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REGULATION OF HEPATIC CHOLESTEROL METABOLISM IN CETP$^{+/-}$/LDLr$^{+/-}$ MICE BY CHOLESTEROL FEEDING AND BY DRUGS (CHOLESTYRAMINE AND LOVASTATIN) THAT LOWER PLASMA CHOLESTEROL

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SUMMARY

1. The hepatic mechanisms involved in the simultaneous regulation of plasma cholesterol concentration and cholesteryl ester transfer protein (CETP) activity were investigated by sharply modifying the hepatic rates of cholesterol synthesis. This was accomplished by cholestyramine, lovastatin and cholesterol feeding in human CETP transgenic mice cross-bred with low-density lipoprotein receptor (LDLr)-knockout mice, generating CETP$^{+/-}$/LDLr$^{+/-}$ mice, which present a plasma lipoprotein profile resembling that of humans.

2. Analyses of pooled data showed that the plasma CETP activity correlated positively with plasma total cholesterol concentration, hepatic CETP mRNA and the liver microsomal cholesterol content; a negative correlation was found between plasma CETP activity and the liver 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and LDLr mRNA levels. These coordinated events represent an efficient control system that stabilizes the cell cholesterol content.

3. Nonetheless, not all cholesterol metabolism regulatory systems seem to fit into a coherent pattern of responses, suggesting that other unknown cellular mechanisms play roles depending on the type of pharmacological intervention.

4. For example, microsomal cholesterol content was not affected by cholestyramine, but was increased on cholesterol feeding (as predicted), and, surprisingly, on lovastatin treatment. Furthermore, although both plasma cholesterol-lowering drugs increased CYP7A1 mRNA and had no effect on CYP27 mRNA, other metabolic components were differentially modified. Cholestyramine and lovastatin, respectively, did not modify and increased both HMG-CoA and sterol responsive element binding protein 1c mRNA, did not modify and lowered liver X receptor α mRNA, lowered and increased ATP binding cassette A1 mRNA and lowered and did not modify scavenger receptor B1 mRNA.

5. That is, different to unabsorbed cholestyramine, lovastatin, as an absorbed plasma cholesterol-lowering drug, may have modified the activity of other unknown genes that play roles in the interaction of CETP with the metabolism of hepatic cholesterol.

Key words: cholesteryl ester transfer protein, cholestyramine, lovastatin, low-density lipoprotein receptor, transgenic mice.

INTRODUCTION

Hepatic cholesterol homeostasis is largely maintained through coordinated cell cholesterol uptake, esterification by acyl cholesterol acyl transferase (ACAT), biosynthesis and export to plasma and bile as cholesterol itself and as bile acids, but how these processes interact with the regulation of the cholesteryl ester transfer protein (CETP) has been largely unknown. Cholesteryl ester transfer protein is a hydrophobic glycoprotein that mediates the plasma cholesteryl ester transfer from high-density lipoprotein (HDL) to apolipoprotein B (apoB)-containing lipoproteins (very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL)) in exchange for triacylglycerol.[1] It plays an important role in the reverse cholesterol transport system, a mechanism whereby cholesterol from the peripheral tissues, including the arteries, is transferred to the liver and excreted in bile.

In humans, CETP mRNA is predominantly expressed in the liver, spleen and adipose tissue and secondarily in the small intestine, adrenal gland, kidney, heart and skeletal muscle.[2–4] Dietary cholesterol raises the plasma CETP concentration in various animal species, such as rabbit,[5] hamster[3] and in humans[6] and brings about an increase in plasma CETP and hepatic CETP mRNA in transgenic mice expressing the simian[7] or the human[8] *CETP* gene. This suggests that transcription of the *CETP* gene in the liver is a major mechanism underlying the dietary cholesterol induced plasma CETP concentration and activity in humans[6] and other species.[3,5]

Expression of the *CETP* gene is controlled by cellular cholesterol via sterol responsive elements.[4] In this regard, it has been shown that the sterol regulatory element binding protein-1 activates the *CETP* gene *in vivo* but is not required for sterol upregulation of gene expression[9] and a nuclear receptor-binding site that is activated by liver X receptor α (LXRα) also mediates the positive sterol response of the *CETP* gene.[10]

The level of expression of human CETP in transgenic mice influences the rate of development of experimental atherosclerosis.[1] In this regard, in experimental animals, both endogenous and

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diet-induced hypercholesterolaemia relate to the plasma CETP concentration.[3,5,8,11]

Several studies have demonstrated that CETP activity in the plasma can also be affected by drugs such as bile acid sequestrants (cholestyramine) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). Furthermore, it has been shown that cholestyramine lowers the plasma CETP mass and LDL particle number in hypercholesterolaemic subjects.[12] Similar results regarding cholestyramine were obtained by Korhonen *et al*.,[13] whereas other studies have demonstrated that statins simultaneously diminish plasma LDL–cholesterol and CETP concentration and activity in normolipidaemic[14] and hyperlipidaemic patients.[13,15,16] Nonetheless, the hepatic mechanisms involved in the simultaneous regulation of plasma cholesterol and CETP by drugs that lower plasma cholesterol are not known. This issue was investigated in the present study using a model of transgenic mice expressing the human *CETP* minigene cross-bred with LDL receptor (LDLr)-deficient mice ($CETP^{+/-}/LDLr^{+/-}$) that present a plasma lipoprotein profile resembling that of humans. Animals were treated with statin and cholestyramine, which are known to reduce the plasma cholesterol concentration[17–19] by increasing the number of LDLr in the liver.[20] However, although cholestyramine is not absorbed by the intestine and stimulates the synthesis of cholesterol and of bile acids, statins are absorbed and competitively inhibit HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. The cholesterol and fat feeding used in the present experiments aimed to check the sensitivity of the biochemical responses in these models because this treatment is known to modify the expression of the *CETP* gene.

METHODS

Animals

The Ethics Committee of the University of São Paulo Medical School approved the present experimental protocol. Transgenic mice expressing the human *CETP* minigene with 3.4 kb (5′) and 2.2 kb (3′) natural flanking sequences (line 5203; C57BL6/J background),[8] provided by Dr A Tall (Molecular Medicine Division, Columbia University, NY, USA), were cross-bred with LDLr-deficient mice (LDLr knockout (KO)) provided by Jackson Laboratories (Bar Harbor, ME, USA) and the resultant male heterozygous $CETP^{+/-}/LDLr^{+/-}$ mice, aged 12–16 weeks, were fed chow diet with or without added 3% cholestyramine, 0.025% lovastatin or 2% cholesterol plus 10% corn oil (high-cholesterol, high-fat diet).

All mice were housed in a room under a 12 h light–dark cycle with free access to food and water. At the end of the treatment period (21 days), overnight-fasted mice were killed and blood was collected into EDTA (1 mg/mL)-containing tubes. Plasma was removed following centrifugation at 1000 *g* for 15 min at 4°C. The liver was rapidly excised, rinsed with ice-cold saline solution (0.9% NaCl), frozen immediately in liquid nitrogen and stored at −70°C for RNA preparation and cholesterol content measurement.

Plasma CETP activity, total cholesterol and triacylglycerol measurements

The activity of CETP was determined by an indirect procedure that measured the percentage transfer of [^{14}C]-cholesteryl oleate from HDL to LDL, as described previously.[12] Briefly, 10 μL mouse plasma as the CETP source was added to a mixture containing 50 μL [^{14}C]-HDL (40 mg/dL HDL–cholesterol) and 200 μL LDL (200 mg/dL LDL–cholesterol) and incubated at 37°C or at 4°C (blank) for 2 h. Samples were then chilled on ice, LDL precipitated with dextran sulphate and magnesium chloride (1 : 1) and the radioactivity in the HDL supernatant measured. Plasma total cholesterol and triacylglycerol concentrations were determined enzymatically using commercially available kits from Roche (Manheinn, Germany). The plasma lipoprotein profile of pools of three to four control animals was checked by fast protein liquid chromatography (FPLC), showing that approximately 90% of the cholesterol distribution corresponded to the LDL fraction.

Measurement of hepatic cholesterol

Frozen liver samples (100–200 mg) were minced in 1 mL ice-cold buffer containing 0.3 mol/L sucrose, 1 mmol/L EDTA, 50 mmol/L potassium fluoride (KF), 50 mmol/L KCl, 5 mmol/L dithiotreitol (DTT), pH 7.4, and then disrupted in a 2-L Potter Elvehjem homogenizer (model MA 099; Marconi Piracicaba, SP, Brazil). Homogenates were centrifuged at 14 000 *g* for 20 min at 4°C and the supernatant was centrifuged again at 150 000 *g* for 70 min at 4°C in an SW-41 rotor (Beckman Instruments, Palo Alto, CA, USA). The microsomal pellet was dissolved in 1 mL buffer (0.1 mol/L K_2HPO_4, 1 mmol/L EDTA, 50 mmol/L KF, 5 mmol/L DTT and 50 mmol/L KCl, pH 7.4). Cholesterol was extracted from the liver and the microsomal fraction according to the method of Carr *et al*.[21] Total and unesterified cholesterol and triacylglycerols were determined by enzymatic methods. Protein concentration was determined according to the method of Lowry *et al*.[22]

Extraction of total RNA

Total mouse liver RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity of all RNA samples was evaluated on borate agarose gel electrophoresis after ethidium bromide staining. Reverse transcription was performed using a Superscript II pre-amplification system (Invitrogen) from 1 μg total RNA to a final volume of 20 μL.

Determination of CETP mRNA levels by competitive reverse transcription–polymerase chain reaction

The competitive reverse transcription–polymerase chain reaction (RT-PCR) assay to determine the concentration of CETP mRNA consisted of a co-amplification of the target single-strand cDNA with known amounts of a cDNA competitor molecule added into the same tube.[23] The cDNA used as a competitor was obtained by reverse transcription from total RNA of hamster kidney. The reverse transcription product was amplified by PCR in a DNA thermal cycler PTC-200 (MJ Research, Watertown, MA, USA) using the CETP sense and antisense primers (GenBank accession no. M63992, 5′-AACGTCATCTCCAACATCATGG-3′ and 5′-CTGCAGGAAGCTCT-GGATGGAC-3′) and purified using the Wisard PCR Preps DNA Purification System (Promega, San Luis Obispo, CA, USA). After quantification at 260 nm, a serial dilution of increasing amounts of DNA (0.001–0.2 amol/μL) was prepared in Tris–EDTA (TE) solution. The choice of hamster *CETP* cDNA as a competitor was due to the presence of a homologous region with a 668 bp between the *CETP* gene of hamster (nucleotides 1–668)[3] and human (743–1411 bp).[2] In humans, this region is located in exon 7 and exons 8–14 and can be amplified using the same primers. However, the restriction enzyme *Ava*I can digest the amplified product from hamster cDNA and produce two fragments of 417 and 251 bp.

The reverse transcription product (2 μL) was amplified together with increasing amounts of competitor using a PCR mix (50 mmol/L KCl, 2 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl, pH 8.3) containing 200 μmol/L dNTP mix, 15 pmol each CETP sense and antisense primer, as described above, and 2.5 U Taq DNA polymerase (Amersham Bioscience, Sunnyvale, CA, USA). The initial denaturation at 94°C for 4 min was followed by a 28 cycle amplification at 94°C for 1 min, 54°C for 90 s and 72°C for 2 min, with a final extension at 72°C for 10 min. For quantitative analysis of the PCR product (10 μL) was incubated with 2.5 U *Ava*I (Invitrogen) for 2 h at 45°C. At the end of the incubation, the entire volume was submitted to electrophoresis in a 3% agarose gel stained with ethidium bromide in Tris–borate–EDTA (TBE) buffer. The gel was photographed and the band densities analysed using Alpha Imager TM 1220 software (Alpha Innotec, San Leandro, CA, USA). The quantification was based on the density ratio of the sum of the two competitor bands (417 + 251 bp) and the target band

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(668 bp). The logarithm of the corrected ratio was then plotted against the initial amount of competitor (amol/L) added to the PCR medium. At the equivalence point (log ratio = 0), the initial amount of target cDNA corresponds to the initial amount of the competitor. Taking into account the dilution factors, the mRNA concentration in the preparation could be calculated.

Determination of mRNA levels of other hepatic lipid regulatory genes

The hepatic mRNA expression of HMG-CoA reductase, LDLr, cholesterol 7α-hydroxylase (CYP7A1), CYP27, LXRα, farnesoid X-receptor (FXR), sterol responsive element binding protein 1c ($SREBP_{1c}$), ATP binding cassette A1 (ABCA1) and scavenger receptor B1 (SRB1) were determined by RT-PCR using mouse glyceraldeyde-3-phosphate dehydrogenase (GAPDH) mRNA as the internal control. The sequences of each primer pair (sense and antisense) used in the RT-PCR reaction were as follows: HMG-CoA reductase (AA009175), 5′-GTATGTGGCACTGTGATGGC-3′ and 5′-TGTGCAAAGAACCTCAGACG-3′ LDLr (GenBank Accession no. NM_010700), 5′-CAGTGTGCAGATGGCTCCT-3′ and 5′-CACTCAGAGCCAATCTTGAGG-3′ CYP7A1 (GenBank Accession no. NM_007824), 5′-GTTTGAAGCCGGATATCTAACG-3′ and 5′-CAAGGTGCGTCTTAGCCTTC-3′ CYP27 (GenBank Accession no. AK004977), 5′-AGAGACCACATGGATCAGTGG-3′ and 5′-TCAGGAATGGAGGGTTTCAG-3′ LXRα (GenBank Accession no. NM_013839), 5′-GGGAACGAGCTGTGCAGT-3′ and 5′-GAAGTGGCTTGAGCCTGTTC-3′ $SREBP_{1c}$, 5′-ATCGGCGCGGAAGCTGTCGGGGTAGCGTC-3′ and 5′-ACTGTCTTGGTTGTTGATGAGCTGGAGCAT-3′[24] ABCA1 (GenBank Accession no. NM_013454), 5′-CATTAGCACGCTCTTCTCCC-3′ and 5′-TGCAGTGGTGAGATTGAAGC-3′ SRB1 (GenBank Accession no. NM_016741), 5′-AAGTGGTCAACCCAAACGAG-3′ and 5′-TCTCCATCAATATCGAGCCC-3′ and GAPDH (GenBank Accession no. M32599), 5′-CTGCATCCACTGGTGCTG-3′ and 5′-AGGGTTTCTTACTCCTTGGAGG-3′.

The RT-PCR reaction was performed using the reverse transcription product coamplified in the presence of sense and antisense primers of GAPDH and each gene (15–25 pmol) in PCR buffer (50 mmol/L KCl, 2 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl, pH 8.3), 200 μmol/L dNTPs mix, 5% dimethylsulphoxide (DMSO) and 2 U Taq DNA polymerase in a final volume of 50 μL. The PCR conditions were 94°C for 4 min and subsequently 30 cycles at 94°C for 1 min, 60°C for 90 s and 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were subjected to electrophoresis in a 2.5% agarose gel, stained with ethidium bromide and the density of the band determined. The relative density of each sample was normalized to the signal of GAPDH and was expressed as the ratio sample/GAPDH.

Drugs and chemicals

Lovastatin was kindly provided by Merck Sharp and Dohme (São Paulo, Brazil) and cholestyramine was supplied by Bristol-Myers-Squibb (São Paulo, Brazil). [4-^{14}C]-Cholesteryl oleate (1.66–2.22 GBq/mmol) was purchased from New England Nuclear (Life Sciences, Boston, MA, USA). All other reagents were obtained from Sigma (St Louis, MO, USA) and Merck (Rio de Janeiro, Brazil) and were of analytical grade quality.

Statistical analysis

Results are presented as the mean±SD. One-way analysis of variance (ANOVA), followed by Newman–Keuls' post hoc test, was used to assess differences between the experimental groups. $P < 0.05$ was considered significant. Spearman's or Pearson's correlation coefficients were used to examine associations among variables. $P < 0.05$ was considered to be significant.

RESULTS

Lovastatin lowered the plasma concentration of cholesterol and increased the microsomal cholesterol content (Table 1), as well as hepatic HMG-CoA reductase mRNA (Fig. 1), whereas plasma CETP activity was not modified, CETP and LXRα mRNA decreased and CYP7A1 mRNA, $SREBP_{1c}$ and ABC1 increased; the mRNA of LDLr, CYP27 and SRB1 was not altered (Fig. 1).

Cholestyramine reduced the plasma cholesterol concentration but, unlike lovastatin, plasma CETP activity and liver CETP mRNA were reduced (Table 1). In addition, despite a greater conversion rate of hepatic cholesterol into bile acids, represented by increased CYP7A1 mRNA expression (Fig. 1), the hepatic cholesterol content was not modified by cholestyramine. This occurred because the reduction of plasma cholesterol may have been due to greater removal of plasma cholesterol as VLDL by the liver, more likely through receptors other than the LDLr[25,26] because the latter was not modified by cholestyramine. Unlike lovastatin, cholestyramine did not modify mRNA levels of CYP27, LXRα, $SREBP_{1c}$ and diminshed the mRNA levels of ABCA1 and SRB1.

Experiments on cholesterol feeding served as controls for cholestyramine and lovastatin. As predicted from previous work,[8] cholesterol feeding increased the plasma cholesterol concentration, CETP activity and liver cholesterol content, although, unexpectedly, liver CETP mRNA was not modified (Table 1). Furthermore, mRNA of HMG-CoA reductase, LDLr, LXRα, $SREBP_{1c}$ and CYP27 was suppressed, whereas that of SR-BI and ABCA1 was stimulated as predicted (Fig. 1).

When pooled data from control and treated animals were then examined in the experimental model (Table 2), correlation was demonstrated between plasma total cholesterol and CETP activity,

Table 1 Plasma lipid concentrations, cholesteryl ester transfer protein (CETP) activity and hepatic CETP mRNA and cholesterol content in transgenic $CETP^{+/-}/LDLr^{+/-}$ mice after 3 weeks on 3% cholestyramine, 0.025% lovastatin or 2% cholesterol

	Control (n = 7)	Cholestyramine (n = 8)	Lovastatin (n = 8)	Cholesterol (n = 8)
TC (mg/dL)	101 ± 9	78 ± 9***	81 ± 5***	144 ± 29***
Triacylglycerols (mg/dL)	173 ± 35	134 ± 29	153 ± 37	87 ± 27***
CETP activity (%/h)	23.8 ± 4.2	20.5 ± 2.3*	24.6 ± 2.2	30.8 ± 4.1**
CETP mRNA (amol/L per μg total RNA)	0.27 ± 0.05	0.12 ± 0.05***	0.17 ± 0.04***	0.27 ± 0.08
Liver TC (mg/g wet liver)	1.6 ± 0.4	1.5 ± 0.3	1.8 ± 0.3	3.6 ± 0.9**
Liver UC (mg/g wet liver)	1.3 ± 0.2	1.3 ± 0.1	1.6 ± 0.1	2.0 ± 0.2**
Microsome TC (μg/mg protein)	11.3 ± 2.5	12.2 ± 2.0	16.6 ± 2.1**	36.5 ± 10.3***
Microsome UC (μg/mg protein)	10.1 ± 2.8	10.1 ± 1.9	14.4 ± 2.5**	22.3 ± 6.3***

Data are represented as the mean ± SD. *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$ compared with control.
TC, total cholesterol; UC, unesterified cholesterol.

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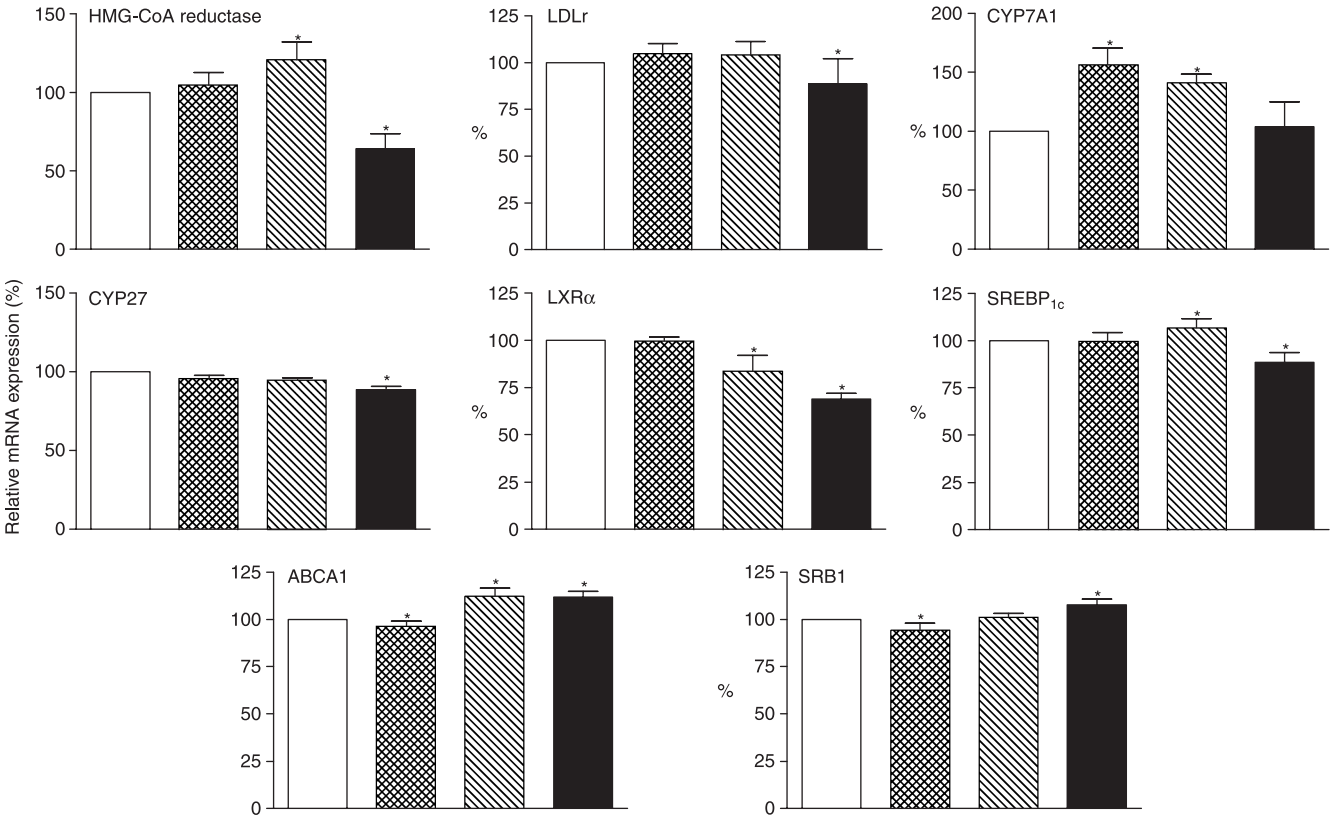

Fig. 1 Hepatic mRNA expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, low-density lipoprotein receptor (LDLr), cholesterol 7α-hydroxylase (CYP7A1), CYP27, liver X receptor α (LXRα), sterol responsive element binding protein 1c ($SREBP_{1c}$), ATP binding cassette A1 (ABCA1) and scavenger receptor B1 (SRB1) in transgenic $CETP^{+/-}/LDLr^{+/-}$ mice after 3 weeks on control chow diet ($n = 7$; □, cholestyramine ($n = 8$; ▨), lovastatin ($n = 8$; ▧) and dietary cholesterol ($n = 8$; ■). Each mRNA (mean ± SD) was normalized to GAPDH mRNA content and is expressed as a relative level. *$P < 0.001$ compared with control.

Table 2 Correlations between plasma total cholesterol and cholesteryl ester transfer protein (CETP) activity with hepatic mRNA expression of CETP, 3-hydroxy-3-methylglutaryl coenzyme A reductase, low-density lipoprotein receptor and CYP7A1 and total cholesterol content in the liver and microsome after 3 weeks in $CETP^{+/-}/LDLr^{+/-}$ mice ($n = 31$)

Parameters	Plasma TC	Plasma CETP activity
Plasma TC		0.71***
Hepatic CETP mRNA	0.43*	0.52**
Hepatic HMG-CoA reductase mRNA	–0.64***	–0.36*
Hepatic LDLr mRNA	–0.46**	–0.25
Hepatic CYP7A1 mRNA	–0.70***	–0.51**
Liver TC	0.47**	0.42*
Microsomal TC	0.48**	0.56**

Transgenic $CETP^{+/-}/LDLr^{+/-}$ mice after 3 weeks on chow diet (control), 3% cholestyramine, 0.025% lovastatin or 2% cholesterol. Spearman's coefficients (*P* values) were calculated considering the mean of duplicate values for each animal. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

TC, total cholesterol; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLr, low-density lipoprotein receptor; CYP7A1, cholesterol 7α-hydroxylase.

as reported by others.[6,11] In addition, there were correlations between plasma total cholesterol and CETP activities with hepatic CETP mRNA as well as with hepatic HMG-CoA reductase mRNA, although the correlation for the latter was negative. Furthermore, plasma total cholesterol correlated negatively with hepatic LDLr mRNA, although the correlation with plasma CETP activity did not reach significance.

DISCUSSION

The negative correlations given in Table 2 reflect the fact that plasma total cholesterol and CETP activities increased as the hepatic cholesterol production rate was impaired and could be interpreted as the presence of the *CETP* gene facilitating the hepatic uptake of cholesterol. Accordingly, plasma total cholesterol and CETP activities were also correlated with the cholesterol content in the total liver, as well as in the microsomal fraction. These biochemical processes were tightly related to the plasma cholesterol concentration in a coherent chain of events.

In the present experimental model, plasma triacylglycerol was not modified by either cholestyramine or lovastatin; however, a 50% decrement was observed on cholesterol feeding. These effects of high-cholesterol, high-fat feeding should bring on faster hepatic rates of VLDL synthesis and output. Therefore, the plasma triacylglycerol-lowering effect demonstrated in the present study and previously by others[27] does not seem to fit into the known hepatic lipid regulatory patterns and suggests that other, as yet unidentified, liver factors may have intervened. An explanation for the response of plasma triacylglycerol to cholesterol treatment must be linked to the expression of $SREBP_{1c}$, which was suppressed.

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Despite the contradictory response shown by the experimental model of hypercholesterolaemia to treatments that profoundly modify the hepatic regulation of cholesterol, several coherent patterns of response emerged. In response to cholestyramine, an increased rate of bile acid synthesis ran together with diminished liver expression of CETP, plasma CETP activity and cholesterol concentration. Responses to cholestyramine and cholesterol treatment were rather coherent in the sense that they opposed each other. Within this frame of responses, the liver microsomal cholesterol content increased markedly on cholesterol feeding, but was not altered by cholestyramine. The latter was quite likely due to the opposing effects of increased CYP7A1 expression and diminished ABCA1 expression on cell cholesterol content.

Because cholestyramine treatment was expected to increase hepatic activity of HMG-CoA reductase, LDLr number and CYP7A1 activity, the finding that only the latter increased seemed incongruent at first. However, once cholestyramine treatment started a combined transient decrease in hepatic cholesterol content, faster rates of *de novo* cholesterol synthesis and greater LDLr-related protein expression may have been circumvented over time by an enhanced plasma LDL–cholesterol uptake through other receptors.[25,26] Accordingly, Einarsson *et al*.[28] have reported that rats fed 5% cholestyramine exhibit a more than threefold increase in cholesterol CYP7A1 activity, with no change in microsomal free cholesterol concentration, suggesting that the cell pool size of free cholesterol is not critical for the regulation of CYP7A1 in rat liver microsomes.

The plasma cholesterol-lowering action of statins has been attributed to a depletion of the unesterified cholesterol content in hepatocytes bringing on a compensatory increase in the activity of HMG-CoA reductase and of LDLr number. Therefore, the increase in microsomal unesterified cholesterol content in response to lovastatin, although of a much less magnitude than that elicited by cholesterol feeding, was unexpected. When compared with the results of others, we have linked this type of response to the duration of the treatment period. In fact, the liver cholesterol content was not lowered when high doses of lovastatin (100 mg/kg) were administered to rats for a long period (2 weeks),[29] whereas in another study where lovastatin and atorvastatin (40 mg/kg) were administered to rats for 3 days, liver HMG-CoA reductase mRNA was stimulated and the hepatic cholesterol content was reduced 30–40%.[30] Some insight on this issue was afforded by experiments on animals expressing CETP because Jiang *et al*.[31] demonstrated in CETP-expressing mice a greater content of liver total and esterified cholesterol and lower mRNA for HMG-CoA reductase, CYP7A1 and LDLr than in non-transgenic mice. Furthermore, in the study of Masucci-Magoulas *et al*.[11] on CETP transgenic cross-bred with apoE-deficient mice, downregulation of the HMG-CoA reductase and LDLr mRNA was attributed to an increased hepatic cholesterol content elicited by the CETP-dependent cholesterol uptake. In this regard, data from our laboratory in mice showed that the expression of the *CETP* gene increased the rate of uptake of intravenously infused esterified cholesterol from HDL, but not LDL, particles.[32] In addition, major hyperplasia of the endoplasmic reticulum is known to be induced by HMG-CoA reductase inhibitors.[33] The increase in the concentration of HMG-CoA reductase protein in endoplasmic reticulum microsomal membranes is due to the competitive inhibition of this enzyme by statins.[34] This accumulation of unesterified cholesterol, limited to the microsomal membranes, should not be related to the storage of cholesteryl ester in the cytosol on cholesterol feeding.

Discrepancies between responses of plasma CETP activity and liver CETP mRNA were also observed in a previous study where lovastatin (0.075%) was administered to rabbits: in spite of markedly lowering the plasma cholesterol concentration, lovastatin did not elicit modifications in either plasma CETP concentration or liver CETP mRNA.[5] Furthermore, because other tissues (e.g. adipose) make CETP and also take up lovastatin, it is possible that, in addition to the liver, other CETP sources contribute to plasma CETP activity. Nonetheless, hepatic synthesis is likely an important source of plasma CETP in transgenic mice[4] taking into account that, according to present data and to previous publications,[5,8] the plasma CETP activity correlated with liver CETP mRNA.

In spite of the present findings, lovastatin treatment elicited coherent metabolic alterations; that is, a simultaneous increase in the hepatic expression of HMG-CoA reductase and a reduction of plasma cholesterol concentration.

Other results of the present study regarding the effects of lovastatin were that the expression of CYP7A1, ABCA1 and SREBP1c increased, whereas that of LXRα decreased. These results suggest that some unknown regulator of bile acid synthesis may be triggered by lovastatin and by the expression of CETP. In this regard, the regulation of CYP7A1 seems a complex system that could vary according to, among other factors, the animal species. For instance, studies have shown that cholesterol-rich diets boost the CYP7A1 mRNA level only in rats[35] and some inbred strains of mice.[36] Recently, Chiang *et al*.[37] showed that FXR exerts an inhibitory effect on CYP7A1 that may overcome the stimulatory action of LXR, which has been considered the dominant regulator of CYP7A1 transcription.[38] In addition, although bile acids regulate the transcription of genes that control cholesterol homeostasis through molecular mechanisms that are poorly understood, an LXR-responsive element in the promoter region of the rat cholesterol *CYP7A1* gene has been identified.[38] These data provide evidence for a new signalling pathway that activates CYP7A1 transcription in response to oxysterols. Because CYP7A1 is regulated by LXRs, these nuclear receptors may help coordinate the HDL cholesteryl ester catabolism through the hepatic excretion of cholesterol as bile acids. In this regard, a nuclear receptor binding site activated by LXR mediating the positive sterol response of the *CETP* gene has been reported by Luo and Tall.[10] They showed that CETP is transactivated by LXRα and LXRβ, a result compatible with these receptors having a role in regulating *CETP* gene expression *in vivo*. Thus, although no clear explanation can be afforded as yet by such variability of the response of CYP7A1 mRNA in the present study, the CYP7A1 mRNA likely was uniquely modified by the accumulation of cholesterol in the liver and in its microsomal fraction. In addition, the presence of apoE and CETP may have synergistically contributed to the storage of hepatic cholesterol.

Two cautionary notes are necessary because some inconsistencies reported here could be explained by the facts: (i) that mRNA levels do not necessarily predict the amount of protein expressed; and (ii) it is possible that statin-related hepatic mechanisms that modify the plasma CETP concentration may be ascribed to biochemical parameters other than those known to control the hepatic cholesterol metabolism that were reported herein.

The relevance to humans of studies in animal models on how hepatic CETP is regulated by drugs that lower plasma cholesterol concentration stems from several population investigations into the responses of CETP polymorphisms to these drugs. Statins would be

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effective in increasing HDL levels in Japanese B1B1 carriers, because of a lower concentration of HDL–cholesterol and higher level of plasma CETP compared with other genotypes.[39] In addition, cardiovascular event reduction by statin therapy is substantially enhanced in the presence of a B2 allele.[40] Therefore, cardiovascular event reduction has been associated with the CETP Taq1B polymorphism enabling more effective pharmacogenetically directed therapy.

Conversely, in the REGRESS study, pravastatin yielded the highest improvement of lipid and angiographic parameters in patients with high baseline CETP independent of baseline lipids, lipid changes and TaqIB genotype, indicating that the plasma CETP level itself is an important determinant of the response to statins.[41] In addition, statin treatment improved the lipoprotein profile in familial hypercholesterolaemic patients, but to a lesser extent in those with high CETP levels, implying that statin treatment does not entirely counteract the lipoprotein abnormalities associated with high CETP levels.[42] Thus, the genetic variation in the *CETP* gene may be one important factor in designing better treatments.

Finally, the issue of CETP hepatic regulation is particularly relevant to the mechanisms involved in reverse cholesterol in humans since drugs that impair CETP activity became available.[43]

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