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Complementary Cholesterol-Lowering Response of a Phytosterol/ α -Lipoic Acid Combination in Obese Zucker Rats

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Abstract

To investigate the cholesterol-lowering effectiveness of a phytosterol/ α -lipoic acid (PS/ α LA) therapy, thirty-two male Zucker rats were randomly assigned to 1 of 4 diets for 30 days: (i) high fat diet (HF, 40% energy from fat); (ii) HF diet supplemented with 3% phytosterols; (iii) HF diet supplemented with 0.25% α LA; or (iv) HF diet supplemented with PS (3%) and α LA (0.25%, PS/ α LA). Compared with the HF diet, combination PS/ α LA proved more effective in reducing non-HDL cholesterol (–55%) than either the PS (–24%) or the α LA (–25%) therapies alone. PS supplementation did not affect LDL particle number, however, α LA supplementation reduced LDL particle number when supplemented alone (–47%) or in combination with PS (–54%). Compared with the HF-fed animals, evidence of increased HDL-particle number was evident in all treatment groups to a similar extent (21–22%). PS-mediated interruption of intestinal cholesterol absorption was evident by increased fecal cholesterol loss (52%) and compensatory increase in HMG-CoA reductase mRNA (1.6 fold of HF), however, α LA supplementation did not affect fecal cholesterol loss. Hepatic mRNA and protein expression patterns suggested that α LA modulated multiple aspects of cholesterol homeostasis including reduced synthesis (HMG-CoA reductase mRNA, 0.7 fold of HF), reduced bile acid synthesis (CYP7a1 expression, 0.17 of HF), and increased cholesterol clearance (reduced PCSK9 mRNA, 0.5 fold of HF; increased LDLr protein, 2 fold of HF). Taken together, this data suggests that PS and α LA work through unique and complementary mechanisms to provide a superior and more comprehensive cholesterol lowering response than either therapy alone.

Keywords

Phytosterols; alpha lipoic acid; lipids; lipoproteins; high fat diet

Introduction

Despite recent improvements in blood cholesterol through effective pharmaceutical management of LDL-C, hypercholesterolemia continues to be a high priority health concern with a prevalence of ~40% in the United States [1].

Phytosterols (PS) are plant-based sterols that reduce cholesterol absorption by competing with dietary and endogenous cholesterol for incorporation into bile salt micelles within the intestinal lumen. PS are well established as ‘natural’ cholesterol-lowering agents with consistent LDL-cholesterol reductions in numerous well-controlled clinical interventions [2] and pre-clinical studies that have defined the molecular mechanisms involved [3]. Intake of PS from naturally occurring plant-based food sources, from fortified foods, or supplements are associated with LDL-C reductions in the range of 10–16% [4,5]. Although PS are effective in reducing LDL-cholesterol, there is controversy as to whether PS supplementation contributes to the development or regression of arterial lesions [6,7]. Alternatively, α -lipoic acid (α LA), a naturally occurring mitochondrial cofactor that has been traditionally recognized for its antioxidant properties, has only recently been touted as an emerging therapy to improve dyslipidemia [8–10]. The total body pool of α LA from synthesis and ingestion of dietary sources including muscle, heart, kidney, and liver is low; therefore, the purported health effects of α LA are only observed following dietary supplementation, which have been investigated in the broad range of 600–1800 mg/d [11]. Data from our laboratory has previously shown that reductions in LDL-cholesterol and LDL-particle number following α LA supplementation were associated with lower circulating concentrations of proprotein convertase subtilisin/kexin type 9 (PCSK9), a primary regulator of LDLr protein turnover [12]. α LA supplementation was recently shown to reduce atherosclerotic plaque formation by reducing aortic lipid and inflammatory cell infiltration in Watanabe heritable hyperlipidemic rabbits in the absence of any change in plasma lipids [13]. However, there is very little additional published data which explores how α LA may regulate cholesterol metabolism.

Combination nutraceutical therapies may offer a feasible approach to effectively manage blood cholesterol concentrations in individuals who do not require immediate pharmaceutical intervention or who respond sub-optimally to standard drug-based interventions [14]. The combination of lipid-lowering nutraceuticals offers a flexible approach to cholesterol management in that they can be specifically formulated to target one or multiple metabolic pathways that regulate cholesterol homeostasis. For example, a nutraceutical blend containing bioactive compounds that reduce intestinal cholesterol absorption and simultaneously stimulate hepatic cholesterol clearance or bile acid synthesis could offer LDL-C lowering potential beyond that observed with any of the components in isolation. Although a PS/ α LA combination has not been examined, both of these nutraceuticals have been successfully incorporated into other lipid-lowering combinations previously. Effective cholesterol-lowering responses have been observed when combining phytosterols with a multi-vitamin mixture [15], probiotics [16], dietary fiber [17], and drugs including statins [18] and ezetimibe [19]. Similarly, combining alpha lipoic acid with eicosapentaenoic acid proved more effective than either therapy alone in ameliorating serum

lipids and cardiac antioxidant defense abnormalities brought on through high cholesterol feeding [20].

Therefore, the primary objective of this study was to determine the effectiveness of combined PS/ α LA supplementation in reducing blood cholesterol concentrations in a Zucker rat model challenged by both a genetic predisposition and dietary induction toward obesity and dyslipidemia. We further aimed to characterize the independent and interactive molecular mechanisms by which both PS and α LA modulated cholesterol balance – focusing specifically on the mRNA/protein expression patterns of important regulators of cholesterol homeostasis.

Experimental Approach

Animals and diets

Thirty-two 160 g male Zucker rats (Harlan Laboratories, Indianapolis, Indiana) were brought to the Animal Care Facility at the University at Buffalo. Rats were housed individually in cages with shavings in a temperature-controlled room (20°C) with a 12h light/dark cycle and had free access to water. At the initiation of the experiment, rats were randomly assigned to 1 of 4 diets (n=8/group) for 30 days according to Table 1: (i) high fat diet (HF, AIN 76A Western Diet, 40% energy from fat); (ii) HF diet supplemented with 3% phytosterols (HF-PS, Vegapure® 95 E, BASF, Florham Park, NJ); (iii) HF diet supplemented with 0.25% α LA (HF- α LA, R enantiomer, α LA R+ SAP, Nutritional Fundamentals for Health, Quebec, Canada); or (iv) HF diet supplemented with PS (3%) and α LA (0.25%, PS/ α LA). Diet ingredients (Dyets, Bethlehem, PA) were mixed on site in two separate batches and stored at 4°C for the duration of the experiment (Figure 1). Rats were fed daily and any leftover feed was discarded. Dietary spillage was included in daily feed intake estimates. As α LA possesses anorectic properties [21,22], all animals were pair-fed to ensure similar feed and caloric intake between the groups. Body weights were obtained 3 times per week. The animals used in this experiment were cared for in accordance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC). All procedures were reviewed and approved by the Animal Care Committee at the University at Buffalo (protocol # PTE25061N).

Sample collection and processing

Following the 30 day feeding period, rats were anesthetized with isoflurane for blood and tissue collection. Fasting (14 h) blood (serum and EDTA plasma) was collected by cardiac puncture and processed as previously described [23] and stored at –80°C. The liver was quickly excised, rinsed in chilled saline (pH 7.4, 154 mM containing 0.1 mM phenylmethylsulfonyl fluoride) and flash frozen in liquid nitrogen. All tissues were stored at –80°C until further processing and analyses.

Blood biochemistry

Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and non-HDL cholesterol were determined by automated enzymatic kits (Sekisui Diagnostics, Lexington, MA, USA) on an ABX Pentra 400 autoanalyzer (Horiba Instruments Inc., Irvine CA, USA).

Direct assessment of lipoprotein particle number and size was conducted by nuclear magnetic resonance spectroscopy (Liposcience, Raleigh, NC) [24].

Hepatic lipids

Hepatic TG were analyzed with a commercial kit (ab65336, Abcam, Cambridge, MA, USA). Hepatic TG were extracted by homogenization in aqueous Triton-X buffer (2%) and measured at OD_{570nm} according to manufacturer instructions. For analysis of hepatic fatty acids, approximately 0.5 g of pulverized liver was spiked with heptadecanoic acid (C17:0) as internal standard. Total lipids were isolated from liver tissue with a modified Dole mixture (3 heptane:12 propanol:3 DDH₂O, vol:vol) followed by extraction with heptane:DDH₂O (3:1 vol:vol) [25]. Fatty acid extracts were methylated with methanolic boron trifluoride (Sigma Aldrich, St. Louis, MO). Fatty acid methyl esters were separated using a Supelcowax 10 column (30 m × 0.25 mm with 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) in a Shimadzu GC-17A gas chromatograph fitted with a flame ionization detector. Relative hepatic fatty acid content was calculated by using individual FA peak area relative to the total area and expressed as the percentage of total fatty acids.

Hepatic and fecal cholesterol was extracted and analyzed according to our previously published procedures [23,26]. Approximately 0.5 g of pulverized liver and 50mg of oven-dried feces was spiked with α-cholestane as internal standard and saponified in freshly prepared KOH–methanol at 100°C for 1 h. The non-saponifiable sterol fraction was extracted with petroleum diethyl ether and dried under N₂ gas. Sterol fractions were analyzed on the same GC system using a SAC-5 capillary column (30m × 0.25mm × 0.25 μm, Supelco, Bellefonte, CA, USA).

RNA preparation and real-time RT-PCR

Total RNA was isolated from whole liver tissue using TRIzol reagent (Invitrogen Inc., Grand Island, NY). RNA concentration and integrity was determined with spectrophotometry (260 nm) and agarose gel electrophoresis, respectively. RNA preparation and real-time RT-PCR was conducted using a one-step QuantiFast SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA) on a Biorad MyiQ real time PCR system according to previously established protocols [27]. Gene expression was analyzed using the 2^{−(delta delta Ct)} method [28]. Sequences of sense and antisense primers for target and housekeeping genes were based on previously published reports for β-actin [29], acetyl-coA carboxylase (ACC) [30], fatty acid synthase (FAS) [31], 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-COAr) [32], proprotein convertase subtilisin/kexin type 9 (PCSK9) [33], and cholesterol 7 α-hydroxylase (CYP7a1) [34].

Immunoblot analysis

Immunoblots were prepared as previously described [27]. Hepatic crude membrane for immunoblot analysis of LDLr was prepared according to previous reported procedures [35]. Briefly, 200 mg of pulverized, frozen liver was homogenized for 2 min with 1 mL of ice cold Homogenization Buffer (10 mM Tris-HCl, 250 mM sucrose, 2 μg/mL of PMSF, pH 7.5). The homogenate was then centrifuged at 1,000 × g for 10 min at 4°C to pellet undisturbed cells and nuclei. The collected supernatant was then centrifuged at 100,000 × g

for 1 hour at 4°C. The resulting pellet (containing the crude membrane fraction) was re-suspended in 500 µl of Re-suspension Buffer (80 mM NaCl, 2 mM of CaCl₂, 50 mM Tris-HCl, 1% Triton-X-100, 2 µg/mL of PMSF, pH 7.5) and stored at -80°C for future analysis. Immunoblots were prepared as previously described [27] using anti-LDLr (Novus Biologicals, NB110-57162) and β-actin as a housekeeping protein. Images were quantified using Image J (National Institutes of Health, Bethesda, Maryland).

Nuclear extracts for immunoblot of SREBP2 and LXR were prepared as follows. Tissue was homogenized in 10 volumes of CHAPS-containing buffer [40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM -glycerophosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamidine, 1 mM DTT, and Roche complete protease inhibitors (#04693116001, Roche, Indianapolis, IN)]. The resulting homogenate was clarified by a 1,000 × g for 5 minutes (at 4°C), and the pellet (nuclear fraction) was washed with CHAPS buffer three times, followed by a 1,000 × g centrifugation for 5 minutes (at 4°C), then resuspended in 60 µl of lysis buffer, and 8.3 µl of 5 M NaCl was added to lyse the nuclei. This mixture was rotated at 4°C for 1 hour and then centrifuged at 12,000 × g for 15 minutes (at 4°C). The subsequent supernatant contained the soluble nuclear-enriched fraction. A small aliquot of each fraction was taken for the determination of protein concentration for each sample. Then, equal volume of 2X sodium dodecyl sulfate loading buffer was added to each fraction for Western analysis. Samples were boiled for 5 minutes, then Western analysis was performed. Nuclear extracts were probed with commercial antibodies for, SREBP2 (ab30682, abcam), and LXR (3641-100, Biovision). Target proteins were normalized to β-actin and quantified using Image lab (version 4.1, Biorad Laboratories, Hercules, CA).

Statistical analyses

Data were analyzed with a general linear model ANOVA using experimental block as a fixed factor [36]. Multiple comparisons between treatment groups were analyzed with Tukey's post-hoc test. Data were analyzed with SPSS 16 for Mac (SPSS Inc, Chicago IL). Data are presented as mean ± SEM. Differences were considered significant at p = 0.05.

Results

Food intake and body weight

Daily feed intake between the groups was matched to allow us to examine metabolic responses to the diets independent of the anorectic effects typically observed with αLA supplementation (Table 2). HF-fed animals gained 121.58% of their starting body weight over the course of the 30 day feeding trial, however, αLA supplementation protected (p<0.05) against body weight gain, either alone (102.05%) or combined with PS (108.92%) (Table 2). As expected, PS supplementation had no effect on body weight gain (113.92%).

Blood, tissue, and fecal lipids

Compared with the HF diet, serum total-C, although not altered in the PS group, was reduced by αLA supplementation (-20%) and to a further extent in the PS/αLA group (-33%) (Figure 2a). Likewise, while both PS and αLA reduced non-HDL cholesterol to a

similar extent compared with the HF group (–23–25%), the combination of PS/αLA was more effective in lowering non-HDL cholesterol than either therapy alone (–55%) (Figure 2b). HDL-C was not influenced by PS supplementation ($p>0.05$) but was reduced in both the αLA and PS/αLA groups compared with the HF group (18–23%) (Figure 2c). LDL particle number was not affected by PS supplementation but was reduced ($p<0.05$) to a similar extent in the αLA and PS/αLA groups (47–54%) compared with the HF group (Figure 3a). LDL particle size was similar ($p>0.05$) between all groups (Figure 3b). Compared with the HF group, HDL particle number was increased ($p<0.05$) comparably in the PS, αLA and PS/αLA groups (21–22%), however, HDL particle size was not affected by any treatment (Figure 3c, d).

Hepatic cholesterol was reduced ($p<0.05$) similarly in the PS (–49%) and PS/αLA (54%) groups but not affected by LA-supplementation compared with the HF group (Figure 4a). Fecal cholesterol concentrations were increased to a comparable extent in the PS (+52%) and PS/αLA (48%) groups but not affected by αLA supplementation compared with the HF group (Figure 4b).

Hepatic TG was reduced ($p<0.05$) to a similar extent in the PS (–63%), αLA (–44%), and PS/αLA (–49%) group compared with the HF group (Figure 4c). The hepatic FA profile is presented in Table 3. PS supplementation increased ($p<0.05$) hepatic SAT FA (+15%), reduced n-6 PUFA (–61%) and the PUFA:SAT ratio (–83%) compared with the HF group. Conversely, αLA supplementation reduced ($p<0.05$) SAT (–18%), and increased ($p<0.05$) n-6 (+84%) and n-3 (+97%) PUFA, and the PUFA:SAT ratio (+118%) compared with the HF group. The combination group ($p<0.05$) demonstrated an increase in PUFA [both n-6 (+31%) and n-3 (+59%)] and increased PUFA:SAT ratio (+36%). The 16:0:18:2n-6 ratio, an indirect index of *de novo* lipogenesis [37], was increased ($p<0.05$) in the PS (+68%) group and reduced ($p<0.05$) by αLA supplementation (–61%), but not altered ($p<0.05$) in the PS/αLA group compared with the HF group.

Gene/protein expression patterns

Compared with the HF group, the rate-limiting enzyme in cholesterol synthesis, hepatic HMG-CoAr mRNA, was increased ($p<0.05$) in response to PS supplementation (1.6 fold of HF), but reduced ($p<0.05$) in the αLA-supplemented group (0.7 fold of HF). HMG-CoAr mRNA was not altered in the combination PS/αLA group compared with the HF group (Figure 5a).

CYP7A1 mRNA, the rate-limiting enzyme in bile acid synthesis, was reduced ($p<0.05$) in the αLA (0.17 of HF) and PS/αLA (0.29 of HF) groups compared with the HF control (Figure 5b). PS supplementation alone did not affect CYP7A1 mRNA expression. αLA, either alone or in combination with PS, reduced ($p<0.05$) FAS mRNA expression (0.18 and 0.23 fold of HF, respectively) and also independently lowered the expression of ACC (0.41 fold of HF), both rate-controlling enzymes in fatty acid synthesis (Figure 5c, d). PS supplementation increased ($p<0.05$) the mRNA expression of FAS compared with the HF group (2.7 fold) but did not affect ($p>0.05$) ACC mRNA expression (Figure 5c, d).

mRNA levels of PCSK9, a main regulator of LDLr protein turnover, was similarly reduced ($p<0.05$) in the α LA (0.6 fold of HF) and PS/ α LA (0.78 of HF) groups compared with the HF-fed animals but not affected ($p=0.08$) by PS supplementation (Figure 6a). Hepatic PCSK9 protein abundance was reduced in the α LA-supplemented group (0.5 fold of HF, $p<0.05$) but not affected ($p>0.05$) in the PS and PS/ α LA groups (Figure 6b). LDLr protein abundance was reduced ($p<0.05$) in the PS and PS/ α LA groups (0.32 and 0.43 fold of HF) but increased ($p<0.05$) by α LA supplementation (2 fold of HF, Figure 6b).

Discussion

Several novel findings regarding the cholesterol-lowering effects of a PS/ α LA combination were observed in this study: 1) Compared with the HF group, the PS/ α LA group resulted in greater reductions in total cholesterol (-33%) and non-HDL cholesterol (-55%) than the PS and α LA groups alone; 2) α LA supplementation complemented the cholesterol-lowering response of PS with additional reductions in LDL-particle number (-47%); 3) the cholesterol-lowering mechanisms of PS and α LA are unique and complementary with PS primarily reducing intestinal cholesterol absorption ($+52\%$ in fecal cholesterol) and α LA modulating several aspects of cholesterol metabolism including reduced cholesterol synthesis (0.7 fold change in HMG-CoAr mRNA), inhibition of hepatic PCSK9 expression (mRNA, 0.6 fold of HF; protein, 0.5 fold of HF), and enhancement of hepatic LDLr abundance (1.4 fold of HF).

The well-documented cholesterol-lowering effects of PS are mainly restricted to the total and LDL-C fraction, with little to no effect observed on HDL-C concentrations or lipoprotein particle number and size [38], although the latter has rarely been investigated. The advanced lipoprotein analyses provides important information regarding the atherogenicity of the blood lipid profile as lipoprotein particle size distribution and number can vary considerably even in individuals with similar LDL-C and HDL-C concentrations [39,40]. Although neither treatment altered HDL particle size, our results suggest that both PS and α LA independently increased HDL-particle number, a novel findings that has not been previously reported to our knowledge. Interestingly, combining PS and α LA together did not bolster this HDL-particle number raising effect, perhaps suggesting that both treatments work through a similar mechanism(s). Recent work suggests that HDL particle number may be a better predictor of cardiovascular risk compared with traditional assessment of HDL-C concentrations, possibly by reflecting reverse cholesterol transport capacity [39,41]. Therefore, future studies should be conducted to corroborate our findings of PS and α LA as potential HDL-particle raising therapies, particularly in at-risk human subjects.

In support of a previous report in humans [38], PS supplementation did not affect LDL particle number in the current study. Alternatively, the number of LDL particles was reduced in the α LA and PS/ α LA groups, suggesting that α LA may be an effective adjunct to PS therapy by providing additional lipoprotein modulating effects beyond that typically observed with PS alone. LDL particle number may be influenced through multiple mechanisms including lipase activity regulating the conversion of VLDL to LDL or through LDL clearance through the LDL receptor. Previous work from our lab [12] and others [42]

suggest that α LA may induce lipoprotein lipase activity, suggesting that the reduction in LDL particle number with α LA supplementation is not mediated through an interference with VLDL metabolism to LDL through lipase remodeling. Alternatively, the reduction in hepatic PCSK9 expression observed in the α LA-supplemented animals in the current study corresponds with an earlier report from our lab showing α LA-associated reductions in plasma PCSK9 concentrations [12]. Taken together, it appears likely that α LA may reduce LDL-particle number by reducing PCSK9-mediated turnover of the LDL receptor and thereby enhancing LDL-particle clearance.

Alternatively, our results suggest that the cholesterol-lowering effects of PS are not mediated through changes in PCSK9 expression and LDLr turnover. Instead, PS supplementation was associated with increased fecal cholesterol loss, reduced hepatic cholesterol concentrations and an increase in hepatic HMG-CoAr mRNA, all supportive of a primary reduction in intestinal cholesterol absorption and a secondary compensation in hepatic cholesterol synthesis as reported in previous investigations [26]. Alternatively, α LA-supplementation had no effect on fecal cholesterol loss, suggesting that its cholesterol-lowering action is not due to an interference with intestinal cholesterol absorption. We are not aware of any studies that have examined intestinal cholesterol metabolism in response to α LA, however, we previously detected no change in intestinal Niemann-Pick C1-Like 1 (NPC1L1) mRNA expression in α LA-supplemented Zucker rats (Rideout, unpublished observation). In contrast to the PS-fed group, α LA-supplemented animals demonstrated a reduction in hepatic HMG-CoAr mRNA expression that suggests that at least part of the cholesterol-lowering response in this group may be attributed to a reduction in cholesterol synthesis, as previously discussed [12]. Thirunavukkarasu et al. (2004) reported increased hepatic HMG-CoA reductase activity with high fructose fed Wistar rats that was normalized in response to intraperitoneal administration of α LA [42]. Although this appears to be the only other examination of the effect of α LA on HMG-CoA reductase, the results are difficult to compare given the vast study differences including model, administration (diet vs. injection), and endpoint (transcription vs. activity measurement).

PS supplementation did not affect hepatic CYP7a1 expression, a result supported by previous work examining CYP7a1 activity measurements following PS supplementation [43,44]. Nevertheless, a recent report suggests that gene variants in CYP7a1 may explain the heterogeneity to lipid responses to PS intakes by modulating the bile acid pool size and hence the efficacy of intestinal cholesterol absorption [45]. Alternatively, LA supplementation, when provided alone or in combination with PS, markedly reduced CYP7a1 expression compared with the HF group. Supporting this observation, a previous microarray transcriptional profiling study previously reported an α LA-induced reduction in CYP7a1 expression in young and old Fischer rats supplemented with 0.2% α LA [46]. Although such a reduction in CYP7a1 transcription could conceivably result in a significantly smaller bile acid pool size and effectively impair intestinal cholesterol absorption to lower blood cholesterol concentrations, as discussed above, our fecal cholesterol data does not support an inhibition of α LA on intestinal cholesterol absorption. Additional studies with direct quantification of cholesterol absorption and bile acid pool size in response to α LA supplementation are required.

Although our primary objective was to examine the effect of the PS/ α LA combination on enterohepatic cholesterol metabolism, we also assessed several indices of hepatic fat metabolism as both PS and LA have been shown to modulate fatty acid and triglyceride responses [23,47]. PS supplementation reduced hepatic TG concentrations and was associated with a shift in the hepatic FA profile toward increased saturated and reduced PUFA compared with the HF group. In a PS-supplemented C57BL/6 mouse model, we previously demonstrated hepatic TG-lowering responses that were linked to enhanced fecal fatty acid excretion with a compensatory increase in hepatic lipogenesis as measured directly with stable isotope tracers [23]. The increase in the ratio of 16:0/18:2n-6 in the current study (an indirect measure of hepatic *de novo* lipogenesis) along with enhanced expression of hepatic FAS in the PS-fed group, supports our previous observation. A compensatory increase in *de novo* lipogenesis may also explain the shift toward a saturated FA profile in the liver, as the saturated fatty acid palmitate is the end product of FAS activity. Alternatively, although the α LA group also displayed a reduction in hepatic TG content, the hepatic FA profile shifted in the opposite direction as the PS-fed animals with a reduction in saturated FA and an increase in long chain PUFA. Furthermore, with a reduction in the 16:0/18:2n-6 ratio and a reduced FAS and ACC mRNA expression, TG reductions in response to α LA are likely through a direct lowering of hepatic lipogenesis, as supported by previous work [48,49]. Given the opposite directions of FAS and ACC expression following PS and α LA supplementation, it may not be surprising that the TG lowering response in the combination group was not greater than either therapy alone.

In summary, combination PS and α LA results in greater blood cholesterol reductions than either therapy alone. Furthermore, as the cholesterol-lowering response of PS is typically restricted to the total and LDL-C fraction, adding α LA to PS complements the cholesterol-lowering action of PS by reducing LDL particle number. The cholesterol-lowering mechanisms of PS and α LA are unique and complementary with PS primarily reducing intestinal cholesterol absorption and α LA modulating several aspects of cholesterol metabolism that may include a reduction in cholesterol synthesis and an up-regulation of LDL-C clearance through modulation of PCSK9 metabolism.

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Figure 1.
Experimental design of feeding trial.

Figure 2. Blood lipid concentrations (mg/dl) in response to consumption of a high fat diet (HF) supplemented with phytosterols (PS), α-lipoic acid (αLA) or combination PS/αLA in Zucker rats. (a) total-cholesterol; (b) non-HDL cholesterol; and (c) HDL-cholesterol. ^{ab}Groups not sharing a superscript are significantly different ($p < 0.05$, $n = 8$ /group).

Figure 3.

Lipoprotein distribution pattern in response to consumption of a high fat diet (HF) supplemented with phytosterols (PS), α-lipoic acid (αLA) or combination PS/αLA in Zucker rats. (a) LDL particle number; (b) LDL particle size; (c) HDL particle number; and (d) HDL particle size. ^{ab}Groups not sharing a superscript are significantly different ($p < 0.05$, $n = 8/\text{group}$).

Figure 4. Hepatic and fecal lipids in response to consumption of a high fat diet (HF) supplemented with phytosterols (PS), α -lipoic acid (α LA) or combination PS/ α LA in Zucker rats. (a) Hepatic triglycerides (mg/g tissue); (b) hepatic cholesterol (mg/g tissue); and (c) fecal cholesterol (mg/g feces). ^{ab}Groups not sharing a superscript are significantly different ($p < 0.05$, $n = 8$ /group).

Figure 5. Hepatic mRNA expression of cholesterol and lipid regulatory genes in response to consumption of a high fat diet (HF) supplemented with phytosterols (PS), α-lipoic acid (αLA) or combination PS/αLA in Zucker rats. (a) HMG-CoA reductase; (b) CYP7a1; (c) FAS; and (d) ACC. ^{ab}Groups not sharing a superscript are significantly different ($p < 0.05$, $n = 8/\text{group}$).

Figure 6. Hepatic expression of PCSK9 [(a) mRNA and (b) protein] and LDLr protein expression (c) in Zucker rats fed a high fat diet (HF) supplemented with phytosterols (PS), α-lipoic acid (αLA) or combination PS/αLA. ^{ab}Groups not sharing a superscript are significantly different ($p < 0.05$, $n = 6$ /group).

Table 1

Diet formulation for Zucker rats

Ingredient	Diet ¹			
	HF	PS	α LA	PS/ α LA
Sucrose	32.12	32.12	32.12	32.12
Milk Fat	19.96	19.96	19.96	19.96
Casein	19.47	19.47	19.47	19.47
Maltodextrin	9.98	9.98	9.98	9.98
Phytosterol ²	-	3	-	3
α LA	-	-	0.25	0.25
Corn starch	4.99	4.99	4.99	4.99
Powdered Cellulose	4.99	4.99	4.74	4.74
AIN76-Mineral Mix	3.49	3.49	3.49	3.49
AIN76-Vitamin Mix	0.99	0.99	0.99	0.99
Corn Oil	3	0	3	0
Calcium Carbonate	0.39	0.39	0.39	0.39
DL-Methionine	0.29	0.29	0.29	0.29
Choline Bitartrate	0.19	0.19	0.19	0.19
Cholesterol	0.14	0.14	0.14	0.14
Ethoxyquin	0.004	0.004	0.004	0.004
Total (g)	100	100	100	100
Energy (kcal/g)	4.65	4.38	4.63	4.38
Energy (%):				
Fat	44.63	41.20	44.63	41.20
Carbohydrate	40.49	42.9	40.49	42.9
Protein	14.87	15.78	14.87	15.78

¹ HF, high fat; PS, high fat + phytosterols (3%); α LA, high fat + alpha lipoic acid (0.25%); PS/ α LA, high fat + phytosterol (3%) + alpha lipoic acid (0.25%).

² 3.6 kcal/g; total sterol content minimum 60% by weight including β -sitosterol (max 80%), β -sitostanol (max 15%), campesterol (max 40%), stigmasterol (30%).

Table 2

Feed intake and body weight changes in Zucker rats fed a high fat diet (HF) supplemented with phytosterols (PS), α -lipoic acid (α LA) or combination PS/ α LA

Item	HF	PS	α LA	PS/ α LA
Feed intake (g/d)	20.81 $\pm$ 0.02	20.87 $\pm$ 0.02	20.33 $\pm$ 0.12	20.57 $\pm$ 1.04
Start weight (g)	185.85 $\pm$ 4.66	184.71 $\pm$ 4.90	188.67 $\pm$ 3.86	184.25 $\pm$ 5.08
Endpoint Body weight (g)	410.65 $\pm$ 5.35 ^a	396.58 $\pm$ 4.08 ^a	386.44 $\pm$ 2.75 ^b	383.62 $\pm$ 3.33 ^b
% change body weight	121.58 $\pm$ 3.68 ^a	113.92 $\pm$ 5.46 ^a	102.05 $\pm$ 3.11 ^b	108.92 $\pm$ 4.47 ^{ab}

Table 3

Hepatic fatty acid profile in Zucker rats fed a high fat diet (HF) supplemented with phytosterols (PS), α -lipoic acid (α LA) or combination PS/ α LA

Fatty acid	HF	PS	α LA	PS/ α LA
SAT	41.90 $\pm$ 1.08 ^a	48.05 $\pm$ 0.50 ^b	35.63 $\pm$ 0.35 ^c	42.02 $\pm$ 0.90 ^a
MUFA	43.77 $\pm$ 2.31	42.94 $\pm$ 1.26	37.77 $\pm$ 0.85	38.55 $\pm$ 2.64
PUFA	14.31 $\pm$ 1.93 ^a	9.00 $\pm$ 0.93 ^b	26.58 $\pm$ 0.71 ^c	19.42 $\pm$ 2.52 ^d
n-3 PUFA	1.95 $\pm$ 0.35 ^a	1.32 $\pm$ 0.19 ^a	3.85 $\pm$ 0.17 ^b	3.11 $\pm$ 0.56 ^b
n-6 PUFA	12.37 $\pm$ 1.60 ^a	7.68 $\pm$ 0.75 ^b	22.74 $\pm$ 0.56 ^c	16.31 $\pm$ 1.98 ^d
n-3:n-6 PUFA	0.15 $\pm$ 0.01	0.17 $\pm$ 0.01	0.17 $\pm$ 0.00	0.18 $\pm$ 0.02
MUFA:SAT	1.06 $\pm$ 0.07	0.90 $\pm$ 0.03	1.06 $\pm$ 0.03	0.92 $\pm$ 0.07
PUFA:SAT	0.34 $\pm$ 0.05 ^a	0.19 $\pm$ 0.02 ^b	0.75 $\pm$ 0.02 ^c	0.46 $\pm$ 0.06 ^d
16:0/18:2n-6	6.19 $\pm$ 0.77 ^a	11.74 $\pm$ 0.76 ^b	1.98 $\pm$ 0.06 ^c	4.40 $\pm$ 0.64 ^a