CLINICAL SCIENCE

Simultaneous transfer of cholesterol, triglycerides, and phospholipids to high-density lipoprotein in aging subjects with or without coronary artery disease

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OBJECTIVE: To verify whether the capacity of high-density lipoprotein (HDL) to simultaneously receive non-esterified cholesterol, triglycerides, cholesteryl esters, and phospholipids changes with aging and the presence of coronary artery disease.

DESIGN: Cross-sectional study with biochemical analyses.

SUBJECTS: Eleven elderly patients with coronary artery disease (74±5 years) were compared with the following groups of non-coronary artery disease subjects (referred to as ''healthy''): 25 young (25±5 years), 25 middle-aged (42±6 years), and 25 elderly subjects (75±8 years).

METHODS: Plasma samples were incubated with a nanoemulsion labeled with radioactive lipids; the transfer of the lipids from the nanoemulsion to the HDL was measured in chemically precipitated HDL. HDL size and paraoxonase-1 activity were also determined.

RESULTS: The transfer of cholesteryl esters and phospholipids to high-density lipoprotein was significantly greater (*p*<0.001) in healthy elderly subjects than in the middle-aged and younger subjects. Non-esterified cholesterol and triglyceride transfer was not different among these three groups. The HDL size was significantly greater (*p*<0.001) in healthy elderly subjects than in the middle-aged and younger subjects. The paraoxonase-1 activity was similar among the groups. Compared with healthy elderly subjects, coronary artery disease elderly subjects had significantly less (*p*<0.05) transfer of non-esterified cholesterol, triglycerides, and cholesteryl esters to the HDL and a significantly smaller (*p*<0.05) HDL size.

CONCLUSION: Because lipid transfer is enhanced in healthy elderly subjects but not in those with coronary artery disease, increasing lipid transfer to HDL may be a protective mechanism against the disease.

KEYWORDS: Aging; cholesteryl ester transfer protein (CETP); transfer proteins; lipoproteins; nanoparticles.

INTRODUCTION

Both the high-density lipoprotein (HDL) cholesterol concentration in the plasma and the concentration of the main HDL apolipoprotein, apo A1, negatively correlate with the incidence of coronary artery disease, and both are biomarkers of healthy aging.[1-4] Data from the Prospective Cardiovascular Münster (PROCAM) study have shown that having a low HDL cholesterol level was the predominant characteristic of subjects older than 60 years with a history of myocardial infarction compared to subjects older than 60 years without previous cardiovascular events.[4]

The HDL fraction is composed of heterogeneous particles with sizes ranging from 7 to 14 nm.[1,5] In addition to its roles in reverse cholesterol transport and cholesterol esterification, HDL has antioxidant activity that is mostly due to its association with paraoxonase 1 (PON 1) and anti-inflammatory, antithrombotic, and vasodilation activities that may account for the antiatherogenic action of the lipoprotein.[6,7]

Nascent HDL is produced in the liver and intestine as discoid particles composed of phospholipids and non-esterified cholesterol. These particles are progressively

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transformed into a spherical shape by the acquisition of lipids and the esterification of cholesterol by lecithin-cholesterol acyltransferase (LCAT), with apo A1 as a cofactor. Because most of the apo A1 that is present in the plasma is contained in the HDL fraction and because LCAT is also associated with HDL, cholesterol esterification occurs mainly in this lipoprotein fraction.[7] HDL is thus constantly being remodeled, and lipid transfers are essential for the roles of this lipoprotein in cholesterol esterification and reverse cholesterol transport. Both processes are intertwined and are necessary for cholesterol homeostasis. Lipid transfers between lipoprotein classes are bidirectional and depend on the structures of the donor and acceptor lipoproteins and the activity of the transfer proteins, i.e., cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). The concentrations of the donor and acceptor lipoprotein classes may also influence the process.[8-11]

Because the lipid transfer process is a determinant of HDL composition and metabolism, it is also possible that in addition to the HDL function in reverse cholesterol transport, other atheroprotective functions of the lipoprotein may be affected by this process. The current study was designed to verify the effects of aging on the capacity of HDL to simultaneously receive cholesteryl esters (CEs), phospholipids (PLs), triglycerides (TGs), and non-esterified cholesterol (NEC). A standard artificial nanoemulsion was used as the donor of radioactive lipids to HDL in an in vitro assay[12] with plasma from subjects within different age ranges: young, middle-aged, and elderly subjects. The HDL size and PON 1 activity were also measured. A group of elderly patients with coronary disease was also studied to investigate whether a different pattern of lipid transfer to HDL was associated with the disease.

METHODS AND MATERIALS

Study subjects

The volunteer participants in the study were recruited at the outpatient clinics and at the Division of Geriatric Cardiology of the Heart Institute of the Medical School Hospital of the University of São Paulo, where they were referred for routine check-ups and clinical and laboratory tests and were pronounced healthy. Three groups of subjects paired for gender and body mass index (BMI) were studied: young (aged 20-25 years; 10 men and 15 women), middle-aged (aged 39-54 years; 9 men and 16 women), and elderly (aged 65-82 years; 11 men and 14 women) subjects. A fourth group (aged 65-81 years; 5 men and 6 women) was composed of 11 elderly patients with coronary artery disease (CAD). The presence of CAD was confirmed by coronary angiography performed within six months prior to the study. None of the participants were smokers; obese; alcoholics; or had liver, renal, metabolic, inflammatory, or neoplastic disease. Their physical characteristics and plasma biochemical parameters are shown in Table 1. All of the patients in the non-CAD elderly and CAD elderly groups were receiving standard maintenance hypertension treatment with a beta-adrenergic blocker or an angiotensin-converting enzyme (ACE) inhibitor. The elderly CAD subjects were also taking statin medications, which were discontinued for at least 30 days before the study.

The Ethics Committee of the Medical School Hospital of the University of São Paulo approved the study, and written informed consent was obtained from all of the subjects following a complete description of the protocol.

Table 1 - Physical characteristics and plasma biochemical parameters of the study subjects.

Parameters	Young (n = 25)	Middle-aged (n = 25)	Elderly (n = 25)	CAD Elderly (n = 11)
Age (years)	25 ± 5	42 ± 6	75 ± 8*	74 ± 5
Gender (M/F)	10/15	9/16	11/14	5/6
BMI (kg/m²)	26 ± 4	25 ± 5	27 ± 3	26 ± 4
Cholesterol				
Total (mg/dL)	113 ± 18	173 ± 43	190 ± 40*	201 ± 30
HDL (mg/dL)	41 ± 6	40 ± 7	50 ± 12*	42 ± 6**
LDL (mg/dL)	96 ± 23	102 ± 44	104 ± 19	143 ± 25**
Triglycerides (mg/dL)	86 ± 15	100 ± 20	101 ± 30	123 ± 46
Apo A1 (mg/dL)	128 ± 30	135 ± 14	141 ± 40	100 ± 60
Apo B (mg/dL)	70 ± 18	88 ± 15	80 ± 15	87 ± 40

The data are expressed as the means $\pm$ S.D.;
*$p < 0.001$ when compared to the young and middle-aged groups;
**$p < 0.05$ when compared to the non-CAD elderly group. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; apo A1, apolipoprotein A1; Apo B, apolipoprotein B.

Plasma lipids and apolipoproteins

Plasma total cholesterol (CHOD-PAP; Roche, Basel, Switzerland) and triglyceride (Triglyceride Rapid; Roche) levels were determined by enzymatic methods using a Cobas Bio analyzer (Roche, Basel, Switzerland). HDL cholesterol was measured using the same method used for total cholesterol after lipoprotein precipitation with magnesium phosphotungstate. The low-density lipoprotein (LDL) cholesterol level was estimated with the Friedewald formula.[13] Apo A1 and apo B levels were determined using an immunoturbidimetric assay (Roche) on a Cobas MIRA analyzer.

Nanoemulsion preparation

The lipid donor nanoemulsion was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg triolein, and 0.5 mg cholesterol, all purchased from Sigma Chemical Company (St. Louis, MO). The lipids were emulsified by prolonged ultrasonic irradiation in aqueous media and a two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain the nanoemulsion, as previously described.[14,15] The nanoemulsion fraction was dialyzed against a 0.9% NaCl solution. Trace amounts of [12, 2d(n)-³H]-cholesteryl oleate and glycerol tri[9, 10(n)-³H] oleate or 4-¹⁴C-cholesterol and L-3-phosphatidylcholine, 1-stearoyl-2-[1-¹⁴C] arachidonyl. (Amersham, Little Chalfont, Buckinghanshier, UK) were added to the initial solution.

Lipid transfer from the nanoemulsion to HDL

This assay was previously described by Lo Prete et al.[12] In brief, the nanoemulsion labeled with ³H-CE and ¹⁴C-PL or with ¹⁴C-NEC and ³H-TG was incubated with whole plasma followed by chemical precipitation of the apo B-containing lipoproteins and the nanoemulsion. The supernatant containing the lipids that shifted from the nanoemulsion to the HDL was then measured for radioactivity in a scintillation solution. The results of the radioactive transfer from the nanoemulsion to the HDL were expressed as the percentage

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of the total incubated radioactivity found in the HDL-containing supernatant.[12]

PON 1 activity

PON 1 activity was assayed using 12-h fasting blood serum, according to the method described by Mackness et al.,[16] by adding serum to Tris-HCl buffer (100 mmol/L, pH 8.0) containing 2 mmol/L $CaCl_2$ and 1.1 mmol/L paraoxon (0,0-diethyl-0-p-nitrophenylphosphate; Sigma). The rate of generation of p-nitrophenol was determined at 37℃ with the use of a spectrophotometer at 405 nm.

HDL diameter

The HDL size was measured by use of a ZetaPALS zeta potential analyzer (Brookhaven Instruments, Holtsville, NY), as previously described.[17]

Statistical analyses

A power calculation indicated that, with n>23 in each group, we would have 80% power (at a significance level of 0.05) to detect a difference in lipid transfer values between the study groups. The differences between the results were evaluated using analysis of variance (ANOVA) and Student's t-test. Significant correlations were identified using ANOVA and Pearson's test. In all of the analyses, a difference of $p<0.05$ was considered statistically significant. The data are expressed as the means $\pm$ standard deviations. GraphPad Prism version 3.0 was used to assist with the analyses.

RESULTS

Table 1 shows the plasma lipid and apolipoprotein concentrations of the study subjects. The HDL cholesterol levels were greater in the non-CAD elderly than in the middle-aged and young subjects ($p<0.001$) but did not differ between the young and middle-aged groups. The LDL cholesterol, triglycerides, apo A1, and apo B levels were not different among these three groups. In the CAD elderly group, the HDL cholesterol level was higher and the LDL cholesterol level was lower than in the non-CAD elderly group ($p<0.001$).

Table 2 shows that the non-CAD elderly group had larger HDL particles when compared with the middle-aged and young subjects. The CAD elderly group had smaller HDL particles than the non-CAD elderly group. The PON 1 activity was similar among the four groups.

Table 2 also shows the transfer of the four lipids from the artificial nanoemulsion to the HDL fraction. The transfer of CE and PL was greater in the non-CAD elderly subjects than in the middle-aged and young subjects ($p=0.0368$), whereas the latter two groups did not differ in the transfer of CE or PL. The NEC and TG transfer rates were equal in these three study groups. Compared with the non-CAD elderly group, the CAD elderly had decreased transfer of NEC, TG, and CE to HDL ($p<0.001$), but the PL transfer was equal.

The CE, TG, and NEC transfers were positively correlated with each other. In contrast, the PL transfer correlated positively with the CE transfer but showed no correlation with the TG or NEC transfer. With respect to the correlation analysis of the lipid transfer values versus the physical characteristics and laboratory data of the subjects, positive correlations were found between age and the CE transfer and between HDL cholesterol, cholesterol total concentration and HDL size, as shown in Figure 1. No correlation was found between age and the TG, PL, or NEC transfer. The PL transfer correlated positively with the HDL cholesterol level, whereas the NEC, CE, and TG transfers did not. With respect to other plasma lipids, a positive correlation was found between the TG transfer and the apo B concentration. No correlation was found between the CE, PL, NEC, or TG transfer and the total, LDL or very-low-density lipoprotein (VLDL) cholesterol or TG. The transfer of the four lipids did not correlate with the plasma glucose level or the patient's BMI.

DISCUSSION

In this study, the group of non-CAD elderly subjects showed greater transfers of CE and PL and larger HDL particle sizes than did the middle-aged and young subjects. In the CAD elderly subjects, less CE, TG, and NEC was transferred, and the HDL particle size was smaller than in the non-CAD elderly subjects. These novel observations were obtained by means of the in vitro method recently described by Lo Prete et al.,[12] in which the simultaneous transfer of all four main lipids is tested, specifically focusing on HDL and measuring the particle size of the entire HDL fraction using laser light scattering.[17]

In reverse cholesterol transport, cholesterol from the peripheral tissues is transported by HDL and taken up by the SR-B1 receptors in the liver, together with the HDL particles.[18] Alternatively, HDL cholesterol is transferred to other lipoprotein classes, such as VLDL and VLDL catabolic products, such as LDL, that are taken up together by the liver, which excretes the lipoprotein cholesterol content into the bile. During this process, the transfer proteins play a major role. CETP and PLTP are the major proteins involved in these lipid shifts. CETP specifically mediates the transfer of cholesteryl esters and triglycerides and is associated with HDL in the plasma.[19] In contrast, PLTP facilitates the transfer of surface phospholipids from other lipoproteins to HDL and influences HDL remodeling by promoting the formation of larger HDL particles, such as HDL2b and HDL2a, at the expense of the small subfraction HDL3 (see ref. 20 for a review). Lipid transfers between lipoprotein classes are bidirectional but frequently result in lipid enrichment or the depletion of lipid species of a given lipoprotein class.[7,21] Cholesterol is stored in cells in its esterified form. After the hydrolysis of CE by cytoplasmic esterases, NEC is transferred from cells to HDL by the ABCA1 system, which comprises membrane transporters that mediate the efflux of NEC and PL to HDL.[22] The cholesterol from peripheral tissues transported in HDL can be transferred to the other lipoproteins by CETP or be taken up by hepatic SR-B1 receptors.[22] Both the ABCA1 system and the SR-B1 receptors, which were not evaluated here, are thus important contributors to the continuously changing HDL composition and may have influenced the results of the lipid transfer assay in this study.

Both the concentration of HDL in the plasma and the qualitative and functional aspects of this lipoprotein are important for its anti-atherogenic actions.[23,24] Patients whose HDL contains the apo A1 Milano mutation exemplify a population that is protected from cardiovascular events despite low levels of circulating HDL. Similarly, individuals with high levels of HDL that is prone to modification may be less protected than individuals with average levels of HDL

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Table 2 - The transfer of lipids from the artificial nanoemulsion to HDL, HDL size and PON1 activity in the study subjects.

PARAMETERS	Young (n = 25)	Middle-aged (n = 25)	Elderly (n = 25)	CAD Elderly (n = 11)
Lipid transfers (%)				
Non-esterified cholesterol	9.6 ± 1.7	10.1 ± 1.2	10.0 ± 2.4	8.2 ± 1.3**
Cholesteryl ester	3.7 ± 1.0	4.1 ± 0.7	5.3 ± 1.8*	3.1 ± 2.3**
Triglycerides	6.5 ± 1.3	6.9 ± 1.3	7.3 ± 2.4	5.1 ± 1.6**
Phospholipids	18.7 ± 4.6	18.3 ± 4.0	20.6 ± 5.3*	19.9 ± 2.3
HDL diameter (nm)	8.9 ± 0.3	8.9 ± 0.3	9.7 ± 1.6*	8.7 ± 0.7**
PON 1 (nmol min^{-1} mL^{-1})	97 ± 44	85 ± 40	85 ± 44	76 ± 42

The data are expressed as the means ± S.D.;
*$p<0.001$ when compared to the young and middle-aged groups;
**$p<0.05$ when compared to the non-CAD elderly group. HDL, high-density lipoprotein; PON 1, paraoxonase 1. The transfer of lipids from the donor nanoemulsion to HDL is expressed as the % of the total radioactivity from the whole plasma that was recovered in the HDL fraction after chemical precipitation of the apo B-containing lipoproteins and the nanoemulsion.

that is more resistant to modification.[9] The capacity to receive lipids is a fundamental feature of HDL because the metabolism and function of this lipoprotein in the reverse cholesterol transport fundamentally depend on lipid exchanges.[25]

In the in vitro assay with whole plasma used in this study, many factors may have influenced the transfer of lipids from the standard donor nanoemulsion to HDL, such as the concentration, composition, and structure of both the HDL and the other acceptor lipoprotein classes, which contain apo B. The latter lipoproteins compete with HDL to receive lipids from the nanoemulsion and may thus influence the HDL lipid acceptance results. CETP and PLTP are paramount in this process because they facilitate the transfer of the lipids. The concentration of apo B, which is present in LDL and VLDL and LDL cholesterol, was not different among the study groups, but the HDL cholesterol level, which was higher in the non-CAD elderly, could have influenced the transfer results, which would favor an increase in lipid acceptance by HDL. There are no consistent reports in the literature on the effect of aging on the action of transfer proteins.

An increase in core lipids, such as CE, may increase the size of the particles,[24] as was found in this study in the elderly group. The increase in the PL transfer found in the
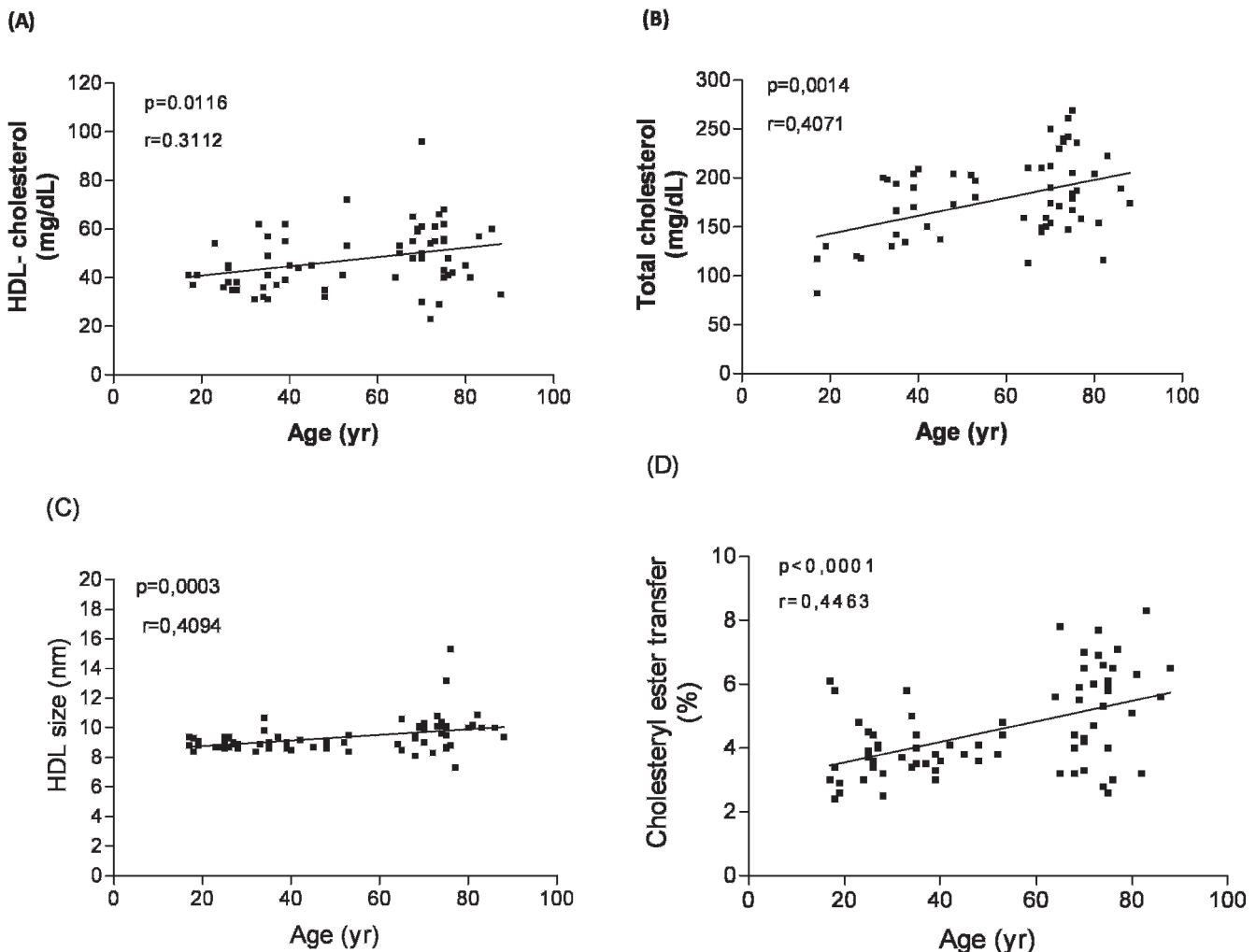
(A)

(B)

(C)

(D)

Figure 1 - Correlations between age and HDL cholesterol (A), total cholesterol (B), HDL size (C), and the transfer of cholesteryl esters (D).

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non-CAD elderly group should favor the expansion of the surface monolayer because the HDL particles swell with the greater influx of core CE. Therefore, the existence of larger HDL particles in the non-CAD elderly subjects can be attributed to the increased PLTP activity observed with the increased transfer of PL to HDL in this group. It is noteworthy that a positive correlation was also observed between age and CE transfer, HDL size and HDL cholesterol. The increase in HDL cholesterol in the absence of apo A1 is in accordance with our hypothesis that the cholesterol content in the HDL particles, and not the particle number, was greater in the elderly.

The smaller amount of transfer of CE, TG, and NEC to HDL in the group of elderly patients with CAD than in the non-CAD subjects suggests that an increase in the transfer of those lipids may protect against CAD. Diminution of HDL size in elderly patients with CAD may be secondary to the decreased lipid transfers. A diminished ability of HDL to receive NEC may reflect disturbances in the function of the lipoprotein; because most of the cholesterol esterification process occurs in the HDL fraction, diminution of the NEC influx to the lipoprotein would correspond to decreased cholesterol esterification. As expected, the HDL cholesterol level was lower in the CAD elderly group, and this result may be one of the causative factors for the decreased lipid transfers to the lipoprotein. Because of the dynamic nature of HDL metabolism, it is difficult to hypothesize whether the diminished influx of lipids was one one of the causes of CAD in the CAD elderly group or only a marker that reflected the exposure of the lipoprotein to the metabolic disturbances occurring in the disease.[26] In any event, the small number of subjects in the CAD group, as indicated by the power analysis, may have been a limitation when comparing that group to the non-CAD group.

In this study, PON 1 activity was not different among the subject groups; however, the activity of this enzyme can widely vary (up to 40-fold) in a given population because of environmental factors and polymorphisms.[27]

CONCLUSION

In conclusion, our results show that in elderly subjects who did not have CAD, the transfer of CE, the major component of the HDL core, and PL, the main HDL surface layer component, to HDL was higher than in younger subjects. In elderly subjects with CAD, the transfer of CE, TG, and NEC to HDL was decreased compared to the non-CAD elderly subjects. Those differences were also correlated with HDL particle size and suggest that aging is accompanied by changes in lipid transfer and HDL size that, in addition to HDL cholesterol levels, could also be determinants of the absence or presence of CAD in the elderly.

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