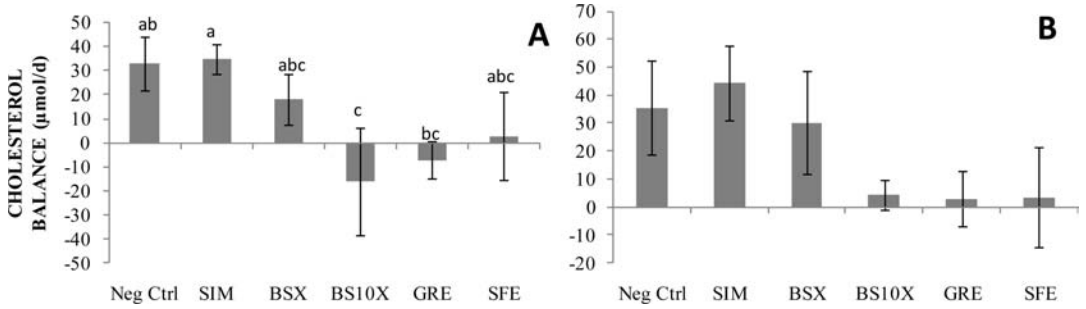

Figure 4. Cholesterol balance in female (A) and male (B) hamsters treated with basal hypercholesterolemic diets (Neg Ctrl), simvastatin (SIM), broccoli sprouts (BSX or BS10X), and broccoli sprout extracts rich in GR (GRE) or SF (SFE). Values are mean ± SEM for four hamsters in each diet. Values with the same letter indicate they are not significantly different ($p < 0.05$).

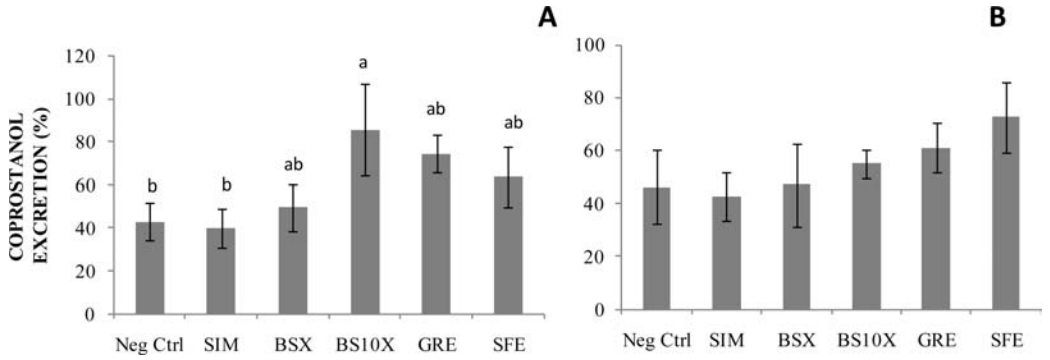

Figure 5. Coprostanol excretion expressed as percentage of cholesterol consumption in female (A) and male (B) hamsters treated with basal hypercholesterolemic diets (Neg Ctrl), simvastatin (SIM), broccoli sprouts (BSX or BS10X), and broccoli sprout extracts rich in GR (GRE) or SF (SFE). Values are mean ± SEM for four hamsters in each diet. Values with the same letter indicate they are not significantly different ($p < 0.05$).

Gene Expression. Real time PCR data showed differences in hepatic gene expression due to supplemented diets. The expression of SREBP-1, SREBP-2, FAS, and ApoB100 genes was significantly affected ($\alpha = 0.05$, $p < 0.05$; Figure 6). Furthermore, we observed that the effects of dietary treatments on the expression of genes involved in hepatic sterol and lipid metabolism differed depending on gender. SREBP-1, SREBP-2, and ApoB100 mRNA levels showed clear opposite responses in female and male animals, especially when broccoli sprouts at a high dose (BS10X) were fed (Figure 6). On the other hand, the differences found when glucosinolate or sulforaphane extracts were added to the diet were not as marked, suggesting that other compounds associated with broccoli sprouts may contribute additionally to the effect observed. These findings are supported by previous papers which showed that cholesterol homeostasis was affected by sex, due in part, to the influence of sex hormones on transcriptional and post-transcriptional regulation of the metabolic pathways.[30−32] Thus, the interactions of food components that influence cholesterol and lipid homeostasis may differ depending on the sex of the animal. Overall, these results are consistent with the observed differences in hepatic cholesterol accumulation in female and male hamsters fed BS10X and SFE diets (Figure 1).

Glucosinolate and sulforaphane extracts had significant impact on the expression of SREBP-1, SREBP-2, and FAS only in female animals. SREBP-1 was down-regulated 0.66- and 0.46-fold by GSE and SF, respectively (Figure 6A). SREBP-1 is a transcription factor that activates the expression of several genes involved in lipid homeostasis, including FAS.[33,34] Hence, the down-regulation of FAS that we observed due to the extracts (Figure 6C) correlates positively with the trend observed in SREBP-1 mRNA levels. However, the down-regulation of both genes observed did not affect plasma triglyceride levels (Figure 3A). We did not generate data related to the accumulation of hepatic fatty acids, which could serve as a better reference for the hepatic gene expression measured. On the other hand, SREBP-2 is another transcription factor isoform that preferentially stimulates transcription of the LDL-receptor and sterol biosynthetic enzymes, including HMGR, the enzyme that catalyzes the rate-limiting step for de novo cholesterol biosynthesis.[33] SREBP-2 mRNA levels were down-regulated as well by both extracts to around half the expression of the negative control (Figure 6B); this correlated positively with the reduction of hepatic cholesterol found when these diets were fed to female hamsters (Figure 1A). Surprisingly, we did not find an effect on HMGR and LDLR as a consequence of low mRNA levels of the transcription factor (data not shown). Most of the proteins involved in cholesterol and lipid homeostasis show a complex regulation at transcriptional, post-transcriptional, and post-translational levels (e.g., SREBP proteins have to be detached by proteolysis from the ER membrane to stimulate transcription in the nucleus). Differences between mRNA levels and protein accumulation in nucleus have been shown previously for both SREBP-1 and -2.[35] In this study we did not measure protein levels; therefore, changes in mRNA accumulation may not reflect the same trend in protein accumulation or activity.

Diets supplemented with broccoli sprouts, particularly at high concentration (BS10X), had an impact on SREBP-1 mRNA levels in females and on ApoB100 gene expression in male hamsters. In female hamsters BS10X caused a 0.43-fold decrease of SREBP-1

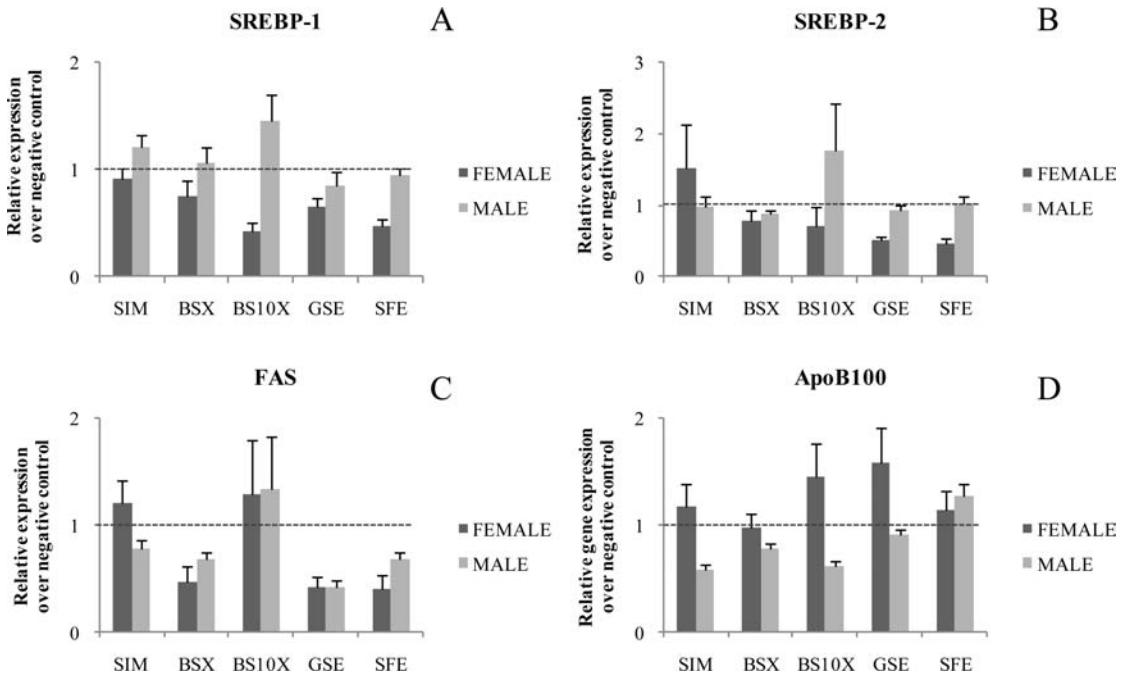

Figure 6. Effect of supplementation of broccoli sprouts and extracts on the expression of genes involved in cholesterol and lipid metabolism in liver. BSX, freeze-dried broccoli sprouts, 2 μmol/day; BS10X, freeze-dried broccoli sprouts, 20 μmol/day; GRE, glucosinolate-rich extract; SFE, sulforaphane-rich extract (n = 4 of each treatment).

gene expression, which did not correlate with FAS and plasma triglycerides (Figure 6A). The reduction of SRBP-2 expression observed for this treatment was not statistically significant (Figure 6B). However, this treatment did reduce significantly the accumulation of hepatic cholesterol (Figure 1A). As previously mentioned, BS10X was the treatment that caused the highest coprostanol excretion (Figure 5A) in female hamsters, and thus it should be considered as an additional factor for the reduction of liver cholesterol when broccoli sprouts are consumed at high dose. On the other hand, BS10X, in addition to simvastatin, was the only treatment that show some significant down-regulation in male hamsters in the expression of ApoB100, the main structural component of LDL (Figure 6D). However, only BS10X caused a reduction of hepatic cholesterol to <50% of the negative control (Figure 1B). In addition, in males, we observed a slight increase in gene expression for SREBPs and FAS, contrary to females. All of these point to additional mechanisms by which broccoli sprout components may interact and affect cholesterol homeostasis in male hamsters.

In this study we showed the hypocholesterolmic effect of broccoli sprouts in the liver of hamsters fed diets with high levels of cholesterol and fat. This effect could be partially explained by the observed impact of BS10X on cholesterol clearance and by changes in the expression of some genes involved in cholesterol homeostasis in liver. These effects, however, were different between male and female animals, supporting previous findings of sex differences in the regulation of cholesterol metabolism.[30−32] Another aspect to consider is that SREBPs and HMGR are regulated in addition by post-translational modifications that affect their activities.[34] This has a deep impact on cholesterol and triglyceride homeostasis and should be addressed in the future to further understand the effects of these phytochemicals on lipid metabolism. This investigation demonstrated that the ingestion of broccoli sprouts reduced liver cholesterol in female Syrian hamsters by increasing coprostanol excretion and down-regulating the levels of expression of SREBP-1 and probably by other mechanisms that were not analyzed in this study. The effects on the expression of genes involved in lipid homeostasis were highly affected by sex. Particularly in females, the extract rich in sulforaphane down-regulated the expression of SREBP-2 and FAS in addition to SREBP-1. These data suggest that glucoraphanin of broccoli sprouts, when it is catabolized to sulforaphane, interacts synergistically with other elements of the food matrix to reduce hepatic cholesterol in hamster. Further studies are necessary to ascertain the minimal dose of sulforaphane that is required to modulate the synthesis of hepatic cholesterol and to understand why it was different in males and females. Results from this work strengthen the significance of the food matrix on the bioactivity of phytochemicals.

■ ABBREVIATIONS USED

ACAT, acyl-CoA:cholesterol acyltransferase; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; DTC, dithiocarbamate; BSX, freeze-dried broccoli sprouts, 2 μmol/day; BS10X, freeze-dried broccoli sprouts, 20 μmol/day; GC-FID, gas chromatography—flame ionization detector; GR, glucoraphanin; GRE, glucoraphanin-rich broccoli sprout extract; HDL, high-density lipoprotein; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; I3C, indole-3-carbinol; LDL, low-density lipoprotein; SF, sulforaphane; SFE, sulforaphane-rich broccoli sprout extract; TC, total cholesterol; TMCS, trimethylchlorosilane; VLDL, very low density lipoprotein; SREBP, sterol regulatory element binding proteins; FAS, fatty acid synthesis; ApoB100, apolipoprotein B100; RT-PCR, real time-PCR.

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DISCLOSURE

Under a licensing agreement between Brassica Protection Products and The Johns Hopkins University, Dr. Fahey may be entitled to a share of royalty received by the University on sales of broccoli sprouts. Dr. Fahey is a founder of, and unpaid advisor to, Brassica Protection Products. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.

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