Metabolic Behavior in Rats of a Nonprotein Microemulsion Resembling Low-Density Lipoprotein

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A protein-free microemulsion (LDE) with a lipid composition resembling that of low-density lipoprotein (LDL) was used in metabolic studies in rats to compare LDE with the native lipoprotein. LDE labeled with radioactive lipids was injected into the bloodstream of male Wistar rats, and plasma kinetics of the labeled lipids were followed on plasma samples collected at regular intervals for 12 h after injection. The 24-h LDE uptake by different tissues was also measured in tissue samples excised after the animals had been sacrificed. We found that LDE plasma kinetics were similar to those described for native LDL [fractional clearance rate (FCR) of cholesteryl ester, 0.42 ± 0.11 h^{-1}]. The major site for LDE uptake was the liver, and the tissue distribution of the LDE injected radioactivity was as one would expect for LDL. To test whether LDE was taken up by the specific LDL receptors, the LDE emulsion was injected into rats treated with 17α-ethinylestradiol, which is known to increase the activity of these receptors; as expected, removal of LDE from the bloodstream increased (FCR = 0.90 ± 0.35 h^{-1}). On the other hand, saturation of the receptors that remove remnants by prior infusion of massive amounts of lymph chylomicrons did not change LDE plasma kinetics. These results indicate that LDE is cleared from plasma by B,E receptors and not by the E receptors that remove remnants. Incorporation of free cholesterol into LDE increased LDE plasma clearance. Incubation studies also showed that LDE incorporates a variety of apolipoproteins, including apo E, a ligand for recognition of lipoproteins by specific receptors. Our data suggest that LDE can be a useful tool to test LDL metabolism and B,E receptor function.

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Low-density lipoprotein (LDL), which carries most of the plasma cholesterol in humans, can be considered a biological microemulsion consisting of a core of cholesteryl esters (45–50% of the particle weight) and residual triglycerides (3–4%) surrounded by a monolayer of phospholipids (16–25%) and unesterified cholesterol (5–8%). The protein moiety of LDL is apolipoprotein (apo) B-100.

LDL is the final product in the lipolysis of very low density lipoprotein (VLDL), a triglyceride-rich lipoprotein synthesized by the liver. VLDL triglycerides are progressively lost from the particles through the action of lipoprotein lipase on the endothelium of capillary vessels of peripheral tissues, such as muscle and adipose tissue. The enzyme is stimulated by a co-factor, apolipoprotein CII, present on the surface of VLDL particles. LDL is removed from plasma by the B,E receptors that recognize the receptor-binding domain of apo B-100. The lipoprotein is then internalized and LDL cholesterol is utilized in various cellular processes (1).

It is possible to model the metabolism of chylomicrons or VLDL with protein-free triglyceride-rich emulsions of defined lipid composition (2–5). In the bloodstream the emulsions acquire apolipoproteins from the circulating lipoproteins undergoing lipolysis. Subsequently, apo E serves as ligand for the binding of the triglyceride-depleted particles to hepatic receptors that take up remnants (E receptors).

In the present study, we tested the hypothesis whether a microemulsion resembling the lipid phase of LDL (6,7), without apo B, could mimic the metabolism of native LDL. The microemulsion (LDE) labeled with radioactive lipids was injected into the bloodstream of control rats to determine the plasma kinetics and the percent uptake of the emulsion by different organs. To confirm the role of the specified LDL receptors in removing LDE from plasma, the LDE plasma kinetics and tissue uptake were also followed in a group of rats treated with 17α-ethynilestradiol, which enhances the activity of these receptors. To test whether the remnant receptors could take up the emulsion, LDE was also injected into rats following infusion of lymph chylomicrons resulting in saturation of these receptors. The effects of hypothyroidism and of LDE supplementation with free cholesterol were also evaluated. Our data show that LDE can be useful to test *in vivo* LDL metabolism and B,E receptor function.

MATERIALS AND METHODS

Preparation of LDE. Egg phosphatidylcholine was purchased from Lipid Products (Surrey, United Kingdom), and triolein, cholesteryl oleate and cholesterol were from Nu-Chek-Prep (Elysian, MN). [4-^{14}C]Cholesteryl oleate, [1α,2α(n)-^{3}H]oleate were from Amersham (Amersham, United Kingdom). Lipids were judged >99% pure by thin-layer chromatography (TLC). The microemulsion utilized in this study was prepared from lipid mixtures composed of 40 mg of egg phosphatidylcholine, 20 mg of cholesteryl oleate and 0.6 mg of triolein, with addition of radioactive lipids. In some experiments, 5% (w/w) cholesterol was also added to the mixture.

The lipid mixtures were sonicated and purified to obtain the microemulsion according to the procedures described by Ginsburg *et al.* (5). Lipid mixtures were dried under an N_2 stream followed by overnight vacuum desiccation at 4°C to remove residual solvent. Dried lipids were resuspended in 10 mL of 0.1 M KCl, 0.01 M Tris HCl at pH 8.0. The suspension was sonicated using a Branson Cell Disruptor model B-30 (São Paulo, Brazil), with 125 Watts output in the "continuous" operating mode, for 180 min under an N_2 atmosphere. The temperature was kept between 53–55°C, as monitored by a thermocouple inserted into the vial. The emulsified lipid suspension was then transferred to clean tubes for ultracentrifugation at 195,000 × *g* (30 min) in an SW 40 rotor of a Beckman

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Abbreviations: AML, acute myeloid leukemia; LDE, microemulsion resembling low-density lipoprotein; LDL, low-density lipoprotein; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

L8-M ultracentrifuge (Palo Alto, CA). The top 10% of the solution, containing particles that float at a background density of approximately 1.006 g/mL, was removed by aspiration with a needle. The remaining solution was adjusted to a background density of approximately 1.22 g/mL by adding solid KBr and was then centrifuged at 195,000 × *g* (120 min) at 4°C. The top 20–30% of the sample was collected by aspiration at room temperature, and dialyzed overnight in buffer to remove KBr from the solution. The microemulsion fraction was analyzed for lipid composition (phospholipids, cholesteryl esters, triacylglycerols) by standard laboratory methods (8–10) and utilized in the experiments described below. Before analysis, the microemulsion containing cholesterol was also submitted to preparative TLC in the solvent system described below to allow separate determination of cholesterol and cholesteryl ester.

Animals. Male Wistar rats, weighing 250–300 g and fed a standard commercial chow (Anderson Clayton, São Paulo, Brazil) *ad libitum*, were utilized in this study. One group of rats was treated with 17α-ethinylestradiol for five days. The drug was dissolved in ethanol (100 μg/μL) and propyleneglycol to a final concentration of 1 mg/mL, and injected subcutaneously at a daily dose of 5 mg/kg of body weight (11). Another group of rats was rendered hypothyroid by addition of propylthiouracil to their drinking water (100 mg/dL) for 30 d (12).

LDE plasma kinetics and tissue uptake. Microemulsions labeled with radioactive core lipids were injected into the bloodstream of control rats and of rats treated with 17α-ethinylestradiol or propylthiouracil to measure the rates of lipid disappearance from plasma.

The animals were anesthetized with diethyl ether, and a polyethylene cannula (Intramedic PE 50) was inserted into the left carotid artery. Clotting inside the cannula was prevented by pretreatment with silicon (Clay Adams, Parsippany, NJ). The animals were kept in individual cages for at least 90 min for recovery from anesthesia. The microemulsion was then injected as a bolus of approximately 3 mg of total lipid, in a volume of approximately 0.5 mL. At intervals of 0.5 g, 1, 2, 4, 7, 9 and 12 h, when the animals were sacrificed by air embolization, blood samples of 0.3 mL were collected into tubes containing 20 μL heparin.

To measure the uptake of LDE by the tissues, the microemulsion was labeled with [1α,2α(n)-^{3}H]cholesteryl oleyl ether. Selected organs were excised 24 h after the injection of the emulsion into three control rats. Plasma samples for determination of emulsion clearance were not taken from these rats.

Determination of radioactivity in plasma and tissues. Lipids were extracted (13) with chloroform/methanol (2:1, vol/vol) from 100 μL aliquots of the separated blood plasma and from approximately 1 g samples of the excised organs. The extracted lipids were concentrated and resolved into classes by TLC using hexane/diethyl ether/acetic acid (70:30:1, by vol) as developing solvent. The cholesteryl ester and triacylglycerol bands were then placed separately into vials with 7 mL scintillation solution [5 g PPO/0.5 g dimethyl POPOP (Sigma, St. Louis, MO)/333 mL Triton X-100/667 mL toluene] (14); and radioactivity was measured by liquid scintillation spectrometry with a LKB model 1211 Spectrometer (Upsala, Sweden).

Competition between LDE and lymph chylomicrons for removal from plasma. Intestinal lymph was collected from male Wistar rats, weighing 300–400 g, over ice with ethylenediaminetetraacetic acid (EDTA) added (final concentration, 1 mM) over 24 h through a cannula implanted into the mesenteric lymph duct. After surgery the rats were maintained in restriction cages, and cottonseed oil was infused (0.035 mL/h) through a gastrostomy tube. Water was accessible *ad libitum.* Saline (5 mL, pH 7.0), d = 1.006 g/mL, containing EDTA (1 mM) was layered on lymph (5 mL) in Beckman SW 40 rotor tubes and centrifuged at 24,500 rpm for 20 min at 20°C (15). Chylomicrons were recovered from 1.5 mL of the creamy top layer aspirated from each tube, and the triglyceride concentration was determined (10). Samples were kept at 4°C and used within 24 h in the experiments.

Lymph chylomicrons were injected into the rats through a carotid cannula, in a bolus corresponding to 7.2 mg of triglyceride every 10 min for 5 h. The labeled microemulsion was injected through the same cannula 5 min after the first injection of lymph chylomicrons. LDE was also injected into three rats in which saline solution (0.8 mg/dL) was substituted for an equivalent volume of lymph chylomicrons (16).

Incubation of LDE with rat plasma high-density lipoprotein (HDL) and with rat plasma HDL apolipoproteins. Microemulsions containing approximately 280 μg of total lipid were incubated with rat HDL (230 μg of HDL protein) obtained by ultracentrifugation of plasma in discontinuous saline gradients (17). Buffer solution (0.01 M Tris-HCl, pH 8.2) was added to attain a 4.0 final volume in each tube. Incubation was for 15 min in a shaking water bath at 37°C. Solid KBr was then added to raise the density to 1.21 g/mL. The mixture was then placed in a Beckman SW41 rotor tube and centrifuged in discontinuous density gradients for 24 h at 20°C (17). The microemulsion particles were recovered in the 1.5 mL top volume of the tube and assayed for protein content (18). Apos associated with the microemulsions were separated by 15% sodium dodecyl sulfate (SDS)-glycerol polyacrylamide gel electrophoresis (19).

Microemulsions (200 μg) were also incubated with rat plasma HDL apo (200 μg), obtained by ultracentrifugation of plasma on discontinuous gradients (17) followed by delipidation of the HDL fraction (20). The incubation, recovery and analysis of the protein associated with LDE were according to the procedures described.

Calculation of LDE removal from plasma. A compartmental model was utilized for the analysis of plasma decaying curves of the emulsion's radioactive lipids (21). The model implies two intravascular (pools 1 and 2) and one extravascular (pool 3) compartment, assuming that the system was in dynamic equilibrium. The emulsion is instantaneously introduced in pool 1. During the elapsed time, a fraction of pool 1 (k_{13}) migrates to pool 3, while another fraction (k_{12}) is transferred to pool 2. A fraction of pool 2 (k_{23}) also migrates to pool 3. Rates of transfer k_{12}, k_{13} and k_{23} were estimated using nonlinear least squares procedures (22,23). Fractional clearance rate (FCR) of the labeled lipids from the intravascular compartment was estimated according to Matthews (24):

$$FCR = \frac{(k_{12} + k_{13}) \cdot k_{23}}{k_{12} + k_{23}} \quad [1]$$

The Student's *t*-test was used to analyze differences between means.

RESULTS

LDE prepared without cholesterol had approximately 63% phospholipids, 36% cholesteryl ester and 1% triacylglycerol, whereas LDE prepared with cholesterol consisted of approximately 61% phospholipids, 35% cholesteryl ester, 2% cholesterol and 3% triacylglycerol. The cholesteryl ester/phospholipid molar ratio was approximately 0.7 in both microemulsions.

When LDE was simultaneously labeled with [4-^{14}C]-cholesteryl oleate and [1α,2α(n)-^{3}H]cholesteryl oleyl ether and injected into four control rats, the removal rate for both labels was nearly identical (FCR = 0.40 ± 0.02 and 0.40 ± 0.04 min^{-1}, respectively). This indicates that practically no recirculation of the LDE cholesteryl ester occurred over 12 h (25). In the rat the cholesteryl ester or ether can indeed be considered the marker of removal of the emulsion from plasma because it is not selectively removed from the emulsion particles (26).

FCR of the LDE cholesteryl ester and triacylglycerols injected in the bloodstream of control, estrogen-treated and hypothyroid rats is shown in Table 1. In control rats, the emulsion triacylglycerols were removed from plasma three times faster than was cholesteryl ester. In the animals treated with estrogen, cholesteryl ester was removed two times faster than in controls, but the removal of triacylglycerols was not affected (Fig. 1). In contrast, treatment with propylthiouracil did not significantly change the removal of cholesteryl ester, but the FCR of triacylglycerols was twice as rapid. The uptake by organs of [^{3}H]cholesteryl ether of emulsions injected in three control rats is shown in Table 2. Most of the emulsion radioactivity was taken up by the liver.

In the rats that were infused lymph chylomicrons, the plasma triacylglycerols raised from 12 to 27 mg/dL 1 h after the beginning of the infusion to 106 mg/dL after 3 h, and 72 mg/dL after 5 h. Figure 2 shows that the clearance of the emulsion cholesteryl ester in these rats (n = 6) was not different from that measured in rats (n = 3) in which the chylomicron infusion was replaced by infusion of an equal volume of isotonic saline solution (0.44 ± 0.19 and 0.51 ± 0.17 h^{-1}, respectively). This suggests that LDE was not removed from the plasma by the mechanisms that remove chylomicron remnants.

When free cholesterol was added to LDE (Fig. 3), FCR of either cholesteryl ester (0.54 ± 0.15 h^{-1}) or triacylglycerols (3.23 ± 0.68 h^{-1}) of the emulsion injected in control rats was significantly increased as compared to LDE

TABLE 1

Effects of Estrogen Treatment and Hypothyroidism on the Removal of Microemulsion Lipids from Plasma[a]

Treatment	Removal from plasma (FCR, h^{-1})	
	Cholesteryl esters	Triacylglycerols
Controls (n = 15)	0.42 ± 0.09	1.40 ± 0.63
Estrogen-treated (n = 10)	0.90 ± 0.35[b]	1.62 ± 0.59
Hypothyroid (n = 7)	0.39 ± 0.09	2.76 ± 0.94[b]

[a]Micromulsions without free cholesterol, and labeled with [4-^{14}C]cholesteryl oleate and glycerol tri[10(n)-^{3}H]oleate were injected into the carotid artery of control rats and rats treated with 17α-ethinylestradiol (5 mg/kg body wt/d during 5 d) or propylthiouracyl (100 mg/dL in drinking water for 30 d). Blood was sampled at regular intervals during 12 h, and the fractional clearance rate (FCR) was calculated from the curves of radioactivity remaining in plasma according to a three-compartmental model. Results are means ± SD.

[b]$P < 0.05$ compared to control values.

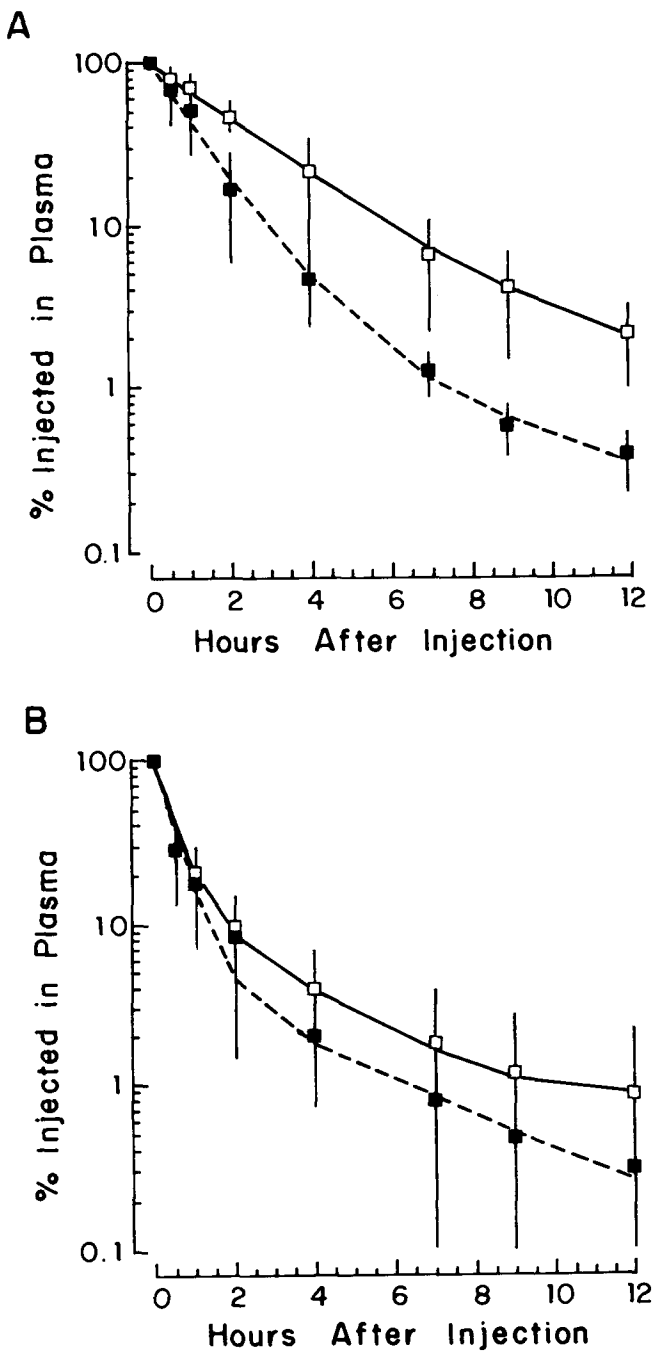

FIG. 1. Removal from plasma of [4-^{14}C]cholesteryl oleate (A) and glycerol tri[10(n)-^{3}H]oleate (B) of the microemulsion in control rats (□) and rats treated with 17α-ethinylestradiol (■).

TABLE 2

Tissue Uptake of the Microemulsion Labeled with [1,2(n)-^{3}H] Cholesteryl Oleyl Ether[a]

Tissue	% of Injected dose	% by Gram of tissue
Liver	83.79 ± 3.98	9.70 ± 1.44
Spleen	1.17 ± 0.25	1.65 ± 0.28
Lung	0.39 ± 0.09	1.73 ± 0.19
Muscle	3.39 ± 1.45	0.03 ± 0.02
Heart	0.11 ± 0.02	0.14 ± 0.03
Kidney	0.13 ± 0.00	0.07 ± 0.01
Adipose	0.74 ± 0.25	0.00 ± 0.00
Adrenal	0.62 ± 0.09	15.52 ± 3.81

[a]The microemulsion did not contain free cholesterol. Tissues were excised for lipid extraction and radioactivity determination 24 h after injection of the emulsion. The results are means ± SD of three experiments.

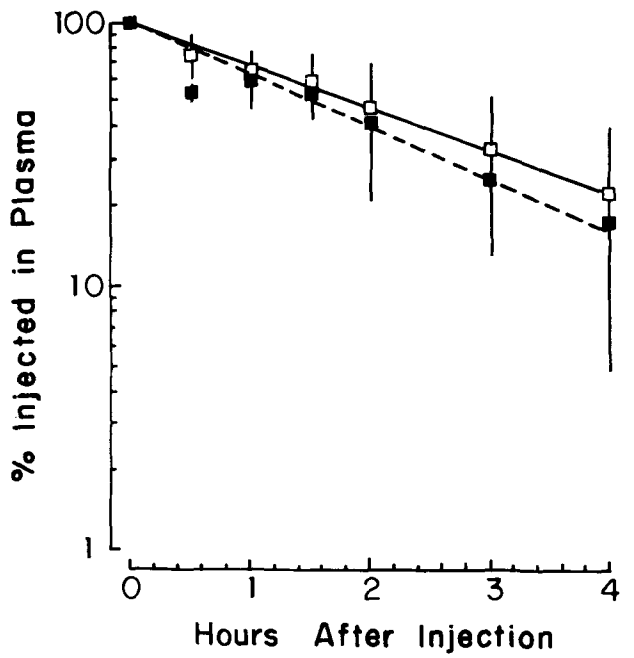

FIG. 2. Removal from plasma of [4-^{14}C]cholesteryl oleate of the microemulsion in rats infused with isotonic saline solution (□) or with lymph chylomicrons (■).

without free cholesterol ($P < 0.05$). FCR of the labeled free cholesterol of the emulsion was 0.92 ± 0.41 h^{-1}.

After incubation with HDL or HDL apo, LDE incorporated apos AI, AIV, E, CII and CIII. As observed in the SDS polyacrylamide gels, no difference was seen in the proportion of incorporated apos in LDE with and without cholesterol.

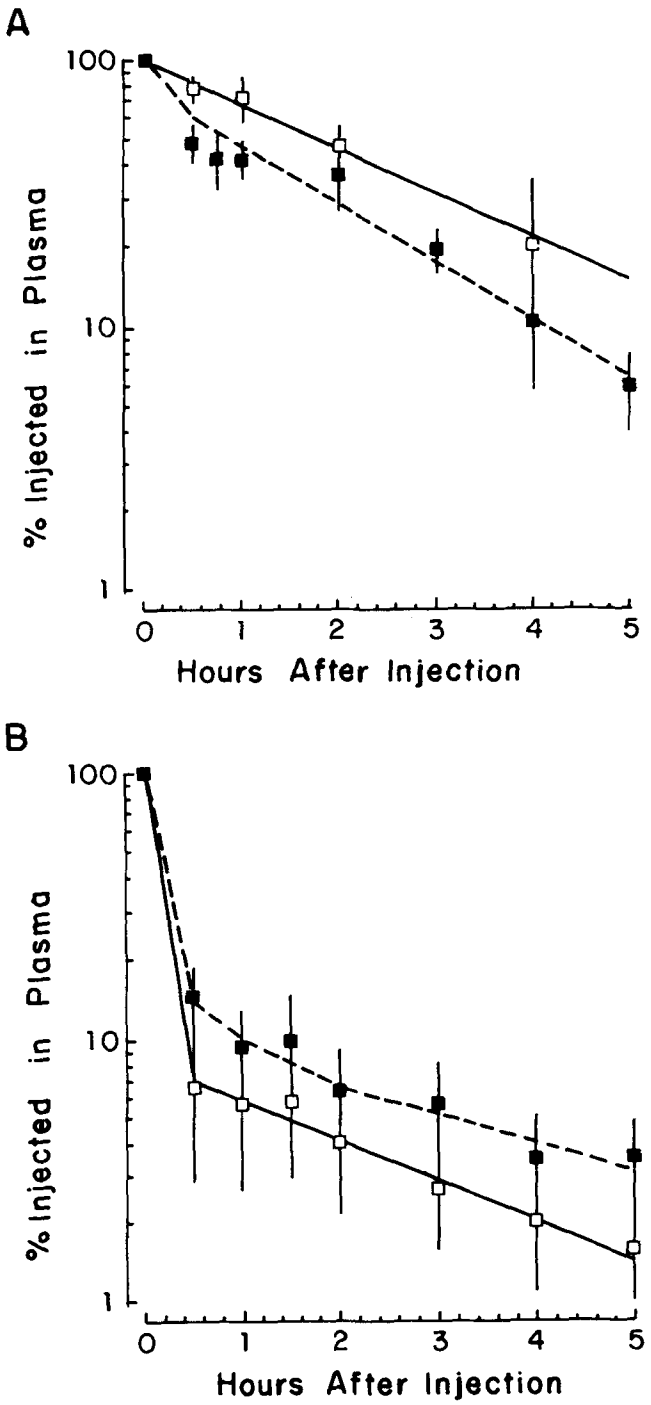

FIG. 3. Removal from plasma of (A) [4-^{14}C]cholesteryl oleate of the microemulsion with (■) or without cholesterol (□) and of (B) [^{14}C]cholesterol (□) and glycerol tri[10(n)-^{3}H]oleate (■) of the microemulsion with cholesterol.

DISCUSSION

The structure of protein-free microemulsions containing egg phosphatidylcholine and cholesteryl oleate, similar to those utilized in the current investigation, has been well-defined previously (5–7). These systems were shown to mimic the lipid portion of native LDL, and incorporation of unesterified cholesterol up to 15% molar did not affect their LDL-like size characteristics (7).

LDL interacts with B,E receptors through apo B-100, but the receptors can also recognize apo E, which is not usually present in the LDL particles. We hypothesized whether LDE, when injected into the bloodstream, could pick up apo E molecules from native lipoproteins, enabling recognition and uptake of the microemulsion by the B,E receptors. In fact, the incubation of LDE with HDL

or HDL apos confirmed that apo E binds the emulsion, together with other exchangeable apos like apos AI and CII and CIII.

The present results show that LDE has a markedly different metabolic behavior in rats as compared to the triglyceride-rich emulsions resembling chylomicrons described in our previous studies (2-4). Whereas the chylomicron-like emulsion was rapidly removed from plasma, LDE was retained for several hours, as native LDL would be. This emphasizes the importance of the lipid portion of these particles for their metabolic behavior, as both emulsions were capable of incorporating the various exchangeable apos present in the plasma. As occurs with native LDL, the liver was the main site of removal of LDE from the circulation. Evidence was gathered here that LDE was taken up by B,E receptors that remove native LDL, instead of by the E receptors that bind chylomicron remnants. Firstly, treatment of the rats with high doses of 17α-ethinylestradiol, which enhances the activity of the B,E receptors severalfold (27,28), resulted in nearly twofold increase in the rate of LDE removal from the plasma. This behavior is identical to that of native LDL injected in estrogen-treated rats (29-31), and different from chylomicron-like emulsions, which were slowly cleared in rats under estrogen treatment (2). Secondly, when LDE was injected in rats infused with amounts of lymph chylomicrons capable of saturating the removal sites of remnants (E receptors) (16), the plasma kinetics of the radioactive cholesteryl ester remained unchanged, suggesting that LDE is not removed by this mechanism.

LDE was also tested in hypothyroidism, a metabolic disorder in which lipoprotein plasma kinetics are well-documented in rats. The results again resembled those of native LDL and were markedly different from the kinetics of chylomicrons and triglyceride-rich emulsions. The plasma clearance of native LDL in hypothyroid rats has been found to be only slightly decreased (32), whereas in our experiments the LDE clearance was unchanged. In contrast, the removal of remnants of chylomicrons and triglyceride-rich emulsions was pronouncedly slowed down (33).

Despite the minimum content of triacylglycerols in LDE, the emulsion underwent lipolysis, as shown by a triacylglycerol FCR greater than that of cholesteryl ester (2,3). Lipolysis was greater in the rats made hypothyroid, probably due to increased lipoprotein lipase activity under this condition (12).

In the current study, we found that addition of free cholesterol to LDE led to acceleration of its removal from plasma. In protein-free lipid model systems for LDL, unesterified cholesterol is located primarily at the particle surface (7). This allows the conjecture that the introduction of cholesterol into LDE could favor the incorporation of apo E into the surface of the microemulsion particles, thus facilitating clearance. Moreover, apo C is known to decrease the binding of apos to receptors, and the addition of free cholesterol could also increase the ratio apo E/C, as previously documented with triacylglycerol-rich emulsions (3,34). However, after incubation with HDL or HDL apos, we found no difference between LDE with and without free cholesterol in regard to the proportion of associated apos.

Plasma kinetics of LDE radioactive, free cholesterol (Fig. 3) were similar to native LDL, as described by Eisenberg *et al.* (35). It is also noteworthy that triglyceride clearance was accelerated by addition of free cholesterol to LDE, which is probably due to enhanced removal of emulsion particles rather than to increased lipolysis.

Extensive studies of LDL plasma kinetics in human subjects could be very useful to understand the pathophysiology of lipid metabolism and atherosclerosis. However, these studies have been restricted by the laborious procedures of isolation and labeling of native LDL and the obligatory use of autologous lipoprotein due to the risk of transmission of HIV or hepatitis virus into recipient subjects. Need of a standard LDL preparation for injection into multiple individuals was already emphasized by Goldstein and Brown in a 1984 review (36) as a requirement for uniform kinetic studies. Our study raises the possibility for the use of LDE as a tool to evaluate LDL metabolism and B,E receptor function. Consistent with this assumption, the rate of removal of LDE from plasma has been shown to be slower in patients with familial hypercholesterolemia, as expected for native LDL (37). In contrast, LDE was rapidly removed from the plasma in patients with acute myeloid leukemia (AML) (38), which is expected because AML cells overexpress B,E receptors and avidly take up the native LDL (39,40). Finally, we would like to suggest that our results provide a rationale for the utilization of LDE to deliver antitumor drugs to neoplastic cells with enhanced expression of LDL receptors.

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