

A Combination of Psyllium and Plant Sterols Alters Lipoprotein Metabolism in Hypercholesterolemic Subjects by Modifying the Intravascular Processing of Lipoproteins and Increasing LDL Uptake^{1,2}

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Abstract

We previously demonstrated that a diet therapy involving consumption of 7.28 g psyllium (PSY) and 2 g of plant sterols (PS) per day reduced LDL cholesterol from 3.6 ± 0.7 to 3.1 ± 0.8 mmol/L ($P < 0.01$) and decreased the number of intermediate density lipoprotein particles and the smaller LDL and HDL subfractions in hypercholesterolemic individuals ($n = 33$). The study design was a randomized double blind crossover. Subjects consumed either 2 test cookies containing PSY+PS or 2 placebo cookies for 1 mo with a 3-wk wash out between treatments. To explore mechanisms of the lipid-lowering effects of combined PSY+PS, we present data related to intravascular and molecular regulation. Intake of PSY+PS decreased the cholesterol concentration in LDL-1 from 2.46 ± 0.66 to 2.26 ± 0.46 mmol/L and in LDL-2 from 0.63 ± 0.24 to 0.54 ± 0.27 mmol/L ($P < 0.05$) in the test compared with the placebo period. An increase in LDL peak size from 27.3 ± 0.8 to 27.5 ± 0.6 nm ($P < 0.05$) and a decrease in the prevalence of LDL pattern B from 27 to 18% ($P < 0.05$) also occurred during the PSY+PS period. Cholesteryl ester transfer protein activity was 11% lower ($P < 0.05$) during the test period. Notably, the abundance of the LDL receptor in circulating mononuclear cells as measured by real time PCR was 26% higher during the test compared with the placebo period ($P < 0.03$). These results indicate that the hypocholesterolemic action of PSY and PS can be explained in part by modifications in the intravascular processing of lipoproteins and by increases in LDL receptor-mediated uptake. *J. Nutr.* 137: 1165–1170, 2007.

Introduction

Hypercholesterolemia, especially elevated LDL cholesterol (LDL-C),⁵ is a major risk factor for coronary heart disease (CHD) (1,2). LDL size and LDL subfractions are now considered as better clinical markers of CHD development. The predominance of small dense LDL is associated with incidence of CHD (3). Therefore, LDL subtyping can provide important information to improve risk prediction and to evaluate the response to lipid therapy (4). Studies suggest that the proportion and cholesterol concentration of small LDL particles (LDL <25.5 nm) possess a powerful and independent risk prediction in middle-aged men (5).

LDL particles comprise 7 density subfractions with diameters between 22 and 27.2 nm (6). These are divided into phenotype A (or pattern A), characterized by predominance of large, buoyant LDL particles with diameters >25.5 nm, and phenotype B (or pattern B), comprising small, dense LDL particles with diameters <25.5 nm (7). Phenotype B is associated with features of the metabolic syndrome such as increased plasma levels of triglyceride (TG) and apolipoprotein B, decreased HDL cholesterol (HDL-C), insulin resistance, high plasma glucose and insulin, and hypertension (8,9). Therefore, a therapeutic approach to decrease LDL pattern B is very important in the management of the metabolic syndrome.

Cholesteryl ester transfer protein (CETP) facilitates intravascular lipoprotein remodeling by promoting the equimolar exchange of cholesteryl esters with TG in HDL and apo B-containing lipoproteins affecting lipoprotein phenotype. Higher CETP activity has been shown to result in the formation of small, dense LDL particles in type 2 diabetes (10). CETP in conjunction with lecithin cholesteryl acyltransferase (LCAT) plays an important role in HDL remodeling, generating HDL particles with a high capacity for cell cholesterol uptake (11).

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⁵ Abbreviations used: CETP, cholesterol ester transfer protein; CHD, coronary heart disease; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL-C, HDL cholesterol; LCAT, lecithin cholesteryl acyltransferase; LDL-C, LDL cholesterol; PS, plant sterol; PSY, psyllium; TG, triglyceride.

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We previously showed that dietary treatment with psyllium (PSY) and plant sterols (PS) significantly decreased LDL-C concentrations and the number of the smaller subfractions of LDL and HDL particles, resulting in a less atherogenic lipoprotein profile in free-living individuals (12). To our knowledge, there are no data to provide an insight on the secondary mechanisms of LDL-C lowering associated with modulation of lipoprotein subfractions resulting from the action of soluble fiber and PS in the intestinal lumen. Although a few studies in humans (13) and animals (14) showed the effect of soluble fiber on the intravascular processing of lipoproteins, the combined action of PSY+PS on lipoprotein subfraction distribution in the intravascular compartment has not been evaluated. A better understanding of the mechanisms at the intravascular as well as at the molecular level would provide an insight on the cardioprotective role of these functional components. The purpose of this study was to assess the secondary mechanisms of plasma LDL-C lowering and the remodeling of lipoprotein subfractions resulting from the action of PSY+PS from our previous study (12). We hypothesized that PSY+PS would modulate the intravascular processing of lipoproteins and affect receptor-mediated uptake of LDL.

Materials and Methods

Placebo and test cookies were obtained from RD Foods. Free cholesterol kits were obtained from Wako Chemicals. CETP kits were obtained from BioVision (Biovision Research Products). Lipoprint System LDL subfraction kits were obtained from Quantimetrix, HBSS and Histopaque 1077 were obtained from Sigma Chemical, and Trizol was obtained from Invitrogen. We obtained the DNA synthesis kit from BioRad (Bio-Rad Laboratories), the DNase treatment and removal kit from Ambion, and the LightCycler Fast Start DNA Master Plus Syber Green I and capillaries from Roche Diagnostic. Primers for LDL-R and HMG CoA reductase were obtained from Sigma Genosys.

Subjects. Thirty-three healthy adults (11 men, 8 premenopausal women, and 14 postmenopausal women) aged 35–65 y with initial plasma LDL-C between 2.6 and 4.1 mmol/L (100 and 160 mg/dL) and a BMI between 25 and 35 kg/m² were recruited. All participants completed the study. The exclusion criteria were diabetes, cardiovascular disease or lipid-lowering drug treatment, and fiber or sterol supplementation. All subjects gave a written informed consent to participate and the Committee on the Use of Human Subjects in Research of the University of Connecticut approved the study protocol.

Study design. As previously described (12), we conducted the study to determine the effects of PS and PSY, provided via cookies, on plasma lipids and on lipoprotein size and subfraction distribution by using a randomized, double blind, crossover, placebo-controlled study design. Briefly, we randomly assigned participants ($n = 33$) to the test cookie (10 g PSY yielding 7.68 g/d soluble fiber and 2.6 g/d PS) group or placebo cookie (0 g PSY + 0 g PS) group for 1 mo. After a 3-wk-wash out period, they changed to the alternate treatment. Subjects were asked to maintain their habitual diet and level of exercise. Dietary intake during both periods was assessed by the completion of 5-d weighted dietary records that included 2 weekend days. We recorded participants' weight, height, and blood pressure at the beginning and end of each treatment period. At the end of each treatment period, 2 blood samples drawn on different days to control for day-to-day variability of plasma lipids were collected. Plasma was separated by centrifugation at 2000 \times g; 20 min, and aprotinin (0.5 mL/100 mL), sodium azide (0.1 mL/100 mL), and phenyl methyl sulfonyl fluoride (0.1 mL/100 mL) were added for preservation. Plasma was stored in individual aliquots at -80°C for analysis of LCAT and CETP activity, LDL peak size and diameter, and LDL oxidation.

Dietary supplement and analysis. A detailed description of diet composition of the experimental cookies was previously reported (12).

Briefly, 2 cookies/d were packed in individually labeled bags and provided to the subjects on a weekly basis. Participants returned empty bags or bags containing the uneaten portion of the cookies and the weight was recorded to calculate the amount of cookies consumed per individual in each dietary period. The Nutrition Data System for Research software version 5.0 (developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN) Food and Nutrient Database 28 was used to calculate dietary fiber intake as a mean of the 5-d dietary records. We previously showed that energy derived from total fat, saturated fat, monounsaturated fat, polyunsaturated fat, carbohydrates, and protein did not differ between periods (12).

Plasma LCAT and CETP activity determinations. Plasma LCAT activity was determined using a previously described method (15), by measuring the reduction in the mass of endogenous free cholesterol after a 6-h incubation, and was expressed as the molar esterification rate (micromole decrease in unesterified cholesterol per liter plasma per hour). Free cholesterol concentrations were measured by an enzymatic method.

Plasma CETP activity was determined by using a CETP activity assay kit following the manufacturer's instruction. Briefly, 3 μL of plasma sample (as the source of CETP) was added to the reaction mixture containing a fluorescent self-quenched neutral lipid as the donor molecule and an acceptor molecule. A CETP-mediated transfer of the fluorescent neutral lipid to the acceptor molecule resulted in an increase in fluorescence, which was read in a fluorescence plate reader at excitation 456 nm and emission 535 nm. CETP activity was expressed as millimoles of neutral lipid transferred per liter plasma per hour. Samples were run in duplicate. All CETP analyses were conducted in the same day to decrease variability. The intra-assay variability was 2.2%.

LDL particle size pattern. The Lipoprint LDL system (16) was used to determine LDL particle size pattern via nongradient, high-resolution PAGE. According to this method, particle distribution with a mean particle size >25.5 nm is characterized as pattern A, whereas pattern B is a mean particle size <25.5 nm (8).

LDL oxidation. LDL oxidation was determined according to Abbey et al. (17). LDL was isolated at a density of 1.09 g/L by ultracentrifugation in a L8-M ultracentrifuge using the rotor VTI 65.2 (Beckman Instruments) at 65,000 \times g; 45 min. Samples were dialyzed overnight in 1.5 g/kg NaCl and 0.1 g/kg Na₂HPO₄, pH 7.4. After dialysis, protein in the LDL samples was assayed as described by Markwell et al. (18). Samples were diluted to 102 μg LDL/0.0012 L using dialysis buffer (10 mmol/L Na₂HPO₄, 0.15 mol/L NaCl, pH 7.4). After the dilution, samples were transferred to a DU-640 UV spectrophotometer (Beckman Coulter) and 25 μL of 0.25 mmol/L CuSO₄ was added to initiate oxidation. Samples were incubated at 37°C for 180 min at 230 nm and absorbance was plotted every 120 s. The rate of oxidation was determined from the slope of the propagation phase. Lag time was determined from the intercept of the lag and propagation phases.

Mononuclear cell isolation. Mononuclear cells were isolated from whole blood by the method of Boyum (19). Briefly, 20 mL blood was diluted with 10 mL HBSS without Ca²⁺ and Mg²⁺, layered over 10 mL Histopaque 1077, and centrifuged at 500 \times g; 30 min (Rotanta 460 R). The mononuclear cell interface was removed, washed with HBBS, and centrifuged at 600 \times g; 10 min twice. The cell pellet was resuspended in 200 μL Tris buffer (10 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L CaCl₂, pH 7.4), and kept at -80°C until RNA was extracted.

RNA extraction and purification. Total RNA was extracted from mononuclear cells according to a method based on that developed by Chomczynski and Sacchi (20). TRIzol reagent was used according to the manufacturer's instructions. The method was slightly modified by using isopropyl alcohol for RNA precipitation. The DNA-Free kit was used to remove trace contaminating genomic DNA following the manufacturer's instructions. RNA was extracted by precipitation using 2.5 volumes of 100% ethanol and 0.1 volume of 3 mol/L sodium acetate of pH 5.2. RNA pellet was washed with 70% ethanol and dissolved in diethyl pyrocarbonate-treated water.

cDNA synthesis. cDNA was synthesized using iScript cDNA synthesis kit following the manufacturer's instruction. Briefly, 20 μL of reaction volume containing 1 μg purified RNA, 5 μL 5 \times iScript reaction mix, 1 μL iScript reverse transcriptase, and the rest of nuclease free water was incubated for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

Real-time PCR. Primers for the target genes, LDL receptor and HMG CoA reductase, and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (21) were designed using LightCycler Probe Design software 2.0 (Roche Diagnostics). The forward and reverse primer sequences for LDL receptor were AAGAACATCAACAGCAT-CAACT and TGGCAAATGTGGACCTCA, respectively. Similarly, for GAPDH, the sequences were ATCCATGACAACTTTGGTATCG and TCTTCTGGGTGGCAGTG, respectively, and for HMG CoA reductase were AGCAGATTTGCACGTCTAC and TGGACTGGAAACGGATA-TAAAGG. The estimated product size of each reaction product of LDL receptor, HMG CoA reductase, and GAPDH was 74, 73, and 70 bp, respectively.

Real-time PCR were performed in duplicate using the LightCycler FastStart DNA Master^{plus} SYBR Green I (Roche Diagnostics) following the manufacturer's instruction. The reactions were performed in Light-Cycler 2.0 instrument under the following reaction condition: polymerase activation at 95°C for 5 min followed by 45 cycles of denaturing at 95°C for 10 s, annealing at 57°C for 10 s, and extension at 72°C for 10 s. After amplification, a melting curve was obtained to determine the optimal PCR conditions. Quantification was done by analyzing the fluorescence curves and detecting the crossing point of samples using LightCycler Software 4.0 (Roche Diagnostics).

Statistical analysis. Because we found no significant differences due to gender or hormonal status as calculated by repeated measures ANOVA, we grouped all the subjects for data presentation. A paired *t* test was used to compare placebo and test periods. Data are presented as means \pm SD. Differences of *P* < 0.05 were considered significant.

Results

Plasma LCAT and CETP activity, LDL oxidation rate, and lag time. Plasma LCAT activity did not differ between the treatment periods, whereas CETP activity was lower in the test period compared with the placebo period (*P* < 0.05; Table 1). In contrast, the rate of LDL oxidation and lag time were not different between periods (Table 1).

The individual responses in CETP activity during the placebo and the test periods are presented in Figure 1.

Plasma LDL size and distribution pattern. Mean and peak LDL particle sizes were greater during the test period compared with the placebo period (*P* < 0.05; Table 2). After the PSY+PS treatment, there was a significant shift of LDL pattern B toward pattern A. In the placebo period, 27% of the subjects had pattern B, whereas only 18% had this pattern in the test period (*P* < 0.05; Table 2).

TABLE 1 LCAT and CETP activity, LDL oxidation rate and lag time of all subjects following the placebo and PSY+PS test periods¹

Parameter	Placebo	Test
LCAT activity, $\mu\text{mol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$	26.3 \pm 13.7	26.7 \pm 8.7
CETP activity, $\text{mmol}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$	8.4 \pm 1.6	7.5 \pm 2.3
LDL oxidation rate, $\text{nmol diene}\cdot\text{mg LDL protein}^{-1}\cdot\text{min}^{-1}$	13.6 \pm 4	13.2 \pm 5
Lag time, min	56.2 \pm 11.6	55.4 \pm 10.3

¹ Data are means \pm SD, *n* = 33.

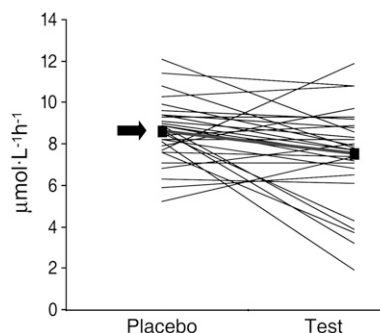


FIGURE 1 Differences in CETP activity for all subjects (8 men, 11 women, and 14 postmenopausal women) between the placebo and PSY+PS test periods. The mean change for all subjects is indicated by the grey line pointed with an arrow.

Cholesterol concentrations in LDL subfractions. Cholesterol concentrations in LDL-1 and LDL-2 subfractions were significantly lower in the test period. Cholesterol concentrations were reduced in LDL-1 from 2.5 \pm 0.7 to 2.3 \pm 0.5 mmol/L and from 0.6 \pm 0.2 to 0.5 \pm 0.3 mmol/L in LDL-2 (*P* < 0.05; Table 2). The cholesterol concentration in the LDL-3 subfraction did not change.

LDL receptor mRNA abundance. The mRNA abundance of the LDL receptor and HMG CoA reductase presented correspond to the 17 subjects for which the integrity of the RNA was verified by gel electrophoresis (Table 3). The abundance of the LDL receptor in circulating mononuclear cells as measured by real-time PCR was 26% greater in the test period compared with the placebo period (*P* < 0.03). The relative quantification analysis obtained by comparing the normalized ratio of LDL receptor genes showed that expression of the LDL receptor gene increased 1.3-fold during the PSY+PS treatment. The individual responses in abundance of LDL receptor mRNA during the test and placebo periods are presented in Figure 2. In contrast, HMG CoA reductase gene expression was not modified by the treatment (Table 3).

Discussion

We previously reported that a combination therapy with PSY and PS resulted in a less atherogenic lipoprotein profile by decreasing plasma LDL-C, number of intermediate density lipoprotein and LDL, and HDL smaller subfractions (12). The

TABLE 2 LDL mean and peak sizes, cholesterol concentration in LDL-1, LDL-2, and LDL-3, and percent phenotype B of all subjects following placebo and PSY+PS test periods¹

Parameter	Placebo	Test
LDL mean size, nm	27.00 \pm 0.41	27.11 \pm 0.37*
LDL peak size, nm	27.30 \pm 0.77	27.50 \pm 0.62*
Phenotype B, %	27	18*
LDL-C, ² mg/dL		
LDL-1	95.6 \pm 25.5	88.2 \pm 18*
LDL-2	24.6 \pm 9.3	21 \pm 10.3*
LDL-3	3.4 \pm 6.7	4.4 \pm 6.3

¹ Data are means \pm SD, *n* = 33. *Different from placebo, *P* < 0.05.

² To convert to mmol/L, divide by 38.67.

TABLE 3 mRNA abundance of the LDL receptor, HMG CoA reductase, and GAPDH in mononuclear cells isolated from subjects during the placebo and PSY+PS test periods¹

mRNA Abundance	Placebo	Test
	<i>Arbitrary units</i>	
LDL receptor	1.72 ± 0.96	2.42 ± 1.09*
HMG CoA reductase	2.8 ± 1.7	3.1 ± 1.8

¹ Data are means ± SD, n = 17. *Different from placebo, P < 0.05.

objective of this study was to probe for secondary mechanisms to clarify the nature of the lipid-lowering effects of PSY+PS.

PSY, PS, and LDL size phenotype and subfraction distribution. Treatment with PSY+PS increased mean and peak LDL particle sizes and shifted the LDL distribution from pattern B to pattern A. This reduction of pattern B from 27% in the placebo to 18% in the test period obtained from the Lipoprint LDL System confirms our previous data that PSY+PS induced a significant reduction in the number of smaller LDL subfractions obtained from H-NMR analysis. In addition, cholesterol concentrations in LDL-1 and LDL-2 subfractions were significantly decreased during the test period, further contributing to the decrease in plasma LDL-C concentrations.

Studies have shown that small, dense LDL particles are associated with the etiology of atherosclerosis, whereas larger, less dense LDL particles are less atherogenic (22,23). There are limited studies on the effects of soluble fiber or PS on lipoprotein subfractions. Behall et al. (24) observed increases in LDL particle size when β-glucan from barley was consumed with a Step 1 diet. However, this effect was pronounced only in postmenopausal women and the LDL particle size of all the participants remained <25 nm. In another study, oat fiber supplementation resulted in lower concentrations of small, dense LDL particles than placebo wheat cereal (25). These studies indicate that soluble fibers modify LDL particle phenotype. PS, on the other hand, has not been shown to affect LDL size (26,27); however, dietary manipulation has been shown to modify LDL electrophoretic characteristics by shifting the LDL particles toward a less atherogenic pattern A (28). Studies by Pedersen et al. (29) and Varady et al. (30) showed that PS had favorable effects on LDL size and subfractions as well as cholesterol content in small, medium, and large LDL particles. A combination of dietary

supplementation including soluble fiber, PS, vegetable protein, and almond has been shown to reduce cholesterol concentrations in almost all LDL subfractions (31). These findings suggest that soluble fiber may affect LDL size distribution, whereas PS may modify cholesterol concentrations in the subfractions. This may explain our finding that the combination of PSY and PS had favorable effects on LDL subfraction distribution and LDL size. As a predominance of small, dense LDL particles, indicative of the pattern B phenotype, has been suggested to result in a 3-fold increase in CHD risk (32) and LDL pattern B is typically seen in those individuals with insulin resistance (9) and metabolic syndrome (33), the significant reduction of pattern B by PSY+PS in this study may suggest PSY and PS as an important dietary strategy for these high-risk populations.

PSY, PS, and intravascular processing of lipoprotein. In this study, we found an 11% reduction of CETP activity and no change in LCAT activity by the combination therapy of PSY+PS. This may explain our previous findings that PSY+PS treatment decreased the number of smaller LDL and HDL particles and improved LDL size and subfraction distribution without affecting plasma levels of HDL-C. Higher CETP activity has been shown to favor the formation of TG-rich smaller LDL subfractions (11) and to result in lower HDL-C and, indirectly, in decreases in HDL size (34) by the formation of TG-rich larger HDL that serve as substrate for hepatic lipase (35). Therefore, the PSY+PS-induced reduction of CETP activity observed in this study may be beneficial in reducing proatherogenic lipoproteins.

We previously showed that PSY supplementation reduces CETP activity without altering LCAT activity (15). A PSY-induced decrease in CETP activity has also been reported in animal models (36,37) and PS has been shown to decrease plasma CETP mass (38).

There is no consistent effect of PS on LDL oxidation. In 1 study, PS reduced the oxidized LDL concentration in plasma (38), whereas in another study, there was no effect (39). Animal studies reported that LDL from soluble fiber fed to animals is less susceptible to oxidation (14,40). In this study, PSY+PS did not affect oxidative modification of LDL despite the increase in LDL size.

PSY, PS, and the LDL receptor. Mononuclear cell LDL receptor mRNA abundance was significantly modified by PSY+PS treatment, whereas the effect on HMG CoA reductase mRNA abundance was not. The abundance of the LDL receptor in circulating mononuclear cells was increased by 26%. Mononuclear cells were used in this study as a surrogate to reflect responses in hepatic cells. Studies show that the mechanisms that regulate mRNA levels in liver and mononuclear cells are similar and suggest that freshly isolated mononuclear cells can be used to predict HMG CoA reductase and LDL receptor mRNA levels in liver (41,42).

Studies in guinea pigs from our laboratory showed that PSY intake increased LDL turnover by upregulation of LDL receptors (43,44). The suggested mechanism for this effect has been demonstrated in animal studies and postulates that PSY intake enhances bile acid excretion (45), resulting in the upregulation of CYP7A (14) that leads to the depletion of hepatic free cholesterol (44).

Plat and Mensink (46) documented that PS supplementation resulted in reduction in plasma LDL-C concentrations that were associated with an increase in mononuclear cell LDL receptor mRNA abundance, with no changes in HMG CoA reductase mRNA. An increase in LDL receptor protein levels was also

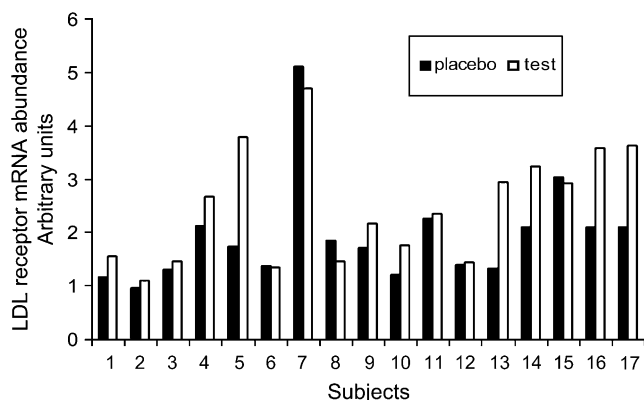


FIGURE 2 Differences in mRNA abundance of the LDL receptor in isolated mononuclear cells in 17 subjects between the placebo (dark bar) and PSY+PS test periods.

observed, indicating PS affects LDL receptor both at transcriptional and translational levels. The authors further suggested that HMG CoA reductase activity may be partly regulated by PS at the transcriptional level. However, Vega-Lopez et al. (47) demonstrated opposite results in which PSY supplementation increased HMG CoA reductase in human mononuclear cells without any effect on LDL receptor mRNA abundance. Differences in the results may be due to the presence of PS in our combination therapy that may lead to the increased LDL receptor mRNA levels. The results of our study indicate that hypocholesterolemic action of PSY+PS is partly achieved by increases in receptor-mediated uptake of LDL-C and HMG CoA reductase does not play an important role in cholesterol homeostasis in this condition.

This study provides a better understanding of how a dietary manipulation with PSY and PS results in improvement of lipoprotein profiles. Our data suggest that the hypocholesterolemic mechanisms of PSY+PS are partially related to reduced CETP activity, resulting in changes in the intravascular processing of lipoproteins that are associated with modifications in LDL composition, size, and potential for atherogenicity. An increase in LDL uptake from circulation through receptor-mediated mechanisms also explains in part the LDL-C lowering caused by PSY+PS.

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