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ORIGINAL RESEARCH

Antioxidant Supplements Improve Profiles of Hepatic Oxysterols and Plasma Lipids in Butter-fed Hamsters

Johanne Poirier[1], Kevin A. Cockell[1,2,3], W.M. Nimal Ratnayake[2], Kylie A. Scoggan[2,3], Nick Hidiroglou[2], Claude Gagnon[2], Hélène Rocheleau[2], Heidi Gruber[2], Philip Griffin[2], René Madère[2], Keith Trick[2] and Stan Kubow[1]

[1]School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9. [2]Nutrition Research Division, Food Directorate, Health Products and Food Branch, Health Canada, Ottawa, Ontario, Canada K1A 0K9. [3]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5. Email: stan.kubow@mcgill.ca

Abstract: Hypercholesterolemic diets are associated with oxidative stress that may contribute to hypercholesterolemia by adversely affecting enzymatically-generated oxysterols involved in cholesterol homeostasis. An experiment was conducted to examine whether the cholesterol-lowering effects of the antioxidants selenium and α-tocopherol were related to hepatic oxysterol concentrations. Four groups of male Syrian hamsters (n = 7–8) were fed high cholesterol and saturated fat (0.46% cholesterol, 14.3% fat) hypercholesterolemic semi-purified diets: 1) Control; 2) Control + α-tocopherol (67 IU all-racemic-α-tocopheryl-acetate/kg diet); 3) Control + selenium (3.4 mg selenate/kg diet); and 4) Control + α-tocopherol + selenium. Antioxidant supplementation was associated with lowered plasma cholesterol concentrations, decreased tissue lipid peroxidation and higher hepatic oxysterol concentrations. A second experiment examined the effect of graded selenium doses (0.15, 0.85, 1.7 and 3.4 mg selenate/kg diet) on mRNA expression of the oxysterol-generating enzyme, hepatic 27-hydroxylase (CYP27A1, EC 1.14.13.15), in hamsters (n = 8–9) fed the hypercholesterolemic diets. Supplementation of selenium at 3.4 mg selenate/kg diet was not associated with increased hepatic 27-hydroxylase mRNA. In conclusion, the cholesterol lowering effects of selenium and α-tocopherol were associated with increased hepatic enzymatically generated oxysterol concentrations, which appears to be mediated via improved antioxidant status rather than increased enzymatic production.

Keywords: selenium, lipid hydroperoxide, glutathione, sterol 27-hydroxylase mRNA, tocopherols, thiobarbituric acid-reading substances

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Introduction

Numerous human and animal studies have demonstrated that intake of high cholesterol and high saturated fat (HCHS) diets results in both increased oxidative stress[1–3] and elevated plasma concentrations of non-high density lipoprotein cholesterol (non-HDL-C), a major risk factor for cardiovascular disease.[4] The association noted between oxidative stress and plasma lipids[5] suggests that antioxidants such as selenium (Se) and α-tocopherol (α-Toc) might beneficially influence plasma lipids.

Dietary Se supplementation in various animal models has been shown to be associated with decreased plasma levels of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), low density lipoprotein cholesterol + very low density lipoprotein cholesterol (LDL-C + VLDL-C) and triglycerides (TG).[6–20] Several studies also indicate that supplementation of Se can lower LDL-C and raise high density lipoprotein cholesterol (HDL-C) in human subjects.[21–24] Human and animal studies have examined the effects of α-Toc on plasma lipids with mixed results. In animal models, α-Toc supplementation is generally hypocholesterolemic[25–30] whereas, in most human trials, α-Toc has typically shown no effect on plasma cholesterol concentrations except for certain circumstances such as in diabetes.[31]

The cholesterol-lowering mechanisms of Se and α-Toc are not well understood. Mechanisms underlying the cholesterol lowering effects of the supplementation of both Se[15–20] and α-Toc[32,33] have been explored, however the antioxidants have not been studied for their association with the liver X receptor (LXR) pathway. Activation of the LXR pathway has been shown to result in the lowering of whole body levels of cholesterol that, in turn, can lead to blood cholesterol lowering action.[34] The most important physiological activators for the LXR are the enzymatically produced oxysterols which include the species 24(S)-hydroxycholesterol (24(S)-OHC), 25-hydroxycholesterol (25-OHC) and 27-hydroxycholesterol (27-OHC).[35–37] The importance of these three oxysterols as *in vivo* ligands for LXR has been strongly implicated in a recent study, which showed that feeding a high cholesterol diet to triple-knockout mice that do not synthesize 24(S)-OHC, 25-OHC and 27-OHC failed to induce hepatic mRNA expression of LXR responsive genes.[38]

Enzymatically generated oxysterols are primarily synthesized via cytochromes P-450 enzymes[39] acting on cholesterol levels that are over and above cell requirements. An important cytochrome P-450 enzyme is 27-hydroxylase that is present in both liver and extra-hepatic tissues and is proposed to be involved in the regulation of cholesterol metabolism.[39] Se induces tissue cytochromes P-450 content,[11,40] which suggests that its supplementation may lead to both an upregulation of the enzyme 27-hydroxylase and its product 27-OHC which would help to explain its hypocholesterolemic effects.

Similar to the enzymatically generated oxysterols that are associated with diets high in cholesterol,[41–43] free radical generated oxysterols are produced via consumption of HCHS diets;[44] however, they do not act as ligands for LXR. Moreover, recent evidence suggests that free radical generated oxysterols can impede the physiological actions of enzymatic oxysterols.[45] The oxysterol 7-ketocholesterol (7-keto) is one of the main free radical oxysterols produced during cholesterol oxidation[46] and is used as a marker of oxidative stress.

There is little knowledge regarding the impact of antioxidant supplements, provided either alone or in combination, on either free radical or enzymatically generated oxysterols. Antioxidants including Se have been shown to inhibit the production of free radical produced oxysterols,[47] which suggests that antioxidant supplements can lead to improved ratios of enzymatic to free-radical generated oxysterols. Such ratio improvements might enhance the physiological actions of enzymatic oxysterols including their cholesterol modulating effects, which could lead to cholesterol-lowering effects of Se and α-Toc supplementation.

In the present work, two experiments were performed. In Experiment 1, the effects of non-toxic pharmacological supplementation of Se and α-Toc in concert with HCHS feeding in adult male Golden Syrian hamsters were examined on: i) plasma lipid concentrations; ii) hepatic glutathione (GSH) content, hepatic Se-dependent GSH peroxidase (SeGSH-Px, EC 1.11.1.9) and non-Se-dependent GSH peroxidase (non-SeGSH-Px, EC 2.5.1.18) activities; and iii) *in vivo* lipid peroxidation and enzymatically and free radical produced hepatic oxysterols. In Experiment 2, adult male Golden Syrian hamsters were fed graded levels of Se for four weeks in order to examine the effect of Se on hepatic cytochrome P-450 27-hydroxylase (*Cyp27a1*) mRNA expression.

Materials and Methods

Experiment 1—Animals, diets, and sample preparation

The feeding and animal handling protocol was followed as previously described.[10] Adult hamsters were chosen for the present study based on similarities with humans with regards to dietary cholesterol[48] and dietary Se metabolism.[49] Following the acclimatization period, hamsters were fed for three wk one of four diets (Table 1) which consisted of: i) Control (CT) containing formulated basal requirements of α-Toc and Se; ii) CT + α-toc (67 IU all-racemic α-tocopheryl acetate/kg diet); iii) CT + Se (3.4 mg Se/kg diet); iv) CT + α-Toc (67 IU all-racemic α-tocopheryl acetate/kg diet) + Se (3.4 mg Se/kg diet). The pharmacological supplemental level of Se (3.4 ppm/kg diet) was chosen to be equivalent to two-thirds of the non-toxic 5 ppm Se/kg diet dose fed previously to Syrian hamsters for 25 wk.[50] Subacute toxicity of Se has been tested in previous work, which found levels of dietary Se up to 10 ppm to be non-toxic to Syrian hamsters.[51,52] The levels of α-Toc in the basal diet were chosen based on National Research Council (NRC) recommendations for the hamster of 27 IU all-racemic α-tocopheryl acetate/kg diet (10 tocopherol equivalents = 10 mg all-racemic α-tocopherol = 15 IU).[53] The α-Toc supplemented diet contained 67 IU all-racemic α-tocopheryl acetate/kg diet which provided 2.5-fold higher levels of α-tocopheryl acetate/kg diet NRC recommendations for the hamster.[53] Care and handling of the Syrian hamsters conformed to the guidelines of the Canadian Council of Animal Care[54] and the protocols for the animal experiments were approved by the McGill University Animal Care Committee. Samples were prepared as previously described.[10]

Table 1. Composition of experimental diets (g/kg)[a]

Ingredients	CT[b]	CT[b] + α-Toc	CT[b] + Se	CT[b] + α-Toc + Se
Casein, vitamin-free	159.1	159.1	159.1	159.1
Cornstarch	285.44	285.34	284.66	284.56
Sucrose	175.3	175.3	175.3	175.3
Dextrose	99	99	99	99
Cellulose	43	43	43	43
Butter[c]	157	157	157	157
Safflower oil[d]	13.76	13.76	13.76	13.76
Cholesterol, USP[e]	4.5	4.5	4.5	4.5
Mineral mix[f]	43	43	43	43
Vitamin mix[g]	8.6	8.6	8.6	8.6
Choline bitartrate	11.2	11.2	11.2	11.2
Sodium selenate	0.046	0.046	0.8229	0.8229
Vitamin E acetate	0.052	0.134	0.052	0.134
Metabolizable energy, MJ/Kg	18.3	18.3	18.3	18.3

[a]All diets were formulated at McGill University and prepared in pellet form by Dyets Inc. (Bethlehem, Penn).
[b]Fatty acid composition of CT is as follows (% by weight) as provided by Dyets: C4:0, 3.4; C6:0, 2.0; C8:0, 1.2; C10:0, 2.7; C12:0, 3.0; C14:0, 10.7; C14:1, 1.6; C16:0, 28.0; C16:1, 2.5; C18:0, 13.0; C18:1, 26.8; C18:2, 2.5; C18:3, 1.5; C20:0, 1.1.
[c]Butter contains 18% H_2O and therefore 157 g fat /kg diet provided 129 g fat/kg diet.
[d]Safflower oil was added to prevent essential fatty acid deficiency. α-Toc concentration of SAFF is 350 ppm of α-tocopherol, 180 ppm of other tocopherols. Fatty acid profile of safflower oil included (% by weight): 14:0, trace; 16:0, 6.9; 16:1, trace; 18:0, 2.9; 18:1, 12.2; 18:2, 78.0; 18:3, trace.
[e]Cholesterol USP was added to butterfat 4.5 g/kg.
[f]The mineral mix was free of Se and was composed of (g/kg): calcium carbonate 336.4; calcium phosphate, monobasic 285.0; magnesium oxide 2.985; potassium iodate (10 mg KI/g) 0.76; potassium phosphate, dibasic 40.76; sodium chloride 11.45; cupric carbonate 0.084; cobalt chloride 0.133; sodium fluoride 0.002; ferric citrate 25.45; manganous carbonate 0.229; ammonium paramolybdate 0.008; zinc carbonate 0.53; sucrose 296.209. Sodium selenate (10 mg/g sodium selenate) was added separately to make the diets; for basal Se diets 0.046; for high Se diets 0.8229.
[g]The vitamin mix was free of α-Toc and was composed of (g/kg): vitamin A palmitate (500,000 IU/g) 0.4263; vitamin D3 (400,000 IU/g) 0.9315; vitamin K1 premix (10 mg/g) 110.0; biotin 0.03; folic acid 0.3; niacin 13.5; pantothenate (Ca) 1.5; riboflavin 2.25; thiamin HCl 3.0; pyridoxine HCl 0.9; vitamin B12 (0.1%) 1.5; sucrose 865.6622. α-Toc acetate (500 IU/g) was added separately to make the diets; for basal α-Toc diets, 0.052; for high α-Toc, 0.129.
Abbreviations: CT, Control; CT + α-Toc, control + α-tocopherol; CT + Se, control + selenium; CT + α-Toc + Se, control + α-tocopherol + selenium.

Experiment 2—Animals, diets, and sample preparation

Forty adult Golden Syrian male hamsters, aged 9–10 wks (approximate weight 110–120 g) were purchased from Charles River. Hamsters were housed in the Animal Resources Division, Food Directorate of Health Canada (Ottawa, ON, Canada) in stainless steel wire-bottom cages and acclimatized to laboratory conditions for 10 days while being fed a standard commercial diet. At the end of the acclimatization period hamsters were weighed and randomized to four groups of ten animals each and fed their respective diets for four wk. The dietary levels of Se were adjusted to four different levels which included: 1) CT; 2) CT + 0.85 ppm Se; 3) CT + 1.7 ppm Se; and 4) CT + 3.4 ppm Se. As in Experiment 1, the minimal level of Se of 0.15 ppm in the basal diet conformed with NRC guidelines of 0.1 ppm[53] (Table 1). The semi-purified diet composition was similar to Table 1 except that cholesterol was lowered from 0.45% to 0.1% w/w and the basal level of α-Toc was increased from 27 IU/kg diet to 45 IU/kg diet in CT diet. The feeding and animal handling protocol was followed as per Experiment 1.[10] At the end of the feeding period, hamsters were fasted overnight and sacrificed within 2 days in a treatment-blocked randomized order. Immediately following removal, the liver pieces were weighed, frozen in liquid nitrogen and stored at −80 °C until further use.

Assays

Plasma cholesterol and triglyceride (TG) analysis, measurement of tissue lipid peroxidation, analysis of hepatic total GSH-Px, SeGSH-Px and non-SeGSH-Px (GSH transferase) activities, hepatic GSH and oxidized GSH (GSSG) analysis, hepatic α-Toc and Se analysis, and hepatic protein

The following assays were carried out as previously described in greater detail:[10] a) plasma lipids were measured using automated enzymatic methodology using the Abbott VP Super System (Abbott, Irving, TX, USA) with Abbott enzymatic reagent kits (Abbott, Irving, TX, USA; b) the extent of lipid peroxidation in plasma was determined as thiobarbituric acid-reacting substances (TBARS) using a modified method of Asakawa and Matsushita[55] and Wong et al;[56] c) tissue supernatants were assayed for total GSH-Px and SeGSH-Px activity using an automated modification[57] of the coupled assay of Paglia and Valentine;[58] d) the detection of GSH and GSSG was completed using a Cayman chemical kit (Ann Arbor, MI, USA); e) the concentrations of α-Toc in liver were analyzed by HPLC; and f) protein concentrations of all supernatants were determined using the Biuret assay (Abbott LN5A13-26) with BSA as a standard. For liver Se analysis, hepatic tissue was digested with nitric acid and the Se content was measured using flame atomic absorption spectrophotometry (Hitachi, Polarized Zeeman AAS, Z-8200, Mississauga, Canada).

Hepatic oxysterol analysis

Oxysterol determination of hepatic tissues was performed by GC-MS as described previously.[59] Briefly, 19-hydroxycholesterol was added to the samples as an internal standard before lipid extraction. Artifactual oxidation of cholesterol during sample processing was minimized by incorporation of L-ascorbic acid and sodium acetate to scavenge oxygen and acidic species, respectively. The lipid extract was saponified and unsaponified lipids were extracted with diethyl ether and free fatty acids were removed by KOH. Bulk cholesterol was removed by solid-phase extraction and oxysterols were eluted with 2-propanol in hexane. Samples were evaporated at room temperature under nitrogen and converted to trimethylsilyl ethers for GC-MS analysis (Agilent 6890 GC System with 5973 Mass Selective Detector, Agilent Technologies, Wilmington, DE, USA) using a J and W DB-1 capillary column with flow rate of helium carrier gas of 1.0 mL/min. The injector was operated in splitless mode and with an initial temperature of 290 °C. After injection, oven temperature began at 80 °C, and then programmed at a rate of 30 °C/min to a final temperature 215 °C, held for 2 min, followed by a rate of 2 °C/min to a final temperature of 280 °C held for 10 min. A volume of 1 μL per sample was injected. Oxysterol analysis was carried out using selected ion monitoring. The multiple ion detector was focused on m/z 145, 353, and 366 for 19-OHC; m/z 367 and 472 for 7-keto; 145, 413, 456 for 24(S)-OHC; 131, 327, 456 for 25-OHC; and m/z 129, 417, and 456 for 27-OHC.

Hepatic RNA extraction and real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen hamster liver samples using two passes of Trizol reagent (Invitrogen Life Technologies, Burlington, ON, Canada) for each sample. The isolated RNA was then purified and DNase I treated on RNeasy mini columns (Qiagen, Mississauga, ON, Canada) as per the manufacturer's recommended conditions. RNA integrity was assessed spectrophotometrically by measuring the absorbance ratio of 260/280 nm and by a conventional agarose gel. RNA was quantified using RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugune, OR, USA). 2 µg of RNA per sample was transcribed to complementary DNA with Retroscript Kit (Ambion) using Oligo dT as per the manufacturer's instructions.

Primers for *Cyp27a1* were designed using PrimerQuest software based on sequence alignment of regions of high homology between rat and human *Cyp27a1* mRNA (Genbank NM_178847 and AY178622, respectively) available through NCBI.[60] The sequences were analyzed using the Basic Local Alignment and Search Tool[60] to verify that the primers were specific for *Cyp27a1*. For glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), appropriate Syrian hamster primers were identified based on previously published rat primers.[61] The forward primer exactly matched Syrian hamster *Gapdh* sequence (Genbank accession number U10983.1). A single-base change was made to the reverse primer to make an exact match with the Syrian hamster. Primer sequences were 5'-GGA TCC AAC ACC CAT TTG GCT CTG-3' and 5'-TGT ATC AGC CTT GAC AGC AGG AGT-3' for *Cyp27a1*, and 5'-TCA AGA AGG TGG TGA AGC AGG C-3' and 5'-GCA TCA AAG GTG GAA GAG TGG G-3' for *Gapdh.*

The quantification of *Cyp27a1* was carried out using SYBR Green Core Reagent Kit (Sratagene, La Jolla, CA, USA). To a microtiter plate well, 5 µL volume of cDNA was added per reaction up to 50 µL final volume per well. Final primer concentration equaled 250 nM, magnesium chloride at 2.5 mM. Duplicate samples were run and averaged. For *Cyp27a1* and *Gapdh* primer sets, two types of negative controls were used: 1) No-template control in which water was added instead of cDNA template; and 2) No-Reverse Transcriptase control, in which the Reverse Transcriptase enzyme was omitted in the cDNA synthesis reaction. Standard curves for *Cyp27a1* and *Gapdh* were used to calculate the relative levels of *Cyp27a1* mRNA. The relative amounts of *Cyp27a1* were normalized using *Gapdh* mRNA expression levels as an endogenous internal standard. Normalized values (*Cyp27a1*/*Gapdh*) were then calibrated to control group, namely, control rats fed the control diet (set as 1.0).

RT-PCR was performed in a model Stratagene Mx4000 detection system using Mx4000 software. The thermal cycler parameters were as follows: 95 °C for 10 min 1 cycle to activate SureStart Taq, 40 cycles of denaturation (95 °C 30 sec), Annealing (60 °C 45 sec) and Extension (72 °C 1 min), 1 cycle for denaturation of amplicons (95 °C 1 min), 81 cycles for dissociation curve (55 °C to 95 °C, 10 sec, 0.5 sec/cycle), 1 cycle for end of assay (25 °C, hold).

Statistical analysis

Results are presented as means ± SEM. Data were tested using the mixed model procedure using SAS software version 9.1.[62] ANOVA was used to determine the effect of treatment and differences between treatment means were identified by least square means. Treatment effects and differences between treatments were considered significant when $P \leq 0.05$. Data was assessed for normality (univariate) and homogeneity of variance using a mixed model procedure. Correlations between biochemical measurements were examined using Spearman's correlation coefficient by rank.

For Experiment 1, to determine whether the α-Toc and Se treatments had independent or interactive effects on the dependent variables tested, a fixed effect, 2 (Se levels) x 2 (α-Toc levels) analysis of variance factorial design was used. *P* values are given as the least square means of the interaction effects (α-Toc x Se) and the main effects (α-Toc, Se) with pooled SEM. Main effects include the effect of basal α-Toc vs. supplemental α-Toc, and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se. Blocking was included in the statistical analysis because it was an integral part of the experimental design due to the staggered feeding which occurred in distinct blocks over time and included one hamster from each dietary

group to make a total of four hamsters per block. Final weight was used in the model to analyze liver weight ($P \leq 0.05$).

For Experiment 2, a fixed effect of one factor with 4 levels factorial design was used. Blocking was considered to be four blocks of ten hamsters from each of four dietary groups determined at time of death of hamsters. The microtiter plate which was used for mRNA analysis was also considered to be a source of variation. Neither blocking nor microtiter plate were used in analysis as they were found to be non-significant in the model. Final weight was included in the analysis of liver weight ($P \leq 0.005$).

Results

Experiment 1—Final weight and liver weight

Adult hamsters consuming CT, CT + α-Toc, CT + Se, and CT + α-Toc + Se diets showed final weights (g) of 121 ± 1.5, 122 ± 1.4, 121 ± 1.4, and 124 ± 1.4, respectively. Final liver weights (g) of adult hamsters consuming CT, CT + α-Toc, CT + Se, and CT + α-Toc + Se diets were 6.8 ± 0.1, 6.8 ± 0.1, 6.7 ± 0.1, and 7.1 ± 0.1, respectively. Neither α-Toc nor Se treatments were associated with any significant effect on final body weight or liver weight. The pharmacological dose of 3.4 ppm Se used in the present study was well tolerated by the hamsters as general health, survival, final body weight, and organ weights of hamsters remained unaffected.

Experiment 1—Plasma cholesterol and TG concentrations

As shown in Table 2, a significant main effect of Se treatment was observed with respect to plasma concentrations of TC ($P \leq 0.05$), non-HDL-C ($P \leq 0.005$) and the ratio of potentially atherogenic non-HDL-C/antiatherogenic HDL-C ($P \leq 0.005$), which were 18%, 32% and 22% lower, respectively, than the CT diet-fed hamsters. The hamster treatment group receiving α-Toc supplements also had significantly ($P \leq 0.05$) lower plasma concentrations of TC and non-HDL-C concentrations (Table 2). Significant interactive effects of α-Toc x Se on plasma HDL-C concentrations were observed ($P \leq 0.05$) (Table 2). The hamsters fed the α-Toc and Se supplemented diet had significantly ($P \leq 0.05$) higher plasma HDL-C concentrations in comparison to the α-Toc-supplemented hamsters. In addition, hamsters receiving the combined supplements of α-Toc and Se showed a significantly lower ratio of non-HDL-C/HDL-C in comparison to all other treatment groups. Plasma TG concentrations did not differ among treatment groups (Table 2).

Experiment 1—Hepatic oxysterols, total hepatic content of enzymatic oxysterols, and hepatic oxysterol/7-keto ratios

A significant main treatment effect of Se supplementation on hepatic concentrations of 25-OHC ($P \leq 0.05$)

Table 2. Effects of dietary α-Toc and Se supplementation on plasma lipid concentrations (TC, non-HDL-C, HDL-C, non-HDL-C/HDL-C ratios and TG) of adult male Syrian hamsters fed HCHS diets for 3 wk.[1]

Variable	Dietary treatment				Main and interaction effects[2]		
	CT	CT + α-Toc	CT + Se	CT + α-Toc + Se	α-Toc	Se	α-Toc x Se
TC (mmol/L)	5.8 ± 0.2[a]	4.9 ± 0.2[b]	4.8 ± 0.2[b]	4.9 ± 0.2[b]	$P \leq 0.05$	$P \leq 0.05$	NS
Non-HDL-C (mmol/L)	2.6 ± 0.2[a]	2.1 ± 0.2[b]	1.8 ± 0.2[b]	1.6 ± 0.2[b]	NS	$P \leq 0.005$	NS
HDL-C (mmol/L)	3.2 ± 0.2[ab]	2.8 ± 0.2[a]	3 ± 0.2[ab]	3.3 ± 0.2[b]	NS	NS	$P \leq 0.05$
Non-HDL-C/HDL-C	0.83 ± 0.08[a]	0.84 ± 0.08[a]	0.65 ± 0.08[b]	0.50 ± 0.08[b]	NS	$P \leq 0.005$	NS
TG (mmol/L)	4.4 ± 0.4	4.1 ± 0.4	3.7 ± 0.4	4.1 ± 0.4	NS	NS	NS

[1]Values are mean ± SEM, n = 8. Means within rows without a common superscript letter differ, $P \leq 0.05$. Diets and abbreviations are as indicated in Table 1. [2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se. Blocking included in statistical model.

Abbreviations: HCHS, high cholesterol and high saturated fat diet; HDL-C, high density lipoprotein cholesterol; Non-HDL-C, non-high density lipoprotein cholesterol; Non-HDL-C/HDL-C, non-high density lipoprotein cholesterol/high density lipoprotein cholesterol ratio; TC, total cholesterol; TG, triglyceride.

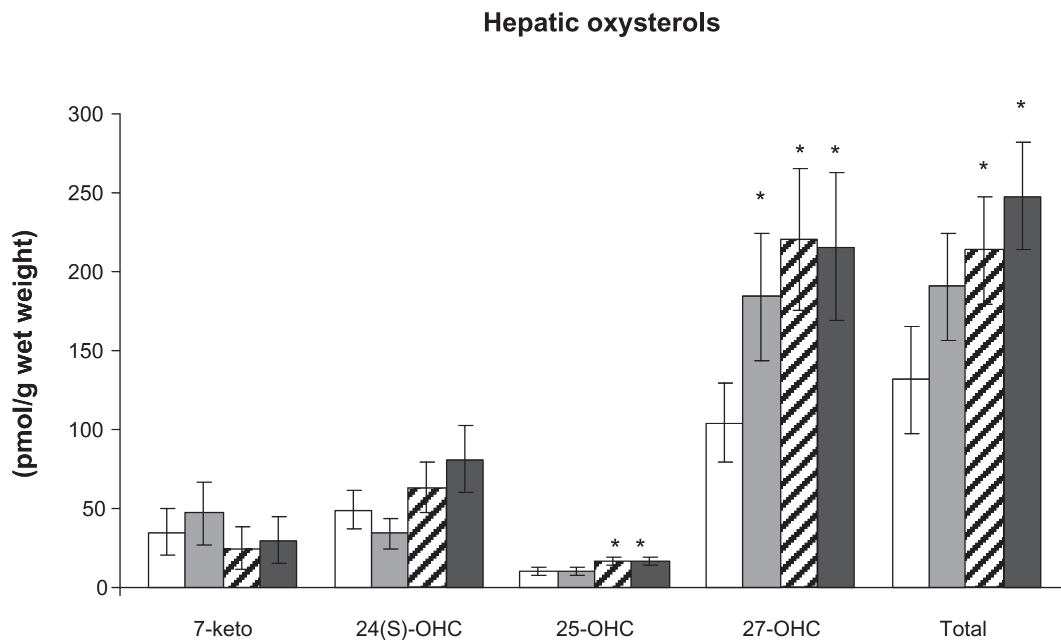

Figure 1. Effects of dietary α-Toc and Se supplementation on liver 7-keto, 24(S)-OHC, 25-OHC, 27-OHC and total oxysterols concentrations of adult male Syrian hamsters fed high cholesterol and high saturated fat diets for 3 wk. (*) Significantly different from control ($P \leq 0.05$). White bars, CT; Light grey bars, CT + α-Toc; Striped bars, CT + Se; Dark grey bars, CT + α-Toc + Se. Values are mean ± SEM (n = 8). Diets and abbreviations are as indicated in Table 1.
Abbreviations: 24(S)-OHC, 24(S)-hydroxycholesterol; 25-OHC; 25-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 7-keto, 7-ketocholesterol; Total, total oxysterols.

and 27-OHC ($P \leq 0.05$) was observed (Fig. 1). In terms of the liver content of 25-OHC, the CT + Se and the CT + α-Toc + Se groups had higher ($P \leq 0.05$) concentrations in comparison to the CT group. Both Se-supplemented diet groups had a significant ($P \leq 0.05$) increase of approximately 200% in the liver content of 27-OHC as compared to livers of hamsters consuming the CT diet. Additionally supplementation of α-Toc alone showed a significant increase ($P \leq 0.05$) in hepatic 27-OHC concentrations relative to the CT diet-fed hamsters. No significant effect of the dietary treatments on hepatic concentrations of 7-keto was observed (Fig. 1). A main treatment effect of Se was noted on total enzymatic oxysterols ($P \leq 0.05$) (Figure 2). Also, the combination of Se and α-Toc was shown to significantly increase total enzymatic oxysterols as compared to controls ($P \leq 0.05$) (Fig. 1).

A significant ($P \leq 0.05$) main treatment effect of Se was noted on hepatic 24(S)-OHC/7-keto. Adult hamsters consuming CT, CT + α-Toc, CT + Se, and CT + α-Toc + Se diets showed 24(S)-OHC/7-keto oxysterol ratios of 1.76 ± 0.49, 1.34 ± 0.53, 2.96 ± 0.53, and 2.78 ± 0.57, respectively. A tendency ($P = 0.07$) for a significant main effect of Se treatment on the total enzymatic oxysterols/7-keto ratio was observed. Hamsters consuming CT, CT + α-Toc, CT + Se, and CT + α-Toc + Se diets showed ratios of 6.63 ± 2.1, 4.95 ± 2.1, 9.30 ± 2.2, and 10.2 ± 2.1, respectively.

Experiment 1—Plasma TBARS concentrations and hepatic LPO concentrations

A significant main effect of Se treatment ($P \leq 0.05$) on plasma TBARS concentrations was observed as hamsters consuming Se supplemented diets showed approximately 19% lower plasma TBARS concentrations as compared to hamsters consuming CT diets (Table 3). In addition, α-Toc supplemented hamsters had significantly ($P \leq 0.05$) lower plasma TBARS concentrations as compared to the hamsters fed the CT diet (Table 3).

A significant main effect of Se treatment on concentrations of LPO was observed in liver ($P \leq 0.05$) (Table 3). Se supplementation resulted in a significant ($P \leq 0.05$) lowering of LPO concentrations of approximately 45% in liver tissue relative to hamsters fed the CT diet. In addition, hamsters fed the CT + α-Toc or CT + α-Toc + Se diets had significantly lower ($P \leq 0.05$) liver concentrations of LPO relative to the CT-fed hamsters.

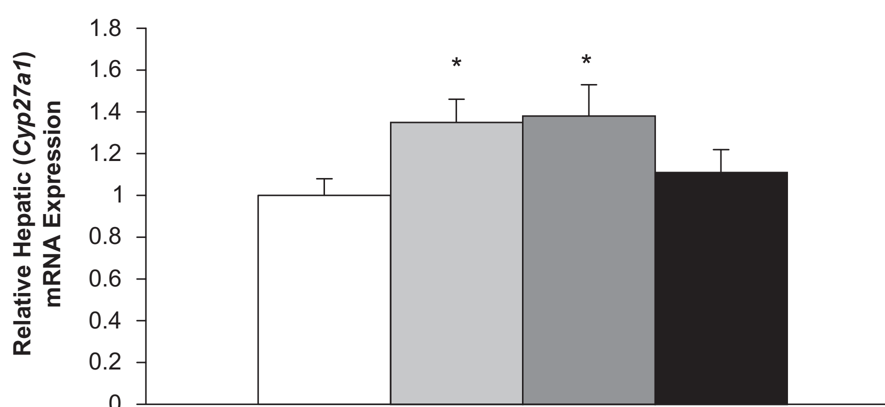

Figure 2. Effects of dietary Se supplementation on liver sterol 27-hydroxylase (*cyp27a1*) mRNA levels of adult male Syrian hamsters fed high cholesterol and high saturated fat diets for 4 wk. (*) Significantly different from control ($P \leq 0.05$). White bar, CT (0.15 ppm); Light grey bar, CT + Se (0.85 ppm); Dark grey bar, CT + Se (1.7 ppm); Black bar, CT + Se (3.4 ppm). Values are SEM (n = 8 or 9).
Abbreviations: CT, control; Se, selenium; ppm, parts per million.

Experiment 1—Tissue concentrations of GSH and GSSG

Significant main effects of Se supplementation were seen with respect to hepatic concentrations of GSH ($P \leq 0.005$) (Table 4). Hamsters receiving diets containing Se supplements had significantly higher hepatic concentrations of GSH relative to non-Se supplemented dietary treatments (Table 4). In the liver, a main effect of Se supplementation was observed with respect to hepatic GSSG concentrations ($P \leq 0.05$) (Table 3). Supplementation of α-Toc did not affect hepatic concentrations of GSSG (Table 3). Hepatic GSH/GSSG ratios were unaffected by dietary treatments (Table 4). Hepatic content of GSH was positively correlated with a) hepatic Se content ($r^2 = 0.54$; $P \leq 0.005$); b) hepatic SeGSH-Px activity ($r^2 = 0.50$; $P \leq 0.005$); c) hepatic concentrations of 24(S)-OHC ($r^2 = 0.42$; $P \leq 0.05$); and d) hepatic total enzymatic oxysterols/7-keto ratios ($r^2 = 0.45$, $P \leq 0.05$).

Experiment 1—Hepatic SeGSH-Px and non-SeGSH-Px (GSH transferase) activities

The hepatic SeGSH-Px activity was significantly higher ($P \leq 0.05$) in association with Se supplementation as the CT + Se and the CT + α-Toc + Se groups had higher activity relative to the CT group (Table 4). Hepatic SeGSH-Px activity was not affected by supplemental α-Toc (Table 4). Non-SeGSH-Px activity was unaffected by the dietary treatments in liver (Table 4).

Experiment 1—Hepatic content of α-Toc and Se

As expected, significant ($P \leq 0.005$) main effects of α-Toc treatment on α-Toc concentrations were

Table 3. Effects of dietary α-Toc and Se supplementation on plasma TBARS and liver LPO of adult male Syrian hamsters fed HCHS diets for 3 wk.[1]

Variable	Dietary treatment				Main and interaction effects[2]		
	CT	**CT + α-Toc**	**CT + Se**	**CT + α-Toc + Se**	**α-Toc**	**Se**	**α-Toc x Se**
TBARS (µmol/L)	1.08 ± 0.06[a]	0.93 ± 0.06[b]	0.87 ± 0.06[b]	0.87 ± 0.06[b]	NS	$P \leq 0.05$	NS
LPO (µmol/g protein)	14.8 ± 1.5[a]	10.4 ± 1.5[b]	8.2 ± 0.5[b]	8.5 ± 1.5[b]	NS	$P \leq 0.05$	NS

[1]Values are mean ± SEM (n = 8). Means within rows with no common superscript roman letter differ significantly ($P \leq 0.05$). Diets and abbreviations are as indicated in Table 1. [2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.
Abbreviations: HCHS, high cholesterol and high saturated fat; LPO, lipid hydroperoxide; TBARS, thiobarbituric acid-reacting substances.

Table 4. Effects of dietary α-Toc and Se supplementation on liver GSH, GSSG, GSH/GSSG ratios, SeGSH-Px activity, Non-SeGSH-Px activity of adult male Syrian hamsters fed HCHS diets for 3 wk.[1]

Variable	Dietary treatment				Main and interaction effects[2]		
	CT	CT + α-Toc	CT + Se	CT + α-Toc + Se	α-Toc	Se	α-Toc x Se
GSH (μmol/g protein)	[3]18 ± 2.4[a]	16 ± 2.0[a]	27 ± 2.0[b]	30 ± 2.0[b]	NS	$P \leq 0.005$	NS
GSSG (μmol/g protein)	[3]8 ± 1.0[a]	9 ± 0.9[a]	11 ± 0.9[b]	11 ± 0.9[b]	NS	$P \leq 0.05$	NS
GSH/GSSG	[3]3 ± 0.55	2 ± 0.55	3 ± 0.45	3 ± 0.45	NS	NS	NS
SeGSH-Px (units/mg protein)	208 ± 9[a]	198 ± 9[a]	238 ± 8[b]	232 ± 9[b]	NS	$P \leq 0.05$	NS
Non-SeGSH-Px (units/mg protein)	54 ± 5	41 ± 5	50 ± 5	49 ± 5	NS	NS	NS

[1]Values are mean ± SEM (n = 8) except where noted, [3]n = 7. Means within rows with no common superscript roman letter differ significantly ($P \leq 0.05$). Diets and abbreviations are as indicated in Table 1.
[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se. Blocking included in statistical model except for GSH/GSSG ratios.
Abbreviations: GSH, glutathione; GSSG, oxidized glutathione; GSH/GSSG, glutathione/oxidized glutathione ratio; HCHS, high cholesterol and high saturated fat diet; SeGSH-Px, Se dependent glutathione peroxidase; Non-SeGSH-Px, non-Se dependent glutathione peroxidase.

observed in liver with α-Toc-supplemented hamsters showing 1.7-fold increases in α-Toc content in liver tissues, respectively, relative to the CT-fed hamsters (Table 5). In liver, the CT + α-Toc and the CT + α-Toc + Se treatment groups had significantly higher ($P \leq 0.05$) α-Toc concentrations relative to the other diet groups. Liver α-Toc was positively correlated with a) 25-OHC ($r^2 = 0.31$; $P \leq 0.05$); b) 27-OHC ($r^2 = 0.33$; $P \leq 0.05$); and c) total enzymatic oxysterols ($r^2 = 0.44$, $P \leq 0.05$).

A significant main effect of Se treatment ($P \leq 0.005$) on liver Se concentrations was observed as consumption of Se was associated with a significant 209% increase ($P \leq 0.005$) in liver Se content in hamsters fed the CT + Se diet in comparison to the liver Se content of hamsters fed the CT diet alone (Table 5). A significant main effect of α-Toc ($P \leq 0.005$) and a significant interactive effect of α-Toc × Se ($P \leq 0.05$) on liver Se content were observed. Hamsters fed the CT + α-Toc + Se diet showed significantly higher liver concentrations of Se as compared to hamsters consuming the CT ($P \leq 0.005$), CT + α-Toc ($P \leq 0.005$) and CT + Se ($P \leq 0.005$) diets. Hepatic Se content was positively correlated with hepatic content of: a) 24(S)-OHC ($r^2 = 0.33$; $P \leq 0.05$); b) 25-OHC ($r^2 = 0.37$; $P \leq 0.05$); c) 24(S)-OHC/7-keto ($r^2 = 0.481$, $P \leq 0.05$); and d) 25-OHC/7-keto ($r^2 = 0.482$, $P \leq 0.05$).

Experiment 2—Hepatic Cyp27a1 mRNA expression

Hamsters consuming the HCHS + 0.85 ppm and HCHS + 1.7 ppm Se diets showed significant ($P \leq 0.05$) increases in hepatic mRNA expression of *Cyp27a1* as compared to livers of hamsters fed the HCHS + 0.15 ppm Se diet (Fig. 2). No significant effect on hepatic *Cyp27a1* mRNA expression was observed in hamsters fed the HCHS + Se (3.4 ppm) diet associated with plasma cholesterol-lowering effects.

Experiment 2—Final weight, liver weight, liver weight/final weight ratio

Adult hamsters consuming CT (0.15 ppm), CT + Se (0.85 ppm), CT + Se (1.7 ppm), and CT + Se (3.4 ppm) diets showed final weights in grams of 116 ± 3, 109 ± 3, 111 ± 3, and 109 ± 3, respectively. Final liver weights in grams of adult hamsters consuming CT (0.15 ppm), CT + Se (0.85 ppm), CT + Se (1.7 ppm), and CT + Se (3.4 ppm) diets were 4.9 ± 0.12, 5.1 ± 0.12, 5.0 ± 0.12, and 5.1 ± 0.12, respectively. Liver weight/final weight ratios of adult hamsters consuming CT (0.15 ppm), CT + Se (0.85 ppm), CT + Se (1.7 ppm), and CT + Se (3.4 ppm) diets were 0.044 ± 0.001, 0.045 ± 0.001,

Table 5. Effects of dietary α-Toc and Se supplementation on liver α-Toc and liver Se of adult male Syrian hamsters fed HCHS diets for 3 wk.[1]

Variable	Dietary treatment				Main and interaction effects[2]		
	CT	CT + α-Toc	CT + Se	CT + α-Toc + Se	α-Toc	Se	α-Toc x Se
α-Toc (nmol/g protein)	[3]21 ± 2[a]	[3]34 ± 2[b]	[3]24 ± 2[a]	36 ± 2[b]	$P \leq 0.005$	NS	NS
Se (nmol/g wet wt)	12 ± 2[a]	13 ± 2[a]	24 ± 2[b]	34 ± 2[c]	$P \leq 0.005$	$P \leq 0.005$	$P \leq 0.05$

[1]Values are mean ± SEM (n = 8) except where noted, [3]n = 7. Means within rows with no common superscript roman letter differ significantly ($P \leq 0.05$). Diets and abbreviations are as indicated in Table 1.
[2]Main effects include the effect of basal α-Toc vs. supplemental α-Toc and basal Se vs. supplemental Se. The four interaction effects included the combinations of basal α-Toc x basal Se, basal α-Toc x supplemental Se, supplemental α-Toc x basal Se, and supplemental α-Toc x supplemental Se.
Abbreviation: HCHS, high cholesterol and high saturated fat.

0.045.0 ± 0.001, and 0.046 ± 0.001, respectively. Se treatment was not associated with any significant effect on final weight, liver weight or liver weight/final weight ratio.

Discussion

The results from this study provide the first evidence that antioxidants can enhance tissue concentrations of important enzymatically-generated oxysterols as supplementation of Se or combined Se and α-Toc supplementation increased hepatic 25-OHC and 27-OHC content and α-Toc supplementation increased hepatic 27-OHC content. The oxysterols 25-OHC and 27-OHC are considered to be the most important physiological activators for LXR receptors.[35–38] Activation of the LXR pathway results in hypocholesterolemic effects.[34] Thus, the increase in hepatic oxysterol concentrations observed in the present study in concert with Se and α-Toc supplementation could play a role in their hypocholesterolemic action.

As tissue oxysterol concentrations can be augmented via increased synthesis, the effect of Se on hepatic *Cyp27a1* mRNA expression was examined. Although Se supplementation at lower levels (0.85 and 1.7 ppm) was associated with significant increases in hepatic mRNA expression, the effective cholesterol-lowering Se supplemental dose of 3.4 ppm was not associated with increased expression of *Cyp27a1* mRNA. Thus, it appears that this supplemental level of Se led to enhanced tissue content of enzymatically-generated oxysterols via decreased oxysterol catabolism, as opposed to increased synthesis. Analogous findings have been observed from the cholesterol loading of human macrophages, which showed cholesterol induced a dose-dependent increase in 27-OHC content without an increase in expression levels of CYP27A1 mRNA.[63]

The improved hepatic ratios of enzymatic to free radical generated oxysterols resulting from Se supplementation could be the result of diminished breakdown of the enzymatically-generated oxysterols by free radical-generated oxysterols. Previous work has shown that treatment of cultured human umbilical vein endothelial cells with oxidized LDL-C that contains the free radical-generated oxysterol, 7β-hydroxycholesterol, diminished cellular 27-OHC concentrations by 78% within 24 h.[45] Tissue content of the important cellular antioxidant GSH was inversely related to free radical produced oxysterols as incubation of murine peritoneal macrophages with 7β-hydroxycholesterol for 24 h led to dose-dependent reduction in cellular GSH.[64] Treatment with Se elevates cellular GSH levels,[64] which was clearly observed in the present work (Table 3). Hepatic GSH content correlated positively with the total enzymatic oxysterol/7-keto ratios, which indicates that the tissue levels of enzymatically generated oxysterols were positively related to antioxidant activity. The positive correlations between hepatic Se and α-Toc levels with hepatic enzymatic oxysterol content also support the notion that enhanced antioxidant protection inhibited breakdown of hepatic enzymatically-generated oxysterols.

HCHS diets are associated with increased oxidative stress and impaired antioxidant capacity, particularly in terms of impaired status of tissue GSH and GSH-dependent antioxidant enzymes.[1,65] The present study shows that pharmacological Se supplementation can at least partially overcome the suppression of both hepatic GSH-Px activity and GSH content consistently noted with HCHS diets.[1,65]

The increase in hepatic concentrations of GSH via Se intake is likely related to increased activities of γ-glutamylcysteine synthetase,[66] the rate-limiting enzyme in GSH biosynthesis. Se supplementation, which decreased tissue lipid peroxidation associated with the HCHS diet (Table 3), has also been demonstrated to diminish lipid peroxidation induced by polyunsaturated fatty acid intake.[67] Along with its effect on SeGSH-Px, the antioxidant efficacy of Se likely involves the enhancement of hepatic GSH content (Table 3), particularly as induction of tissue GSH is well characterized to decrease tissue lipid peroxidation.[68]

The major decrease in concentrations of plasma TC and non-HDL-C observed with Se supplementation in the context of HCHS feeding extends previous Syrian hamster studies demonstrating hypolipidemic effects of Se supplements with low-fat standard laboratory diets[8] and cholesterol-supplemented fish oil-based semi-purified diets[10] and HCHS diets in other rodent studies.[15–19] The lack of significant plasma TG lowering with Se supplementation in the present work contrasts with previous studies showing significant decreases in plasma TG concentrations in humans[21] and rodents in conjunction with HCHS diets[11,20] but concurs with previous observations in Se-supplemented hamsters[8,10] and other rodents.[13] The lack of effect of Se on plasma HDL-C levels agrees with the findings of a previous study in hamsters showing no effect of Se on plasma HDL-C levels,[8] but contrasts with small human intervention studies supplementing healthy volunteers with Se and that have shown beneficial effects of Se on HDL-C concentrations.[22,23] In concert with previous findings noting α-Toc-mediated lowering effects on plasma TC and non-HDL-C concentrations involving hamsters[30] and other species such as rabbits,[33] α-Toc supplementation was associated with the lowering of plasma TC and non-HDL-C concentrations (Table 2).

In conclusion, the present study indicates that in Syrian hamsters the improved enzymatic oxysterol hepatic profiles observed with Se and α-Toc supplements might play a role in their hypocholesterolemic effects in association with HCHS diets. Further studies are needed to define the effects of Se and α-Toc supplementation on plasma lipid and oxysterol concentrations in apparently healthy humans. To date, no major human trials have been designed to specifically examine the effect of supplemental Se alone on plasma lipids. A recent cross-sectional study has revealed an association between plasma lipids and Se in human subjects[69] that could be interpreted as being an adverse relationship although cholesterol-lowering medication could be a confounding influence. Similarly, α-Toc supplementation has not generally been shown to be efficacious in preventing cardiovascular disease.[70] Two large randomized trials are progressively examining the effects of Se and α-Toc supplementation on cancer related endpoints.[71,72] The results from these latter two studies could also be used to examine the effect of the antioxidants on plasma lipids; however, further evidence is needed from large appropriately designed randomized trials with relevant antioxidant baselines and cardiovascular endpoints to clarify the effects of Se and α-Toc supplements on human cholesterol metabolism. In that regard, the use of lipid lowering medication commonly used in intervention studies could act as a confounding factor in human trials examining the effect of the antioxidants on plasma lipids.[73]

The enhancement of hepatic cholesterol-modulating oxysterols via Se and α-Toc supplementation likely occurred through inhibition of free radical-mediated oxysterol catabolism rather than via increased enzymatic biosynthesis. Further research is needed to determine whether the hyperlipidemia associated with HCHS feeding is associated with depressed production of the enzymatically-generated oxysterols involved in cholesterol synthesis and metabolism. Studies are also needed to examine whether the higher hepatic levels of oxysterols induced by Se and α-Toc supplementation might downregulate the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) via a sterol regulatory element binding protein-2 responsive gene, since oxysterols are potent suppressors of the cleavage of sterol regulatory element binding protein.[46] Although the activity or mRNA expression of HMG-CoA was not assessed, it is conceivable that these parameters were reduced as shown in a previous study with Se-supplemented rodents.[15] To clarify these latter aspects we are conducting studies to

examine the effect of Se supplementation on mRNA expression of cholesterol metabolizing genes including 3-hydroxy-3-methylglutaryl-coenzyme A reductase and on several LXR-responsive genes such as the ATP-binding cassette transporters G5 and G8. We are also conducting studies on Se influenced bile acid metabolism to further examine the possible mechanism of hypocholesterolemic action involving Se-induced hepatic oxysterols.

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Abbreviations

CT, control; CYP27A1 and *Cyp27a1*, sterol 27-hydroxylase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; HCHS, high cholesterol and high saturated fat; HDL-C, high density lipoprotein cholesterol; 24(S)-OHC, 24(S)-hydroxycholesterol; 25-OHC, 25-hydroxycholesterol; 27-OHC, 27-hydroxycholesterol; 7-keto, 7-ketocholesterol; LXR, liver X receptor; LPO, lipid hydroperoxide; non-HDL-C, non-high density lipoprotein cholesterol; non-SeGSH-Px, non-selenium dependent glutathione peroxidase; NRC, National Research Council; Se, selenium; SeGSH-Px, selenium dependent glutathione peroxidase; TBARS, thiobarbituric acid-reacting substances; α-Toc, α-tocopherol; TC, total cholesterol; TG, triglyceride.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest.

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