

Lack of stimulation of cholesteryl ester transfer protein by cholesterol in the presence of a high-fat diet

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Abstract Cholesteryl ester transfer protein (CETP) is a key protein involved in the reverse cholesterol transport pathway. The regulation of CETP by dietary fats is not clearly understood. Transgenic mice expressing human CETP under the control of its natural flanking region were fed low- or high-fat diets enriched in monounsaturated fatty acids (MUFAs) or saturated fatty acids in the presence or absence of cholesterol. Addition of cholesterol to the low-fat MUFA diet increased CETP activity and mRNA expression, whereas addition of cholesterol to the high-fat MUFA diet led to a decrease in CETP activity and mRNA expression. In SW 872 cells, oleic acid and cholesterol stimulated CETP gene expression when given alone. However, addition of fatty acids along with cholesterol interfered with the stimulatory effect of cholesterol on CETP gene regulation. Cholesterol-mediated stimulation of CETP involves the transcription factor liver X receptor α (LXR α). High-fat MUFA diets inhibited the expression of LXR α , and addition of cholesterol to the high-fat MUFA diet did not rescue LXR α expression. **Therefore, we present evidence for the first time that inhibition of LXR α expression by a high-fat MUFA diet leads to inhibition of CETP stimulation by cholesterol.**—Cheema, S. K., A. Agarwal-Mawal, C. M. Murray, and S. Tucker. **Lack of stimulation of cholesteryl ester transfer protein by cholesterol in the presence of a high-fat diet.** *J. Lipid Res.* 2005. 46: 2356–2366.

Supplementary key words transgenic mice • dietary fat and cholesterol • liver X receptor α • peroxisome proliferator-activated receptor α

Cholesteryl ester transfer protein (CETP) is considered a key component in regulating cholesterol homeostasis as it transfers cholesteryl esters from HDL to apolipoprotein B-containing lipoproteins (1–4). Increased plasma CETP levels result in low plasma HDL and high plasma LDL or VLDL levels (5, 6). HDL plays a major role in reverse cholesterol transport, a process that involves the movement of cholesterol from peripheral cells to the liver for removal from the body. Studies in transgenic mice, and in humans

with alterations in CETP levels, show that an absence of CETP is associated with the disruption of cholesterol efflux from cell membranes, of cholesterol esterification, and of cholesteryl ester transfer to apolipoprotein B-containing lipoproteins (1). These disruptions impair cholesterol movement to the liver; thus, a high CETP activity might be antiatherogenic as it removes excess cholesterol from the arterial wall. On the other hand, CETP produces VLDL particles that are cholesteryl ester-enriched and decreases the level of antiatherogenic HDL cholesterol; thus, CETP is considered proatherogenic (1–4). In humans, CETP deficiency is associated with high HDL levels and a low prevalence of coronary heart disease (7, 8). The absence of atherosclerosis in some of the first CETP-deficient Japanese patients provided support for the initial hypothesis that CETP is proatherogenic (7). Transgenic mice expressing human (9) or simian (10) CETP exhibit a marked reduction in HDL cholesterol, also suggesting that high levels of CETP protein impart increased risk of coronary artery disease.

Plasma CETP activity increases in response to high-fat, high-cholesterol diets in hamsters (11), rabbits (12, 13), monkeys (14–16), and humans (4) and also in transgenic mice expressing human CETP (17, 18). The increase in CETP activity in response to an atherogenic diet is associated with an increase in hepatic mRNA abundance (19), which in CETP transgenic mice fully accounts for the increase in CETP activity (17). Although dietary cholesterol is likely to be the major factor contributing to an increase in plasma CETP activity, synthetic diets that lead to increased hypercholesterolemia, without the addition of cholesterol, also result in a similar increase in plasma CETP activity (20). There are very few reports available to show

Abbreviations: CAT, chloramphenicol acetyl transferase; CETP, cholesteryl ester transfer protein; CETP-TG, transgenic mice expressing human cholesteryl ester transfer protein; cyp7, cholesterol 7- α hydroxylase; LXR, liver X receptor; LXRE, liver X receptor response element; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; SFA, saturated fatty acid.

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the effect of dietary fats on CETP regulation (16, 18, 20–24). Although saturated fatty acids (SFAs) have been consistently shown to increase CETP, the effects observed with unsaturated fatty acids are variable. Reports indicate that monounsaturated fatty acids (MUFAs) decrease (20, 21, 23), have no effect (18), or increase (15, 24) plasma CETP activity. These different findings might be attributable to variations in the design of the diets, in which the amount of fat and the presence or absence of cholesterol in the diet was overlooked.

We have shown previously that dietary fats modify the regulation of cholesterol 7- α hydroxylase (*cyp7*) by dietary cholesterol (25). The regulation of *cyp7* is similar to that of CETP in that both genes are induced by dietary cholesterol (17, 26). Cholesterol-mediated induction involves the binding of liver X receptor α /retinoid X receptor (LXR α /RXR) to the proximal promoter region of both *cyp7* (27) and CETP (28) genes. Fatty acid-mediated induction of the *cyp7* gene involves peroxisome proliferator-activated receptor α (PPAR α)/RXR, and the peroxisome proliferator response element (PPRE) site is embedded within the LXR α /RXR binding site (29). On the other hand, the regulation of CETP by dietary fats is not clear. It is also not known whether fatty acids interfere with the cholesterol-mediated induction of CETP.

In this study, we investigated the regulation of CETP in transgenic mice expressing the human CETP gene under the control of its natural flanking region (CETP-TG mice) by the quantity and quality of dietary fats in the absence or presence of dietary cholesterol. We further investigated whether the quantity and quality of dietary fats alter the expression of LXR α , which in turn might modulate the regulatory potential of cholesterol on CETP expression. We established that a high-fat diet enriched in MUFAs inhibited LXR α expression and that addition of cholesterol to this diet did not rescue the expression of LXR α . We further established that cholesterol added to a high-fat diet enriched in MUFAs suppressed CETP gene expression. Therefore, this study reports for the first time that the quantity and quality of dietary fats inhibit the expression of LXR α , which is responsible for the decreased stimulation of CETP by dietary cholesterol, when given along with high-fat MUFAs. The possibility of regulation of CETP activity by the cross-talk between PPAR α and LXR α is discussed.

MATERIALS AND METHODS

Animals and diets

Transgenic mice expressing human CETP under the control of its natural flanking region (3.4 kb) were obtained from Dr. Alan Tall (Columbia University, New York) (17). C57BL6/J female mice were purchased from Jackson Laboratories (Bar Harbor, ME). The transgenic mice were crossbred with C57BL6/J mice to obtain heterozygotes (30). Animals were housed in a temperature-controlled (25°C) and humidity-controlled room with a 12 h light/dark cycle. Animals were given rodent chow diet and water for ad libitum consumption during the entire period. Eight week old CETP-TG mice were fed a semipurified diet (custom-made basal mix without fat; ICN Biomedicals, Inc., Quebec, Canada)

containing either low fat (5%, w/w) or high fat (20%, w/w) from olive oil (enriched in 18:1; a MUFA diet) or beef tallow (enriched in 18:0; a SFA diet) in the presence or absence of 1% cholesterol. Fatty acid analysis of the diets showed that the MUFA diet contains 63% (w/w) 18:1, whereas the SFA diet contains 52% (w/w) 18:0. The animals were fed the specified diets for 2 weeks. Body weight was determined at the beginning and end of the diet study. Food was replenished every other day, and food consumption was recorded. At the end of the diet study, food was withdrawn from all groups 12 h before euthanasia. Mice were euthanized, and blood was collected by cardiac puncture in tubes containing EDTA. Livers were removed, weighed, and quick-frozen in liquid nitrogen. Liver samples were stored at -70°C until further use. All procedures were in accordance with the principles and guidelines of the Canadian Council on Animal Care and the Principles of Laboratory Animal Care (National Institutes of Health) and were approved by the Memorial University of Newfoundland Animal Welfare Committee.

Plasma and hepatic lipid analyses

Fasting blood was collected via cardiac puncture in a syringe containing 1 g EDTA/l, and plasma was separated. Lipids were extracted from liver samples as described previously (31). Plasma and liver lipid samples were assayed for total cholesterol using enzymatic methods (kit 352; Sigma-Aldrich, St. Louis, MO), and triacylglycerol levels were assayed using kit 339 (Sigma-Aldrich). The plasma HDL-cholesterol concentration was determined after precipitating β -lipoproteins with phosphotungstate and measuring cholesterol concentration in the supernatant by enzymatic methods (kit 352; Sigma-Aldrich). The plasma LDL-cholesterol concentration was calculated from plasma total cholesterol, HDL-cholesterol, and triacylglycerol concentrations (32).

CETP activity, mass, and mRNA levels

Plasma CETP activity was assayed using a commercial CETP activity assay kit (Roar Biomedical Research, Inc., Columbia University) to identify transgenic mice expressing human CETP at 5 and 8 weeks of age. Cholesteryl ester transfer activity assays for all other experiments were performed using the radioisotope assay as described previously with modifications (30). The percentage transfer of radioactivity from HDL to LDL in a control sample was subtracted from that in the test samples, and the values for CETP activity were expressed as percentage cholesteryl ester transfer per hour.

CETP mass in plasma samples was quantitatively assayed using the CETP ELISA DAIICHI kit (Daiichi Pure Chemicals Co.) as described previously (30). Concentrations of the samples were calculated using a standard curve developed using a CETP stock solution of known concentration.

To detect changes in CETP mRNA abundance, total RNA from mouse livers was purified according to standard procedures (33). Hepatic CETP mRNA levels were determined by reverse transcription and in vitro DNA amplification (30). The amount of CETP mRNA was normalized to β -actin mRNA content and expressed as arbitrary units.

Cloning of the human CETP gene 5' regulatory region, and chimeric gene construction

Chimeric gene constructs, harboring serial deletions of the human CETP gene 5' regulatory region linked to chloramphenicol acetyl transferase (CAT) as a reporter, were designed (34) using the published sequence of the human CETP gene (GenBank accession numbers U71187 and M32992). A 1,520 bp PCR fragment containing 360 bp of the 5' regulatory region, exon 1, intron 1, and exon 2 was generated in which the *Hind*III site was inserted in exon 1. The PCR product was digested with *Hind*III,

and the resulting fragment containing 300 bp of the CETP gene 5' regulatory region was linked to CAT in pCAT.Basic vector (Promega) and designated 300CETP.CAT. Fragment 138CETP.CAT was constructed by digesting 300CETP.CAT with *Xba*I. For the longer CETP gene 5' regulatory region, the 1,520 bp PCR fragment was used to screen a human chromosome 16 genomic library. An *Xba*I-*Xba*I fragment containing sequences from -3,420 to -138 was obtained and cloned into 138CETP.CAT to generate 3400CETP.CAT. 3400CETP.CAT was digested with *Kpn*I to generate 570CETP.CAT (Fig. 1). The sequences of all cloned CETP fragments were confirmed, and restriction mapping of 3400CETP.CAT, 570CETP.CAT, and 138CETP.CAT chimeric gene constructs was performed to confirm the identity of the plasmids.

Cell culture and transfections

SW 872 cells (human liposarcoma cells) were purchased from the American Tissue Culture Collection (Manassas, VA). The cells were standardized to grow in a humidified atmosphere of 5% CO₂/95% air in DMEM/F12 (3:1), 5% FBS, 10 mM HEPES, and 50 µg/ml gentamycin at 37°C according to previously published methods (35). When cells reached 70% confluence, they were differentiated as described below. Briefly, medium was removed and replaced by DMEM/F12 (3:1), 10 mM HEPES, 50 µg/ml gentamycin, 100 µg/ml oleic acid complexed to fatty acid-free BSA, 1 µg/ml insulin, and 1 µg/ml transferrin for 24 h. After 24 h, cells were washed with PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 1.5 mM KH₂PO₄, pH 7.4) and fresh regular growth medium (without serum) containing DMEM/F12 (3:1), 10 mM HEPES, and 50 µg/ml gentamycin was added. Cells were grown under these conditions for 24 h before transfection.

To investigate the effect of fatty acids and cholesterol on CETP gene regulation, the CETP chimeric gene constructs (138CETP.CAT, 570CETP.CAT, and 3400CETP.CAT) were transfected into SW 872 cells according to the CaPO₄ transfection method (29). The total amount of plasmid DNA was kept constant by adding sonicated salmon sperm DNA. The cells were cotransfected with β-galactosidase to control for transfection efficiency. Regular growth medium containing delipidated serum (2.5% FBS) was added to the transfected cells. Oleic acid (a monounsaturated fatty acid; 18:1) was complexed to fatty acid free-BSA (29), and 25-OH cholesterol (Sigma-Aldrich) was dissolved in DMSO

(Sigma-Aldrich) and added to the transfected cells (50 µM for fatty acids and 4 µg/ml for 25-OH cholesterol). Control cells received the vehicle alone. After 24 h, cells were washed twice with PBS, replenished with regular growth medium containing delipidated serum (5% FBS), oleic acid, or 25-OH cholesterol for an additional 24 h. Cells were harvested, and the cell pellet was suspended in 250 mM Tris-HCl, pH 8.0, and freeze/thawed six times. Cell lysates were centrifuged at 9,000 *g* for 20 min at 4°C. The supernatant was assayed for CAT activity and β-galactosidase activity (29).

Western blotting

To perform Western blot analysis, liver extracts were prepared from frozen liver samples (*n* = 8 on each diet) as follows. Briefly, 300 mg of liver tissue was homogenized in 1 ml of homogenization buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 50 mM DTT at 4°C. Samples were sonicated for 30 s and centrifuged at 9,000 *g* for 20 min at 4°C. Supernatant was collected, and the protein was estimated by the method of Lowry et al. (36). Samples containing 50 µg of protein were subjected to sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis. Protein fractions on the gel were transferred to nitrocellulose membranes. For the detection of LXRα and PPARα, the membranes were incubated overnight at 4°C with a 1:500 dilution of polyclonal goat anti-LXRα and goat anti-PPARα antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) dissolved in Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk. The membranes were then washed with 1× Tris-buffered saline containing 0.05% Tween 20 and incubated with a 1:5,000 dilution of the peroxidase-labeled anti-goat secondary antibodies (Santa Cruz Biotechnology) for 2 h at room temperature. Protein bands in the membranes were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology). The Western blots for each sample were repeated at least five times.

Statistical analysis

The data from the mouse experiments were analyzed using three-way ANOVA and a Tukey's post hoc test. The differences between groups were considered significant at *P* < 0.05 (37). Where indicated, values without common letters are significantly different.

RESULTS

Food intake and body weight change

CETP-TG mice were fed low-fat (5%) or high-fat (20%) diets containing either olive oil (enriched in 18:1; MUFA) or beef tallow (enriched in 18:0; SFA) with or without cholesterol. There was no change in body weight or food intake over the treatment period. The average body weight at the end of the treatment, under various dietary conditions, was 24 g, and the average food intake was 2.6 g/day.

Effect of dietary fats and cholesterol on plasma lipid levels

To evaluate the effect of variation in quantity and quality of dietary fats and cholesterol on plasma lipid levels, total, LDL-, and HDL-cholesterol as well as triacylglycerol concentrations were measured in plasma and are given in Table 1. Feeding a high-fat diet enriched in MUFAs or SFAs had no significant effect on plasma total cholesterol concentrations compared with feeding a low-fat MUFA or

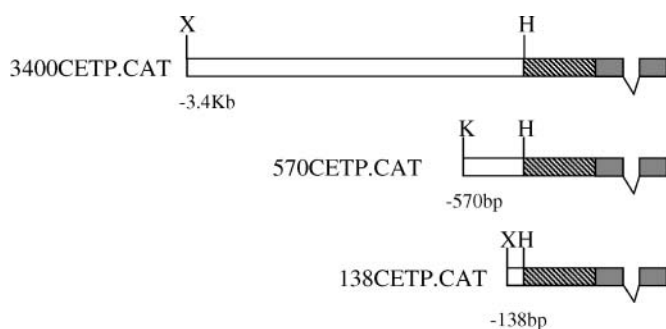


Fig. 1. Structure of the human cholesteryl ester transfer protein (CETP). The chloramphenicol acetyl transferase (CAT) gene chimeras 3400CETP.CAT, 570CETP, and 138CETP.CAT contain 3.4 kb, 570 bp, and 138 bp, respectively, of the CETP gene 5' regulatory region linked to CAT as a reporter. White boxes represent the human CETP gene promoter; hatched boxes represent the CAT structural gene sequence; gray boxes represent the SV40 region. X represents the digestion site for *Xba*I, K represents the digestion site for *Kpn*I, and H represents the digestion site for *Hind*III.

TABLE 1. Plasma lipid levels of CETP-TG mice fed various diets

Fat Level	Diet	Total Cholesterol	LDL-Cholesterol	HDL-Cholesterol	Total Triacylglycerol
<i>mmol/l</i>					
5%	MUFA	1.73 ± 0.13 b	0.70 ± 0.09 a	1.06 ± 0.13 a	0.17 ± 0.07 a
	MUFA + cholesterol	1.87 ± 0.21 b	0.98 ± 0.10 b	0.87 ± 0.11 b	0.19 ± 0.05 a
20%	MUFA	1.75 ± 0.25 b	0.79 ± 0.09 a	0.79 ± 0.13 c	0.20 ± 0.04 a
	MUFA + cholesterol	1.91 ± 0.33 b, c	0.93 ± 0.08 a, b	0.74 ± 0.15 c	0.24 ± 0.06 a
5%	SFA	1.28 ± 0.10 a	0.52 ± 0.03 c	0.72 ± 0.08 c	0.35 ± 0.04 b
	SFA + cholesterol	1.39 ± 0.11 a	0.63 ± 0.07 d	0.75 ± 0.07 c	0.52 ± 0.07 c
20%	SFA	1.48 ± 0.38 a, b	0.57 ± 0.03 c	0.59 ± 0.13 d	0.61 ± 0.23 c
	SFA + cholesterol	1.61 ± 0.42 a, b	0.77 ± 0.04 a	0.57 ± 0.12 d	0.72 ± 0.12 c, d

SFA, saturated fatty acid. CETP-TG mice (transgenic mice expressing human cholesteryl ester transfer protein) were fed a low-fat (5%, w/w) or a high-fat (20%, w/w) diet enriched in MUFAs or SFAs with and without 1% dietary cholesterol for 2 weeks. Blood was collected by cardiac puncture, and plasma was assayed for total, LDL-, and HDL-cholesterol and total triacylglycerol concentrations as described in Materials and Methods. Values shown are means ± SD for eight mice on each diet. Values without common letters are significantly different ($P < 0.05$) using three-way ANOVA.

SFA diet. Addition of cholesterol to the low- or high-fat MUFA and SFA diets also showed no significant effect on plasma total cholesterol concentrations compared with mice fed the MUFA or SFA diets alone. Plasma cholesterol concentrations were lower in mice fed the low-fat SFA diets with or without added cholesterol compared with mice fed the low- or high-fat MUFA diets.

LDL-cholesterol concentrations did not differ between mice fed the low- or high-fat MUFA diets. Addition of cholesterol to the low-fat MUFA diet significantly increased plasma LDL-cholesterol concentrations (~1.30-fold; $P \leq 0.02$), whereas, at high-fat levels, no significant effect of addition of cholesterol was seen. Mice fed the SFA diets did not show changes in LDL-cholesterol levels between the low- and high-fat diets. However, addition of cholesterol to both low- and high-fat SFA diets significantly increased LDL-cholesterol levels. LDL-cholesterol levels were lower in mice fed all types of SFA diets compared with the MUFA diets.

HDL-cholesterol concentrations were lower in mice fed the high-fat MUFA diets with or without cholesterol compared with mice fed the low-fat MUFA diets. Addition of cholesterol to the low-fat MUFA diet decreased the HDL-cholesterol concentrations, whereas addition of cholesterol to the high-fat MUFA diet had no effect. HDL-cholesterol concentrations were lower in mice fed the high-fat SFA diet with or without cholesterol compared with mice fed the low-fat SFA diets. HDL-cholesterol concentrations were lower in high-fat SFA-fed mice compared with low- and high-fat MUFA-fed mice with and without added dietary cholesterol.

There was no significant effect of low- or high-fat MUFA diets or addition of dietary cholesterol to the MUFA diets (low or high fat) on plasma total triacylglycerol concentrations. A significant increase in total triacylglycerol levels was seen when mice were fed high-fat SFA diets compared with the mice fed low-fat SFA diets. Addition of cholesterol to the low-fat SFA diets increased total triacylglycerol levels significantly, whereas it had no significant effect on total triacylglycerol levels in mice fed the high-fat SFA diets. Total triacylglycerol levels were 2- to 3-fold higher in mice fed all types of SFA diets compared with MUFA diets.

Effect of dietary fat and cholesterol on hepatic lipid levels

Changes in hepatic lipid levels of CETP-TG mice fed diets enriched in MUFAs or SFAs, with or without dietary cholesterol, are given in **Table 2**. There was no significant difference in hepatic total cholesterol concentrations between CETP-TG mice fed low- or high-fat MUFA diets or low- or high-fat SFA diets. Addition of cholesterol to both low- and high-fat MUFA and SFA diets significantly increased hepatic total cholesterol concentrations (~2- to 3-fold increase; $P \leq 0.006$), indicating that dietary cholesterol is delivered to the liver. There was an ~2-fold increase in hepatic total cholesterol when mice were fed SFA diets compared with MUFA diets (low and high fat, with and without cholesterol).

Hepatic total triacylglycerol concentrations of mice fed high-fat MUFA diets were significantly higher compared with those in mice fed low-fat MUFA diets. Addition of cholesterol to both low- and high-fat MUFA diets caused a significant increase in hepatic total triacylglycerol concentrations (~2-fold increase; $P \leq 0.007$). Total triacylglycerol levels did not differ between mice fed the low- or high-fat SFA diet, with or without cholesterol. Moreover, hepatic total triacylglycerol levels were 3.5-fold lower in mice fed the low-fat SFA diets compared with mice fed the

TABLE 2. Hepatic lipid levels of CETP-TG mice fed different diets

Fat Level	Diet	Total Cholesterol	Total Triacylglycerol
<i>mmol/mg liver</i>			
5%	MUFA	5.36 ± 0.8 a	30.34 ± 7.97 a
	MUFA + cholesterol	18.61 ± 4.12 c	76.33 ± 17.98 b
20%	MUFA	7.82 ± 1.67 a, b	61.01 ± 4.12 b
	MUFA + cholesterol	17.59 ± 4.34 c	141.15 ± 24.21 c
5%	SFA	14.61 ± 1.12 c	8.61 ± 0.72 d
	SFA + cholesterol	28.16 ± 3.02 d	10.06 ± 1.20 d
20%	SFA	16.43 ± 3.37 c	7.19 ± 2.47 d
	SFA + cholesterol	35.27 ± 6.93 d	9.76 ± 2.05 d

CETP-TG mice were fed a low-fat (5%, w/w) or a high-fat (20%, w/w) diet enriched in MUFAs or SFAs with and without 1% dietary cholesterol for 2 weeks. Liver samples were collected, and lipids were extracted and assayed for total cholesterol and total triacylglycerol concentrations as described in Materials and Methods. Values shown are means ± SD for eight mice on each diet. Values without common letters are significantly different ($P < 0.05$) using three-way ANOVA.

low-fat MUFA diets. Addition of cholesterol to low-fat SFA diets further decreased the hepatic total triacylglycerol levels (7.5-fold) compared with that in MUFA diets. This difference in hepatic total triacylglycerol levels between SFA- and MUFA-fed mice became more prominent when fat levels were increased (8.5-fold and 14.46-fold, without and with cholesterol, respectively).

Effect of dietary fats and cholesterol on plasma CETP activity, mass, and mRNA levels

In addition to being the major source of energy, fatty acids also regulate many genes. To investigate whether the quantity and quality of fat in the diet regulates CETP, we measured the plasma CETP activity, CETP mass, and hepatic CETP mRNA levels in CETP-TG mice fed low- and high-fat MUFA and SFA diets. Plasma CETP activity, CETP mass, and hepatic CETP mRNA levels were significantly higher in CETP-TG mice fed a high-fat MUFA diet compared with CETP-TG mice fed a low-fat MUFA diet (Fig. 2A, B, C, respectively). In mice fed the high-fat SFA diets, these three parameters showed a trend toward an increase compared with mice fed the low-fat SFA diet; however, the difference was not statistically significant (Fig. 2A, B, C, respectively). In addition, mice fed the MUFA diet had higher plasma CETP activity, mass, and mRNA levels compared with mice fed the SFA diets at both low and high fat levels (Fig. 2). The difference in CETP activity between the MUFA and SFA diets became much more significant with high-fat diets. These findings clearly suggest that the quantity and quality of fat regulates CETP activity, mass, and gene expression.

Cholesterol is known to induce CETP activity and hepatic CETP gene expression in transgenic mice expressing human CETP, and saturated fats further enhance this effect (17). However, it is not clear whether cholesterol given along with other dietary fats has a similar effect on CETP regulation. We examined the effects of dietary cholesterol given along with a diet enriched in MUFAs or SFAs on CETP activity, mass, and gene expression in CETP-TG mice fed a low- or a high-fat diet. Addition of cholesterol to the low-fat MUFA diet increased plasma CETP activity (Fig. 3A), with parallel increases in CETP mass and hepatic CETP mRNA levels (Fig. 3B, C). On the other hand, addition of cholesterol to the high-fat MUFA diet significantly inhibited plasma CETP activity (Fig. 3D), with a parallel decrease in hepatic CETP mRNA levels (Fig. 3F) and no major change in CETP mass (Fig. 3E). Based on these results, we conclude that addition of cholesterol to the high- or low-fat MUFA diet has opposite effects on CETP gene regulation.

When cholesterol was added to the low-fat SFA diet, there was a trend toward an increase in CETP activity, mass, and mRNA levels (Fig. 3A, B, C, respectively); however, the increase was not significant. In contrast to what we observed with high-fat MUFA diets, cholesterol given with a diet enriched in high-fat SFA had no effect on CETP activity, mass, or mRNA levels (Fig. 3D, E, F, respectively). Together, these results demonstrate that the regulation of CETP by dietary cholesterol is influenced by the amount and type of dietary fat.

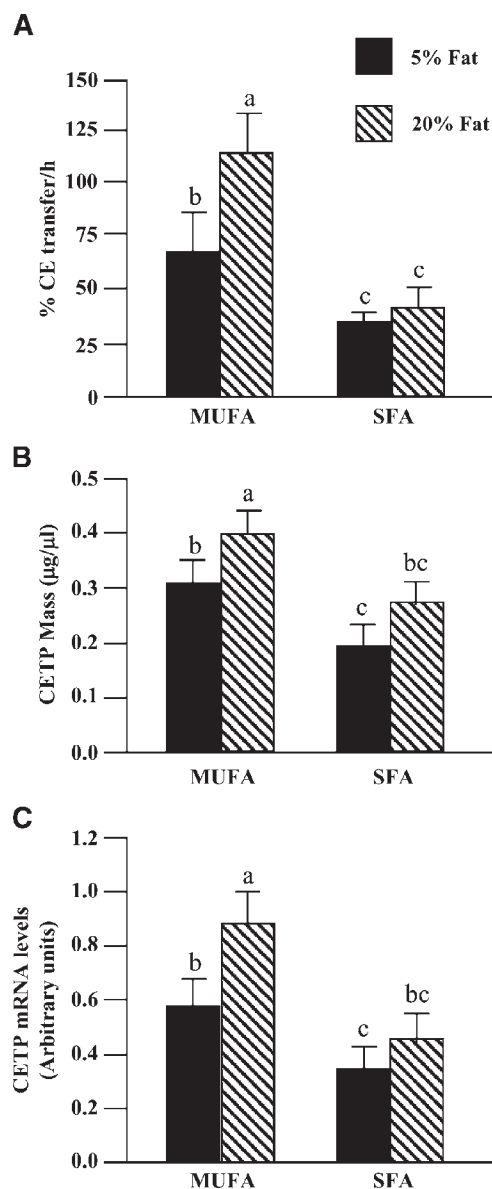


Fig. 2. Regulation of CETP activity, mass, and gene expression by MUFAs and saturated fatty acids (SFAs) in transgenic mice expressing human CETP (CETP-TG mice). The CETP-TG mice were fed diets enriched in MUFAs or SFAs containing 5% (w/w; black bars) or 20% (w/w; hatched bars) fat for 2 weeks. Blood and liver samples were collected and assayed for plasma CETP activity (A), plasma CETP mass (B), and hepatic CETP mRNA expression (C). Plasma CETP activity was assayed using the radioisotope method as described in Materials and Methods. The values are expressed as percentages of cholesteryl ester (CE) transfer per hour. CETP mass in plasma samples was quantitatively assayed using the CETP ELISA DAIICHI kit as described in Materials and Methods. Hepatic CETP mRNA abundance was measured using RT-PCR. The amount of CETP mRNA was normalized to β -actin mRNA and expressed as arbitrary units. Values shown are means \pm SD for eight mice on each diet. Values without a common letter are significantly different ($P < 0.05$) using three-way ANOVA.

Regulation of the CETP gene by dietary fats and cholesterol: in vitro studies

To further understand the molecular mechanisms of regulation of the CETP gene by fatty acids and cholesterol,

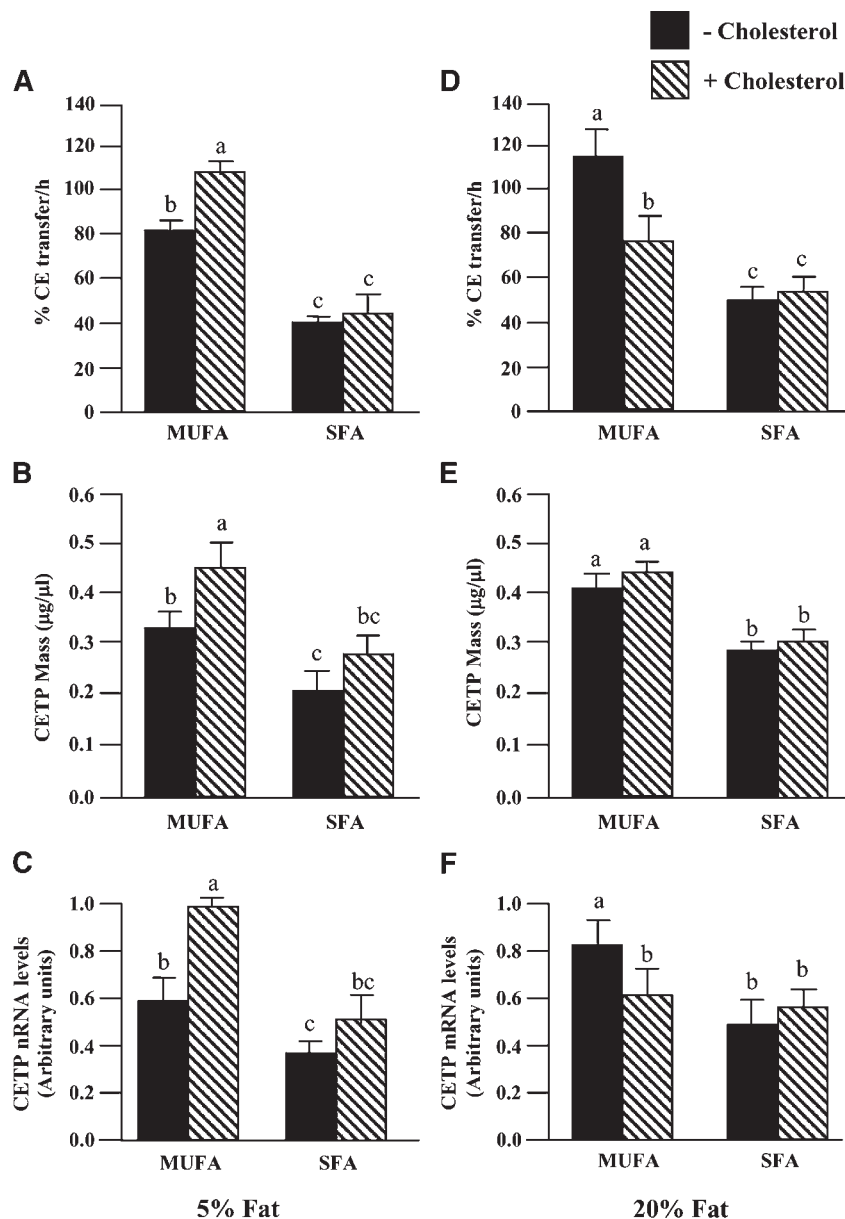


Fig. 3. Effect of variation in the quantity of MUFAs and SFAs on cholesterol-mediated regulation of CETP activity, mass, and mRNA level. The CETP-TG mice were fed diets enriched in 5% (A–C) or 20% (D–F) MUFA or SFA in the absence (black bars) or presence (hatched bars) of 1% cholesterol for 2 weeks. Blood and liver samples were collected and assayed for plasma CETP activity (A, D), plasma CETP mass (B, E), and hepatic CETP mRNA expression (C, F). Plasma CETP activity was assayed using the radioisotope method. The values are expressed as percentages of cholesteryl ester (CE) transfer per hour. CETP mass in plasma samples was quantitatively assayed using the CETP ELISA DAICHI kit as described in Materials and Methods. Hepatic CETP mRNA abundance was measured using RT-PCR. The amount of CETP mRNA was normalized to β -actin mRNA and expressed as arbitrary units. Values shown are means \pm SD for eight mice on each diet. Values without a common letter are significantly different ($P < 0.05$) using three-way ANOVA.

SW 872 cells were transfected with various CETP chimeric gene constructs containing regulatory elements of the CETP gene. Previous findings have demonstrated that cholesterol-mediated stimulation of the CETP gene is contained within 400 bp of the promoter region and involves the transcription factors LXR α /RXR (28). We confirmed these previous findings in SW 872 cells transfected with CETP chimeric gene constructs. The transfected cells were treated with 4 μ g/ml 25-OH cholesterol. Expression of 570CETP.CAT

was increased 3.3-fold ($P \leq 0.002$) when 25-OH cholesterol was added to the growth medium (Fig. 4A). These findings confirm that cholesterol-mediated induction of the CETP gene is contained within 570 bp of the promoter.

To determine whether CETP regulation by oleic acid is at the molecular level, SW 872 cells were transfected with CETP chimeric gene constructs. The transfected cells were treated with oleic acid complexed to fatty acid-free bovine serum albumin. Oleic acid at a concentration of 50

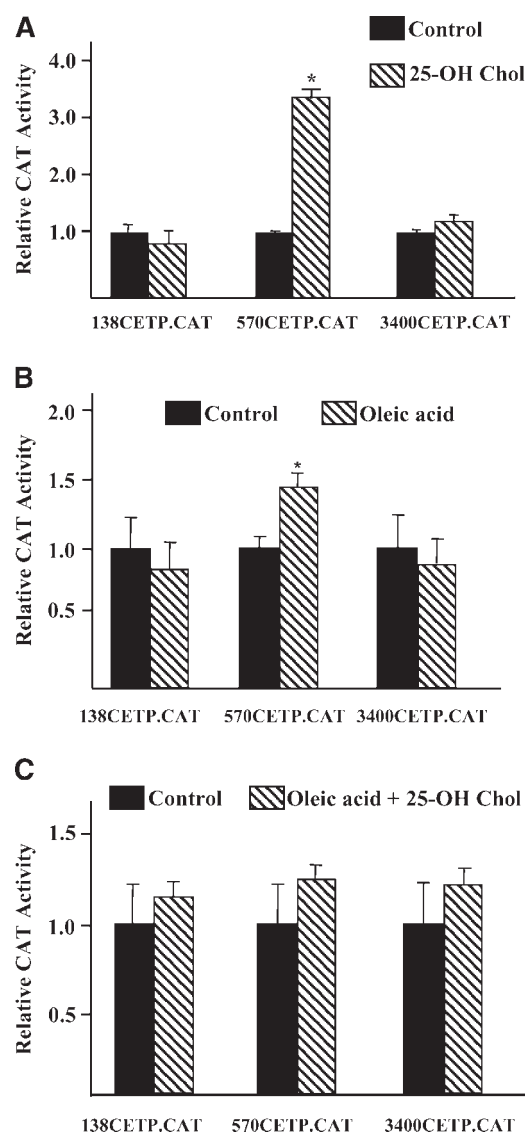


Fig. 4. Oleic acid- and cholesterol-mediated induction of the CETP gene is contained within 570 bp of the CETP 5' regulatory region. **A:** The response of 138CETP.CAT, 570CETP.CAT, and 3400CETP.CAT gene chimeras to 25-OH cholesterol was tested in SW 872 cells. The CETP.CAT gene chimeras were transfected into SW 872 cells. After 24 h, 25-OH cholesterol in DMSO was added to the culture medium to a final concentration of 4 μ g/ml (hatched bars). Control cells received DMSO alone (black bars). **B:** The response of the CETP.CAT gene chimeras to oleic acid (18:1). Oleic acid complexed to fatty acid-free BSA was added to the cell culture medium of the transfected cells to a final concentration of 50 μ M (hatched bars). Control cells received fatty acid-free BSA alone (black bars). **C:** The response of the CETP.CAT gene chimeras to oleic acid (18:1) and 25-OH cholesterol. Oleic acid complexed to fatty acid-free BSA (50 μ M) and 25-OH cholesterol dissolved in DMSO (4 μ g/ml) was added to the cell culture medium of the transfected cells (hatched bars). Control cells received fatty acid-free BSA and DMSO (black bars). For all panels, CAT activity was normalized to β -galactosidase activity encoded by the β -gal expression vector. The average normalized CAT activity value in the absence of ligands was assigned a value of 1. The results shown represent averages \pm SEM of three independent experiments performed in triplicate. * Significantly different from the treatments without ligands ($P < 0.05$).

μ M significantly increased the expression of 570CETP.CAT (Fig. 4B), suggesting that the fatty acid-mediated regulation of the CETP gene is also contained within 570 bp of the 5' regulatory region.

In our *in vivo* experiments, we observed that the quantity and quality of dietary fats altered the cholesterol-mediated regulation of CETP. To explore the molecular mechanisms of this phenomenon, we transfected CETP chimeric gene constructs into SW 872 cells and treated the transfected cells with 50 μ M oleic acid complexed to fatty acid-free bovine serum albumin along with 4 μ g/ml 25-OH cholesterol (Fig. 4C), then measured the CAT activity. In contrast to the results obtained previously, addition of oleic acid along with cholesterol abolished the stimulatory effect of cholesterol on 570CETP.CAT. These results demonstrated that oleic acid and cholesterol stimulate CETP gene expression when given alone. However, addition of fatty acids along with cholesterol interferes with the stimulatory effect of cholesterol on CETP gene regulation.

Regulation of LXR α and PPAR α expression by dietary fats and cholesterol

Fatty acids and cholesterol are generally found together in the diet. Fatty acids are native ligands of PPAR α , and cholesterol is a native ligand for LXR α . LXR α and PPAR α regulate lipid homeostasis by regulating several genes that are involved in cholesterol and fatty acid metabolism, respectively (38–43). To understand the mechanism of fatty acid interference with regulation of the CETP gene by cholesterol in CETP-TG mice, we wanted to investigate whether this interference is at the level of regulation of the expression of the transcription factors PPAR α and/or LXR α . Liver is one of the major sites for CETP expression in CETP-TG mice under the control of its natural flanking region (17); the protein expression of PPAR α and LXR α was measured in the livers of mice fed various diets by Western blotting (Fig. 5A, B). Mice fed high-fat MUFA diets showed significantly increased expression of PPAR α compared with low-fat MUFA-fed mice. Inclusion of cholesterol in the high-fat MUFA diet decreased PPAR α protein expression compared with feeding the high-fat MUFA diet alone. At the same protein concentration, the expression of PPAR α was undetectable in low-fat MUFA-fed CETP-TG mice in the absence or presence of cholesterol (Fig. 5A). The amount of fat or the addition of cholesterol had no effect on PPAR α protein expression in SFA-fed mice (data not shown). These results indicate that PPAR α is induced by high-fat MUFA diets and that the addition of cholesterol to the high-fat MUFA diets decreases the expression of PPAR α .

In contrast to PPAR α , LXR α protein was detected in mice fed the low-fat MUFA diet, and addition of cholesterol to the low-fat MUFA diet increased LXR α protein expression (Fig. 5B). Increasing the amount of fat to 20% inhibited the expression of LXR α protein. When cholesterol was added to high-fat MUFA diets, it did not rescue the inhibitory effect of high-fat MUFAs, even though the nuclear receptor LXR α has been shown to act as a sensor for cholesterol (28). There was no effect of SFA diets on LXR α protein expression (data not shown). These data

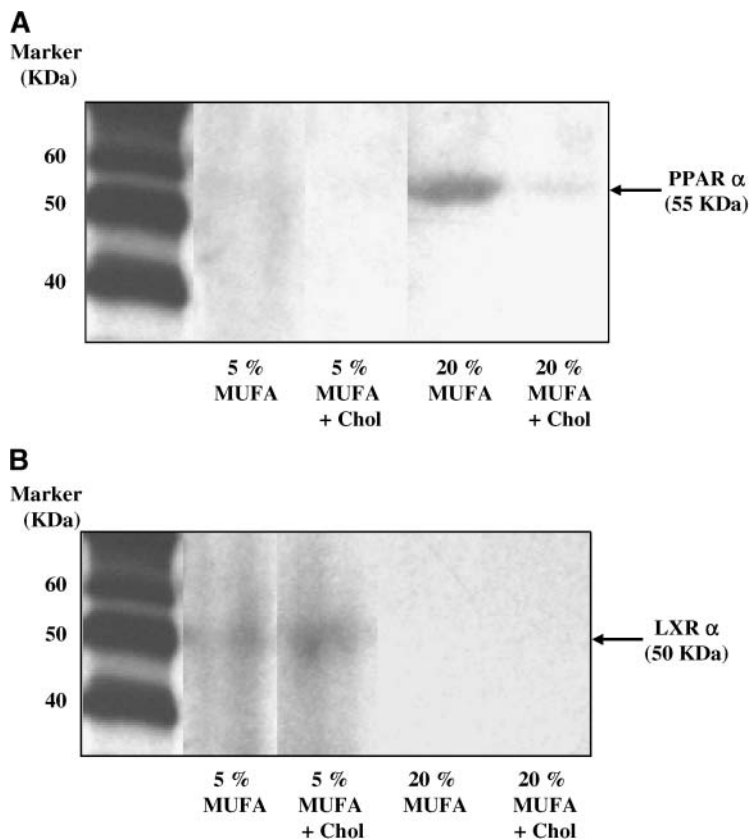


Fig. 5. Regulation of peroxisome proliferator-activated receptor α (PPAR α) and liver X receptor α (LXR α) protein expression in the livers of CETP-TG mice by dietary fats and cholesterol (Chol). The CETP-TG mice were fed diets enriched in 5% or 20% MUFAs in the absence or presence of 1% cholesterol for 2 weeks. Protein extracts were prepared from the livers of these mice as described in Materials and Methods. Samples containing 50 μ g of protein were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblotting with goat anti-PPAR α (A) and goat anti-LXR α (B) antibodies (1:500).

clearly demonstrate that high-fat MUFA diets suppress LXR α protein expression and that the addition of cholesterol does not rescue LXR α protein expression.

DISCUSSION

Dietary cholesterol and saturated fats are known to increase CETP activity and mRNA levels (4, 11–16). However, the effect of unsaturated fatty acids (e.g., monounsaturated fatty acids) on the regulation of CETP is highly controversial (15, 16, 20–24). MUFA diets have been shown to decrease plasma CETP activity (20, 21, 23), although others have reported an increase in CETP activity with MUFA-enriched diets (15, 24). In these studies, the amounts of MUFA varied and many times the diet contained other types of fatty acids along with MUFAs. In addition, the diets were fed for longer time periods. The present study was undertaken to clarify some of the controversial issues related to CETP regulation by diets rich in MUFAs and to further investigate whether dietary fats interfere with cholesterol to regulate CETP, using the well-established mouse model expressing the human CETP gene under the control of its natural flanking region (17).

CETP-TG mice were fed a diet enriched in either MUFAs or SFAs at both low (5%, w/w) and high (20%, w/w) fat levels with and without 1% dietary cholesterol for 2 weeks. CETP activity, mass, and mRNA levels were higher in mice fed MUFA-enriched diets compared with mice fed SFA-enriched diets at both low and high fat levels. These observations are similar to earlier reports by Khosla et al.

(15) and Gupta et al. (24), who found increases in CETP activity by MUFA-enriched diets. It was interesting to observe that the addition of cholesterol to the low-fat MUFA diet increased CETP activity, mass, and gene expression, as expected, whereas the addition of cholesterol to the high-fat MUFA diet suppressed CETP activity and mRNA expression. This effect was not observed in mice fed SFA diets. It appears that at low fat levels, the effect of cholesterol overrides the effect of dietary fatty acids; however, at high fat levels, there is interference of fat with cholesterol to regulate CETP. Kurushima et al. (20, 22) previously reported that addition of oleic acid to a cholesterol-supplemented diet in hamsters prevents cholesterol-induced increases in CETP activity, suggesting that oleic acid interferes with the cholesterol-mediated induction of CETP activity. These findings are similar to our observation that oleic acid interferes with cholesterol to regulate CETP. Previously, we showed that the regulation of *cyp7* by cholesterol depends on the type of dietary fat (25). Cholesterol generally increases *cyp7* expression, although the stimulatory effect of cholesterol was abolished in the presence of a diet enriched in MUFAs, and this effect was not seen with a diet enriched in polyunsaturated fatty acids. The current observations indicate that the cholesterol-mediated regulation of CETP is influenced by the amount and type of dietary fat, similar to the observations made for *cyp7*.

The cholesterol-mediated induction of the CETP gene is contained within 400 bp of the 5' regulatory region (28). We confirmed these findings in SW 872 cells, in which the expression of 570CETP.CAT increased 3.3-fold when 25-OH cholesterol was added to the growth medium. The level of

fat in the diet has been shown to regulate the expression of genes involved in lipid metabolism (43, 44), in which high-fat diets generally increase the expression. In the present study, CETP activity and mRNA levels were significantly higher in mice fed the high-fat MUFA diet compared with low-fat MUFA-fed mice, indicating that the amount of fat regulates CETP expression. To address the question of whether the effect of dietary fats on CETP regulation is a direct effect, we used in vitro transfection experiments, using sequential deletions of the 5' regulatory regions of the human CETP gene. As seen with 25-OH cholesterol, oleic acid (18:1) also induced the expression of the human CETP gene, and the induction was contained within the 570 bp of the human CETP 5' regulatory region. These findings suggest that fatty acids directly regulate the CETP gene, and the fatty acid response elements are also situated within 570 bp of the CETP 5' regulatory region. The response of CETP to dietary cholesterol when given along with high-fat MUFA diets was unexpected in our in vivo experiments using CETP-TG mice. These observations were further confirmed in the in vitro experiments using SW 872 cells transfected with CETP.CAT chimeric gene constructs. Treatment of transfected cells with oleic acid and cholesterol given together abolished the stimulatory effect of fatty acids and cholesterol alone.

It is well established that the regulation of CETP by cholesterol involves the binding of LXR α /RXR to the 5' regulatory region of the CETP gene (28). To further understand the observations that the presence of a high-fat MUFA diet inhibited the cholesterol-mediated induction of CETP, we investigated whether the effect of MUFAs was mediated by altering the expression of LXR α . Western blot analysis revealed that the high-fat MUFA diet significantly suppressed the expression of LXR α and that addition of cholesterol to the high-fat MUFA diet was unable to rescue this inhibition. Thus, inhibition of LXR α expression by the high-fat MUFA diet, alone or in combination with cholesterol, might explain the lack of stimulation of CETP under these conditions. It is likely that a basal level of LXR α is essential to maintain the expression of CETP. Others have also shown that unsaturated fatty acids interfere with LXR activation, which in turn leads to a decrease in the expression of genes (45, 46). These authors found that the effect of fatty acids on the regulation of LXR α expression depends on the degree of unsaturation. The regulation of LXR α by fatty acids is at the transcriptional level, with unsaturated fatty acids being highly efficient and SFAs having no effect. We also observed no effect of a high-fat SFA diet on LXR α expression when given alone or in combination with cholesterol (data not shown). This explains why the SFA diet had a different effect on CETP regulation.

We also observed an increase in PPAR α expression by the high-fat MUFA diet. It is possible that the increased expression of PPAR α interferes with LXR α to regulate CETP. Previous studies have shown that LXR α and PPAR α interact in vitro (29, 47–53). PPAR α inhibits the binding of LXR α /RXR α to liver X receptor response element

(LXRE), whereas LXR α inhibits the binding of PPAR α /RXR α to PPRE and antagonizes peroxisome proliferator signaling. We have shown previously that the LXRE motif (DR-4 site) in the *cyp7* gene has a hidden PPRE motif (DR-1 site) (29). The *cyp7* gene was induced by activated LXR α and PPAR α ; however, the coexpression of the two nuclear receptors abolished the response of the *cyp7* promoter, indicating interaction between PPAR α and LXR α to regulate *cyp7* expression (47). Tobin et al. (51) have shown the presence of five possible PPRES in the mouse LXR α 5' regulatory region, which were required to maintain the in vitro response to a PPAR α agonist. Thus, it is likely that the induction of PPAR α by the high-fat MUFA diet is responsible for the inhibition of LXR α by binding of PPAR α to a PPRE in the LXR α 5' regulatory region.

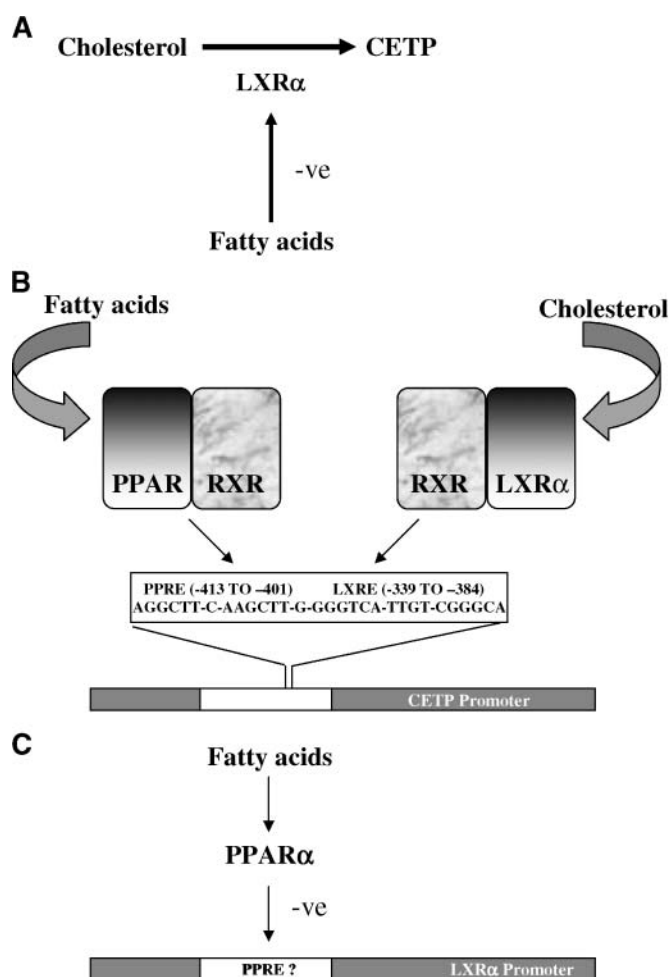


Fig. 6. Possible mechanisms involved in the fatty acid- and cholesterol-mediated regulation of CETP. **A:** Regulation of CETP by indirect regulation of LXR α by fatty acids. Cholesterol induces CETP via LXR α , whereas fatty acids downregulate LXR α , thereby interfering with the cholesterol-mediated induction of CETP. **B:** The fatty acid-mediated regulation of CETP might involve PPAR α /retinoid X receptor (RXR), which could compete for the binding of LXR α /RXR to the CETP promoter region. A potential peroxisome proliferator response element (PPRE) sequence is shown adjacent to the liver X receptor response element (LXRE) sequence in the CETP 5' regulatory region. **C:** PPAR α induced by fatty acids might bind to a possible PPRE present in the promoter region of LXR α and might inhibit the expression of LXR α . ve, negative regulation.

Whether or not fatty acid-mediated regulation of CETP involves the PPRES present in the 5' regulatory region(s) of LXR α and/or CETP is a topic of future investigation. **Figure 6** gives diagrammatical representations of the possible mechanisms involved in fatty acid and cholesterol interaction to regulate CETP. Data presented in this study have demonstrated that fatty acids interfere with the cholesterol-mediated regulation of CETP (Fig. 6A). Whether the effect of fatty acids and cholesterol on CETP regulation is attributable to the interaction of PPAR/RXR/LXR directly with the CETP promoter (Fig. 6B), or to the indirect regulation of LXR α by fatty acids (Fig. 6C), is not clear and needs to be explored. We have identified a potential PPRE site in the human CETP 5' regulatory region adjacent to the LXRE site, as shown in Fig. 6B. Studies are under way in our laboratory to address whether PPAR α /RXR binds to this site and to unravel the mechanism(s) of PPAR/LXR/RXR interaction.

Plasma CETP levels are highly correlated with CETP production during liver perfusion, indicating that the regulation of CETP production in the liver affects CETP availability in plasma (14). The levels of CETP in turn influence the relative degree of atherogenicity by altering the levels of LDL- and HDL-cholesterol concentrations. Comparison of the plasma LDL-cholesterol and HDL-cholesterol concentrations from low-fat MUFA-fed animals shows that they correlate well with changes in CETP activity. On the other hand, high-fat MUFA diets fail to show a trend with the CETP activity. Although there is a decrease in CETP activity when cholesterol is added to the high-fat MUFA diet, the LDL-cholesterol shows a minor increase, with no change in HDL-cholesterol concentrations. Fusegawa et al. (16) reported no significant differences in plasma CETP activity of St. Kitts vervet monkeys fed diets enriched in saturated, monounsaturated, or polyunsaturated fatty acids. However, addition of cholesterol to any of these diets increased plasma CETP activity, which is not in agreement with our findings. Those authors used 16% (w/w) dietary fat and 0.8 mg/kcal cholesterol in the diets, and the diets were fed for much longer time periods; however, we used 5% and 20% (w/w) fat content in our diets. The diet regimens we used are from two extremes; it is possible that there is a threshold point that triggers the dominant effect of fat over cholesterol. Thus, it is important to investigate the effect of dietary cholesterol on CETP regulation when given along with 10% (w/w) or 15% (w/w) fat. We did not find a significant effect of the SFA diet on CETP activity or mRNA expression, which is not consistent with previous observations. The high-fat SFA diet used in our study is rich in stearic acid (18:0), which is considered to be a neutral fatty acid. Diets rich in stearic acid have been shown to have no effect on plasma total cholesterol and LDL-cholesterol concentrations (54).

In conclusion, it is difficult to interpret from the current observations whether the regulation of CETP by dietary fats has an influence on atherogenicity. However, the observations that cholesterol does not have a singular effect on CETP regulation, and that the cholesterol-mediated regulation depends on the quantity and quality of fat

in the diet, are made here for the first time. It is suggested that cholesterol and fatty acids interact to regulate CETP expression, which might involve the interaction of transcription factors such as PPAR α and LXR α . The understanding of the regulation of metabolic pathways by cholesterol together with various fatty acids is crucial because these dietary components are normally consumed simultaneously. **BB**

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