Plasma kinetics of a cholesterol-rich emulsion in subjects with or without coronary artery disease

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Abstract A cholesterol-rich emulsion (LDE) that resembles the LDL lipidic structure is taken-up by LDL receptors after intravenous injection by means of apolipoprotein E it acquires in the circulation and can be used to probe LDL metabolism. In this study, LDE was labeled with $[^{14}C]cholesterol$ oleate and $[^{3}H]cholesterol$ and injected into 19 patients with coronary artery disease (CAD) and into 14 subjects without CAD to verify whether the kinetic behavior of the radioactive lipids is different in CAD. Blood was sampled over 24 h for radioactivity measurement after lipid extraction and separation by thin-layer chromatography. Fractional clearance rate (FCR, in h$^{-1}$) of $[^{14}C]cholesterol$ was not different in CAD and nonCAD expressed as median (25%; 75%): 0.08 (0.062; 0.134) h$^{-1}$ versus 0.06 (0.04; 0.083) h$^{-1}$, $P=0.167$. However, $[^{3}H]cholesterol$ FCR was greater in CAD than in nonCAD (mean ± SEM): 0.163 ± 0.016 h$^{-1}$ versus 0.077 ± 0.014 h$^{-1}$, $P<0.001$. Esterification of the LDE $^{3}H$cholesterol was also greater in CAD subjects than nonCAD at 10 h and 24 h after emulsion injection ($P=0.029$ and 0.024 respectively). In conclusion, both removal from the plasma and esterification of the LDE-cholesterol were increased in CAD. These findings may contribute for unraveling proatherogenic mechanisms and the establishment of novel CAD markers.—Santos, R. D., W. Hueb, A. A. Oliveira, J. A. F. Ramires, R. C. Maranhão. Plasma kinetics of a cholesterol-rich emulsion in subjects with or without coronary artery disease. J. Lipid Res. 2003. 44: 464–469.

Supplementary key words cholesterol esterification • phospholipids • low density lipoprotein receptors • apolipoproteins • radioisotopes • dyslipidemias • coronary heart disease

Emulsions of defined composition resembling the lipidic structure of the plasma lipoproteins are a practical and efficient tool to investigate the lipid intravascular metabolism (1–6). Due to the risk of infection and immune reactions, autologous plasma lipoproteins must be isolated and reinjected into recipient subjects, which is a laborious and time-consuming procedure. In contrast, the same emulsion preparation can be used in an undetermined number of subjects. Furthermore, emulsions are easy to prepare and label with radioactive, fluorescent, or other markers, and bear the advantage of being standard uniform preparations (1–10). In contrast, native lipoproteins comprise different subclasses that vary widely among subjects, each one with distinct biological and pathophysiological properties. All these operational advantages greatly facilitate the execution of systematic studies on the plasma kinetics of lipids. Emulsion models of lymph chylomicrons have been used to clarify the chylomicron metabolism status in several disease states and the action of antilipidemic drugs upon this metabolism (6–10).

The metabolism of LDL can also be probed with artificial emulsions (1–5, 11–13). In this regard, we have previously studied the metabolic behavior of a cholesterol-rich emulsion termed cholesterol rich emulsion (LDE) that roughly resembles the LDL lipidic structure. LDE is made without protein, but when injected into the blood stream it acquires several small molecular weight apolipoproteins, including apoE (2–3). ApoE endows LDE particles to bind to the LDL receptors, since those receptors recognize not only the apoB present in LDL, but also apoE that is not found in the LDL fraction (14). LDE may be used as a probe to verify LDL intravascular metabolism and removal and LDL receptor function (3–5).

The adequacy of LDE as a tool to test in vivo the mechanisms of LDL removal from the plasma was demonstrated in previous clinical studies, wherein the plasma kinetic results obtained with LDE were as expected for native LDL. In this respect, removal of LDE labeled with radioactive cholesteryl esters was slower in patients with familial hypercholesterolemia (4), wherein LDL receptor function is

Abbreviations: CAD, coronary artery disease; FCR, fractional catabolic rate; LDE, cholesterol rich emulsion; TLC, thin layer chromatography.

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defective and accelerated in untreated patients with acute myeloid leukemia (3) due to the LDL receptor upregulation existing in the neoplastic cells (15). Conversely, after disease remission achieved by chemotherapy, LDE removal decreased due to destruction of the neoplastic cells with receptors upregulation, together with increase in LDL cholesterol (LDL-C) concentration (3). Recently, we were able to show that LDE fractional catabolic rate (FCR) negatively correlates with age (16). This also corroborates the validity of LDE to mimic the behavior of the native lipoprotein, because in several studies it was shown indeed that the LDL plasma removal progressively diminishes with aging due to the age-related decrease in LDL receptor expression (17). Due to its property of binding to LDL receptors that are upregulated in several cancers, LDE has been proposed as a vehicle to target chemotherapeutic agents against neoplastic tissues (3, 11–13).

In the current study, LDE was labeled with radioactive free and esterified cholesterol and injected into patients with coronary artery disease (CAD) and into control subjects without the disease and with LDL levels in the same range as CAD patients. Presence or absence of CAD was angiographically documented in the two study groups. The study aim was to verify whether or not the plasma kinetics of the labels differ between the two groups. The results unravel a possible new marker for the disease and may shed new light in the understanding of the disease mechanisms.

MATERIALS AND METHODS

Study subjects

The participants of the study were selected from the outpatient clinics of the Heart Institute of the São Paulo University Medical School Hospital. All patients had been submitted to coronary angiography that was indicated by their primary physicians to rule out obstructive coronary artery disease in the last 10 days before the kinetic study. The coronary angiography was considered normal if there was no visible obstruction according to the evaluation of two independent invasive cardiologists. Following this criterion patients were allocated to two groups: group CAD with visible obstructions on angiography (n = 19) and group nonCAD without visible obstructions on angiography (n = 14). None of the participants were addicted to alcohol consumption or had systemic arterial hypertension, diabetes, liver, renal, thyroid, or cardiac failure, or had inflammatory or neoplastic disease. No patient was using lipid lowering drugs on the last 3 months before this study.

Plasma lipids and apolipoproteins

Blood samples for determination of plasma lipids and apolipoproteins were collected after a 12 h fast on the same day the kinetic studies were performed. Commercial enzymatic methods were used for the determination of total cholesterol (Boeckinger-Mannheim, Penzberg, Germany) and triglycerides (Abbott Laboratories). HDL-C was determined by the same method used for total cholesterol after lipoprotein precipitation with magnesium phosphonatogstate. VLDL-C and LDL-C were calculated by the formula of Friedewald (18). Plasma apoA-I and apoB were assayed by radial immunodiffusion (Lipo-Partigen R-ApoA-I and Nor-Partigen R-ApoB plates, Behing, Marburg, Germany).

LDE preparation

LDE was prepared from a lipid mixture composed of 40 mg cholesteryl oleate, 20 mg egg phosphatidylcholine, 1 mg trilein, and 0.5 mg cholesterol purchased from Nu-Check Prep (Elysian), [14C]cholesteryl oleate and [3H]cholesterol purchased from Amersham International (Amersham, UK) were added to the mixture. Emulsification of lipids by prolonged ultrasonic irradiation in aqueous media and the procedure of two-step ultracentrifugation of the crude emulsion with density adjustment by addition of KBr to obtain LDE microemulsion was carried out by the method of Ginsburg et al. (1) modified by Maranhão et al. (2). LDE was dialysed against saline solution and passed through 0.22 μm filter for injection into the patients.

LDE plasma kinetics

The participants were fasting for 12 h at the beginning of the test at ~9 AM, but they were allowed two standard meals (total of ~1,800 kcal) during the study at ~12:30 PM and 7 PM. LDE containing 37 kBq of [14C]cholesteryl oleate and 74 kBq of [3H]cholesterol in a volume 500 μL was intravenously injected in a bolus. Plasma samples were collected during 24 h, in intervals of 5 min and 1, 4, 10, and 24 h after the injection. Aliquots (1.5 ml) of blood plasma were extracted with chloroform-methanol-water (2:1:1, v/v/v) overnight at 4°C (2). The organic phase was transferred to a test tube and dried under nitrogen flux. Lipids were resuspended with 5 ml of the Folch’s solution (chloroform-methanol, 2:1, v/v) and transferred for test tubes. Next, the tubes were dried under nitrogen flux, resuspended with 300 μL of Folch’s solution and submitted to separation by thin layer chromatography (TLC) (silica-gel 60H, 0.5 mm thickness), with a solvent system containing hexane-ethylisothyl ether-acetic acid (70:30:1, v/v/v). The bands that corresponded to cholesteryl ester and to free cholesterol, after being developed in the mobile phase existing in the neoplastic cells (15). Conversely, after disease remission achieved by chemotherapy, LDE removal decreased due to destruction of the neoplastic cells with receptors upregulation, together with increase in LDL cholesterol (LDL-C) concentration (3). Recently, we were able to show that LDE fractional catabolic rate (FCR) negatively correlates with age (16). This also corroborates the validity of LDE to mimic the behavior of the native lipoprotein, because in several studies it was shown indeed that the LDL plasma removal progressively diminishes with aging due to the age-related decrease in LDL receptor expression (17). Due to its property of binding to LDL receptors that are upregulated in several cancers, LDE has been proposed as a vehicle to target chemotherapeutic agents against neoplastic tissues (3, 11–13).

In the current study, LDE was labeled with radioactive free and esterified cholesterol and injected into patients with coronary artery disease (CAD) and into control subjects without the disease and with LDL levels in the same range as CAD patients. Presence or absence of CAD was angiographically documented in the two study groups. The study aim was to verify whether or not the plasma kinetics of the labels differ between the two groups. The results unravel a possible new marker for the disease and may shed new light in the understanding of the disease mechanisms.
pected cumulative distributions (21). Using this procedure, the following parameters presented a non-Gaussian distribution in our population: plasma triglyceride levels, VLDL, and HDL-C, and the [14C]cholesterol oleate FCR. Data with non-Gaussian distribution, as well as the compared data of [3H]cholesterol FCR and [14C]cholesterol oleate FCR, are expressed as median (25%; 75%). The parametric data are expressed as mean ± SE of the mean. Categorical data regarding the comparison between CAD and nonCAD were evaluated by Fisher’s exact test. Variables with non-Gaussian distribution were log transformed and all parameters were evaluated by the Student’s t-test. The correlation between the FCR of the emulsion radiolabels with the plasma lipids and apolipoproteins was made by the Pearson’s test. If there was correlation between more than one variable and the FCRs, stepwise multiple linear regression was performed. In all analysis, difference of two-tail P < 0.05 was considered statistically significant. All statistical calculations were performed with the software SPSS® for windows version 10.0.

Informed consent and radiological safety

The experimental protocol was approved by the Ethical Committee of the Heart Institute and a written informed consent was given by all participants. The safety of the radioactive dose intravenously injected into the patients was assured according to the regulations of the International Commission on Radiological Protection (22) as described in our previous study (11). The injected dose on each experiment was 0.03 mSV, well below the 50 mSV annual limit for intake of radionuclides (22).

RESULTS

Table 1 shows the physical and clinical characteristics and the plasma concentration of lipids and apolipoproteins of the studied subjects. There were no differences between the two groups regarding age, sex, and body mass index. CAD patients had lower HDL-C (P = 0.0017) and apoA-I (P = 0.009) concentration than the nonCAD subjects. There was a trend to higher triglyceride levels in CAD patients, but this did not attain statistical significance (P = 0.068). There were no differences between the two groups regarding total and LDL cholesterol and apoB.

[3H]cholesterol FCR was positively correlated with log plasma triglyceride concentrations (r = 0.37, P = 0.002) and negatively correlated with LDL (r = −0.35, P = 0.03) and log HDL-C (r = −0.047, P = 0.002). A positive correlation was also found between [3H]cholesterol FCR and log triglycerides/HDL ratio (r = 0.44, P = 0.0054). [3H]cholesterol FCR was not correlated with apoB and apoA-I concentrations. After the stepwise linear regression procedure, only log plasma triglyceride levels kept influencing the [3H]cholesterol FCR (adjusted r² = 0.18, P = 0.008).

The log [14C]cholesterol ester FCR was not correlated with either plasma lipid or apolipoprotein data (data not shown).

Figure 1 shows the plasma decay curves of the [14C]cholesterol oleate of CAD patients and their controls. There were no differences regarding [14C]cholesterol oleate FCR: 0.08 (0.062; 0.134) h⁻¹ and 0.06 (0.04; 0.083) h⁻¹ P = 0.167, respectively for CAD and nonCAD.

The plasma decay curves of [3H]cholesterol are shown in Fig. 2. The FCR of [3H]cholesterol was greater in CAD patients than in nonCAD: 0.163 ± 0.016 h⁻¹ versus 0.077 ± 0.014 h⁻¹, P < 0.001. In order to evaluate the influence of plasma triglycerides and LDL-C levels, in our results the comparison of the kinetic data was performed

| TABLE 1. | Clinical characteristics, and mean and standard error of the means of plasma lipids (mg/dl) and apolipoproteins (g/l) in subjects with CAD or without (nonCAD) |
|-----------|-----------------|-----------------|---------|
| CAD (n = 19) | NonCAD (n = 14) | P     |
| Age (years) | 55.3 ± 2.1      | 54.4 ± 3.2      | 0.82   |
| Female Sex n (%) | 5 (17.5%)      | 5 (35.7)        | 0.23   |
| Body mass index (kg/m²) | 26.8 ± 1.0      | 26.8 ± 1.2      | 0.98   |
| Total cholesterol | 242 ± 11        | 225 ± 8         | 0.281  |
| LDL-C | 164 ± 10        | 145 ± 8         | 0.134  |
| HDL-C | 30 (28; 36)     | 44 (36; 57)     | <0.0001|
| VLDL-C | 48 (27; 56)     | 31 (24; 46)     | 0.19   |
| Triglycerides | 240.0 (145; 302) | 156 (121; 231) | 0.068  |
| ApoA-I | 1.73 ± 0.084    | 2.14 ± 0.128    | 0.090  |
| ApoB | 1.52 ± 0.051    | 1.55 ± 0.051    | 0.760  |

Fig. 1. Decaying curves of the emulsion [14C]cholesterol oleate obtained from coronary artery disease (CAD, n = 19) and non-coronary artery disease (nonCAD, n = 14) subjects. The labeled emulsion was intravenously injected in a bolus and blood samples were drawn in pre-established intervals over 24 h for measurement of the radioactivity in a scintillation solution. Data expressed as mean ± SEM.
TABLE 2. Clinical characteristics, plasma lipids (mg/dl), apolipoproteins (g/l), and fractional catabolic rates (FCR, in min⁻¹) of the emulsion lipid labels in subjects with (CAD) or without coronary artery disease (NonCAD) paired by plasma triglyceride and LDL-C levels (values are means and standard error of the means)

<table>
<thead>
<tr>
<th></th>
<th>CAD (n = 9)</th>
<th>NonCAD (n = 10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>54.8 ± 10.4</td>
<td>53.0 ± 9.20</td>
<td>0.69</td>
</tr>
<tr>
<td>Female Sex n (%)</td>
<td>4 (36.6)</td>
<td>2 (22.2)</td>
<td>0.64</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2 ± 5.29</td>
<td>26.8 ± 1.21</td>
<td>1.0</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>206 ± 26</td>
<td>222 ± 35</td>
<td>0.27</td>
</tr>
<tr>
<td>LDL-C</td>
<td>135 ± 12</td>
<td>140 ± 29</td>
<td>0.86</td>
</tr>
<tr>
<td>HDL-C</td>
<td>30 (28; 33)</td>
<td>41 (35; 53)</td>
<td>0.007</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>38 (25; 52)</td>
<td>36 (28; 46)</td>
<td>0.97</td>
</tr>
<tr>
<td>ApoA1</td>
<td>1.66 ± 0.43</td>
<td>2.11 ± 0.39</td>
<td>0.022</td>
</tr>
<tr>
<td>ApoB</td>
<td>1.44 ± 0.23</td>
<td>1.56 ± 0.21</td>
<td>0.21</td>
</tr>
<tr>
<td>[14C]Cholesteryl oleate FCR</td>
<td>0.085 (0.065; 0.157)</td>
<td>0.07 (0.035; 0.091)</td>
<td>0.13</td>
</tr>
<tr>
<td>[3H]Cholesterol FCR</td>
<td>0.165 ± 0.017</td>
<td>0.08 ± 0.017</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 2 shows that there was a trend for increased esterification ratio in CAD when compared with nonCAD in all experimental points that was, however, statistically significant only at times 10 h and 24 h after emulsion injection (P = 0.029 and 0.024, respectively).

DISCUSSION

In this study, it was shown that while the removal from the plasma of LDE cholesterol esters is similar comparing CAD with nonCAD controls, the removal of LDE free-cholesterol is faster in CAD. In addition, the esterification of the emulsion cholesterol was greater in CAD subjects.

Relative to native LDL, LDE is more rapidly removed from the plasma (5). This is related to the differences in apolipoprotein profile on the surface of those particles.

TABLE 3. Emulsion [3H]cholesterol esterification ratio expressed as means and standard error of the mean in plasma ([3H]cholesterol/ [3H]cholesterol esters) in subjects with CAD or nonCAD

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>CAD (n = 19)</th>
<th>NonCAD (n = 14)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08</td>
<td>2.51 ± 0.17</td>
<td>2.82 ± 0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>1</td>
<td>1.97 ± 0.11</td>
<td>2.09 ± 0.17</td>
<td>0.55</td>
</tr>
<tr>
<td>4</td>
<td>1.57 ± 0.064</td>
<td>1.66 ± 0.094</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>1.26 ± 0.029</td>
<td>1.40 ± 0.06</td>
<td>0.029</td>
</tr>
<tr>
<td>24</td>
<td>1.15 ± 0.015</td>
<td>1.22 ± 0.028</td>
<td>0.024</td>
</tr>
</tbody>
</table>

ApoE that binds to LDE particle surface has been shown to possess several times greater affinity for the receptor than the unique LDL apolipoprotein, namely apoB-100 (23). In fact, when apoB-100 was incorporated to LDE before the injection into rats, the removal from the plasma of the emulsion was markedly slowed down (5). The fact that LDE is removed from the plasma faster than native LDL is a methodological advantage because it shortens the period required for performance of the plasma kinetics test.

The plasma concentration of LDL is determined by the balance between the lipoprotein production rate and the rates of removal from the plasma (14). LDL production comprises the hepatic synthesis of the precursor lipoprotein VLDL and VLDL catabolism with generation of LDL. Removal from the plasma and LDL uptake by the body tissues are mediated by cell membrane receptors that recognize apoB-100 (24). It is possible that groups of subjects with equal LDL-C concentration have different LDL removal rates compensated by different LDL production rates. This situation would be similar to that we recently described in groups of young, middle-aged, and elderly subjects (16). LDL-C concentration was not different in the three groups, but the capacity of removing LDE was negatively correlated with age. Similarly, in athletes and sedentary groups of subjects, LDE removal was greater in the athletes despite LDL-C concentration was similar (25).

It is valid to assume that in groups of subjects with smaller LDL removal, the lipoprotein is more prone to undergo deleterious changes such as peroxidation or increased oxidizability (26). We then speculated whether in CAD patients the removal of LDL as tested with the help of LDE would be diminished, regardless the plasma concentration of the lipoprotein. Nonetheless, the results of the plasma clearance of the emulsion cholesterol ester, indicating that the removal of the LDE particles is not different between CAD and nonCAD subjects, our hypothesis could not be supported. The emulsion cholesterol ester that makes up the emulsion core is conceivably the marker of the LDE particles removal from the plasma (2, 4). However, as in native LDL, the emulsion cholesterol ester moiety may partially shift to other lipoprotein classes by the action of the cholesterol transfer ester protein (27). It is worthwhile to point out that there are no studies in the literature exploring the plasma kinetics of native LDL in normolipidemic subjects with CAD.

In contrast with the cholesterol esters, unesterified cholesterol may freely diffuse from the emulsion to the plasma, and thus the shift from the emulsion particles does not depend on transfer proteins (28). In this study, the fact that the plasma decay curve of the emulsion [3H]cholesterol was faster than that of the [14C]cholesterol esters in all CAD and nonCAD subjects indicates that the cholesterol independently shifts from the emulsion to the plasma and is more rapidly removed than the emulsion particles.

Besides apoE, in contact with plasma LDE also gains apoCs and apoAs that are also small, exchangeable proteins (2, 3). ApoA-I binds to and stimulates LCAT, allow-
ing the esterification of the emulsion cholesterol (29). ApoA-I, the main apolipoprotein in the HDL fraction, is absent from the LDL fraction. Because it also acquires apoA-I (2, 3), LDE can probe the in vivo esterification of cholesterol occurring in the plasma. The plasma decay curves of the [3H]cholesterol was faster in the CAD than in the nonCAD subjects. Furthermore, the esterification ratio was greater in CAD than in nonCAD subjects at 10 h and 24 h after emulsion injection. This indicates first that the free cholesterol escapes from the emulsion and comes out of the plasma compartment more intensively in CAD. Second, that the esterification process suffered by the emulsion free cholesterol is more intense in CAD than in nonCAD.

In a recent study, LDE doubly labeled with [14C]cholesterol oleate and [3H]cholesterol was intravenously injected into CAD patients 24 h before the coronary bypass surgery. In the excised fragments of aorta, radial, and mammary arteries, the uptake of the emulsion [3H]cholesterol was pronouncedly greater than that of the [14C]cholesterol oleate, which suggests that, indeed, the free cholesterol dissociates from the emulsion particles and deposits in arterial wall (unpublished observations).

Regarding the increased plasma cholesterol esterification in the CAD patients observed in our study, this finding was also reported in previous studies by others (30–32). In those studies, the increased cholesterol esterification was evaluated in vitro by using radiolabeled HDL. Greater cholesterol esterification rates have been also associated with increased LDL-HDL ratio (33), increased log triglyceride/HDL-C ratio (34), high plasma triglyceride levels (34), and the presence of small size LDL (33), all linked with atherogenesis (35), CAD (32), and conditions that predispose to CAD such as diabetes mellitus (36–37) and arterial hypertension (31). The confirmation in vivo in CAD, patients of the existence of an increased plasma cholesterol esterification status constitutes a further validation of the emulsion approach to lipid intravascular metabolism and demonstrates the ability of those emulsions to signal abnormalities in this metabolism.

It is relevant that the [3H]cholesterol FCR was positively correlated with plasma triglycerides and with the log triglycerides-HDL ratio and negatively with HDL-C in the univariate analysis and with log plasma triglycerides in the multivariate analysis because all those parameters have been shown to correlate with the presence of CAD (38). Thus, the existence of those correlations with [3H]cholesterol FCR further strengthen the possibility that this emulsion kinetic parameter be a marker of the disease and perhaps reflects a contribution to the atherogenesis process. Nonetheless, the difference in [3H]cholesterol FCR between the two groups persisted even when the paired analysis for triglyceride and LDL-C levels was performed, which means that triglyceride levels were not determinant in this result.

In conclusion, the use of LDE as a probe to test the lipoprotein intravascular metabolism of CAD patients shows that important alterations may be present in the disease regarding the cholesterol component of lipoprotein particles. Both removal from the plasma and esterification of cholesterol are increased. These findings may contribute to unraveling of new proatherogenic mechanisms and also for the establishment of novel disease markers.21

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