ELSEVIER

J. Biochem. Biophys. Methods 32 (1996) 45–57

JOURNAL OF
biochemical and
biophysical
methods

Research article

'In vitro' cholesteryl ester bidirectional flow between high-density lipoproteins and triglyceride-rich emulsions: effects of particle concentration and composition, cholesteryl ester transfer activity and oleic acid

Helena C.F. Oliveira *, Eder C.R. Quintão

Lipids Lab. (University of São Paulo Medical School) and School of Physical Education (UNICAMP), São Paulo, Brazil

Abstract

The cholesteryl ester flow between high-density lipoproteins (HDL) and triglyceride-rich lipoproteins was investigated utilizing HDL fractions and synthetic lipid emulsion particles (EM). HDL was labeled in vitro with [1,2-^{3}H(n)]cholesteryl ester or with [1,2,6,7-^{3}H(n)]cholesteryl oleate, whereas EM were made with [4-^{14}C]cholesteryl oleate (CO) or [carboxyl-^{14}C]triolein (TO). The cholesteryl ester (CE) transfer rate between HDL and EM was spontaneous to some extent (because it occurred in saline medium), saturable, enhanced in a dose-dependent manner by the plasma fraction at $D > 1.21$ g/ml ascribed to its CETP activity, and greater for HDL_3 than for HDL_2. Unesterified fatty acids in the medium elicited two opposing effects: (1) enhanced the spontaneous cholesteryl oleate shift to EM and inhibited the reverse flow from EM to HDL_3 but not to HDL_2; (2) partially impaired the plasma $D > 1.21$ g/ml-induced bidirectional cholesteryl oleate flow. Approx. 2 mol TO from EM exchange for 1 mol CO from HDL_3. Net cholesteryl ester balance was dependent on the concentration of HDL-cholesteryl ester, and independent from EM-cholesteryl oleate, possibly due to the much smaller concentration of the latter. These in vitro experiments shed light on the complex physical chemistry of transport of cholesteryl ester and

* Corresponding author. Lipid Lab. LIM\10, Faculdade de Medicina da USP, Av. Dr. Arnaldo, 455-3°s-52. CEP 01246-903, Sao Paulo, Brazil. Fax: (55) (11) 852 1255.

SSDI 0165-022X(94)00060-3

triolein between HDL and TG-rich lipoproteins which occurs during the metabolism of chylomicrons in plasma.

Keywords: Cholesterol ester transfer protein (CETP) activity; HDL_2; HDL_3; Unesterified fatty acid; Cholesteryl oleate; Triolein

1. Introduction

In vitro and in vivo experiments have shown that HDL-cholesteryl ester is transferred to lighter density lipoproteins [1,2]. This process may have opposite consequences concerning atherogenesis. It may protect against it because apoB containing lipoproteins (chylomicrons, VLDL and LDL) are taken up by specific liver receptors [3,4]. On the other hand, it may elicit plasma accumulation of remnant lipoproteins that also are atherogenic [5,6] particularly in the hyperlipidemias [7]. In this regard, animal species containing high plasma levels of cholesteryl ester transfer protein activity (CETP), such as humans, non-human primates and rabbits, are more prone to diet-induced atherogenesis as opposed to low CETP activity species, such as dogs and rats, that are relatively resistant although pigs are an exception [7,8].

The enzyme lipoprotein lipase enhances the HDL-esterified cholesterol shift to VLDL mediated by CETP [9]. During lipolysis, unesterified fatty acid concentration increases in VLDL remnant particles and in HDL while the latter also gains phospholipids [10]. These processes induce a greater binding of CETP to the lipoproteins [10] and lipoprotein composition and size modifications [11]. In this regard, as compared to the fasting state, in post-alimentary plasma the transfer of cholesteryl ester is markedly increased [2,12].

Several authors have utilized different lipid emulsions to investigate possible factors controlling the transfer of apoLP and lipids [16,17] between plasma lipoproteins in vitro [13–17] and in vivo [18] but these mechanisms are not fully understood. For instance, CETP promotes the heteroexchange of cholesteryl ester and triglycerides [17,19], however, whenever donor and recipient lipoproteins bear the same triglyceride/esterified cholesterol ratios an exchange of cholesteryl ester predominates between these lipoproteins [2,20,21].

The present experiments were designed to characterize in vitro the cholesteryl ester flow between different HDL fractions (HDL_2 and HDL_3) and a triglyceride-rich lipoprotein emulsion (EM) that exhibit a metabolic behavior in vivo that is similar to that of natural chylomicrons [22–24]. Specific objectives were to: (1) compare HDL_3 with HDL_2 in regard to their capacity of exchanging cholesteryl ester with EM: (a) in the absence and in the presence of plasma containing CETP activity; (b) along different concentrations of the latter; (c) under the influence of unesterified fatty acids; (2) measure net balance of cholesteryl ester in HDL and in EM: (a) along variable HDL-cholesteryl ester and EM-triolein concentrations; b) under the influence of modifications in the EM composition, such as presence or absence of cholesteryl oleate; (3) quantify the molecular exchange between HDL-cholesteryl ester and EM-triolein.

2. Material and methods

2.1. Radioactive materials

[1,2-^{3}H(n)]Cholesterol, [1,2,6,7-^{3}H(n)]cholesteryl oleate, [4-^{14}C]cholesteryl oleate, [carboxyl-^{14}C]triolein (N. England Nuclear, Boston, MA) were more than 99% pure as shown by silica gel TLC with the solvent system hexane/ethyl ether/acetic acid (70:30:1, v/v).

2.2. Triglyceride-rich emulsion

Cholesterol, cholesteryl-oleate and triolein were obtained from NuChek Prep (Elysian, MN, USA) and lecithin from Lipid Products (Surrey, UK). They were more than 99% pure as shown by TLC. Lipid mixtures (2% cholesterol, 6% cholesteryl oleate, 23% lecithin and 69% triolein, by weight) together with [^{14}C]cholesteryl oleate or with [carboxyl-^{14}C]triolein were sonicated in a sodium bromide water solution ($D = 1.101$ g/ml) utilizing a Branson Cell Disruptor (Branson Ultrasonics Corp, Danbury, CT) model B-30, with a 1 cm probe, at 70–80 W for 30 min under nitrogen flow. Triglyceride-rich particles were purified after discontinuous gradient ultracentrifugation as previously described [25]. The triglyceride-rich emulsion composition achieved was: 4.5% cholesterol, 6.8% cholesteryl oleate (CO), 9.5% lecithin and 80.3% triolein (TG), by weight.

2.3. Lipoprotein isolation and labeling

Blood was drawn from a pool of normolipidemic donors on EDTA (1.5 mg/ml of blood) and immediately centrifuged at 2000 rpm at 4°C for plasma separation. Plasma lipoproteins were obtained by sequential ultracentrifugation utilizing a 50Ti rotor in an L7-55 ultracentrifuge (Beckman Instruments, Palo Alto, CA) [26]. Light lipoproteins were removed by spinning at $D = 1.063$ g/ml for 20 h and thereafter, HDL_2 and HDL_3 were obtained by spinning sequentially at $D < 1.125$ g/ml and at $D < 1.210$ g/ml, respectively.

[1,2-^{3}H(n)]Cholesterol (50 μCi) dissolved in ethanol (100 μl) was added dropwise to the HDL_3 fraction ($D > 1.125$) (10 ml) with gentle mixing and incubated at 37°C for 20 h in a tube rotator (Scientific Equipment Products, Baltimore, MD). After adjustment to $D = 1.21$ g/ml, labeled HDL_3 was purified by ultracentrifugation. About 25–30% of the radioactivity was recovered in HDL_3. After TLC on silica gel H, unesterified and esterified cholesterol bands were scraped into counting vials and radioactivity was measured in Aquasol (New England Nuclear, Boston, MA) in the beta scintillation counter (LS 6000TA, Beckman Instruments, Fullerton, CA). About 77% to 88% of the HDL_3 radioactivity was present as esterified cholesterol. An identical procedure was utilized to label HDL_2 and HDL_3 with 50 μCi of [1,2,6,7-^{3}H(n)]cholesteryl oleate. Labeling efficiency was 30% but 97–99% of the radioactivity was present as cholesteryl oleate ([^{3}H]CO).

2.4. In vitro studies

The following assays of cholesteryl ester transfer between HDL and TG-rich emulsions were developed: (1) time course study; (2) effects of lipoprotein and emulsion concentrations; (3) influence of CETP-rich plasma fraction ($D > 1.21$); (4) influence of the emulsion composition; (5) influence of unesterified fatty acids in the incubation medium.

All incubations were done at 37°C utilizing a tube rotator. After incubation, mixtures were adjusted to $D = 1.101$ g/ml utilizing a NaCl 1.53 g + KBr 3.54 g/ml water solution of $D = 1.346$ g/ml [26]. Samples were immediately transferred to ultracentrifuge tubes, the volume was adjusted to 7 ml with NaBr solution, $D = 1.101$ g/ml, and overlayered with 4 ml saline, $D = 1.006$ g/ml. This gradient was ultracentrifuged at 40 000 rpm in an SW41Ti rotor at 4°C, for 30 min. The top phase (3 ml) containing EM was vacuum aspirated and radioactivity measured in this phase and in the infranatant HDL fraction. Less than 1.0% of the HDL radioactivity added to duplicate blank tubes (incubation without EM) in all experiments was found in the $D < 1.006$ g/ml layer.

The following incubation media were utilized: (1) 0.15 M NaCl, pH 7.0–7.2; (2) fatty acid-free bovine albumin (Sigma, Chemical Co., St. Louis, MO) 4.0 g% in saline; and (3) ultracentrifuged plasma $D > 1.21$ g/ml fraction drawn from a pool of healthy blood donors. Before incubation, this fraction was submitted to exhaustive dialysis against saline and shown to be cholesterol-free by the Chod Pap enzymatic method (Boehringer-Mannhein, Germany) and triglyceride-free by the ENZ-color method (Biodiagnostica, Paraná, Brazil).

In the studies utilizing HDL labeled with [^{3}H]cholesteryl ester ([^{3}H]CE) prepared by incubation of the lipoprotein with [1,2-^{3}H(n)]cholesterol, EM and HDL fractions obtained by ultracentrifugation after the incubation were extracted with chloroform/methanol (2:1, v/v), and the esterified and unesterified cholesterol radioactivity bands separated by TLC were measured by liquid scintillation as described above. In studies utilizing [1,2,6,7-^{3}H(n)]cholesteryl oleate ([^{3}H]CO), or [4-^{14}C]cholesteryl oleate ([^{14}C]CO), aliquots of EM and HDL drawn from ultracentrifuged tubes were directly counted in Aquasol.

Results were expressed as percent of radioactivity or as mass transferred. The latter was calculated as radioactivity (dpm) in the acceptor particle measured after incubation, divided by the specific activity (dpm/μg) in the donor particle measured before the incubation.

3. Results

The [^{3}H]CE content transferred from HDL_3 to EM or from EM to HDL_3 seems to increase along time in saline but could not be acompanied for periods much longer than 8 h due to EM clumping. However, incubations were stable for 2 to 24 h in the presence of plasma $D > 1.21$ g/ml, which, stimulated the transfer rate from HDL_3 to EM about four times (Table 1). On the other hand, average [^{14}C]CO flow from EM to HDL_3 in the presence of plasma $D > 1.21$ g/ml had already reached a plateau at 2 h, and at 4 or 8 h

Table 1
Time course of esterified cholesterol transfer between HDL_3 containing [1,2-^{3}H(n)]cholesteryl ester ([^{3}H]CE) and TG-rich emulsions (EM) made with [4-^{14}C]cholesteryl oleate ([^{14}C]CO) in saline and in plasma $D > 1.21$ g/ml media

Media	Time(h)	Esterified cholesterol transferred(μg)		
		HDL_3 to EM	EM to HDL_3	Net balance in EM
Saline	2	3.3	17.1	−13.8
	4	4.3	32.7	−28.4
	8	5.6	40.8	−35.2
Plasma($D > 1.21$ g/ml)	2	18.3	28.0	−9.7
	4	19.8	26.2	−6.4
	8	20.3	34.0	−13.7
	24	21.8	24.7	−2.9

HDL_3 was labeled with [^{3}H]cholesterol that had been esterified in vitro by previous incubation with plasma. [^{3}H]cholesteryl ester transfer was measured after separation from ^{3}H-unesterified cholesteryl by TLC. Amounts of esterified cholesterol (μg) in each incubation were: HDL_3 = 29; EM = 126 (containing 1405 μg of triolein as the major lipid). Incubation conditions: 1.1 ml, 37°C. Data shown as μg (mean of duplicates).

was not greater than the corresponding values in the presence of saline. Consequently, net gain by HDL_3 was considerably smaller in the plasma $D > 1.21$ g/ml incubation than in the saline medium. Nonetheless, transfer activity in saline might to some extent be ascribed to residual CETP attached to HDL_3.

During 2-h incubations the effect of variable HDL/EM ratios on the bidirectional transfer of cholesteryl ester between these particles, in the presence of plasma $D > 1.21$ fraction, is shown in Table 2. Either HDL_3 or HDL_2 were utilized in the assays. Cholesteryl ester flows were greater with HDL_3 than with HDL_2. The CE flow from

Table 2
Effects of amounts of lipoproteins on the transfer of esterified cholesterol (CE) between HDL_2 or HDL_3 (containing [1,2-^{3}H(n)]cholesteryl ester) ([^{3}H]CE) and TG-rich emulsions (EM) (made with [4-^{14}C]cholesteryl oleate) ([^{14}C]CO) incubated in plasma $D > 1.21$ g/ml (1.1 ml) at 37°C for 2 h.

Quantity of lipids (CE and TG) in incubations(μg)			Esterified cholesterol transferred(μg)		
[^{3}H]CE-HDL_2	[^{14}C]CO-EM		HDL to EM	EM to HDL	Net balance in EM (% variation)
CE	CE	TG			
20	120	1601	9.2	17.1	−7.9 (−6.6)
40	40	533	12.9	12.8	+0.1 (+0.2)
120	20	267	10.7	6.8	+3.9 (+19.5)
[^{3}H]CE-HDL_3	[^{14}C]CO-EM				
CE	CE	TG			
29	126	1405	18.3	28.0	−9.7 (−7.7)
49	40	446	16.8	15.3	+1.5 (+3.7)
120	20	223	6.3	12.1	+4.2 (+21.0)

Labeling procedures are described in Table 1. Data in μg are means of duplicates.

Table 3
Transfer of cholesteryl ester (CE) and triolein (TO) between HDL_3 and TG-rich emulsions incubated in plasma $D > 1.210$ g/ml during 2 h at 37°C in the presence or absence of inhibitor of the enzyme lecithin-cholesterol acyl transferase (DTNB)

Lipoproteins		DTNB (1.5 mM)	Transfer of CE (μg)
Donor	Receptor		
[^{3}H]CE-HDL_3 [a]	to EM [b]	+	15.76
		−	16.30
[^{3}H]CE-HDL_3 [a]	to EM(−CO) [c]	+	17.42
		−	15.75
		Mean	16.31
[^{14}C]CO-EM [d]	to HDL_3	+	11.69
		−	12.53
		Mean	12.11
		Net transfer to chylomicrons	+ 4.20 [e]
			Transfer of TG (μg)
[^{14}C]TO-EM [f]	to HDL_3	+	43.55
		−	39.04
[^{14}C]TO-EM(CO) [g]	to HDL_3	+	40.87
		−	40.34
		Mean	40.95

In two incubations triglyceride emulsion was made without cholesteryl oleate (−CO). All experiments in triplicate assays

[a] HDL_3 was labeled in vitro with [1,2-^{3}H(n)]cholesterol which had previously been 77–88% esterified: [^{3}H]CE. HDL_3 incubated contained cholesteryl ester (120 μg) and triglycerides (23 μg).

[b] Triglyceride-rich emulsions in incubation: triglycerides (223 μg) and cholesteryl oleate (20 μg).

[c] Triglyceride-rich emulsions without cholesteryl oleate mass: triglycerides (230 μg).

[d] [4-^{14}C]Cholesteryl oleate added during the emulsion preparation.

[e] $p < 0.001$ (Student's t-test of the difference) between the two means (16.31–12.11).

[f] [*carboxyl*-^{14}C]triolein added during the emulsion preparation.

[g] [^{14}C]TO-EM(−CO) = emulsion without cholesteryl oleate mass.

HDL to EM did not change significantly as the HDL/EM cholesteryl ester ratio radically increased from 0.16 or 0.23 to 6. Nonetheless, there was a simultaneous decrease of cholesteryl oleate mass flow from EM to both HDL fractions bringing on a positive net cholesteryl ester balance in EM.

A net shift of cholesteryl ester from HDL_3 to EM was also shown at 2-h incubation when the HDL_3/EM cholesteryl ester proportion was high in the plasma $D > 1.21$ g/ml incubation medium (Table 3), namely, the contents of cholesteryl ester were 120 μg in HDL_3 and 20 μg in EM. Addition of DTNB, an inhibitor of the enzyme lecithin-cholesterol-acyltransferase (LCAT), as well as incubations of HDL_3 with EM made without cholesteryl oleate, did not influence the CE net flow. This assay also disclosed that the mass transfer to HDL_3 of EM-triolein labeled as [carboxyl-^{14}C]triolein ([^{14}C]TO) was roughly about three times greater than the cholesteryl ester mass (identified as [1,2-^{3}H(n)]cholesteryl ester) that shifted from HDL_3 to EM.

At 2-h incubation periods the influence of increasing amounts of plasma fraction of

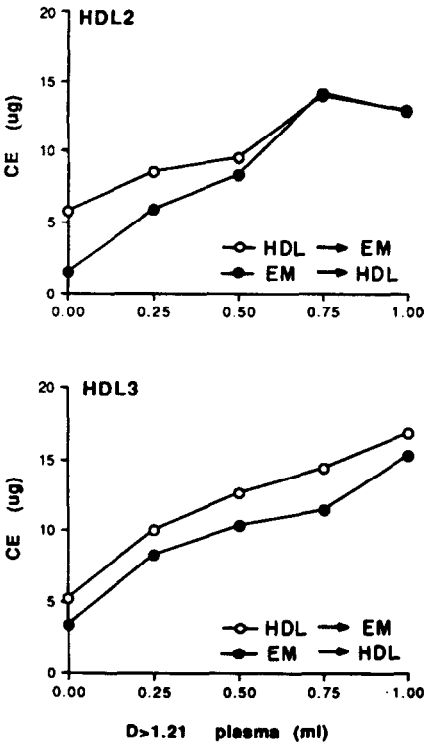

Fig. 1. Influence of plasma fraction of $D > 1.21$ volume on the bidirectional transfer of cholesteryl oleate between TG-rich emulsion (EM) and HDL_2 or HDL_3. HDL labeled in vitro with [1,2,6,7-^{3}H(n)]cholesteryl oleate ([^{3}H]CO) and EM made with [4-^{14}C]cholesteryl oleate ([^{14}C]CO). Incubations contained cholesteryl ester (40 μg) as HDL and as EM; total volume 1.1 ml, at 37°C, for 2 h. Results in μg are means of duplicates.

$D > 1.21$ g/ml on the bidirectional transfer of cholesteryl ester is shown in Fig. 1. Cholesteryl ester flow from EM to HDL, and vice versa, was proportional to the plasma $D > 1.21$ volume in the incubation either with HDL_2 or HDL_3. Nonetheless, as the plasma fraction volume varied from zero to 1 ml, cholesteryl ester flow value from EM approached the flow value from HDL_2 so that the net balance transfer was no longer observed. In regard to HDL_3, there occurred a small but significant (2.07 μg) net gain of esterified cholesterol by the EM particles.

It had previously been shown that free fatty acids can influence the cholesteryl ester transfer process between HDL and triglyceride-rich lipoproteins [7,10,27] and also the size and composition of these lipoproteins [11]. The role of unesterified fatty acids in our assay conditions was investigated in incubations utilizing oleic acid either added to plasma at $D > 1.21$ g/ml or in albumin solution (molar ratio of free fatty acids/albumin = 5) (Table 4). This ratio was chosen because lipoproteins effectively compete with albumin for fatty acid binding only when the molar ratio is greater than 4 [28]. Oleic acid induced a spontaneous transfer of esterified cholesterol from HDL_2 or from HDL_3 to EM and diminished the reverse flow toward HDL_3 but not HDL_2. Further-

Table 4
Role of oleic acid on the bidirectional transfer of cholesteryl oleate between HDL_2 or HDL_3 and TG-rich emulsion (EM)

Incubation medium	Cholesteryl oleate transferred		
	[^{3}H]CO-HDL to [^{14}C]CO-EM	[^{14}C]CO-EM to [^{3}H]CO-HDL	Net balance in EM (μg)
	HDL_2 to EM	**EM to HDL_2**	
Saline	5.83	1.52	+4.32
Albumin (4 g%)	3.10	1.80	+1.30
Albumin/oleic acid [a]	8.24	1.72	+6.52
Plasma $D > 1.21$ g/ml	12.88	12.80	+0.08
$D > 1.21$ g/ml + oleic acid [b]	6.36	5.45	+0.91
	HDL_3 to EM	**EM to HDL_3**	
Saline	5.20	3.34	+1.86
Albumin (4 g%)	3.12	3.70	−0.58
Albumin/oleic acid [a]	12.84	0.80	+12.04
Plasma $D > 1.21$ g/ml	16.82	15.32	+1.50
$D > 1.21$ g/ml + oleic acid [b]	5.56	5.10	+0.46

HDL labeled in vitro with [1,2,6,7-^{3}H(n)]cholesteryl oleate ([^{3}H]CO) and EM made with [4-^{14}C]cholesteryl oleate ([^{14}C]CO). Incubations contained 40 μg of cholesteryl ester as HDL and EM in 1.1 ml, at 37°C, for 2 h. Data as means of duplicates in μg.

[a] Albumin 4 g% solution containing 6 mM oleic acid (molar ratio 1:5).

[b] Oleic acid (6 mM) in chloroform/methanol (2:1, v/v) was dried down under N_2 flow. Plasma $D > 1.21$ g/ml fraction (1 ml) was added to the fatty acid and vortex stirred prior to incubation.

more, in the presence of plasma $D > 1.21$ g/ml, this amount of oleic acid impaired the esterified cholesterol exchange between EM and both HDL fractions.

4. Discussion

Unesterified cholesterol spontaneously exchanges between plasma lipoproteins [29,30]. However, equilibration of the neutral lipids cholesteryl ester and triglycerides among plasma lipoproteins is slow and requires the intervention of the cholesteryl ester tranfer protein (CETP) [7,17,31]. Furthermore, several physiological factors might intervene in this exchange process, such as: (1) relative concentration of the acceptor and donor lipoproteins [16]; (2) size and composition of the lipoproteins [32–35]; (3) concentration of the plasma fraction of $D > 1.21$ g/ml containing CETP activity [19]; (4) presence in the incubation medium of unesterified fatty acids [7,10,11,27]. Nonetheless, it is not known how these factors influence shifts of cholesteryl ester between triglyceride-rich particles and specific HDL fractions, namely HDL_2 and HDL_3.

In the presence of a large quantity of the TG-rich emulsion particles (EM) in relation to that of HDL_3, as opposed to what occurred in the saline medium, the plasma fraction at $D > 1.21$ gml did not influence the largely predominant cholesteryl ester flow from EM to HDL at 4 and 8 h; and even at the 24-h plasma incubation the flow rate value

was not greater than at 4- or 8-h values as obtained in saline (Table 1). Although the reverse flow from HDL_3 was stimulated 3.6 to 5.5 times by plasma $D > 1.21$, the net balance attained still favored a shift of cholesteryl ester from EM to HDL_3 (Table 1). On the other hand, as the HDL/EM cholesteryl ester mass ratio increased (Table 2), the net balance of the cholesteryl ester mass favored EM in regard to both HDL fractions and in spite of a decrement in the acceptor EM particle number, that is, in the concentration of triglycerides. Nonetheless, although the HDL/EM cholesteryl ester ratio rose in the incubations a steady cholesteryl ester flow toward EM was observed, simultaneous to a progressive decrement in the reverse flow from EM. Also, since the TG/CE ratio was kept constant in the three incubations the diminished flow observed toward HDL must be ascribed to a greater cholesteryl ester concentration in HDL as compared to that in EM. Present data confirm those of Martins et al. [16] which showed that emulsion-cholesteryl ester transfer to HDL, but not the reverse, was unrelated to the lipid emulsion concentration.

In incubations utilizing EM made without cholesteryl oleate, the uptake of cholesteryl ester by EM from HDL_3 was not additionally favored (Table 3). This further indicates that the the emulsion uptake of cholesteryl ester is independent from the concentration of cholesteryl ester in the EM particle.

Since CETP promotes an exchange of cholesteryl ester and triolein between lipoproteins, triglyceride flow from EM to HDL_3 was quantified in incubation containing plasma $D > 1.21$ g/ml (Table 3). Admitting the cholesteryl ester of HDL_3 mainly as cholesteryl oleate (CO, Mol.wt. = 651), the stoichiometry of the CO shift from HDL_3 to EM was about 25 nmol/ml and of triolein (TO, Mol.wt. = 885) from EM to HDL_3 was 46 nmol/ml, that is, a molar exchange ratio CO/TO = 0.55. In other words, HDL_3 gains 2 molecules of TO in exchange for 1 CO molecule transferred to EM. On the other hand, the cholesteryl ester molecular flow from HDL_3 to EM and from EM to HDL_3 disclosed a 1.4 ratio, that is, two cholesteryl ester molecules from EM shift to HDL_3 in exchange for three cholesteryl ester molecules displacing in the opposite direction. In short the molecular exchanges CO/CO and CO/TO were not equimolar as had been described by other authors [34–36]. Weinberg and Scanu [17] also found heteromolar exchange of cholesteryl ester-HDL_3 and triglyceride-Intralipid close to 1.4. However, Rajaram et al. [19] also showed that on low CETP concentration the CO/TO exchange ratio between HDL and VLDL was equimolar, but high CETP concentration elicited three times more CO than TO exchange. The experimental factors responsible for such variable results could have also been the quantity of unesterified cholesterol, non-esterified fatty acids and CETP because these factors are known to influence the exchange of cholesteryl oleate and triolein. Gain of unesterified cholesterol by the triglyceride-rich particles favors the CE shift from HDL to VLDL [34], whereas, as demonstrated by Sparks and Pritchard [37], enrichement of HDL with unesterified cholesterol may reduce the efficiency of HDL in gaining cholesteryl ester from LDL. Thus, nonesterified cholesterol modulates the rates of exchange of nonpolar lipids by decreasing the availability of core cholesteryl ester or triglycerides to the lipoprotein surface for interactions with CETP. However, since varying the concentration of cholesterol modifies both size and composition of the EM particle this work was limited at investigating the influences both of the plasma volume containing CETP activity and of non-esterified

fatty acids. The present study demonstrated (Fig. 1) that bidirectional cholesteryl ester flow between HDL_3 or HDL_2 and TG-rich emulsions also was to some extent proportional to the plasma $D > 1.21$ concentration. Some degree of transference that occurred in the absence of plasma may have been ascribed to residual CETP activity present in the HDL fraction. Nonetheless, as the plasma $D > 1.21$ g/ml volume increased the net gain by EM of cholesteryl ester from HDL_2 flattened out whereas the gain from HDL_3 persisted along a wide range of plasma volume values (Fig. 1). In general, the CE bidirectional flows between HDL and EM (Table 2), and the CE net balance (Fig. 1), were greater when HDL_3 instead of HDL_2 was used. In vivo, HDL_2 is thought to be converted into the smaller HDL_3 particle through a process mediated by CETP and hepatic lipase, where TG from VLDL is exchanged for CE from HDL [7]. In our in vitro assay HDL_3 seems better than HDL_2 as CE donor over a wide range of CETP plasma activity. This result can be attributed to a lower TG/CE ratio in HDL_3 as compared to HDL_2, or to variable interactions of CETP with the different HDL species that can be attributed to their specific apolipoprotein compositions [38], so that the CE/TG heteroexchange would be more favorable in regard to HDL_3 than to HDL_2 in a system that lacks lipases.

In conclusion, our results show that the transfer rate of esterified cholesterol between HDL and TG-rich emulsion particles occurs spontaneously in saline medium (probably attributable to residual CETP inherent in HDL), is saturable and greatly facilitated in a dose dependent manner by the quantity of the plasma fraction of $D > 1.21$ containing CETP. The net CE balance between EM and HDL ultimately results from different driving forces: the CE flow from HDL to EM is mostly driven by the amount of the plasma fraction containing CETP (Table 1, Fig. 1), an effect that is more distinct for HDL_3 than for HDL_2, while the CE flow from EM to HDL depends both on the concentration of HDL-cholesteryl ester (Table 2), and on the CETP plasma activity (Table 4).

Plasma lipolytic activity brought about by the enzyme lipoprotein lipase modifies the CETP-mediated changes in triglyceride/cholesteryl ester proportions in the HDL core [2,10,12,39,40]. Addition of lipoprotein-lipase in vitro, or previous partial lipolysis of VLDL was known to facilitate the cholesteryl ester mass shift from HDL to VLDL [9,41]. Also, Barter [27] reported that sodium oleate incubated with HDL, VLDL and CETP enhances the esterified cholesterol shift from HDL to VLDL but inhibits the reverse shift of triglycerides. Greater CETP binding to the lipoproteins was ascribed to the enrichment either of LDL or VLDL with unesterified fatty acids [10,42], as well as of HDL with phospholipids and free fatty acids [10]. Conversely, lowering the pH of the medium or adding albumin to it, interfered negatively with the CETP mediated esterified cholesterol transfer [41]. Also when HDL gains phospholipids, or a small quantity of nonesterified fatty acids in post-alimentary plasma, the transfer of cholesteryl ester from HDL to the triglyceride-rich particles is stimulated when compared to the fasting state [2,12]. In the present report, oleic acid in albumin solution markedly enhanced the spontaneous transfer of cholesteryl ester from HDL_2 and HDL_3 to EM (Table 4). The net quantity transferred was far greater than that brought about by plasma $D > 1.21$ alone because the reverse flow from EM to HDL_3 was markedly inhibited. Addition of a similar unesterified fatty acid (NEFA) concentration to plasma $D > 1.21$ g/ml partially

impaired the bidirectional cholesteryl ester flow. In spite of the enhancement of the CETP activity by nonesterified fatty acids, Tall et al. [7] reported that CETP incubations with lipid emulsion containing NEFA facilitated the CETP-emulsion binding while simultaneously inactivating the CETP. Loss of transfer activity was time and NEFA concentration dependent but did not occur when the fat emulsion was absent. Such inactivation was attributed to the NEFA-derived peroxides produced during the emulsion preparation [7] and could have explained the partial reduction of the cholesteryl ester flow in the medium plasma $D > 1.21$ g/ml containing oleic acid when compared to plasma $D > 1.21$ alone. Therefore, the NEFA action on the CETP-mediated cholesteryl ester shift seems rather complex and dependent on medium NEFA concentration and its partition between albumin and lipoprotein particles, and also on the NEFA oxidized state [7,19,42].

These experimental data in vitro show how physiological variations of several factors, such as relative concentrations of the incubated lipoproteins and presence of NEFA or CETP activity, can affect the CE distribution between heavy lipoproteins and light TG-rich LP, and in addition have quantitatively different effects depending on the type of HDL fraction, thus working as a model for the steps of cholesteryl ester transport that occur in plama in vivo.

Acknowledgements

This work was partially supported by grants from CNPq, FAPESP and Hospital das Clínicas (LIM). The authors are thankful to Miss Jussara C. Rocha for technical assistance, and to Senária Eguti and Dr. Elettra Greene for the preparation of the manuscript.

References

[1] Nichols, A.V. and Smith, L., Effects of very low density lipoproteins on lipid transfer in incubated serum. J. Lipid Res., 6 (1965) 206-210.

[2] Tall, A.R., Sammett, D. and Granot, E., Mechanisms of enhanced cholesteryl ester transfer from high density lipoproteins to apolipoprotein B-containing lipoproteins during alimentary lipemia. J. Clin. Invest., 77 (1986) 1163–1172.

[3] Brown, M.S. and Goldstein, J.L., The LDL receptor concept: clinical and therapeutic implications. Atheroscler. Rev., 18 (1988) 85–93.

[4] Havel, R.J., Functional activities of hepatic lipoprotein receptors. Annu. Rev. Physiol., 48 (1986) 119–134.

[5] Patsch, J.R., Karlin, J.B., Scott, L.W., Smith, L.C. and Gotto, Jr., A.M., Inverse relationship between blood levels of high density lipoprotein subfraction 2 and magnitude of postprandial lipemia. Proc. Natl. Acad. Sci. USA, 80 (1983) 1449–1453.

[6] Zilversmit, D.B., Atherogenesis: a postprandial phenomenon. Circulation, 60 (1979) 473–484.

[7] Tall, A.R., Swenson, T., Hesler, C. and Granot, E., Mechanisms of facilitated lipid transfer mediated by plasma lipid transfer proteins. In: A.M. Gotto Jr. (Ed.), Plasma Lipoproteins. Amsterdam, Elsevier Science Publishers, 1987. pp. 277–297.

[8] Goldberg, D.I., Beltz, W.F. and Pittman, R.C. Evaluation of pathways for the cellular uptake of high density lipoprotein cholesterol esters in rabbits. J. Clin. Invest., 87 (1991) 331–346, 1991.

[9] Tall, A.R., Sammett, D., Vita, G.M., Deckelbaum, R. and Olivecrona, T., Lipoprotein lipase enhances the cholesteryl ester transfer protein-mediated transfer of cholesteryl esters from high density lipoproteins to very low density lipoproteins. J. Biol. Chem., 259 (1984) 9587–9594.
[10] Sammett, D. and Tall, A.R., Mechanisms of enhancement of cholesteryl ester transfer protein activity by lipolysis. J. Biol. Chem., 260 (1985) 6687–6697.
[11] Musliner, T.A., Michenfelder, H.J. and Krauss, R.M. Interactions of high density lipoproteins with very low and low density lipoproteins during lipolysis. J. Lipid Res., 29 (1988) 349–361.
[12] Castro, G.R. and Fielding, C.J., Effects of postprandial lipemia on plasma cholesterol metabolism. J. Clin. Invest., 75 (1985) 874–882.
[13] Derksen, A., Ekman, S. and Small, D.M., Oleic acid allows more apoprotein-I to bind with higher affinity to large emulsion particles saturated with cholesterol. J. Biol. Chem., 264 (1989) 6935–6940.
[14] Derksen, A. and Small, D.M., Interaction of Apo-Ai and Apo-E with triglyceride-phospholipid emulsions containing increasing cholesterol concentrations. Modes of triglyceride-rich nascent an remnant lipoproteins. Biochemistry, 28 (1989) 900–906.
[15] Tajima, S., Yokoyama, S. and Yamamoto, A., Effect of lipid particle size on association of apolipoproteins with lipid. J. Biol. Chem., 258 (1983) 10073–10082.
[16] Martins, I.J., Hopkins, L. and Joll, C.A., Redgrave, T.G., Interactions between model triacylglycerol-rich lipoproteins and high-density lipoproteins in rat, rabbit and man. Biochim. Biophys. Acta, 1081 (1991) 328–338.
[17] Weinberg, R.B. and Scanu, A.M., In vitro reciprocal exchange of apoproteins and nonpolar lipids between human high density lipoproteins and an artificial triglyceride-phospholipid emulsion (intralipid). Atherosclerosis, 44 (1982) 141–152.
[18] Connelly, P.W. and Kuksis, A., Effect of core composition and particle size of lipid emulsions on apolipoprotein transfer of plasma lipoprotein 'in vivo'. Biochim. Biophys. Acta, 666 (1980) 80–89.
[19] Rajaram, O.V., Chang, L.B.F. and Barter, P.J., Factors influencing the hetero-exchange of cholesteryl esters and triglyceride between high density lipoproteins and triglyceride-rich lipoproteins. In: International Symposium on Atherosclerosis, 9th, Rosemont, 1991, Int. Atherosclerosis Soc., Abstracts, p. 238.
[20] Morton, R.E. and Zilversmit, D.B., Inter-relationship of lipids transferred by the lipid-transfer protein isolated from human lipoprotein-deficient plasma. J. Biol. Chem., 258 (1983) 11751–11757.
[21] Barter, P.J. and Jones, M.E., Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. J. Lipid Res., 21 (1980) 238–249.
[22] Hirata, M.H., Oliveira, H.C.F., Quintão, E.C.R., Redgrave, T.G. and MaranhÃO, R.C., The effects of Triton WR1339, protamine sulphate and heparin on the plasma removal of models of chylomicrons and remnants in rats. Biochim. Biophys. Acta, 917 (1987) 344–346.
[23] Oliveira, H.C.F., Hirata, M.H., Redgrave, T.G. and Maranhão, R.C., Competition between chylomicrons and their remnants for plasma removal: a study with artificial emulsion models of chylomicrons. Biochim. Biophys. Acta, 958 (1988) 211–217.
[24] Zerbinatti, C.V., Oliveira, H.C.F., Wechsler, S. and Quintão, E.C.R., Independent regulation of chylomicron lipolysis and particle removal rates: effects of insulin and thyroid hormones on the metabolism of artificial chylomicrons. Metabolism, 40 (1991) 1122–1127.
[25] Redgrave, T.G., Roberts, D.C.K. and West, C.E., Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal. Biochem., 65 (1975) 42–49.
[26] Havel, R.J., Eder, H.A. and Bragdon, J.H., The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest., 34 (1955) 1345–1353.
[27] Barter, P.J., Chang, L.B.F. and Rajaram, O.V., Sodium oleate dissociates the heteroexchange of cholesteryl esters and triacylglycerol between HDL and triacylglycerol-rich lipoproteins. Biochim. Biophys. Acta, 1047 (1990) 294–297.
[28] Spector, A.A, Fatty acid binding to plasma albumin. J. Lipid Res. 16 (1975) 165–179.
[29] Kakis, G., Kuksis, A. and Breckenridge, W.C., Redistribution of cholesterol and beta-sitosterol between intralipid and rat plasma lipoproteins and red blood cells 'in vivo'. Biochem. Cell Biol., 66 (1988) 1312–1321.
[30] Velazquez, E., Montes, A. and Ruiz-Albusac, J.M., Free cholesterol transfer from human lower-density lipoproteins ($d < 1.063$) to lipoprotein-deficient serum and high-density lipoproteins. Metabolism, 39 (1990) 1263–1266.

[31] Tall, A.R., Abreu, E. and Shuman, J., Separation of a plasma phospholipid transfer protein from cholesterol ester/phospholipid exchange protein. J. Biol. Chem., 258 (1983) 2174–2180.
[32] Dullaart, R.P.F., Groener, J.E.M. and Erkelens, D.W., Effect of the composition of very low density lipoprotein on the rate of cholesteryl ester transfer from high density lipoproteins in man, studied 'in vitro'. Eur. J. Clin. Invest., 17 (1987) 241–248.
[33] Eisenberg S., Preferential enrichement of large-sized very low density lipoprotein populations with transferred cholesteryl esters. J. Lipid Res., 26 (1985) 487–494.
[34] Morton, R.E., Free cholesterol is a potent regulator of lipid transfer protein function. J. Biol. Chem., 263 (1988) 12235–12241.
[35] Milner, T.G., Ko, K.W.S., Ohnishi, T. and Yokoyama, S., Enhancement of the human plasma lipid transfer protein reaction by apolipoproteins. Biochim. Biophys. Acta, 1082 (1991) 71–78.
[36] Ko, K.W S., Ohnishi, T. and Yokoyama, S., Triglyceride transfer is required for net cholesteryl ester transfer between lipoproteins in plasma by lipid transfer protein. Evidence for a hetero-exchange transfer mechanism demonstrated by using novel monoclonal antibodies. J. Biol. Chem., 269 (1994) 28206-28213.
[37] Sparks, D.L. and Pritchard, P.H., Transfer of cholesteryl ester into high density lipoprotein by cholesteryl ester transfer protein: effect of Hdl lipid and apoprotein content. J. Lipid Res., 30 (1989) 1491–1498.
[38] Lagrost, L., Persegol, L., Lallemant, C. and Gambert, P, Influence of apolipoprotein composition of high density lipoprotein particles on cholesteryl ester transfer protein activity. Particles containing various proportions of apolipoproteins AI and AII. J. Biol. Chem., 269 (1994) 3189–3197.
[39] .Dullart, R.P.F., Groener, J.E.M., van Wijk, H., Sluiter, W.J. and Erkelens, D.W., Alimentary lipemia-induced redistribution of cholesteryl ester between lipoproteins-studies in normolipidemic, combined hyperlipidemic, and hypercholesterolemic men. Arteriosclerosis, 9 (1989) 614–622.
[40] Franceschini, G., Moreno, Y., Apebe, P., Calabresi, L., Gatti, E., Noe, D., De Fabiani, E., Zoppi, F. and Sirtori, C.R., Alterations in high-density lipoprotein subfractions during postprandial lipidaemia induced by fat with and without ethanol. Clin. Sci., 75 (1988) 135–142.
[41] Tall, A.R., Plasma lipid transfer proteins. J. Lipid Res., 27 (1986) 361–367.
[42] Lagrost, L., Barter and Phillip J., Cholesteryl ester transfer protein promotes the association of HDL apolipoprotein A-I and A-II with LDL: potentiation by oleic acid. Biochim. Biophys. Acta, 1127 (1992) 255–262.