Effect of Stanol Ester on Postabsorptive Squalene and Retinyl Palmitate

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Stanol ester dissolved in margarine inhibits cholesterol absorption in general and, despite increasing cholesterol synthesis, decreases serum total and low-density lipoprotein (LDL) cholesterol levels, but its effects on postprandial lipid metabolism are unknown. We performed fat tolerance tests in 11 men at baseline and during short-term stanol ester consumption without and with stanol esters added to the test meal also containing retinol and squalene. Cholesterol, triglycerides, retinyl palmitate, and squalene were analyzed in plasma, chylomicrons, and very–low-density lipoprotein (VLDL) at baseline and 3, 4, 6, 9, 12, and 24 hours after the test meal. Serum total and LDL cholesterol only tended to diminish after the 2-week stanol ester consumption. However, the proportion of plasma plant sterol and cholesterol-precursor sterol to cholesterol was significantly altered, suggesting that cholesterol absorption was diminished and cholesterol synthesis was increased. Postprandial peak times of squalene and retinyl palmitate in plasma, chylomicrons, and VLDL were significantly reduced by stanol esters, but their concentrations in chylomicrons were unchanged. Stanol esters reduced the VLDL squalene peak concentration by 23% ($P < .05$) and the incremental area under the curve (AUIC) in plasma and VLDL by 22% and 32% ($P < .01$ for both). Chylomicron remnant metabolism measured with triglycerides only tended to diminish. The effects of stanol esters in the diet only and both in the diet and with supplementation did not differ significantly. We conclude that dietary stanol esters reduce postprandial lipoproteins measured with dietary retinyl palmitate and especially squalene, and the reduction is observed even though serum total and LDL cholesterol are only inconsistently decreased after short-term stanol ester consumption.

SITOSTEROL, a plant sterol, has been known since the 1950s to reduce serum cholesterol to some extent[1] by inhibiting cholesterol absorption. Sitostanol, a nonabsorbable 5α-saturated derivative of sitosterol, reduces serum cholesterol and the intestinal absorption of cholesterol even more effectively than sitosterol.[2-7] A fat-soluble form of sitostanol (containing small amounts of campestanol; thus, the term stanols is preferable in the following) has been developed by esterification with rapeseed oil fatty acids and dissolved in mayonnaise or margarine. Stanol esters in mayonnaise or margarine reduce serum total and low-density lipoprotein (LDL) cholesterol levels from 10% to 20% in hypercholesterolemic[8-11] and normocholesterolemic[12] populations, in type 2 diabetics,[13-15] in children with familial hypercholesterolemia,[16] and in postmenopausal women with coronary artery disease.[17] The exact mechanism of the stanol-induced inhibition of cholesterol absorption is not known in humans. In rats, crystalline stanol decreases the micellar solubility of cholesterol and reduces the concentration of cholesterol in micelles.[7] It may be assumed that the fat-soluble sitostanol ester may more easily enter intestinal micelles than the crystalline stanol, resulting in cholesterol malabsorption and a compensatory increase in cholesterol synthesis.[9,13,15,17] Stanol esters do not interfere with fat absorption.[13] Tetrahydrolipstatin, a pancreatic lipase inhibitor, has been shown to improve postprandial lipemia.[18]

The question now arises as to whether stanol ester affects postprandial lipid metabolism by interfering with the micellar concentration of cholesterol or increasing the removal of postprandial lipoproteins. We investigated this question by measuring postprandial concentrations of retinyl palmitate and squalene, indicators of postprandial lipoprotein absorption and removal,[19,20] and cholesterol and triglycerides in plasma, chylomicron, and very–low-density lipoprotein (VLDL) fractions at baseline and during dietary intake of stanol esters without and with stanol esters also added to the fat load.

SUBJECTS AND METHODS

Subjects

The study group consisted of 11 men with a mean age of 58 ± 7 years (mean ± SE) and a body mass index of 25.8 ± 0.9 kg/m^2. One subject had the apoprotein E2/3 phenotype, 6 subjects had the apoprotein E3/3, phenotype, and 4 had the apoprotein E4/3 phenotype. Because the comparisons were made intraindividually, the different apoprotein E phenotypes were not a confounding factor. The subjects had no renal, liver, thyroid, or gastrointestinal diseases. One subject had stable coronary artery disease treated with a β-blocking agent, nitrate, and acetosalicylic acid without any change in the dosage. The study protocol was approved by the Ethics Committee of our hospital.

Study Design

The subjects visited the outpatient clinics 3 times 1 week apart, and a 24-hour postprandial fat clearance test was performed during each visit. Subjects kept a 7-day food record, from which the dietary constituents were calculated by a computer-based quantitation.[21] The margarine was provided in containers with 8 g rapeseed oil margarine in each, in which 1 g stanol was dissolved as rapeseed oil fatty acid esters (Raisio Inc, Rasio, Finland). The subjects used one container, usually on a slice of bread, 3 times per day at breakfast, lunch, and dinner such that the intake of stanol was planned to be 3 g/d. The subjects were instructed by the dietitian to replace 24 g fat from their normal diet with stanol ester margarine and otherwise to have the diet unchanged for 2 weeks. This was accomplished by replacing the home diet margarine on a piece of bread with stanol ester margarine. At baseline, the fat intake was 73.4 ± 5.0 g/d, of which 33.4 ± 4.2 g was saturated and 23.4 ± 1.6 g monounsaturated fatty acids. Cholesterol intake was 281 ± 27 g/d. The polyunsaturated to saturated fatty acid ratio was 0.4 ± 0.1. The fatty acid composition of the diet changed only insignificantly as in the prior

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studies.[17] Dietary saturated and total fat intake and cholesterol intake were inconsistently changed during the intervention. The postprandial fat clearance test was repeated after 1 and 2 weeks with stanol ester margarine in the diet. The two test meals during dietary stanol ester consumption contained, in random order, margarine without or with stanol esters. After the third fat clearance test, the study was completed.

Experimental Procedures

The postprandial fat clearance test was started at 8 AM after a 12-hour fast. After baseline blood sampling, the subjects received a fatty meal containing 90 g fat, 432 mg cholesterol, 345,000 IU aqueous vitamin A, and 0.5 g squalene. In the fat-loading tests, 8 g margarine without and with stanol (1 g) esters was added in random order to the fat load. The meal was prepared as a cream-eggshake. After the meal, the subjects fasted for 9 hours, after which they consumed a standard low-cholesterol, low-fat hospital meal. Postprandial blood samples were drawn after 3, 4, 6, 9, 12, and 24 hours into dark heparin-containing tubes.

Analytical Methods

Commercial kits were used for enzymatic analysis of serum total and lipoprotein cholesterol and triglycerides (Boehringer Diagnostica, Mannheim, Germany). Chylomicrons were separated from the plasma, after careful overlayering with 1.0063-g/mL NaCl salt solution, by ultracentrifugation in a fixed-angle Beckman (Fullerton, CA) rotor for 30 minutes, followed by density-gradient separation of VLDL (<1.006 g/mL).[22]

Squalene and serum cholesterol-precursor sterols Δ8-cholestenol, desmosterol, and Δ7-lathosterol, indicators of cholesterol synthesis,[23] and serum plant sterols campesterol and sitosterol, indicators of cholesterol absorption,[23] were quantified from nonsaponifiable lipids with gas-liquid chromatography on a 50-m capillary column[24,25] (Ultra-1; Hewlett-Packard, Palo Alto, CA). A simplified cholesterol synthesis chain is demonstrated in Fig 1. Apoprotein E phenotyping was performed by isoelectric focusing from serum.[26] Retinyl palmitate was analyzed with high-performance liquid chromatography.[27] All retinyl palmitate procedures were completed in subdued light.

Postprandial incremental concentrations of cholesterol, triglycerides, retinyl palmitate, and squalene were calculated by subtracting the respective fasting value from each postprandial value. In addition, the postprandial responses were quantified by calculating the incremental area under the curve (AUIC) between 0- and 24-hour levels for retinyl palmitate and squalene and between 0- and 9-hour levels for cholesterol and triglycerides (positive values), respectively, for every patient. To eliminate variation in serum cholesterol values, serum noncholesterol sterols are standardized and presented as proportions in relation to cholesterol or to plant sterols.

Statistical significance was tested with ANOVA, ANOVA and analysis of covariance for repeated measures, Student's 2-sided *t* test, and paired *t* test. Correlation coefficients were calculated with Pearson's product-moment correlation test. Logarithmic transformations were used with skewed distributions (AUICs for triglycerides, retinyl palmitate, and squalene). A P value less than .05 was considered statistically significant.

RESULTS

Serum cholesterol was practically unchanged, but the serum campesterol concentration (data not shown) and proportion to cholesterol were significantly decreased by 20% during the 2-week stanol ester margarine consumption (Table 1). The respective decrease for the proportion of sitosterol to cholesterol was 11% (nonsignificant [NS]). Simultaneously, the proportions of serum Δ8-cholestenol, desmosterol, and Δ7-lathosterol

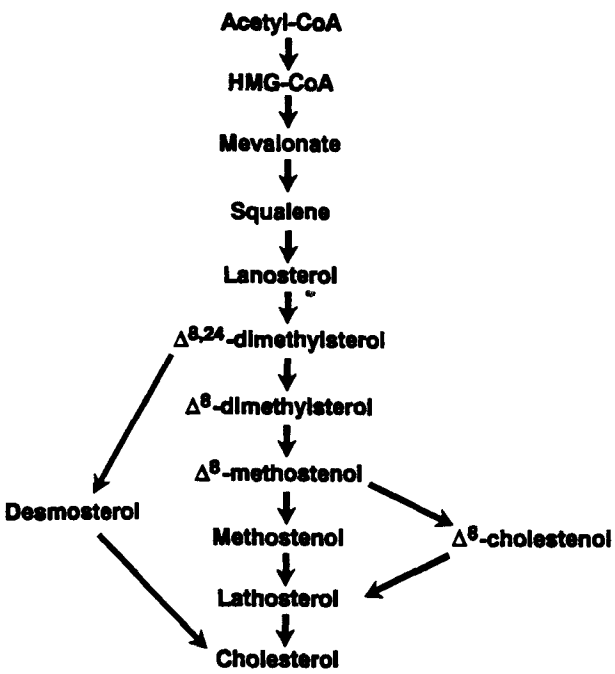

Fig 1. Simplified cascade of cholesterol biosynthesis from acetyl-coenzyme A (acetyl-CoA).

to cholesterol were increased by 26% to 68%, and even more markedly, by 41% to 54%, when the respective proportions to sitosterol were calculated (Table 1).

The effects of stanol esters in the diet only and in the diet and supplementation did not differ significantly in plasma or in the lipoprotein fractions measured for any of the variables (Fig 2), so the mean values of the two fat-loading stanol experiments were combined in the following. The postprandial cholesterol peak time, peak concentration, and AUICs were similar at baseline and during the stanol ester studies, except that the chylomicron cholesterol AUIC was significantly increased

Table 1. Serum Sterols at Baseline on the Home Diet and Percent Change After Two Weeks on Stanol Ester Margarine in 11 Men

Variable	Baseline	Sitostanol Ester Diet (% change)
Serum cholesterol (mg/dL)	177.4 ± 11.1	−2.6 ± 5.1
Squalene (10^2 mmol/mol cholesterol)	126.8 ± 7.8	+2.2 ± 17.6
Δ8-cholestenol (10^2 mmol/mol cholesterol)	19.0 ± 2.3	+68.0 ± 17.7*
Δ8-cholestenol/sitosterol (μg/dL)	14.4 ± 2.6	+50.8 ± 14.9*
Desmosterol (10^2 mmol/mol cholesterol)	89.1 ± 6.3	+26.3 ± 13.1
Desmosterol/sitosterol (μg/dL)	67.0 ± 10.2	+41.1 ± 10.2*
Δ7-lathosterol (10^2 mmol/mol cholesterol)	158.1 ± 12.0	+35.5 ± 11.6*
Δ7-lathosterol/sitosterol (μg/dL)	117.1 ± 14.5	+53.7 ± 12.8*
Campesterol (10^2 mmol/mol cholesterol)	282.3 ± 45.5	−19.6 ± 5.0*
Sitosterol (10^2 mmol/mol cholesterol)	158.2 ± 24.1	−10.9 ± 5.4
Cholestanol (10^2 mmol/mol cholesterol)	157.8 ± 8.9	−6.5 ± 3.6

NOTE. Results are the mean ± SE.
*P < .05 or less *v* baseline.

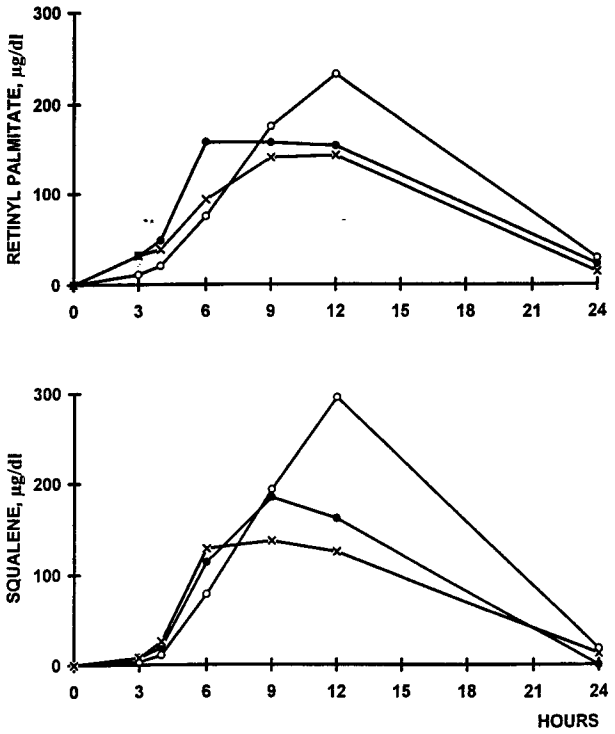

Fig 2. Incremental postprandial VLDL retinyl palmitate and squalene in 11 healthy men at baseline (○) and during intake of stanol ester margarine with stanol ester in the fat load test (×) and without stanol ester in the fat load test (●). Mean values are shown. $P < .05$, baseline *v* stanol esters; P = NS, fat load supplemented *v* not supplemented with stanol ester.

during stanol esters. The latter tended to reduce the peak time and AUIC of triglycerides in plasma and VLDL, with no effect on chylomicrons (Table 2).

Stanol esters significantly shortened the peak time of retinyl palmitate in plasma, chylomicrons, and VLDL by 2.9, 2.2, and 1.2 hours, respectively (Table 2 and Fig 3). The chylomicron peak concentration and AUIC were not affected by stanol esters.

Stanol esters significantly shortened the peak time of squalene in plasma, chylomicrons, and VLDL by 2.0, 2.2, and 2.3 hours, respectively (Table 2 and Fig 4). The peak concentration of squalene was diminished in VLDL by 23%, and the AUIC in plasma and VLDL by 22% and 32%, respectively ($P < .01$ for both). The chylomicron peak concentration and AUIC were unchanged by stanol esters.

DISCUSSION

The novel observation in the present study is that daily consumption of stanol ester margarine diminished postprandial squalene and retinyl palmitate peak times, and especially postprandial squalene concentrations and AUICs. Postprandial cholesterol and triglyceride concentrations only tended to diminish. The addition of stanol esters to the test meal during the daily stanol ester feeding did not consistently further reduce postprandial lipids. This may indicate that it is not the acute stanol ester–induced impairment of absorption but the altered metabolic phenomena caused by daily treatment that is responsible for the altered postprandial lipid metabolism.

Two weeks' consumption of stanol ester margarine only tended to reduce the serum cholesterol level and had no effect on triglyceride or high-density lipoprotein (HDL) cholesterol levels (data not shown). These results indicate that the improved values for postprandial lipoprotein markers were not caused by an altered lipid pattern but were a true stanol effect. A significantly altered lipid pattern would in fact have been considered a potential confounding factor. In the present study, a 2-week stanol ester consumption seemed insufficient to affect serum cholesterol levels. In earlier studies with serum total and LDL cholesterol reductions, the treatment periods have been markedly longer, from 6 weeks to 14 months.[2,8-17] However, in the present study, the proportion of serum plant sterol to cholesterol decreased and that of precursor sterols increased significantly, suggesting that the adherence to stanol ester margarine consumption was good and the subsequent cholesterol absorption efficiency was inhibited and cholesterol synthesis was increased. Accordingly, these results suggest that the

Table 2. Postprandial Peak Time, Incremental Peak Concentration, and AUIC for Retinyl Palmitate and Squalene in Plasma, Chylomicrons, and VLDL at Baseline and During Stanol Esters (in diet only and diet + supplementation in fat load) in 11 Men

	Peak Time (h)			Peak Concentration (mmol/L)			AUIC (9 h, mmol/L · h)		
Variable	Plasma	Chylomicrons	VLDL	Plasma	Chylomicrons	VLDL	Plasma	Chylomicrons	VLDL
Cholesterol									
Baseline	9.0 ± 3.1	4.2 ± 0.4	8.5 ± 2.5	0.25 ± 0.07	0.17 ± 0.03	0.16 ± 0.04	−0.7 ± 0.8	0.8 ± 0.2	0.3 ± 0.4
SEM	8.1 ± 1.8	4.7 ± 0.5	5.5 ± 1.0	0.21 ± 0.04	0.30 ± 0.05	0.13 ± 0.03	−0.4 ± 0.5	1.4 ± 0.2*	0.5 ± 0.2
Triglycerides									
Baseline	4.9 ± 0.8	4.4 ± 0.4	7.3 ± 1.9	1.57 ± 0.30	1.11 ± 0.20	0.38 ± 0.07	6.6 ± 2.0	4.7 ± 1.1	1.7 ± 0.7
SEM	4.8 ± 0.5	5.0 ± 0.6	5.7 ± 1.0	1.19 ± 0.17	1.03 ± 0.18	0.33 ± 0.07	5.0 ± 1.0	4.6 ± 0.8	1.1 ± 0.4
				µg/dL			24 h AUIC, µg/dL · h		
Retinyl palmitate									
Baseline	10.6 ± 0.5	9.4 ± 0.9	10.1 ± 0.6	484 ± 83	247 ± 34	264 ± 61	5,163 ± 928	2,223 ± 362	2,676 ± 599
SEM	7.8 ± 0.6*	7.1 ± 0.5*	8.9 ± 0.5*	421 ± 67	320 ± 54	211 ± 29	3,733 ± 587	2,334 ± 342	2,102 ± 308
Squalene									
Baseline	10.4 ± 0.6	9.8 ± 0.8	10.9 ± 0.4	688 ± 151	301 ± 58	319 ± 89	6,581 ± 1,501	2,763 ± 619	3,118 ± 827
SEM	8.3 ± 0.4*	7.6 ± 0.5*	8.6 ± 0.5*	628 ± 80	367 ± 52	218 ± 33*	5,098 ± 701*	2,598 ± 366	1,945 ± 342*

NOTE. Results are the mean ± SE.
Abbreviation: SEM, stanol ester margarine.
*$P < .05$ *v* baseline.

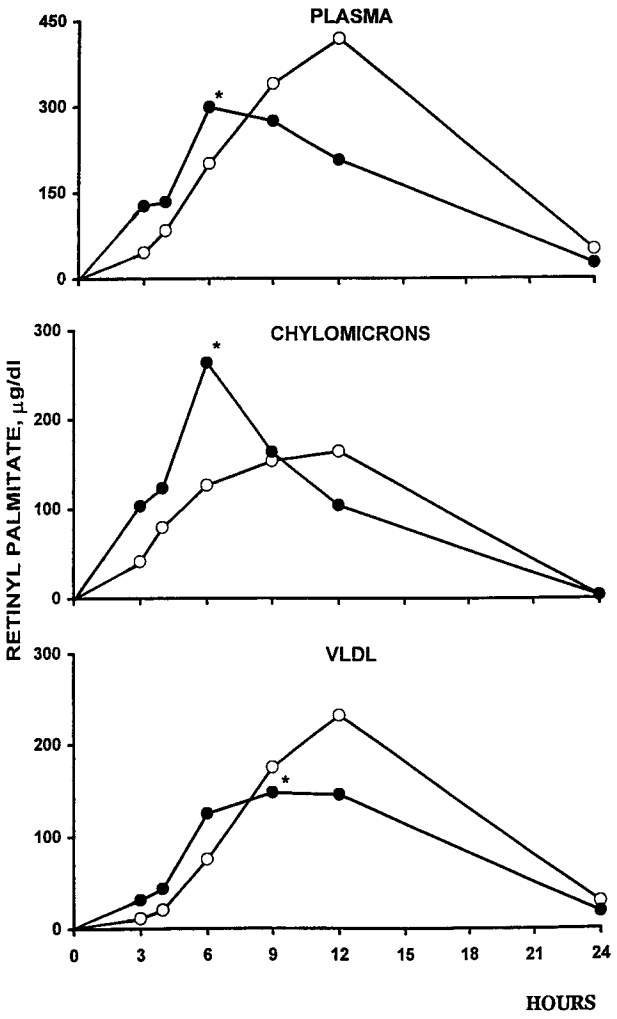

Fig 3. Incremental postprandial retinyl palmitate in plasma, chylomicrons, and VLDL in 11 healthy men at baseline on the home diet (○) and during stanol ester margarine intake (●). Mean values are shown. *$P < .05$ for peak times.

2-week stanol ester consumption altered the whole-body cholesterol metabolism but the serum cholesterol level was only slightly reduced, perhaps due to exclusively increased cholesterol synthesis (Table 1). In addition, the results demonstrate the good compliance of the subjects.

Squalene has been shown to reflect postprandial lipoprotein clearance.[19,20,28] Postprandial apolipoprotein B48 and squalene AUICs also have been shown to correlate significantly.[29] Accordingly, retinyl palmitate and especially squalene can be used as indicators of postprandial lipoprotein clearance. In the present study, postprandial squalene and retinyl palmitate values were significantly interrelated at every time point ($r = .747$ to $.978$) and their relative changes were not significantly different. However, stanol ester had more frequent significant effects on postprandial squalene versus retinyl palmitate values. The hydrocarbon squalene is more fat-soluble than retinol palmitate, which may lead to a more effective replacement of squalene from intestinal micelles by stanols. In addition, we have shown previously that stanol esters do not affect serum levels of retinol, whereas serum levels of β-carotene, which is less polar than retinol, are reduced.[30]

The fasting serum triglyceride concentration is an important determinant of postprandial lipoproteins.[31,32] In fact, the normalization of serum lipids by hypolipidemic agents also normalizes the impaired removal of postprandial lipids.[33] However, in type III hyperlipidemia, this apparently does not occur.[20] Stanol ester margarine did not decrease serum triglyceride in the present study, as well as most[8-16] but not all[17] normotriglyceridemic populations studied. Thus, it can be assumed that the effect of stanol esters on postprandial lipid metabolism is not mediated by an alteration of serum triglyceride or HDL cholesterol levels, which was one of the supposed mechanisms of tetrahydrolipstatin by improving postprandial lipemia.[18] However, in the present study, the effect of stanol esters on the chylomicron remnant AUICs measured by triglycerides, albeit not statistically significant, resembled that obtained with squalene.

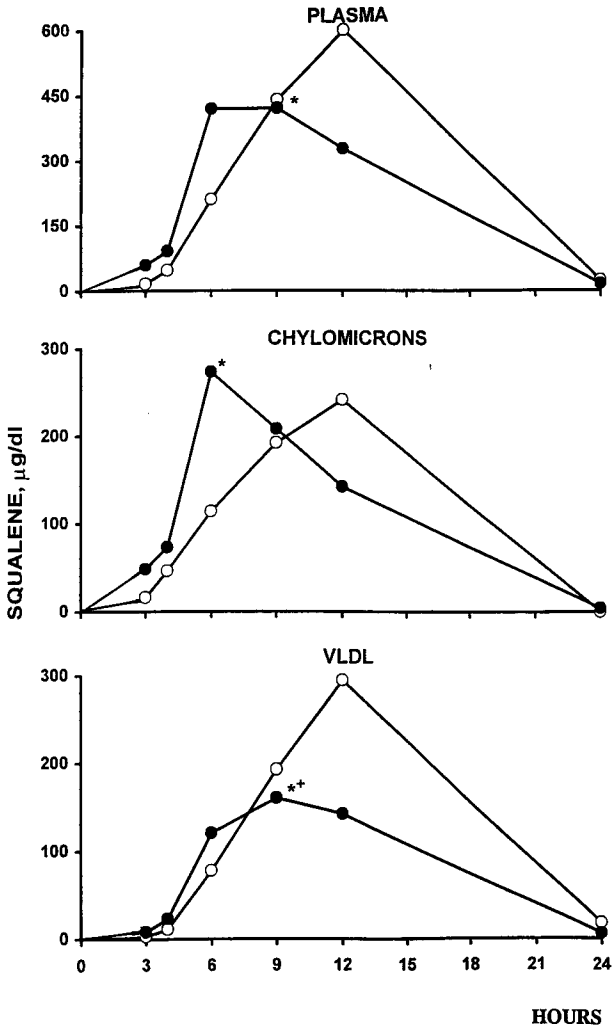

Fig 4. Incremental postprandial squalene in plasma, chylomicrons, and VLDL in 11 healthy men at baseline on the home diet (○) and during stanol ester margarine intake (●). Mean values are shown. *$P < .05$ for peak times; + $P < .05$ for maximal concentrations.

Stanol esters inhibit the absorption of both dietary and biliary cholesterol.[13,34] The underlying mechanism of the inhibited absorption is supposedly related to the micellar solubilization of cholesterol.[7,35] Thus, a reduction of postprandial chylomicron cholesterol, squalene, or retinyl palmitate was expected. Instead, dietary stanols shortened the squalene and retinyl palmitate peak times in chylomicrons and VLDL but unexpectedly significantly increased the chylomicron cholesterol AUIC, suggesting that chylomicron formation was not impaired. The similarity of the postprandial cholesterol curves at baseline and with stanol esters suggested that increased cholesterol synthesis, most likely also in the intestinal mucosa, compensated for the stanol-induced inhibition of cholesterol absorption. The increase in precursor sterols could partly originate from the intestine. Activated mucosal cholesterol synthesis by stanol ester feeding could be due to a decrease of mucosal cholesterol and also to an increase in the conversion of dietary squalene to mucosal cholesterol, increasing the cholesterol AUIC in chylomicrons. The actual increment of chylomicron cholesterol lasted up to 6 to 7 hours, followed by decrements.

The reduction of the postprandial VLDL squalene peak concentration and AUIC, as well as squalene and retinyl palmitate peak times, by dietary stanol ester consumption suggests that chylomicron remnant formation was reduced and/or hepatic remnant removal was enhanced. Shorter squalene and retinyl peak times can also be considered to reflect more effective postprandial particle catabolism. Intestinal function itself was unaltered. Despite the suggested increase in mucosal cholesterol synthesis, inhibited cholesterol absorption is supposed to lead to an overall diminished cholesterol flux to the liver, since stanol esters also inhibit the absorption of cholesterol secreted by the liver into bile.[34] Reduced cholesterol absorption after postprandial chylomicronemia might decrease the hepatic cholesterol pool despite a compensatorily enhanced cholesterol synthesis.[8-10,13,14,17] Stanol esters reduce the LDL apolipoprotein B transport rate,[13,15] possibly due to reduced VLDL synthesis and/or increased hepatic uptake of precursor lipoprotein fractions by upregulated receptor activity. The removal of chylomicron or intestinal VLDL remnants, containing apoprotein E, can only be speculated to be increased. In experimental animals, the upregulation of cholesterol synthesis increased the hepatic removal of chylomicron remnants and VLDL through the LDL receptor.[36]

Accordingly, one probable explanation for the present results is that chylomicron remnant catabolism is enhanced during dietary stanol ester treatment. Since postprandial lipoproteinemia is considered atherogenetic,[37-39] stanol esters seem to offer an efficient means to reduce not only total and LDL cholesterol concentrations but also postprandial lipoproteinemia.

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