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David M. Guderian Jr.
University of Nebraska–Lincoln

Heather E. Rasmussen
University of Nebraska–Lincoln, heather.rasmussen@unl.edu

Curtis A. Wray
University of Nebraska–Lincoln

Patrick Dussault
University of Nebraska-Lincoln, pdussault1@unl.edu

Timothy P. Carr
University of Nebraska-Lincoln, tcarr2@unl.edu

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Cholesterol-lowering properties of plant sterols esterified with beef tallow fatty acids in hamsters

David M. Guderian Jr.,[1] Heather E. Rasmussen,[1] Curtis A. Wray,[2] Patrick H. Dussault,[2] and Timothy P. Carr [1]

1. Department of Nutrition and Health Sciences, University of Nebraska–Lincoln, Lincoln, NE 68583-0806, USA
2. Department of Chemistry, University of Nebraska–Lincoln, Lincoln, NE 68583-0806, USA

Corresponding author — T. P. Carr, tel 402 472-7940, fax 402 472-1587, email tcarr2@unl.edu

Abstract
Plant sterol esters (PSE) reduce plasma low-density lipoprotein cholesterol concentration by inhibiting cholesterol absorption. Previous work in our laboratory indicated that PSE containing stearic acid (SA), compared to linoleic acid, were significantly more effective at reducing cholesterol absorption. We tested the cholesterol lowering properties of PSE containing fatty acids from beef tallow, a natural and abundant source of SA. Male hamsters were fed diets containing 0.0%, 0.5%, 1.0%, or 5.0% beef tallow PSE for 4 weeks. Dose-dependent reductions ($P < .05$) were observed in cholesterol absorption, liver esterified cholesterol, and plasma nonhigh-density lipoprotein cholesterol concentration; hamsters fed 5.0% PSE exhibited reductions of 56%, 98%, and 38%, respectively, compared to controls. Concurrent increases ($P < .05$) in fecal sterol excretion were also observed. In a second hamster study, the cholesterol-lowering properties of beef tallow PSE were compared to hamsters fed molar equivalents of free plant sterols (PS) and free SA. Beef tallow PSE were significantly more effective at reducing liver and plasma cholesterol concentrations than PS and SA consumed individually. Whether PS and SA act through independent or synergistic mechanisms will require further research, although the present findings support the use of dietary PSE containing beef tallow fatty acids in the management of hypercholesterolemia.

Keywords: cholesterol absorption, plant sterols, stearic acid, beef tallow, hamsters

1. Introduction

Coronary heart disease (CHD) continues to be a major cause of death in the United States and other developed countries. Elevated plasma low-density lipoprotein (LDL) cholesterol concentration is a primary risk factor for CHD; thus, treatment strategies for CHD often involve drug therapy as well as dietary and other lifestyle changes that reduce plasma LDL cholesterol concentration [1].

There is particular interest among researchers and consumers in natural bioactive substances that lower plasma LDL cholesterol concentration. Clinical studies have repeatedly shown that plant sterols (PS), when consumed at 1 to 3 g/d, significantly reduce plasma LDL cholesterol concentration [2, 3]. Plant sterols can

also be used as a secondary therapy; the combination of PS and statin medication is more effective than doubling the statin dose [3]. Accordingly, the National Cholesterol Education Program [4] recommends 2 g/d of PS or stanol esters as a therapeutic option for reducing plasma LDL cholesterol concentration.

Plant sterols elicit their cholesterol lowering response by interfering with intestinal absorption of cholesterol [5]. However, as high melting solids with limited solubility in both water and oil, pure PS have yielded inconsistent results in animal and human studies [6]. Mattson and coworkers [7, 8] discovered that esterifying PS with long-chain fatty acids increased their solubility in oil from about 2% to more than 20% and that esterification did not impair their ability to inhibit cholesterol absorption. Commercial synthesis of plant sterol esters (PSE) uses common vegetable oils, such as canola, soybean, and sunflower, as the fatty acid source.

The extent to which the fatty acid moiety of PSE influences cholesterol absorption is vastly understudied. We recently discovered that hamsters consuming PSE enriched with stearic acid (SA), compared to linoleic acid, had significant reductions in both cholesterol absorption and plasma nonhigh-density lipoprotein (non-HDL) cholesterol concentration [9]. We now report the results of 2 hamster feeding studies designed to further elucidate the role of the fatty acid moiety. Study 1 examined the dose response of PSE made with beef tallow fatty acids—an abundant source of SA—on plasma and liver lipid concentrations, cholesterol absorption, and sterol excretion. Study 2 compared the effects of beef tallow PSE vs consuming equivalent amounts of free PS and free SA.

2. Methods and Materials

2.1. Animals and diets

Two studies were conducted using male F_1B Syrian hamsters (BioBreeders, Watertown, Mass) weighing approximately 90 to 100 g. Upon arrival, the hamsters were randomly divided into treatment groups and given free access to food and water throughout both 4-week studies. Hamsters were housed individually in polycarbonate cages with sawdust bedding and kept in a humidity-controlled room at 25°C, using a 12-hour light/dark cycle for the duration of the study. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

In study 1, hamsters were fed a control diet devoid of PSE or diets containing 5, 10, or 50 g/kg PSE esterified with fatty acids from beef tallow (Table 1). Cornstarch was adjusted to accommodate the addition of PSE. In study 2, hamsters were fed a control diet devoid of PSE (0% PSE) or diets containing 50 g/kg PS esterified with fatty acids from beef tallow (5% PSE) or 30 g/kg free PS plus 20 g/kg free SA (3% PS + 2% SA) (Table 2). The control diet in study 2 contained 20 g/kg beef tallow, which acted as a control for the 20 g/kg of fatty acid consumed as a free moiety (3% PS + 2% SA diet) or as part of the PSE compound (5% PSE diet). The control diet also contained 30 g/kg more cellulose, which served as a nonabsorbable substitute for the 30 g/kg PS consumed as a free moiety or as PSE. The dietary protein source was lean ground beef purchased at a local grocery store and freeze-dried at the Food Processing Center at the University of Nebraska. The freeze-dried beef was pulverized and sifted to provide a homogenous powder. The composition of freeze-dried beef for protein, fat, and ash was 64, 32, and 3 g/100 g, respectively. A high content of beef was used in all diets to approximate the macronutrient distribution of a high-protein, low-carbohydrate diet (eg, the Atkins diet), in which the primary protein source is animal protein. The energy distribution of protein, carbohydrate, and fat in the control diets was 32%, 41%, and 28% in study 1, and 34%, 37%, and 29% in study 2. The AIN-93 mineral mix, vitamin mix, and beef tallow were purchased from Dyets, Inc (Bethlehem, Pa). Ingredients were combined and mechanically mixed; the beef tallow was first melted at low temperature before being added to the control diet in study 2. Diets were stored at −20°C.

Table 1. Composition of hamster diets containing PS esterified with fatty acids from beef tallow (PSE), study 1

	0.0% PSE (g/kg)	0.5% PSE (g/kg)	1.0% PSE (g/kg)	5.0% PSE (g/kg)
Ground beef[a]	500	500	500	500
Cornstarch	255	250	245	205
Sucrose	100	100	100	100
PSE	0	5	10	50
Fiber[b]	50	50	50	50
Soybean oil	50	50	50	50
AIN-93 mineral mix[c]	35	35	35	35
AIN-93 vitamin mix[c]	10	10	10	10

a. Ninety percent lean, freeze-dried.
b. Solka-Floc 200 cellulose powder (International Fiber Corp., North Tonawanda, NY).
c. Reeves et al [10].

Table 2. Composition of hamster diets containing PS esterified with fatty acids from beef tallow (PSE) or free PS and free SA, study 2

	0% PSE (g/kg)	5% PSE (g/kg)	3% PS + 2% SA (g/kg)
Ground beef[a]	500	500	500
Cornstarch	305	305	305
Sucrose	50	50	50
PSE	–	50	–
Free PS	–	–	30
Free SA	–	–	20
Fiber[b]	80	50	50
Beef tallow	20	–	–
AIN-93 mineral mix[c]	35	35	35
AIN-93 vitamin mix[c]	10	10	10

a. Ninety percent lean, freeze-dried.
b. Solka-Floc 200 cellulose powder.
c. Reeves et al [10].

Table 3. Plasma cholesterol, liver weight, and liver lipid concentration in hamsters fed diets containing PS esterified with fatty acids from beef tallow (PSE), study 1

	0.0% PSE	0.5% PSE	1.0% PSE	5.0% PSE
Non-HDL cholesterol (mmol/L)	1.68 ± 0.15^{b}	1.65 ± 0.13^{b}	1.69 ± 0.09^{b}	1.05 ± 0.09^{a}
HDL cholesterol (mmol/L)	1.89 ± 0.10^{b}	1.58 ± 0.07^{a}	1.39 ± 0.04^{a}	1.53 ± 0.07^{a}
Liver weight (g/100 g body wt)	4.35 ± 0.06^{bc}	4.50 ± 0.08^{c}	4.18 ± 0.09^{ab}	4.00 ± 0.06^{a}
Liver free cholesterol (μmol/g)	9.67 ± 0.73^{b}	6.62 ± 0.16^{a}	6.21 ± 0.45^{a}	5.03 ± 0.06^{a}
Liver esterified cholesterol (μmol/g)	8.07 ± 1.38^{c}	1.41 ± 0.25^{bc}	0.72 ± 0.15^{ab}	0.18 ± 0.04^{a}
Liver phospholipid (μmol/g)	24.2 ± 0.4	23.3 ± 0.7	23.2 ± 1.2	22.4 ± 0.5

Means ± SEM (n = 11-12). Means within the same row having different superscripts are significantly different ($P < .05$).

Table 4. Cholesterol absorption and fecal sterol excretion in hamsters fed diets containing PS esterified with fatty acids from beef tallow (PSE), study 1

	0.0% PSE	0.5% PSE	1.0% PSE	5.0% PSE
Cholesterol absorption (%)	61.4 ± 1.9^{c}	62.0 ± 3.3^{c}	45.7 ± 3.0^{b}	26.9 ± 2.2^{a}
Fecal neutral sterol excretion (μmol/[d 100 g body wt])	3.82 ± 0.42^{a}	6.78 ± 0.14^{b}	7.46 ± 0.43^{b}	15.99 ± 0.29^{c}
Fecal bile acid excretion (μmol/[d 100 g body wt])	2.63 ± 0.12^{a}	3.40 ± 0.26^{bc}	3.60 ± 0.09^{c}	2.88 ± 0.16^{ab}
Fecal total sterol excretion (μmol/[d 100 g body wt])	6.45 ± 0.43^{a}	10.18 ± 0.33^{b}	11.06 ± 0.48^{b}	18.89 ± 0.40^{c}

Means ± SEM (n = 11-12). Means within the same row having different superscripts are significantly different ($P < .05$).

2.2. Plant sterol ester preparation

Unesterified (free) sterols from soybeans were obtained from Archer Daniels Midland Company (Decatur, Ill) and were composed of approximately 48% sitosterol, 27% campesterol, and 21% stigmasterol as determined by gas chromatography using a DB-5 capillary column (Agilent, Wilmington, Del). Before esterification, free fatty acids were prepared from the beef tallow triglycerides, then esterified to PS as previously described [9].

2.3. Experimental procedures

Body weight and food intake were recorded weekly. Cholesterol absorption efficiency was determined during the third week, and feces were collected during the fourth week to quantify fecal neutral steroid and bile acid excretion. Food was removed on day 28, and the hamsters were euthanized on day 29 by carbon dioxide asphyxiation. The abdomen and thorax were opened by incision, and blood was collected by cardiac puncture. Plasma was obtained by centrifuging for 30 minutes at 4°C at 2000 × *g*. The liver was perfused with saline via the hepatic portal vein to remove residual blood, and the liver was excised, weighed, and immediately frozen in liquid nitrogen.

Cholesterol absorption and fecal neutral steroids were measured as previously described [11]. Fecal bile acids were measured enzymatically (Wako Chemicals, Richmond, Va) after extraction and solubilization in Triton X-100 [12]. Liver lipids were determined as previously described [12], using enzymatic reagents for total cholesterol (Roche Diagnostics, Indianapolis, Ind), free cholesterol (Wako), and phospholipids (Wako). Liver esterified cholesterol was calculated as the difference between total and free cholesterol. Plasma total cholesterol was measured enzymatically (Roche); HDL cholesterol was quantified after precipitating apoB-containing lipoproteins (Precipitating Reagent 1335-250; Thermo Electron Corp, Louisville, Colo). Non-HDL cholesterol concentration was calculated as the difference between total and HDL cholesterol.

2.4. Statistical analyses

All data are expressed as means ± SEM. Treatment differences were determined by 1-way analysis of variance, followed by the Tukey multiple comparison procedure to identify differences in treatment means. Analysis of variance on ranks was used for liver esterified cholesterol concentration because of unequal variance. Differences were considered significant at $P < .05$. Data were analyzed using SigmaStat 3.0 (SPSS, Chicago, Ill).

3. Results

3.1. Study 1

Body weight gain and food intake were not significantly different among treatment groups. The overall mean body weight at the end of the study was 123 ± 1 g, and food intake was 6.3 ± 0.1 g/d.

Plasma non-HDL cholesterol concentration was significantly reduced about 37% in hamsters fed 5.0% PSE compared to other treatments (Table 3). Non-HDL cholesterol in hamsters fed 0.5% PSE and 1.0% PSE was not significantly different than controls, indicating that a higher dose PSE was required to elicit a significant reduction in non-HDL cholesterol. Plasma HDL cholesterol concentration was highest in the control group, but was not significantly different among hamsters fed PSE. Liver free cholesterol concentration was significantly lower in hamsters fed PSE at all levels compared to control (0.0% PSE). Liver esterified cholesterol was incrementally reduced as dietary PSE was increased, with the lowest concentration observed in hamsters fed 5.0% PSE.

Table 5. Plasma cholesterol, liver weight, and liver lipid concentration in hamsters fed diets containing PS esterified with fatty acids from beef tallow (PSE) or free PS and free SA, study 2

	0% PSE	5% PSE	3% PS + 2% SA
Non-HDL cholesterol (mmol/L)	2.79 ± 0.13[c]	0.59 ± 0.05[a]	1.34 ± 0.33[b]
HDL cholesterol (mmol/L)	1.78 ± 0.07[b]	1.36 ± 0.05[a]	1.31 ± 0.14[a]
Liver weight (g/100 g body wt)	3.40 ± 0.10[b]	3.04 ± 0.06[ab]	2.97 ± 0.13[a]
Liver free cholesterol (μmol/g)	8.34 ± 0.17[c]	4.57 ± 0.12[a]	5.75 ± 0.29[b]
Liver esterified cholesterol (μmol/g)	27.00 ± 1.80[b]	0.85 ± 0.09[a]	1.88 ± 0.26[a]
Liver phospholipid (μmol/g)	24.0 ± 0.5	21.3 ± 0.5	24.0 ± 2.6

Means ± SEM (n = 8). Means within the same row having different superscripts are significantly different ($P < .05$).

Table 6. Fecal sterol excretion in hamsters fed diets containing PS esterified with fatty acids from beef tallow (PSE) or free PS and free SA, Study 2

	0% PSE	5% PSE	3% PS + 2% SA
Fecal neutral sterol excretion (μmol/[d 100 g body wt])	3.88 ± 0.13[a]	14.90 ± 0.75[b]	13.03 ± 0.98[b]
Fecal bile acid excretion (μmol/[d 100 g body wt])	1.91 ± 0.17[b]	1.12 ± 0.27[a]	1.09 ± 0.08[a]
Fecal total sterol excretion (μmol/[d 100 g body wt])	5.79 ± 0.27[a]	16.02 ± 0.88[b]	14.12 ± 1.00[b]

Means ± SEM (n = 8). Means within the same row having different superscripts are significantly different ($P < .05$).

The magnitude of change was much greater for liver esterified cholesterol (mean range, 0.18-8.07 μmol/g) than for liver free cholesterol (5.03-9.67 μmol/g). No significant differences were found in liver phospholipid concentration among any of the treatment groups.

Cholesterol absorption was significantly reduced in hamsters fed 1.0% PSE and 5.0% PSE compared to controls (Table 4). Consistent with reduced cholesterol absorption, fecal neutral sterol excretion was increased in hamsters fed PSE; fecal neutral sterol excretion was about 4 times greater in the 5.0% PSE group compared to controls (0.0% PSE). Fecal neutral sterol excretion in hamsters fed 0.5% PSE and 1.0% PSE was about 2 times greater compared to controls. Fecal bile acid excretion was also affected by dietary PSE, although bile acid output was quantitatively less then neutral sterol excretion. When fecal bile acid and neutral sterol were combined, total sterol excretion was significantly increased in all treatment groups relative to controls, and was directly correlated with PSE intake ($r = 0.936$, $P < .001$).

3.2. Study 2

Body weight gain and food intake were not significantly different among treatment groups. The overall mean body weight at the end of the study was 117 ± 1 g, and food intake was 6.8 ± 0.1 g/d.

Plasma non-HDL cholesterol concentration in hamsters fed the 5% PSE diet was significantly reduced by 79% compared to controls (Table 5). Non-HDL cholesterol concentration in the 3% PS + 2% SA group was also reduced ($P < .05$), but to a lesser extent (52%). Plasma HDL cholesterol concentration was significantly lower in hamsters fed 5% PSE and 3% PS + 2% SA, although the magnitude of reduction (24%-26% compared to controls) was less than the changes in non-HDL cholesterol. Significant reductions in liver free and esterified cholesterol were observed in the 5% PSE and 3% PS + 2% SA groups compared to controls; esterified cholesterol was reduced 93% to 97%. Reductions in liver weight were also observed in hamsters fed 5% PSE and 3% PS + 2% SA. Liver weight was directly correlated with liver esterified cholesterol concentration ($r = 0.82$, $P < .001$) when all data were plotted, suggesting that changes in liver weight were mainly due to the esterified cholesterol content. Liver phospholipid concentration was not significantly different in any of the treatments.

Fecal neutral sterol excretion in hamsters fed the 5% PSE and 3% PS + 2% SA diets was significantly increased compared to controls (Table 6). Although fecal bile acid excretion was significantly lower in hamster fed 5% PSE and 3% PS + 2% SA, bile acid excretion represented less than 8% of total sterol excretion in these groups. Consequently, fecal total sterol excretion was increased 177% and 144% in the 5% PSE and 3% PS + 2% SA groups, respectively.

4. Discussion

Consumption of PSE is known to reduce plasma LDL cholesterol concentration by inhibiting intestinal cholesterol absorption [3, 5]. Commercially available PSE are usually prepared by esterifying free sterols to fatty acids from edible plant oils (eg, canola, soybean, and sunflower), thus resulting in sterol esters containing high proportions of unsaturated fatty acids. We recently reported that PSE enriched with SA, compared to linoleic acid, were significantly more effective in reducing intestinal cholesterol absorption and plasma non-HDL and liver cholesterol concentration in hamsters [9]. This research extends these findings in hamsters by testing the dose response of PSE containing fatty acids from beef tallow, a natural and abundant source of SA. Our findings demonstrate dose-dependent reductions in choles-

terol absorption and concurrent increases in fecal sterol excretion, thus resulting in lower plasma and liver cholesterol concentrations.

Total sterol excretion was directly correlated with PSE intake ($r = 0.936$, $P < .001$). In addition, liver cholesterol concentration was also correlated with PSE intake ($r = -0.591$, $P < .001$), indicating that PSE promoted an increased flux of liver cholesterol into the biliary pathway for excretion. However, plasma non-HDL cholesterol was not linearly related with PSE intake and was significantly reduced only in hamsters fed 5.0% PSE. These observations suggest that reductions in liver cholesterol may have been compensated for by increased hepatic cholesterol synthesis, thus maintaining hepatic lipoprotein secretion into plasma. Our previous study [9] demonstrated that, depending on the fatty acid composition of PSE, significant changes in both liver cholesterol concentration and fecal sterol excretion can occur without altering the non-HDL cholesterol concentration. We speculate that the limits of this compensatory response were exceeded only at the highest level of dietary PSE used in the present study.

Clinical studies have established that PS intakes of 1 to 3 g/d reduce plasma LDL cholesterol concentration up to 15% and that higher intakes have little added influence [2, 3]. Most human studies included PSE made with fatty acids from unsaturated plant oils, although the contribution of the fatty acid moiety to the cholesterol-lowering ability of PSE has been vastly understudied. We recently reported that PSE containing 97% SA or beef tallow fatty acids (19% SA, 2% linoleic acid) reduced plasma non-HDL cholesterol to a significantly greater extent than PSE containing soybean oil fatty acids (3% SA, 60% linoleic acid) [9]. Our previous results also indicated that PSE enriched with SA reduced cholesterol absorption and liver cholesterol concentration, and increased fecal cholesterol excretion to a greater extent than PSE deficient in SA [9]. Only 1 other study has directly compared PSE preparations differing in their fatty acid composition. AbuMweis et al [13] reported that human beings consuming PSE made with fish oil or sunflower oil fatty acids had no significant differences in plasma triglyceride, LDL, or HDL cholesterol concentration. Both PSE preparations contained a high proportion of polyunsaturated fatty acids and relatively little SA [13].

Beef tallow is a rich source of SA, containing about 19% SA by weight [14]. Studies examining plasma LDL cholesterol concentration have demonstrated cholesterol-lowering properties of dietary beef tallow compared to soybean oil [15], coconut oil [16], butter fat [17], and palm and palm kernel oil [18]. Studies have also shown beef tallow to elicit equivalent or "neutral" effects on LDL cholesterol levels compared to safflower oil [16], high-oleic sunflower oil [19], and chicken fat [20]. Newbold [21] reported that hypercholesterolemic patients who consumed diets in which most of the calories came from beef fat (and no sucrose, milk, or grains) had significant reductions in serum triglyceride and total cholesterol levels (35% and 28% decrease, respectively). The pioneering studies by Keys et al [22] and Hegsted et al [23] indicated that SA, a major component of beef tallow, is distinctly different than other saturated fatty acids (ie, palmitic, myristic, and lauric) because SA does not elevate serum cholesterol when consumed. Numerous studies have since confirmed the cholesterol-lowering or neutral effects of SA compared to other dietary fatty acids [24, 25]. Although current dietary recommendations encourage individuals to limit saturated fat intake [4, 26], the American Heart Association acknowledges that SA is unique among the common dietary saturated fatty acids because of its cholesterol-lowering or neutral properties, but that "there is no simple means of incorporating this information into dietary guidelines" [26].

Stearic acid elicits its hypocholesterolemic effects by reducing intestinal cholesterol absorption [11, 27–30], accompanied by increased fecal cholesterol excretion [11, 31, 32]. Plant sterols also inhibit cholesterol absorption [33–35]. Because the extent of PSE hydrolysis in the small intestine remains uncertain [36, 37], we included a treatment group in the present study in which hamsters were fed free PS and free SA compared to PSE (study 2). Compared to PSE, consumption of a combination of free sterols and free SA produced a much smaller decrease in non-HDL cholesterol concentration, suggesting that the observed cholesterol lowering may be the result of multiple mechanisms involving intact PSE, free PS, and/or free SA. We recently reported that free PS have the ability to displace cholesterol from micelles in vitro [38], suggesting an intralumenal mechanism that would likely decrease cholesterol solubility and absorption in vivo. The extent to which PSE incorporate into micelles has not been reported. We also reported that intestinal FHs 74 Int cells incubated with SA, compared to palmitic acid, had significantly reduced gene expression of the cholesterol transporter Niemann-Pick C1 Like 1, indicating intracellular regulation of cholesterol absorption by SA [39]. Whether PS and SA act through independent or synergistic mechanisms will require further study.

In conclusion, our results indicate that consumption of PSE containing beef tallow fatty acids decreases cholesterol absorption, decreases liver and plasma cholesterol concentration, and increases fecal cholesterol excretion. Consumption of PSE was more effective at reducing plasma non-HDL cholesterol concentration compared to molar equivalents of free PS and free SA. Our findings support the use of dietary PSE containing beef tallow fatty acids in the management of hypercholesterolemia.

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