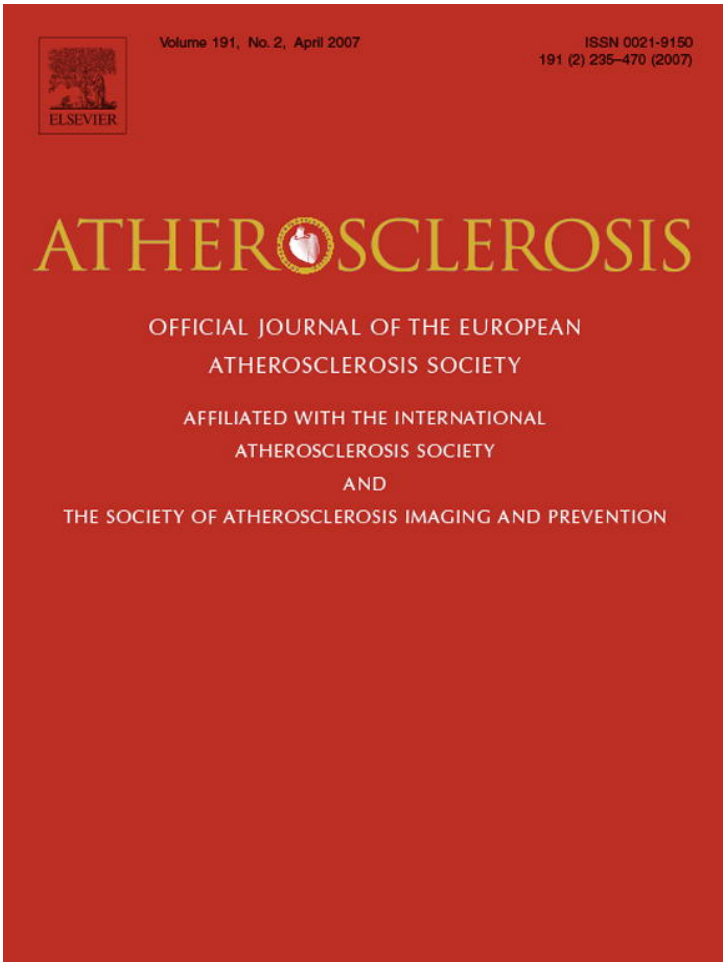

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Atherosclerosis 191 (2007) 313–318

ATHEROSCLEROSIS

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CETP expression enhances liver HDL-cholesteryl ester uptake but does not alter VLDL and biliary lipid secretion

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Received 5 March 2006; received in revised form 17 April 2006; accepted 12 May 2006
Available online 27 June 2006

Abstract

The aim of this work was to study how CETP expression affects whole body cholesterol homeostasis. Thus, tissue uptake and plasma removal rates of labeled HDL-cholesteryl ester (CE), VLDL secretion rates, and biliary lipid secretion and fecal bile acid content were compared between human CETP transgenic (Tg) and non-transgenic (nTg) mice fed with a standard diet. CETP Tg mice exhibited increased HDL-CE plasma fractional catabolic rate and uptake by the liver, adrenals, adipose tissue and spleen. HDL fractions from both CETP Tg and from nTg mice were removed faster from the plasma of CETP expressing than from nTg mice, suggesting a direct role of CETP in accelerating tissue CE uptake. However, neither hepatic output of VLDL cholesterol and triglycerides nor biliary lipid and fecal bile acid excretion were changed in CETP Tg compared to nTg mice. CETP Tg mice also showed enhanced hepatic cholesterol content. Steady state cholesterol homeostasis was probably preserved through the downregulation of hepatic HMG-CoA reductase and LDL receptor expression. In conclusion, although CETP expression facilitates cholesteryl ester tissue uptake, it does not alter biliary lipid and fecal bile acid excretion, the mandatory final step of the reverse cholesterol transport.

Keywords: Cholesterol metabolism; Bile acid metabolism; Reverse cholesterol transport; Hepatic HDL uptake; VLDL metabolism; CETP transgenic mice

1. Introduction

High density lipoproteins (HDL) mediates the transfer of cholesterol from extra hepatic tissues to the liver playing a critical role in cholesterol homeostasis that seems to account for the protective effect of HDL against atherosclerosis. This reverse cholesterol transport system involves several steps. It starts with apoAI secreted directly from the liver or shed off from the large triglyceride containing particles by the action of the enzyme lipoprotein lipase forming preß-HDL. These particles interact with ABCA1, a specific cell membrane transporter that promotes phospholipid and cholesterol efflux [1]. Spherical HDL particles originate after conversion of unesterified cholesterol to cholesteryl esters mediated by the enzyme lecithin cholesterol acyl transferase (LCAT) on the discoidal HDL particles [2,3]. Spherical HDL can further promote cellular cholesterol efflux via ABCG1 [4] and scavenger receptor class B type I, SR-BI [5].

Cholesteryl ester (CE) present in mature spherical HDL can have different metabolic fates: the transfer of CE to apoB-containing lipoproteins mediated by the cholesteryl ester transfer protein (CETP), as well as direct delivery to the liver mediated by the HDL receptor SR-BI receptor [6,7]. Liver cholesterol can then be reutilized in VLDL assembly or secreted into bile either as cholesterol or transformed into bile acids [8].

CETP may influence several metabolic steps of cholesterol metabolism. It accelerates cellular cholesterol efflux either indirectly, through stimulation of LCAT activity [9]

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doi:10.1016/j.atherosclerosis.2006.05.036

or directly, as shown in cultured macrophages [10,11]. CETP also facilitates cellular selective cholesteryl ester uptake by SR-BI expressing cells [12], liver [13] and adipose cells [14,15]. However, the role of CETP in controlling whole body cholesterol homeostasis remains to be elucidated [16]. This is due in part to the fact that in vivo quantitative analyses of cholesteryl ester kinetics are difficult to interpret because it requires a complex multicompartmental approach in species with CETP activity, such as rabbits and humans [17,18]. Thus, in this work, we studied the role of CETP expression on overall cholesterol metabolism by measuring plasma removal rate and tissue uptake of HDL-cholesterol, hepatic VLDL production as well as biliary lipid and fecal bile acid excretion rates in human CETP transgenic as compared to wild type mice.

2. Material and methods

2.1. Animals

The Ethics Committee of the University of São Paulo Medical School approved this experimental protocol. Transgenic (Tg) mice founders expressing the human CETP minigene with 3.4 kb (5′) and 2.2 kb (3′) natural flanking sequences (line 5203, C57BL6/J background) [19] were kindly provided by Dr. Alan Tall (Molecular Medicine Division, Columbia University, NY, USA). Male and female heterozygous CETP and wild type non-transgenic (NTG) C57BL/6J control mice, aged 8–12 weeks, were utilized. All mice were housed in a temperature controlled room under a 12-h light–dark cycle with free access to standard chow diet (Nuvital, PR, Brazil) and water.

2.2. HDL metabolic studies

HDL was isolated from pooled plasma of fasting healthy humans or from CETP Tg and NTG mice by sequential ultracentrifugation in a 50Ti rotor using a L-8 Beckman ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA). HDL labeled with ^{3}H-cholesteryl oleoyl ether, ^{3}H-cholesteryl ester or ^{14}C-cholesteryl oleate (10^{6} dpm/animal) [20] were administered intraperitoneally. The injected HDL mass was less than 5% of the mouse total HDL pool. Blood samples (50 μL) were drawn from the tail vein into heparinized capillary tubes at 0.5, 1, 2, 3, 4, 6, 8 and 24 h for radioactivity determination. The fractional removal of labeled HDL from plasma was calculated from the radioactivity disappearance curve from the peak value up to 8 h after the HDL injection adjusting the exponential decay curve with the program Microcal™ Origin™ 4.1. The amount of labeled cholesteryl ester remaining in plasma was calculated as the area under the curve [21]. After 24 h, mice were anesthetized and exsanguinated by subclavian artery puncture and infused with saline (10 mL) through the left ventricle. The following tissues were collected: liver, small intestine, spleen, heart, adipose tissue, abdominal muscles, lungs, adrenals, kidneys and gonads. Adrenals and gonads from animals belonging to the same experimental group were pooled and stored as well as other organs at −20 °C. A sub-group of plasma samples were fractionated by FPLC to isolate plasma lipoproteins as previously described [22]. Frozen tissues (0.1–0.5 g) were minced and methanol (3.5 mL) and chloroform (7 mL) were added. After an overnight period, tissues were filtered and water (2.3 mL) was added to the organic phase. The aqueous phase was then substituted by chloroform/methanol/water (3:48:47, v/v) and left sitting overnight. The aqueous phase was discarded; the solvent transferred to counting vials, evaporated under nitrogen, and liquid scintillation solution was added for radioactivity measurement (Beckman LS 6000 TA).

2.3. Biochemical analyses

Total cholesterol and triglycerides were measured by commercially available enzymatic procedures (Chod Pap, Merck SA, SP, Brazil and Enz-Color, Biodiagnostica, Paraná, Brazil, respectively). Determination of hepatic VLDL secretion was measured as previously described [23]. Briefly, fasting mice were bled to measure baseline plasma triglyceride concentration. The CETP transgenic and non-trasgenic mice were anesthetized and Triton WR-1339 (Sigma, St. Louis, MO) at a dose of 500 mg/kg dissolved in 0.9% NaCl was injected intravenously to inhibit lipoprotein lipase activity. Thereafter, blood was drawn at 15, 30, 60, 90 and 120 min to measure accumulation of plasma triglycerides and cholesterol.

2.4. Biliary lipid secretion determination

Hepatic bile was collected for 30 min through a common bile duct fistula in mice kept under anesthesia at 37 °C. After hepatic bile sampling, mice were euthanized and liver was removed. Hepatic bile flow was calculated by dividing the volume of bile to the collected time and liver weight and it was expressed as μL/min/g of liver. Bile was kept at −20 °C before biochemical analysis. Biliary lipid concentrations were measured by enzymatic assays, as previously described [24]. Biliary lipid outputs were derived from biliary lipid concentrations and measured hepatic bile flows.

2.5. Bile acid metabolism analyses

Bile acid pool size was determined as the sum of total bile acids measured in gallbladder bile, hepatic bile and ethanolic extracts of liver and small intestine [24]. Fecal bile acid excretion was measured in stools collected for 72 h from mice that were housed in individual cages. Bile acid contents were analyzed using routine enzymatic assays and corrected for recovery of the appropriate internal standard [24].

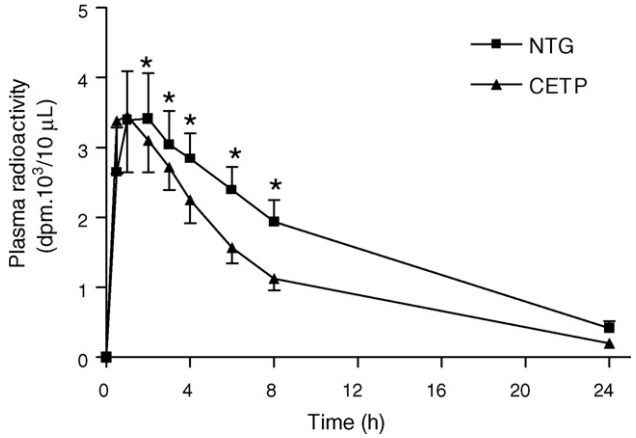

Fig. 1. Plasma radioactivity removal of human HDL labeled with ^{3}H-cholesteryl oleoyl ether after intraperitoneal injection into male non-transgenic (NTG) and CETP-transgenic mice. Data expressed as mean ± S.D. ($n = 10$). Mann–Whitney test: NTG vs. CETP mice: $^{*}P < 0.05$.

2.6. Measurement of the hepatic cholesterol content

Frozen liver samples (±200 mg) were minced in 2 mL of isopropanol with a Potter-Elvehjem homogenizer, stored for 1 h at 37 °C and kept overnight at 4 °C. They were then centrifuged for 10 min, at 3000 rpm, at 4 °C and aliquots of the supernatant used for measurement of total and unesterified cholesterol by enzymatic methods.

2.7. Statistical analyses

The results are presented as mean ± S.D. Student's *t*-test or nonparametric Mann–Whitney *U*-test were used to compare differences between data means. Differences between groups were considered significant at $P < 0.05$.

3. Results and discussion

Plasma radioactivity kinetic curves of human ^{3}H-cholesteryl oleoyl ether-HDL injected into CETP transgenic and control mice (Fig. 1 and Table 1) reveals that overall HDL cholesterol catabolism is faster in the presence of the CETP gene expression. Accordingly, tissue uptake of radioactive cholesteryl ester derived from HDL was significantly increased in liver (47%, $P < 0.005$), adrenals (50%, $P < 0.005$), adipose tissue (42%, $P < 0.05$) and spleen (42%, $P < 0.05$) of CETP transgenic compared to control mice (Fig. 2). Minor effects on the plasma radioactive HDL cholesterol decay curve can be attributed to smaller organs like the spleen and adrenals (Fig. 2). Interestingly, most of the ^{3}H-cholesteryl ether remained in the plasma HDL fraction throughout the kinetic study in both groups of mice. However, as a consequence of the CETP plasma activity, ^{3}H-cholesteryl ether found in LDL fraction was higher in CETP Tg than in control plasma: 21% versus 10% at 8 h, and 27% versus19% at 24 h post injection in CETP Tg versus control mice, respectively, as measured by FPLC of pooled plasma samples.

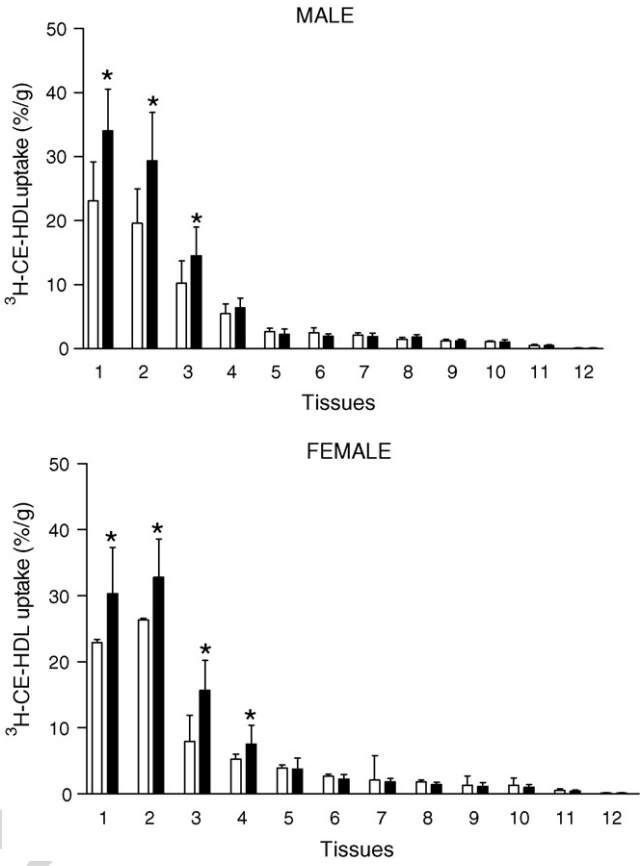

Fig. 2. Tissue ^{3}H-cholesteryl oleoyl ether uptake measured 24 h after intraperitoneal injection of radiolabeled human HDL in CETP transgenic and non-transgenic (NTG) mice. Data expressed as percentage of the injected dose per gram of tissue, mean ± S.D. ($n = 10$). Mann–Whitney test: NTG vs. CETP: $^{*}P < 0.05$. 1: liver; 2: adrenal; 3: adipose tissue; 4: spleen; 5: lung; 6: testis (or ovaries); 7: small intestine; 8: heart; 9: kidney; 10: abdominal skeletal muscle; 11: leg skeletal muscle and 12: brain.

Table 1
Area under the plasma radioactivity vs. time curve (AUC) and fractional catabolic rate (FCR) of human HDL labeled with ^{3}H-cholesteryl oleoyl ether during 8 h after intraperitoneal injection into non-transgenic (NTG) and CETP-transgenic mice

Parameters	Male		Female	
	NTG	CETP	NTG	CETP
AUC (dpm h 10^{6})	7.45 ± 0.78	4.41 ± 0.66[a]	5.76 ± 0.91	3.91 ± 1.12[b]
FCR (h^{-1})	0.040 ± 0.009	0.074 ± 0.008[a]	0.068 ± 0.013	0.092 ± 0.018[c]

Data expressed as mean ± S.D. ($n = 10$). Mann–Whitney test: NTG vs. CETP mice: [a] $P < 0.0001$; [b] $P < 0.0007$; [c] $P < 0.05$.

Table 2
Area under the plasma radioactivity vs. time curve (dpm h 10^6) of labeled HDL-cholesteryl ester obtained from CETP Tg (^{3}H-HDL$_{CETP}$) and non-transgenic mice (^{14}C-HDL$_{NTG}$) during 6 h after simultaneous injection of both HDL into non-transgenic (NTG) and CETP-Tg mice

	NTG mice	CETP Tg mice	P^*
Male			
^{14}C-HDL$_{NTG}$	0.98 ± 0.16	0.70 ± 0.09	<0.003
^{3}H-HDL$_{CETP}$	2.05 ± 0.22	1.44 ± 0.19	<0.002
$P^{\#}$	<0.001	<0.001	
Female			
^{14}C-HDL$_{NTG}$	0.89 ± 0.16	0.76 ± 0.15	n.s.
^{3}H-HDL$_{CETP}$	2.02 ± 0.18	1.47 ± 0.29	<0.001
$P^{\#}$	<0.001	<0.001	

Mean ± S.D. (n = 10). Mann–Whitney test: *NTG mice vs. CETP mice, #^{14}C-HDL$_{NTG}$ vs. ^{3}H-HDL$_{CETP}$.

Table 3
Plasma, liver and biliary lipid concentrations and secretion rates, bile acid pool size and fecal excretion in CETP transgenic and control non-transgenic (NTG) mice

	NTG (n = 9)	CETP (n = 10)
Plasma cholesterol (mg%)	102 ± 16	79 ± 12*
Plasma triglycerides (mg%)	95 ± 20	120 ± 26*
Liver weight (g)	1.0 ± 0.2	1.1 ± 0.2
Liver total cholesterol (mg/g liver)	2.52 ± 0.21	3.47 ± 0.47*
Bile flow (μL/min/g liver)	2.3 ± 0.7	2.0 ± 0.3
Biliary cholesterol concentration (mM)	0.67 ± 0.08	0.59 ± 0.1
Biliary bile salt concentration (mM)	37 ± 9	34 ± 7
Biliary phospholipid concentration (mM)	5.3 ± 0.8	5.2 ± 1.2
Bile cholesterol secretion (nmol/min g liver)	1.6 ± 0.6	1.2 ± 0.3
Bile salt secretion (nmol/min g liver)	88 ± 42	66 ± 17
Bile phospholipid secretion (nmol/min g liver)	13 ± 5	10 ± 3
Bile acid pool size (μmol/100 g BW)	85 ± 17	85 ± 19
Fecal bile acid excretion (μmol/day/100 g BW)	32 ± 6	28 ± 10

Mean ± S.D. (n = 10). *NTG vs. CETP Tg mice: $P < 0.05$ or better.

The plasma removal data apparently disagree with a previous report where the hepatic uptake of rat HDL-CE was delayed by previous in vitro treatment of HDL with CETP [25]. However, additional studies have reported that CETP facilitates selective HDL-cholesteryl ester uptake in several cell types, including SR-BI transfected cells, as well as HepG2 and adipose cells [12–15] and in vivo [12,26]. In transgenic mice overexpressing simian CETP, Stein et al. [27] reported no substantial effect of CETP expression on in vivo removal rates of labeled exogenous LDL-cholesteryl ester injected into muscle as compared to non-transgenic mice. However, a two-fold increase in labeled cholesterol liver uptake was found in transgenic mice 4 and 8 days after the injection. The authors argued that CETP does not modify the cholesterol efflux from peripheral cell but may act on the extracellularly located cholesteryl ester facilitating its selective liver uptake.

In order to test if CETP effect on the plasma HDL cholesterol removal rate was secondary to its action on the HDL particles or depended primarily on its presence in vivo, we injected simultaneously cholesteryl ester labeled HDL obtained from CETP Tg (HDL$_{CETP}$) and non-transgenic (HDL$_{NTG}$) mice into each group of mice (Table 2). In both groups of mice, cholesteryl ester from HDL$_{NTG}$ was removed from plasma more efficiently than from HDL$_{CETP}$. This probably occurred because HDL$_{NTG}$ was a better cholesteryl ester donor particle than HDL$_{CETP}$, as suggested by their differential chemical composition. Total, unesterified, and esterified cholesterol and triglyceride content (mg/dL) of HDL are 47.0, 12.6, 34.4, 11.4, respectively, for CETP transgenic and 61.9, 13.4, 48.5, 4.6, respectively, for non-transgenic mice (mean of two preparations from pooled mice plasma). However, both type of HDL were removed faster from CETP transgenic than from non-transgenic mice plasma (Table 2). Thus, the presence of CETP, either in the plasma compartment or in cells, does accelerate plasma removal rate of HDL-CE. A likely mechanism to explain this CETP effect is provided by a recent work from Gauthier et al. [28]. The authors presented strong evidence for a novel role of cell associated CETP in stimulating cholesteryl ester selective uptake by liver cells. They demonstrated that both exogenous CETP and hepatocyte-associated CETP mediate selective uptake independently of other known lipoprotein receptors (LDL receptor, SR-BI and LRP). The former but not the latter process is susceptible to inhibition by torcetrapib.

It is of interest that adipose tissue uptake of ^{3}H-cholesteryl ether was also increased by 42% in CETP transgenic mice since adipose tissue CETP expression is very small in this particular transgenic strain [19]. These data might support a role for CETP in transferring HDL-CE directly to a plasma membrane microdomain.

Our tissue uptake data showed that the expression of CETP might increase tissue cholesterol content. In fact, the hepatic cholesterol content was significantly greater (37%, $P < 0.02$) in CETP transgenic mice than in non-transgenic mice (Table 3). Consequently, a new steady state condition for cholesterol metabolism must be reached in the liver through some of its highly regulated metabolic pathways, such as hepatic production of VLDL-cholesterol, biliary cholesterol and bile acid secretion or regulation of HMGCoA reductase, the key enzyme of *de novo* cholesterol synthesis. Fig. 3 shows that the hepatic output of VLDL-triglycerides and cholesterol was not significantly changed by the CETP expression. Neither the excretion of biliary lipids and fecal bile acids were influenced by the presence of CETP (Table 3). This latter finding agrees with a recent study in humans using torcetrapib treatment, which markedly impaired plasma CETP activity but did not significantly change serum markers of cholesterol or bile acid synthesis or fecal sterol excretion [29]. Another recent study also reported similar biliary cholesterol con-

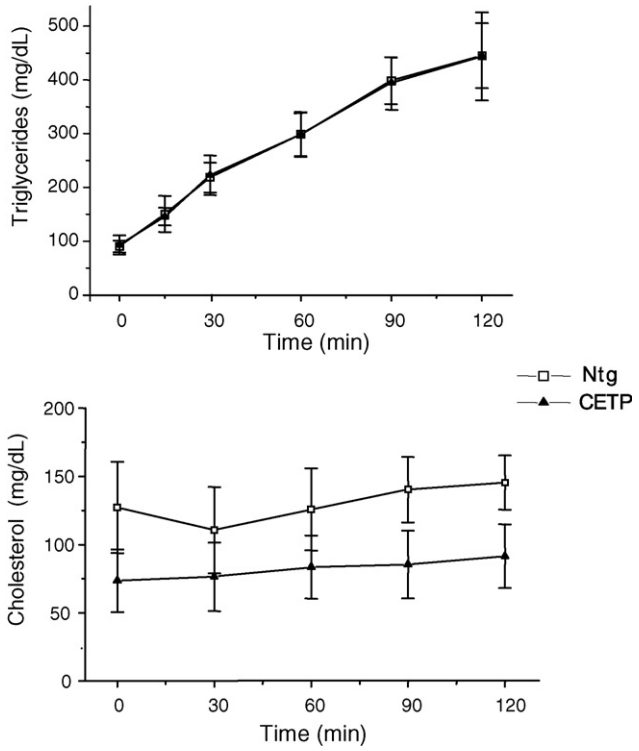

Fig. 3. Hepatic secretion of VLDL-triglycerides and VLDL-cholesterol in fasting non-transgenic and CETP transgenic female mice after endovenous administration of Triton WR 1339. Data are presented as mean ± S.D. ($n = 6$). VLDL secretion rates (mg/dL/min), calculated as the slope of the curves, were not significantly different between CETP and NTG mice, respectively: 3.2 ± 0.2 vs. 3.1 ± 0.2 (VLDL-TG) and 0.10 ± 0.05 vs. 0.126 ± 0.05 (VLDL-cholesterol).

centrations in naive CETP transgenic and control mice [30]. Therefore, either the expression of CETP in mice or its inhibition in humans does not seem to modify the biliary excretion of steroids.

Nonetheless, the response of biliary cholesterol excretion in the presence or absence of CETP may be different in other metabolic contexts. For instance, liver X receptor α (LXRα) agonist treatment increased biliary cholesterol concentration more efficiently in CETP transgenic than in non-transgenic mice [30]. Since dietary cholesterol upregulation of the CETP gene expression [31] is also mediated by the LXR [32], it is conceivable that, upon high cholesterol diet feeding, CETP transgenic mice present higher biliary cholesterol excretion than non-transgenic mice. Further studies are required to evaluate these parameters in human CETP expressing mice when challenged with a cholesterol-enriched diet.

On the other hand, we should also consider important species-dependent differences (besides CETP expression) in cholesterol metabolism when extrapolating findings from mice to other mammals. For example, CYP7A1 is positively regulated by oxysterols via LXR in some rodents, but not in hamsters, monkeys, or humans [33]. In fact, the LXR binding site in the human CYP7A1 gene promoter is mutated, thus preventing binding and activation by LXR/RXR complex [33]. This species-specific difference may explain why mice and rats are very resistant to cholesterol-rich diets, in contrast to humans and other primates [34,35]. In fact, the effect of cholesterol feeding is noticeably species-dependent: cholesterol 7α-hydroxylase activity increases in cholesterol-fed rats, but does not change in guinea pigs, and is inhibited in rabbits and monkeys [36,37].

Previous studies clearly showed that CETP expression increased liver cholesterol content and lowered both the hepatic HMGCoA reductase and LDL receptor mRNA [38]. Together with higher tissue cholesterol uptake and unchanged biliary cholesterol excretion, these findings suggest that CETP could favour an overall positive cholesterol balance in some tissues, perhaps even in the arterial wall. Although speculative, this positive cholesterol balance offers an explanation for the pro-atherogenic effect of CETP reported in some animal and human studies.

In summary, even though human CETP expression in mice stimulated hepatic HDL cholesterol uptake, the hepatic output of VLDL-cholesterol and triglycerides as well as biliary lipid secretion and fecal bile acid excretion were similar in CETP expressing and control mice. The combination of these effects resulted in enhanced cholesterol content in the liver. The steady state cholesterol homeostasis is probably preserved in CETP expressing mice through the downregulation of HMGCoA reductase and LDL receptor expression. Therefore, these findings show that CETP expression does not facilitate the last and mandatory step of the reverse cholesterol transport.

Acknowledgements

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de Sao Paulo (Fapesp #01/11228-5 and #02/07882-4) and Conselho Nacional para o Desenvolvimento Científico e Tecnológico (CNPq #302410/2004-0), Brazil, to HCFO, and by Fondo Nacional de Desarrollo Científico y Tecnológico, Chile (Grant #1030416) to AR.

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