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Consumption of a Novel Dietary Formula of Plant Sterol Esters of Canola Oil Fatty Acids, in a Canola Oil Matrix Containing 1,3-Diacylglycerol, Reduces Oxidative Stress in Atherosclerotic Apolipoprotein E-Deficient Mice

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The antiatherogenic properties of a novel dietary formula (PS-CO) of plant sterol esters of fatty acids, produced by enzymatic interesterification of plant sterols with canola oil (CO), in a CO matrix containing 1,3-diacylglycerol, were evaluated in apolipoprotein E-deficient mice. PS-CO consumption strongly tended to lower total plasma cholesterol levels by 21%, compared to the placebo group. Blood triglycerides were reduced by 38% and 36% compared to CO and placebo-fed mice, respectively. Serum lipid peroxide levels were lowered following PS-CO administration by 62% and 63%, compared to CO and placebo administration, respectively. Unlike CO supplementation, PS-CO consumption preserved serum paraoxonase (PON1) activity. Mouse peritoneal macrophages from PS-CO-fed mice exhibited reduced cellular uptake of oxidized-LDL compared to those from placebo-fed mice and demonstrated a tendency toward a decreased capability to release superoxide anions. These findings indicate that PS-CO supplementation is beneficial in reducing serum lipid levels, and serum and macrophage oxidative stress, thus contributing to the reduction in atherogenic risk factors.

KEYWORDS: Plant sterols; diacylglycerol; oxidative stress; canola oil; macrophage foam cells; Apo E^0 mice

1. INTRODUCTION

The role of atherosclerosis in the development of cardiovascular disease (CVD) is well documented (*1*). A number of processes are implicated in the development of atherosclerosis, especially oxidative stress, i.e., oxidation of lipids in LDL by plasma components and arterial wall cells (*2*). Oxidized LDL (Ox-LDL) may aggregate, adhere to the arterial wall, or be taken up by arterial macrophages, with the latter leading to the development of foam cells (*3*). All of these processes are well-documented features of early atherogenesis (*4*). Serum lipids can be protected against peroxidation by endogenous antioxidants (*5*), as well as by serum paraoxonase 1 (PON1), a lipolactonase which is associated in serum with high-density lipoprotein (HDL) (*6*). PON1 hydrolyzes specific oxidized lipids (*7*) and thus reduces oxidative stress in serum lipoproteins, as well as in macrophages and in atherosclerotic lesions (*8*). PON1 undergoes inactivation by oxidative stress, and its activity was shown to be preserved by dietary antioxidants (*9*)

Dietary supplements have been used to counteract and prevent atherosclerosis (*10*), particularly in those individuals who are at risk of developing CVD, sufferers of metabolic syndrome, hypercholesterolemic individuals, and diabetics, in addition to those who already have CVD. Among the dietary approaches, administration of nutritional supplements with strong antioxidant properties has been widely pursued (*11*). Some of these antioxidant compounds are hydrophobic (vitamin E, carotenoids, certain classes of flavonoids) and are believed to incorporate into LDL, preventing oxidation of its protein and lipid components, while others are hydrophilic (vitamin C, other classes of flavonoids) and function as scavengers of oxidizing species in the blood.

In addition to antioxidants, plant-derived lipids, especially olive oil and canola oil (CO), which are rich in monounsaturated fatty acids (MUFA), and plant sterols and stanols, compounds that chemically resemble cholesterol (*12*) (*13*), have been reported to lower total cholesterol and LDL levels in humans and animals. It is widely assumed that the mechanism by which plant sterols lower the concentration of plasma cholesterol is by interfering with intestinal cholesterol absorption (*14*).

In the present study we have evaluated a novel proprietary formulation (PS-CO) of plant sterol esters of fatty acids, produced by *in situ* enzymatic interesterification of plant sterols with CO, in a matrix of CO containing 1,3-diacylglycerol (DAG), for its antiatherogenic properties, by using the atherosclerotic apolipoprotein E-deficient (ApoE^0) mice model. These mice demonstrate increased LDL oxidation and develop exten-

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sive atherosclerosis within a few months of age (*15*). The effect of PS-CO was evaluated on plasma lipid levels and oxidative stress both in serum and in peripheral macrophages, and compared to the effect of CO and of the placebo.

2. MATERIALS AND METHODS

2.1. Materials. Chemicals for enzymatic analysis were purchased from Sigma (St., Louis, MO). Tissue culture supplies for the culture of macrophages were purchased from Biological Industries (Beth Haemek, Israel).

2.2. PS-CO Preparation. The novel dietary PS-CO was prepared by *in situ* enzymatic interesterification of soybean plant sterols with canola oil. The immobilized surfactant-coated lipase was prepared as previously described (*16*). The enzymatic interesterification was initiated by adding 100 mg of plant sterols (ϕYTO, ABITEC Corporation) (consisting of 48% β-sitosterol, 26% campesterol, 22% stigmasterol, 2% brassicasterol, and 2% other plant sterols) to 450 mg of canola oil (Shemen, Haifa, Israel). A 100 mg amount of immobilized lipase-surfactant complex were added to the mixture. The reaction mixture was then heated at 60 °C for 14 h. At the end, the enzyme was filtered out and the reaction products were analyzed by gas chromatography (Agilent Technologies, Little Falls, DE) equipped with a 15-m capillary column (J&W Scientific, Folsom, CA) and a flame ionization detector.

The product mixture consist of 25% sterol esters of canola oil fatty acids, 15% diglycerides (1,2-DAG:1,3-DAG = 3:7 (w/w)), 8% free fatty acids, 2% free sterols, monoglycerides (less than 2%), and the rest are canola oil triglycerides (approximately 50%).

2.3. Animals and Diet. ApoE^0 mice were generously provided by Dr. Jan Breslaw (Rockefeller University, New York). Fifteen such animals (8 weeks old at the start of the experiment) were randomly divided into three groups. Mice in each group received their regular chow diet (supplied by Koffolk Ltd, Tel-Aviv, Israel, and its ingredients are the following: 210 g/kg total protein, 40 g/kg total fat, 45 g/kg cellulose, 70 g/kg ash, 8−12 g/kg calcium, 7−9 g/kg phosphorus, 3 g/kg chlorides, and 2.5 g/kg sodium), and in addition they were fed by gavage every third/fourth day for 10 weeks, 40/60 μL, respectively, of the following dietary supplement:

Group A: PS-CO, soybean sterols esterified to fatty acids from CO, providing the equivalent of 2.5 mg/day free plant sterols, an amount equivalent to 0.3% (w/w) of the mouse total food intake, combined with 2.08 mg/day diacylglycerol in a matrix of CO. The level of free plant sterols administered to the mice was calculated based on the recommended phytosterols dosage for humans, which is 1.5 g of phytosterols/day. Taken into consideration 60 000 g for the human body weight compared to 20 g for the mouse body weight, the daily dosage for mouse is 0.5 mg/day/mouse. However, since the experiment is done for a limited period, we used a dosage ×5 fold higher; thus, each mouse was administered 2.5 mg/day free plant sterols, equivalent to approx 15 μL/day/mouse. Since the mixture was given by gavage, we fed the mice once in 3 or 4 days (45 μL/3 days/mouse or 60 μL/4 days/mouse).

Group B: Canola oil.

Group C: Placebo phosphate-buffered saline (PBS). Supplementation of the canola oil or the PS-CO did not cause the mice to lose their appetite and had no effect on body weight. The body weight of mice before study entry was 20 ± 2 g, and at the end of the study (after 10 weeks) it was 23 ± 2 g.

The experimental protocol was approved by the Animal Care and Use Committee of the Technion, No. IL-066-10-2001

2.4. Determination of Plasma Lipid Profile. Murine serum samples were analyzed for their lipid profile. Total cholesterol and triglyceride concentrations were measured by automated methods on the multianalyzer Dimension RxL Max utilizing enzymatic Flex reagents (Dade Behring Diagnostic, Marburg, Germany).

2.5. Serum Lipid Peroxidation. Serum from the ApoE^0 mice was diluted 1:4 with PBS, and its susceptibility to oxidation was determined, based on an established protocol (*17*). Briefly, serum sample was incubated with 100 mM of the free radical generating compound, 2′,2′-azobis-2′-amidinopropane hydrochloride (AAPH), which is a water-soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate. The formation of thiobarbituric acid reactive substances (TBARS) was measured and compared to serum that was incubated under similar conditions, but without AAPH.

2.6. Paraoxonase 1 (PON1) Activity. The enzymatic activity of PON1 in serum from ApoE^0 mice was determined by measuring arylesterase activity, using phenylacetate as the substrate (*18*). Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 5 μL of serum, 1.0 mM phenylacetate, and 0.9 mM $CaCl_2$ in 20 mM Tris HCl, pH 8.0. The rate of nonenzymatic hydrolysis of phenylacetate was evaluated in a parallel reaction and subtracted from the total rate of hydrolysis. The E_{270} for the reaction is 1310 M^{-1} cm^{-1}. One unit of arylesterase activity is equal to 1 μmol of phenylacetate hydrolyzed/min/mL. Purified enzyme has nearly 2000 units of arylesterase activity per mg protein.

2.7. MPM Preparation. Macrophages were harvested from the peritoneal fluid of the PS-CO-fed and control-fed ApoE^0 mice (15−25 g) 4 days after intraperitoneal injection of 3 mL of thioglycolate (24 g/L) in saline. The cells (10 to 20 × 10^6/mouse) were washed and centrifuged three times with PBS at 1000*g* for 10 min and then resuspended to a concentration of 10^9 cells/L in DMEM containing 15% horse serum (heat-inactivated at 56 °C for 30 min), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. The cell suspension was dispensed into 35 mm plastic Petri dishes and incubated in a humidified incubator (5% CO_2, 95% air) for 2 h. The dishes were washed once with 5 mL of DMEM to remove nonadherent cells, and the monolayer was then incubated under similar conditions for 18 h, prior to the beginning of the experiment.

2.8. Ox-LDL Uptake by Macrophages. MPM were incubated with ^{125}I-labeled Ox-LDL (10 μg of protein/mL) prepared as described (*17*), and lipoprotein−cell association and degradation were determined as follows: Lipoprotein degradation was measured in the collected medium as the trichloroacetic acid-soluble, nonlipid radioactivity, which was not due to free iodide. Lipoprotein degradation in a cell-free system was measured under identical conditions and was subtracted from the total degradation. The remaining cells were washed three times with cold PBS and dissolved in 0.1 N NaOH for protein and cell-associated lipoprotein determination.

2.9. Macrophage Oxidative Status. Cellular oxidative stress was examined by the release of superoxide anions in response to PMA, measured photometrically as reduction of cytochrome C inhibitable by superoxide dismutase as described elsewhere (*18*).

2.10. Histopathology of Aortic Atherosclerotic Lesion. The histopathological analysis was performed in the laboratories of the Division of Morphological Sciences and in the Unit of Electron Microscopy, Bruce Rappaport Faculty of Medicine. At the end of the experimental period, the mice were anesthetized with ethyl ether in a local nasal container. The heart and entire aorta were rapidly dissected out and immersion-fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer with 0.01% calcium chloride, pH 7.4, at room temperature, and histopathological development of the lesions was analyzed as previously described (*19*).

2.11. Statistical Analyses. All data are reported as means ± SD. A *P* value of 0.05 was taken as statistically significant. Nonpaired Student's *t*-test, assuming equal variance, was used to compare endpoint values of different dietary treatments.

3. RESULTS

3.1. Effect of PS-CO Consumption by ApoE^0 Mice on Plasma Lipid Levels. ApoE^0 mice were supplemented with PS-CO once every 3 days for 10 weeks as described in the Methods section. Plasma lipid levels were measured at the end of the treatment period. PS-CO reduced the cholesterol levels by 21% as compared to placebo, while no significant difference was observed between the CO and placebo treatments (**Figure 1A**); although the statistical significance of the cholesterol data is borderline ($p = 0.0517$), these results indicate a trend and confirm the cholesterol lowering potency of plant sterol preparations. Treatment with PS-CO significantly reduced the level of triglycerides by 38% compared to CO treatment ($p = 0.0003$) and by 36% compared to placebo control ($p = 0.0003$). CO

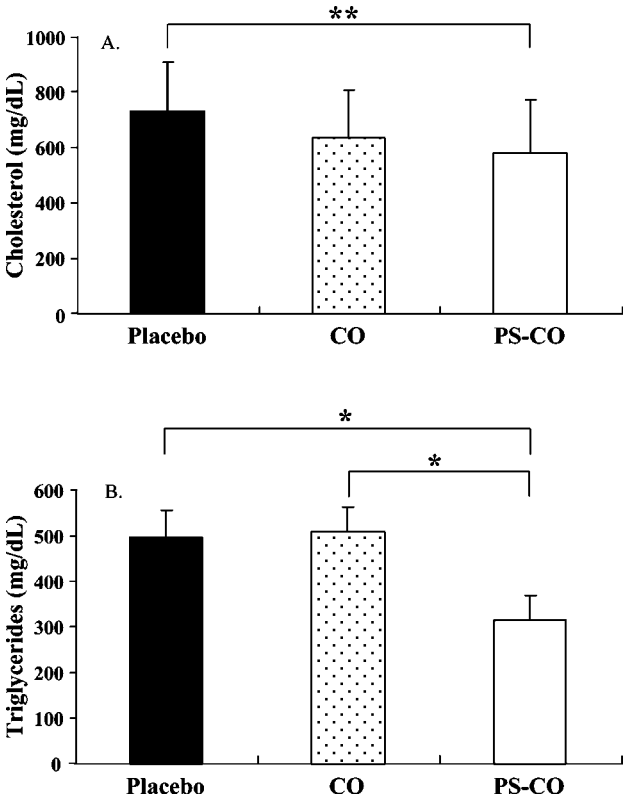

Figure 1. Effect of PS-CO consumption by ApoE^0 mice on plasma lipid levels. ApoE^0 mice were fed for 10 weeks with placebo (closed bars), CO (dotted bars), or PS-CO (open bars) as described in the methods section. Cholesterol (A) and triglyceride (B) levels in the serum were analyzed at the end of the treatment period. Results are means ± SD ($n = 5$) per treatment. Statistical significance between endpoint values of placebo and PS-CO treatments is **P*-value < 0.05, and ***P*-value < 0.1. CO: canola oil; PS-CO: plant sterols esterified with canola oil fatty acids.

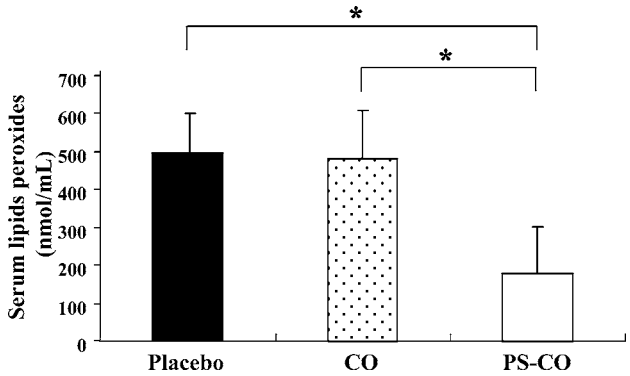

Figure 2. Effect of PS-CO consumption by ApoE^0 mice on serum total antioxidant capacity. ApoE^0 mice were fed for 10 weeks with placebo (closed bars), CO (dotted bars), or PS-CO (open bars) as described in Materials and Methods. Serum lipid peroxide levels (TBARS) were measured using a spectrophotometric assay with or without AAPH. Results are means ± SD ($n = 5$). Statistical significance between endpoint values of placebo and PS-CO treatments is **P*-value < 0.05. CO: canola oil; PS-CO: plant sterols esterified with canola oil fatty acids.

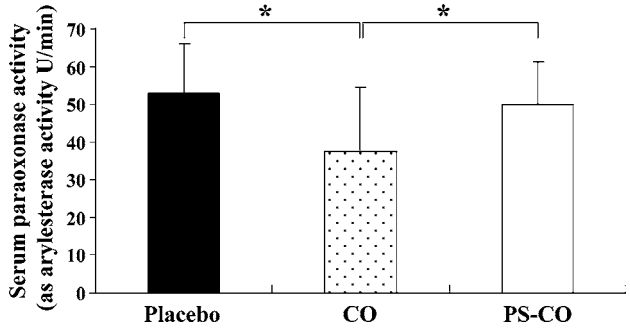

Figure 3. Lack of effect of PS-CO consumption by ApoE^0 mice on PON1 activity. ApoE^0 mice were fed for 10 weeks with placebo (closed bars), CO (dotted bars), or PS-CO (open bars) as described in Materials and Methods. Serum PON1 activity was measured using a spectrophotometric assay. Results are means ± SD ($n = 5$). Statistical significance between endpoint values of CO and PS-CO treatment is **P*-value < 0.1. CO: canola oil; PS-CO: plant sterols esterified with canola oil fatty acids.

treatment did not significantly affect triglyceride levels (**Figure 1B**).

3.2. Consumption of PS-CO by ApoE^0 Mice Increases the Total Serum Antioxidant Capacity. The level of lipid peroxides in serum is a measure factor of oxidative stress level in the cardiovascular system (*18*). The amount of lipid peroxides in the serum of the PS-CO, CO, and placebo-fed ApoE^0 mice were measured at the endpoint. Treatment with PS-CO strikingly reduced the amount of serum peroxides by 62% and 63%, compared to CO ($p = 0.0008$) and placebo ($P = 0.0001$), respectively. Canola-fed mice did not show any reduction in the levels of serum peroxides compared to placebo-fed controls (**Figure 2**).

3.3 Serum Paraoxonase1 (PON1) Activity Is Not Affected by PS-CO Consumption. We measured the level of PON1 activity in the serum of the mice in the three treatment groups. PS-CO treatment did not affect this enzyme's activity, while PON1 activity was significantly ($p = 0.03$) reduced by 29% after consumption of CO in comparison to placebo (**Figure 3**). On the contrary, consumption of PS-CO demonstrated anti-atherogenic property, as it preserved PON1 activity at levels that were similar to those obtained in the placebo group.

3.4. PS-CO Reduces the Oxidative Status of Macrophages. MPM were harvested from the mice in each of the treatment groups. The MPM were incubated with labeled Ox-LDL, and the released degradation products and the residual LDL associated with the cells were analyzed and quantified. The association of Ox-LDL to MPM was not statistically different between the three treatment groups. Degradation of Ox-LDL by MPM, derived after PS-CO consumption, was reduced by 16% in comparison to MPM from the placebo group ($p = 0.0313$) and showed a tendency ($p = 0.0903$) toward a 26% reduction in comparison to MPM from the CO treatment group (**Figure 4**). CO consumption did not statistically affect the degradation of Ox-LDL by MPM.

An additional measure of macrophage oxidative status is the release of superoxide anions. Whereas CO consumption did not cause a reduction in superoxide anion release from MPM in response to PMA, PS-CO consumption tended to decrease superoxide anion release from MPM by 24% as compared to MPM from placebo-fed mice (**Figure 5**).

Likewise, *in vitro* exposure of macrophages from naive ApoE^0 mice to soybean sterols esterified to high oleic oil in a high oleic oil matrix protected them from angiotensin II-induced oxidative stress, as determined by measuring MPM oxidation status and superoxide release (data not shown).

The size of the atherosclerotic lesions in the aortas of the mice in the three treatment groups was measured at the end of

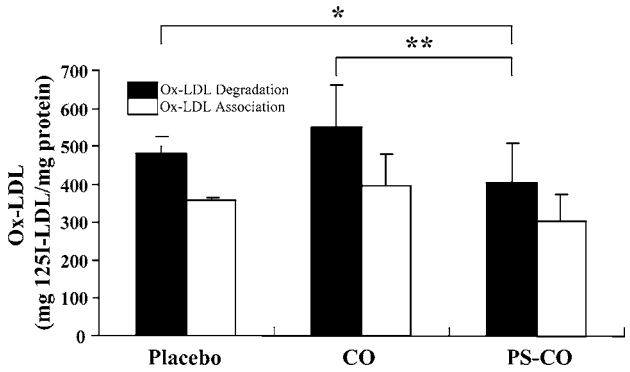

Figure 4. Effect of PS-CO consumption on Ox-LDL retention from Apo E^0's MPM. MPM harvested from $ApoE^0$ mice after 10 weeks of placebo, CO, or PS-CO supplementation were incubated with labeled Ox-LDL. LDL degradation products (closed bars) and associated LDL (open bars) were recovered and analyzed. Results are means ± SD ($n = 3$). Statistical significance between LDL degradation products endpoint values of placebo or CO and PS-CO treatments are *, ** P-value < 0.05 and < 0.1, respectively. CO: canola oil; PS-CO: plant sterols esterified with canola oil fatty acids.

the experiment. No significant effects of PS-CO treatment were observed (data not shown).

4. DISCUSSION

We present here an evaluation of a novel antiatherogenic preparation of plant sterols esterified to CO fatty acids. To date, the most common process to enhance the solubility of plant sterols in order to incorporate them into fatty foods is to esterify them with n-6 polyunsaturated fatty acids (PUFA), such as soybean oil and sunflower oil derived fatty acids, which are susceptible to oxidation. However, in this study, we produced a novel formulation of plant sterols esterified with monounsaturated fatty acids (MUFA), which in contrast to n-6 PUFA, are not readily oxidized.

Plant sterols have been intensely studied in recent years and have shown interesting results, particularly in lowering total cholesterol and LDL cholesterol (*20*). A beneficial effect in counteracting atherogenesis in animal models of atherosclerosis was also reported (*21*). These effects are obtained through mechanisms that are different from the mechanisms employed by other cholesterol lowering drugs and supplements and therefore may constitute both an alternative and an adjuvant therapeutic option. In light of the results obtained on patients that require reduction in their LDL cholesterol levels, the NCEP/ATP III has recommended 2 g/day of plant stanols/sterols as an effective dose to reduce LDL cholesterol (*22*).

In this study, treatment with PS-CO strongly tended ($p = 0.0517$) to reduce total cholesterol levels by 21% as compared to placebo. Our results are in agreement with a human study performed on hypercholesterolemic, mildly overweight individuals (Chan, Y.-M., et al., personal communication), which demonstrated that administration of soybean sterols esterified to high oleic oil in a high oleic oil matrix, significantly reduced LDL cholesterol. These results indicate that plant sterols maintain their cholesterol lowering property when esterified to high oleic acid oil.

The lack of statistical significance in the cholesterol reduction following PS-CO treatment may be due to various factors. One explanation may be related to the fact that the plant sterol esters were given once every 3 days, while other studies on $ApoE^0$ mice administrate plant sterols every day (*21*). Another possibility is that the plant sterol dosage in the present study was not adequate in order to cause a significant effect. Yeganeh et al. (*21*) observed a significant cholesterol lowering effect following the administration of a diet supplemented with 2% (w/w) plant sterols to $ApoE^0$ mice. As mentioned before, the $ApoE^0$ mice in the present study were supplemented with an amount equivalent to only 0.3% (w/w) of their diet.

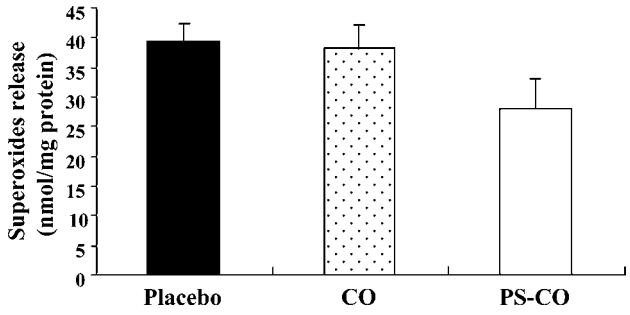

Figure 5. Effect of PS-CO consumption on superoxide anion release from Apo E^0's MPM. Superoxide release was assayed in MPM, isolated from $ApoE^0$ mice fed for 10 weeks with placebo (closed bars), CO (dotted bars), or PS-CO (open bars). Results are means ± SD ($n = 2$). CO: canola oil; PS-CO: plant sterols esterified with canola oil fatty acids.

PS-CO significantly reduced the level of blood triglycerides by 38% and 36% compared to CO and placebo treatments, respectively. There are few indications that plant sterols may possess a triglyceride lowering effect. Lukic et al. (*23*) reported that administration of a soluble phytostanol ester to $ApoE^0$ mice tended to decrease plasma triglyceride levels. In another study a soluble phytostanol ester has been shown to reduce triglyceride levels in cholesterol-fed Golden Syrian hamsters (*24*). In addition, 1,3-DAG, a component of PS-CO, has been previously reported to lower triglyceride levels in experimental animals (*25*). However, these results were obtained following feeding the animals with higher dosage of 1,3-DAG than the present study dosage. Whether this beneficial effect of PS-CO is attributed to the plant sterol component, 1,3-DAG component, or some form of synergistic effect between the two components, remains to be elucidated.

By far the most striking result of this study is the effect of PS-CO administration on the oxidative status. The notion that oxidative status is a major cause of atherosclerosis is supported by a large body of evidence: the presence of oxidized lipoproteins in atherosclerotic lesions, the greater susceptibility of LDL from atherosclerotic patients to oxidation (*26*), and the antiatherogenic effect of antioxidants (*27*). We show here that the amount of lipid peroxides in the serum of PS-CO-fed mice was greatly reduced. Furthermore, CO reduced the activity of serum PON1, while administration of the PS-CO did not, suggesting that plant sterols esterified to high oleic acid oil preserve PON1 activity and hence contribute to antiatherosclerotic pattern in serum. Since PON1 activity was shown to be inhibited by oxidative stress (*9*), the beneficial effect of PS-CO on preserving PON1 activity in serum can be attributed to its antioxidative effects. In addition, cellular uptake of Ox-LDL by MPM from PS-CO-fed mice was reduced, and the release of superoxide anions from these cells tended to be reduced. These results are also reinforced by our previous finding that treating macrophages from adult $ApoE^0$ mice (already displaying atherosclerosis symptoms) with plant sterols esterified to high oleic oil in an high oleic matrix, *in vitro*, renders them less susceptible to oxidative damage mediated by angiotensin II.

The effect of lipid supplements on oxidative stress has been similarly observed in human studies. In the aforementioned

human study (Chan, Y.-M., et al., personal communication), treatment with plant sterol esterified to high oleic oil in a high oleic oil matrix also demonstrated clear antioxidative effects: a significant reduction in the levels of serum LDL peroxides and a lack of increase in serum lipoprotein (a), a proatherogenic factor that was shown to increase upon administration of lipid supplements (*27*). In other studies, a reduction of Ox-LDL in plasma by olive oil administration in humans was observed (*12*). Homma et al. (*28*) have shown a reduction of Ox-LDL in healthy humans fed spreads containing plant stanols for 4 weeks. Summanen and co-workers (*29*) have administered a microcrystalline plant sterol suspension in CO to obese rats, and a marked reduction in lipid peroxidation was detected. A mechanism that can account for these findings cannot be readily outlined. An indication that plant sterols may directly affect the lipid peroxidation levels may also be gathered from in vitro studies (*30*), which have indicated that plant sterols at low concentrations inhibit lipid peroxidation of platelets.

The data presented here clearly indicate that PS-CO has a beneficial effect in mitigating some of the factors that cause atherosclerosis: a positive effect in the blood lipid composition and a marked reduction in oxidative stress, as determined by several parameters. On the other hand a direct and immediate effect of PS-CO on atherosclerotic lesions was not detected. This may be due to the fact that the mice were already 8 weeks old at the start of the study and the lesions may already have been irreversible. In studies where a size reduction of the atherogenic lesions was observed, younger mice were used (*18*).

In conclusion, we have presented evidence that PS-CO supplement, which exerted the effects of its components on blood lipid profile and lipid peroxidation, possesses considerable antiatherogenic capacities and thus has an advantage over the standard formulations of PS. Therefore, our study presents substantial new information in the field of dietary supplementation with plant sterols, which can be beneficial for atherosclerotic patients and individuals susceptible to oxidative stress in general.

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