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Macrophages Take Up Triacylglycerol-Rich Emulsions at a Faster Rate Upon Co-Incubation With Native and Modified LDL: An Investigation on the Role of Natural Chylomicrons in Atherosclerosis

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Abstract Chylomicrons play a role in atherosclerosis, however, because the mechanisms involved in the cell uptake of these particles are not fully understood, investigations were carried out using a radioactively labeled protein-free triacylglycerol-rich emulsion incubated with peritoneal macrophages obtained from normal and apoE-knockout mice. Experiments were done in the presence of substances that inhibit several endocytic processes: EDTA for low density lipoprotein receptor, fucoidan for scavenger receptor, cytochalasin B for phagocytosis, and a lipopolysaccharide for lipoprotein lipase. In addition, triacylglycerol-rich emulsions were also prepared in the presence of native or modified radioactively labeled low density lipoprotein particles that are known to accumulate in the arterial intima. Probucol was also used to prevent the possible role played by an antioxidant in triacylglycerol-rich emulsion uptake. We have shown that triacylglycerol-rich emulsion alone is taken up by a coated-pit-dependent mechanism, mediated by macrophage secretion of apolipoprotein E. Furthermore, native, aggregated, acetylated, and moderately macrophage-oxidized low density lipoprotein stimulate the uptake of a triacylglycerol-rich emulsion through several mechanisms such as an actin-dependent pathway, scavenger receptors, and lipolysis mediated by lipoprotein lipase. On the other hand, in spite of the interaction of low density lipoprotein forms with a triacylglycerol-rich emulsion, the cellular triacylglycerol-rich emulsion uptake is impaired by copper-oxidized low density lipoprotein, possibly due to its diminished affinity towards lipoprotein lipase. We have also shown that macrophages take up aggregated low density lipoprotein better than the acetylated or oxidized forms of low density lipoprotein. J. Cell. Biochem. 84: 309–323, 2002.

Key words: triacylglycerol-rich emulsions; ethylenediaminetetraacetic acid; low density lipoprotein; fucoidan; cytochalasin B; lipopolysaccharide

Low density lipoprotein (LDL) is the major atherogenic lipoprotein [Kruth, 1997] as shown in genetic forms of hypercholesterolemia [Goldstein and Brown, 1982], epidemiological studies [Castelli et al., 1986], clinical drug trials in humans [Shepherd et al., 1995], and in

Abbreviations used: LDL, low density lipoprotein; NaLDL, native LDL; OxLDL, oxidized LDL; AgLDL, aggregated LDL; AcLDL, acetylated LDL; HDL, high density lipoprotein; VLDL, very low-density lipoprotein; TAG, triacylglycerol; EM, triacylglycerol-rich emulsion; EDTA, ethylenediaminetetraacetic acid; [^{3}H]-COE, [1α,2α (n)-^{3}H]-cholesteryl oleoyl ether; [^{14}C]-CO, [4-^{14}C]-cholesteryl oleate; apoE-KO mice, apolipoprotein E-deficient mice; TC, total cholesterol, LPL, lipoprotein lipase; SR-A, scavenger receptor A; LPS, lipopolysaccharide; FPLC, fast protein liquid chromatography; PMSF, phenylmethyl-sulfonyl fluoride; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances.

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experimental animals [Strong, 1976; Felgines et al., 1995]. Other atherogenic components include the lipid triad consisting of small LDL particles, low serum concentration of high density lipoprotein (HDL) cholesterol [Austin et al., 1990], and high triacylglycerol (TAG) levels. These are often associated with hypertension, insulin resistance, and a prothrombotic state [Grundy, 1998]. Nonetheless, the process of atherogenesis extends beyond the accumulation of lipids within the arterial wall and consists of highly specific cellular and molecular responses that can best be described as an inflammatory disease [Ross, 1999]. In this regard, in hypercholesterolemia, the intimal influx of monocyte-derived macrophages and T lymphocytes is preceded by extracellular deposition of amorphous and membranous lipids [Napoli et al., 1997]. Other causes of atherosclerosis include modifications of LDL, free radical production due to cigarette smoking, arterial hypertension, diabetes mellitus, high plasma homocysteine concentrations, coagulation factors, and microorganisms [Ross, 1999]. LDL modified by aggregation, acetylation, or oxidation resulting in the formation of foam cells is a major cause of injury to the arterial intima endothelium and to its underlying smooth-muscle cells and macrophages [Steinbrecher et al., 1984; Khoo et al., 1988; Kruth et al., 1995; Maor et al., 1997].

Several studies have also shown that TAG-rich lipoprotein levels in plasma are associated with the risk of coronary artery disease and atherosclerosis [Austin, 1991; Hokanson and Austin, 1996; Grundy, 1998]. Benlian et al. [1996] analyzed patients with familial hyperchylomicronemia caused by a defined mutation of the enzyme lipoprotein lipase (LPL) and concluded that there were signs and symptoms of premature atherosclerosis. According to a recent work, the presence of the enzyme LPL inhibits the development of diet-induced atherosclerosis, and this fact is unrelated to hyperlipidemia [Yagyu et al., 1999]. In hyperchylomicronemic patients, as well as in those lacking the apolipoprotein C-II (apoC-II) gene, a cofactor for the enzyme LPL, eruptive xanthomatosis, usually is present early in life [Benlian et al., 1996]. Experimental atherosclerosis caused by large TAG-rich particles seems to depend on their cholesterol content [Minnich and Zilversmit, 1989] and may be related to formation of eruptive xanthomata and premature atherosclerosis in humans. In this regard, human monocyte-macrophages increase their cholesterol content when incubated with chylomicron remnants [van Lenten et al., 1985].

Premature atherosclerosis occurs in familial primary combined hyperlipidemia, a genetic disease in which plasma cholesterol and TAG levels are elevated [Wijsman et al., 1998], and small very low density lipoproteins (VLDL) are enriched in cholesterol possibly due to a mutation of LPL [Yang et al., 1996].

TAG-rich chylomicrons and VLDL enter the arterial intima less easily than the much smaller atherogenic LDL and antiatherogenic HDL particles. On the other hand, chylomicrons are a major cholesterol carrier selectively retained early in atherosclerotic lesions [Mamo et al., 1998]. However, as liver cells, fibroblasts, and macrophages bind and internalize chylomicron remnants via the LDL receptor [Floren et al., 1981; Ellsworth et al., 1987; Chei et al., 1991; Gianturco and Bradley, 1999], as well as via a distinct receptor [Gianturco and Bradley, 1999], it is likely that LDL and chylomicrons either interact synergistically, or compete with each other for uptake by macrophages.

In vitro, native LDL does not elicit macrophage cholesteryl ester accumulation, although these cells and human monocyte-derived macrophages have high affinity LDL receptors. However, when incubated with modified LDL these cells accumulate cholesteryl ester. Recently, we have shown that mouse peritoneal macrophages take up more labeled cholesteryl ether from EM when co-incubated with native LDL or delipidated HDL (apoHDL) than when co-incubated with copper-oxidized LDL and HDL [Carvalho et al., 2000a]. All of these considerations indicate that the mechanisms involved in chylomicron uptake by macrophages remain largely unknown and may be influenced by other lipoproteins that prevail in the arterial intima.

In order to investigate these interactions, we have studied the uptake of EM alone or co-incubated with native and modified LDL by mouse peritoneal macrophages. In other words, instead of natural chylomicrons, protein-free EM particles previously utilized in our laboratory [Redgrave et al., 1993; Nakandakare et al., 1994; Oliveira et al., 1988; Carvalho et al., 2000a] and by others [Park et al., 2001; Saito

et al., 2001] were used here to investigate the influence of apolipoproteins belonging to other plasma lipoproteins or produced by the macrophage itself on the macrophage uptake of chylomicrons. To these experimental settings we added several substances whose inhibitory activities are shown in parenthesis: EDTA (LDL receptor activity) [Mamo et al., 1996], fucoidan (a scavenger receptor) [Mamo et al., 1996], cytochalasin B (phagocytosis) [Mamo et al., 1996], and lipopolysaccharide (lipoprotein lipase) [Hill et al., 1995]. In addition, the effect of fucoidan was also investigated when EM or native LDL was pretreated with probucol, an inhibitor of LDL oxidation [Parthasarathy et al., 1986].

MATERIALS AND METHODS

Materials

Cholesterol, cholesteryl oleate, and triolein were purchased from Nucheck Prep. (Elysian, MN); phosphatidylcholine (egg lecithin) was from Lipid Products (Surrey, UK), [1α,2α (n)-^{3}H]-cholesteryl oleoyl ether (specific activity 37 mCi/mmol) from Amersham Life Science (Buckinghamshire, UK), and [4-^{14}C]-cholesteryl oleate (specific activity 45–60 mCi/mmol) from New England Nuclear (Boston, MA). Isotope purity was checked by silica gel thin-layer chromatography (TLC) and shown to be higher than 95%. RPMI-1640 tissue culture medium and Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum, fucoidan, probucol, cytochalasin B, lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Petri dishes were obtained from Flow Laboratories (Mc Lean, Virginia), tissue culture 24-well plates from Becton Dickinson Labware (Lincoln Park, NJ), sterile 0.22 and 0.45 μm filters from Millipore Corp. (Belford, MA), and silica gel 60G for TLC and the enzymatic kit for determining TAG from Merck (Darmstadt, Germany). The enzyme kit for total cholesterol (TC) determination (CHOD-PAD Monotest) was bought from Boehringer–Mannheim (Mannheim, Germany). Mouse peritoneal macrophages were harvested from the peritoneal fluid of Swiss mice (University of São Paulo Medical School Animal House) and from C57Bl/6J apolipoprotein E (apoE)-deficient mice (Jackson Laboratory, Bar Harbor, ME).

Preparation of the Triacylglycerol-Rich Emulsion (EM)

The following components were utilized (% by weight): cholesterol (2%), cholesteryl oleate (6%), phosphatidylcholine (23%), and triolein (69%) dissolved in a chloroform/methanol solution (2:1, v/v), together with 30 μCi of the [1α,2α, (n)-^{3}H]-cholesteryl oleoyl ether ([^{3}H]-COE). The latter cannot be re-secreted by the cell, thus measuring the true particle uptake rate by cells [Glass et al., 1983]. The solvent was evaporated under a nitrogen stream and the lipids were maintained at 4°C for 12 h in a vacuum chamber to eliminate the residual solvent. The lipid mixture was sonicated in an NaCl solution ($d = 1.101$ g/ml, pH 7.3) using a Branson Cell Disrupter (model 450, Branson Sonifier, Danbury, CT), with a 1 cm probe, at 70–80 W for 30 min under a nitrogen flow. EM were purified by discontinuous gradient ultracentrifugation [Oliveira et al., 1988; Zerbinatti et al., 1991] and filtered through a 0.45 μm filter. Particle diameter was about 0.1 μm [Redgrave and Maranhão, 1985]. Emulsion TC and TAG were determined enzymatically using commercial kits.

Isolation and Modifications of the Plasma Lipoprotein Fractions

After approval by the Ethics Committee of the General Hospital, University of São Paulo Medical School, plasma was obtained from fasting normolipidemic blood donor volunteers after centrifugation at 1,000*g* and 4°C for 15 min. The following preservatives were added to the plasma: 2 mM benzamidine, 0.5% gentamicin plus 0.25% chloramphenicol, 0.5 mM PMSF, and 0.1 unit/ml aprotinin. LDL ($d = 1.006–1.063$ g/ml) and the infranatant ($d > 1.21$ g/ml) were isolated by sequential preparative ultracentrifugation at 100,000*g* and 4°C [Havel et al., 1955] using a 50 Ti rotor (L-8 ultracentrifuge, Beckman Instruments, Palo Alto, CA), and dialyzed at 4°C against PBS, pH 7.4 (0.9% NaCl, 0.3% Na_2HPO_4, 0.2% NaH_2PO_4, 0.38% NaOH and 0.01% EDTA). LDL and the infranatant were filter sterilized through a 0.22 μm filter. TC and TAG contents were determined enzymatically and the total protein concentration was determined by the method of Lowry et al. [1951]. LDL was labeled with [4-^{14}C]-cholesteryl oleate ([^{14}C]-CO) by the method of Gavish et al. [1987]. Labeling

efficiency was about 30–35% and more than 95% of the radioactivity was present as [^{14}C]-CO. Native LDL (NaLDL), labeled and unlabeled, was aggregated, acetylated, or copper-oxidized according to the following procedures: (1) vortexing aggregation for 30 s (AgLDL) [Khoo et al., 1988]; (2) acetylation (AcLDL) as described by Basu et al. [1976], with repeated additions of acetic anhydride. The extent of LDL acetylation was confirmed by electrophoresis on agarose gel in barbital buffer, pH 8.4; (3) copper-oxidation (OxLDL) upon overnight dialysis against PBS without EDTA, followed by incubation with 1 mM $CuSO_4$ for 8 h at 37°C by the method of Heinecke et al. [1984]. Oxidation was terminated by the addition of 1 mM EDTA. The extent of LDL oxidation was determined as malondialdehyde (MDA) equivalents using the TBARS assay in the range of 30–40 nmol MDA/mg lipoprotein protein [Puhl et al., 1986]. AcLDL and OxLDL preparations were sterilized by filtration through a 0.22 μm filter. Their TC, TAG, and protein contents were measured as described [Lowry et al., 1951].

Culture of Mouse Peritoneal Macrophages

Resident mouse peritoneal exudate cells were harvested in PBS (0.8% NaCl, 0.06% Na_2HPO_4, 0.02% KCl and 0.04% KH_2PO_4), pH 7.4. Pelleted cells obtained after centrifugation at 500*g*, 4°C for 30 min had their final concentration adjusted in RPMI 1640 medium containing 10% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 IU/ml), and fungizone (2.5 μg/ml) [Sparrow et al., 1989; Harada et al., 1998]. Macrophages were cultured either on Petri dishes (5×10^6 peritoneal exudate cells in 4 ml), or on 24 well-tissue culture plates (1.5×10^6 peritoneal exudate in 0.5 ml) and incubated in a humid 5% CO_2 atmosphere at 37°C for 12 h. Thereafter, each Petri dish or well was washed twice with RPMI 1640 medium to remove non-adherent cells [Hara and Yokoyama, 1991]. Proper control Petri dishes or wells were set up either containing macrophages alone or with [^{3}H]-COE-EM added, or else with NaLDL, AgLDL, AcLDL or OxLDL added, under the conditions described above.

Influence of LP Inhibitors on the Macrophage Uptake of [^{3}H]-COE-EM

Adherent cells were then incubated for 4 h at 37°C in a humid 5% CO_2 atmosphere in DMEM containing [^{3}H]-COE-EM (TC = 7.5 μg/ml and TAG = 450 μg/ml) in the presence or absence of: (1) 20 mM EDTA [Mamo et al., 1996]; (2) fucoidan (50 μg/ml) [Mamo et al., 1996]; (3) cytochalasin B (10 μg/ml) [Mamo et al., 1996]; or (4) LPS (500 ng/ml) [Hill et al., 1995]. In addition, [^{3}H]-COE-EM pretreated with probucol (5 μM) for 24 h [Parthasarathy et al., 1986] was incubated in the presence or absence of fucoidan. As a control, [^{3}H]-COE-EM was incubated with macrophages in DMEM in all experiments.

Native or Modified LDL Protein Co-Incubated With TAG-Rich Particles Co-Elutes Upon Fast Protein Liquid Chromatography Separation (FPLC)

Native (Na), aggregated (Ag), acetylated (Ac), or oxidized (Ox) LDL protein (0.6 mg/ml) preparations were incubated at 37°C for 4 h in PBS, with or without EM particles (TAG = 1.5 mg/ml) and were next separated through a mono-Q column in an FPLC system (Pharmacia, Uppsala, Sweden) with an LCC-500 programmer used to control two P-500 pumps. Two solutions were used for elution: A, 0.01 M Tris-HCl, pH 7.4, and B, 1 M NaCl in buffer A [Vedie et al., 1991]. The sample was filtered through a 0.45 μm filter and introduced via a 0.5 ml-loop into an anion-exchange column (mono-Q HR 5/5) and eluted at a flow rate of 1 ml/min by a linear gradient of 5–42% buffer B during the first 47 min, followed by a 42–100% gradient of buffer B, from 47 to 52 min; 100% buffer B, from 52 to 53 min; and at 53–55 min, 0%. The effluent was monitored with a single-path ultraviolet monitor at 280 nm and 1.5 ml fractions were collected with a Frac-200 fraction collector. Buffers were degassed before use and when necessary the system was operated at room temperature. The total protein concentration in the fractions was determined by the method of Lowry et al. [1951].

Uptake by Macrophages of [^{3}H]-COE-EM Together With Increasing NaLDL, AgLDL, AcLDL, or OxLDL Total Cholesterol Concentrations

Adherent cells drawn from different pools of donor mice on different occasions were incubated under the conditions described above with [^{3}H]-COE-EM together with increasing concentration of TC belonging to NaLDL, AgLDL, AcLDL, or OxLDL relative to the TC

concentration of the emulsion, that is, [1 ×], five-fold [5 ×] or ten-fold [10 ×]. This procedure was required in order to standardize the ideal LDL-TC concentrations used in the ensuing experiments. As a control, [^{3}H]-COE-EM was incubated with macrophages in DMEM alone.

Influence of LP Inhibitors on the Macrophage Uptake of [^{3}H]-COE-EM Co-Incubated With NaLDL, AgLDL, AcLDL, or OxLDL

Adherent cells were incubated under the conditions described above with [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL, AcLDL, or OxLDL, in the presence or absence of: 1) EDTA (20 mM); 2) fucoidan (50 μg/ml); 3) cytochalasin B (10 μg/ml); or 4) LPS (500 ng/ml). In addition, NaLDL pretreated for 24 h at 37°C with probucol (5 μM) was incubated in the presence or absence of fucoidan. As a control, [^{3}H]-COE-EM alone was incubated with macrophages in DMEM.

Uptake by Macrophages of [^{3}H]-COE-EM Together With [^{14}C]-CO-LDL

Adherent cells were incubated under the conditions described above, with [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL, AcLDL, or OxLDL labeled with [^{14}C]-CO. As a control, incubations were carried out with [^{3}H]-COE-EM alone, or with [^{14}C]-CO-NaLDL, -AgLDL, -AcLDL, or -OxLDL alone.

Measurement of Cell-Associated Radioactivity

After incubation, the cells were washed in PBS and solubilized in 0.2 M NaOH in order to measure cell-associated radioactivity with a beta scintillation counter Beckman LS-6000 TA (Beckman Instruments, Palo Alto, CA), with the uptake data being expressed for cell protein content [Carvalho et al., 2000a,b]. Percent cell [^{3}H]-COE-EM uptake was calculated as [(radioactivity in plaques with cells)−(radioactivity in plaques without cells)]/total radioactivity added to the plates × 100 × cell protein mg^{-1} (Equation 1), or as an uptake index: [(percent cellular [^{3}H]-COE-EM uptake in the presence/in the absence of lipoprotein)−1] (Equation 2).

Statistical Analysis

Results were expressed as medians and percentiles (25–75). The Kruskal-Wallis test with the Student Newman-Keuls contrast post-test was used for statistical comparison among groups. The Mann–Whitney test was used for statistical comparison between two groups. A 0.05 confidence level was considered significant.

RESULTS

The mechanisms involved in the uptake of EM by macrophages were investigated by incubating [^{3}H]-COE-EM in the presence or absence of several components that inhibit specific cell functions (written in parenthesis), as follows: EDTA (LDL receptor) [Mamo et al., 1996], fucoidan (scavenger receptor) [Mamo et al., 1996], cytochalasin B (phagocytosis) [Mamo et al., 1996], or LPS (lipoprotein lipase) [Hill et al., 1995]. As shown in Table I, except for the EDTA group of data, percent uptake of [^{3}H]-COE-EM was similar to that of the respective controls. Thus, the uptake of EM by macrophages depends in part on the LDL receptor. Because of this EDTA effect, the influence of apoE production on the uptake of [^{3}H]-COE-EM was analyzed using macrophages from normal and from apoE-knockout mice (apoE-KO) incubated with [^{3}H]-COE-EM (Table II). The latter was taken up by macrophages from normal mice

TABLE I. Macrophage Percent Uptake of the [^{3}H]-COE Labeled Triacylglycerol-Rich Emulsion (EM) Over a Period of 4 h ($n = 8$)

			EM	
Inhibitors	Concentration	To inhibit	Without inhibitors	With inhibitors
EDTA	20 mM	LDL receptor	4.70[a] 4.05/4.90	1.95 1.65/2.20
Cytochalasin B	10 μg/ml	Phagocytosis	6.10 4.90/7.00	5.95 5.45/6.30
Fucoidan	50 μg/ml	Scavenger receptor	4.70 4.05/4.90	4.00 3.75/5.05
Lipopolysaccharide	500 ng/ml	Lipoprotein lipase	3.10 2.45/3.75	3.10 2.85/3.30

Mann-Whitney test with $P < 0.05$.
[a]EM vs. EM + EDTA.

TABLE II. Percent Uptake Over a Period of 4 h of the [^{3}H]-COE Labeled Triacylglycerol-Rich Emulsion (EM) by Macrophages From Normal and apoE Deficient Mice (apoE-KO)*

Mice	EM	EM + EDTA
Normal	1.98[a,b]	0.42
	1.64/2.10	0.22/0.52
ApoE-KO	1.02[b]	0.18
	0.92/1.23	0.14/0.24

*20 mM EDTA was added to hinder the LDL receptor ($n = 8$). Kruskal–Wallis test with the Student Newman–Keuls contrast post-test ($P < 0.05$).
[a]ApoE-KO vs. Normal.
[b]Normal vs. Normal + EDTA, and ApoE-KO vs. ApoE-KO + EDTA.

more efficiently than by macrophages from apoE-KO mice. Thus, apoE produced by macrophages facilitates the uptake of EM alone. EDTA also reduced EM uptake by apoE-KO mouse macrophages.

Because of the influence of macrophage-produced apoE on cellular EM uptake and considering that LDL is the most important atherogenic particle present in the intima, we tested the possibility that LDL particles interact with EM and modify its uptake rate by the cell. Therefore the interaction of EM with NaLDL, AgLDL, AcLDL, or OxLDL was measured by chromatographic analysis (Fig. 1) in which EM, NaLDL, AgLDL, AcLDL, or OxLDL alone (control), or EM co-incubated with these LDL particles were analyzed. After co-incubation with EM particles the LDL protein content of the preparation decreased by 48% and by 39% in the NaLDL and AgLDL corresponding elution regions, respectively, with the rest being bound to the EM particles that eluted at the end of the analysis. The highest peak observed in the NaLDL control separation ($t_{ret} = 24$ min) represented 25% of total protein and only 7% after incubation with EM. The highest peak observed in the AgLDL control separation ($t_{ret} = 24$ min) represented 17% of total protein and only 1.7% after incubation with EM. Similar incubations were carried out with AcLDL and OxLDL that bound almost completely to EM, 100% and 91%, respectively, displacing the EM to the positions corresponding to these LDL. The highest peak observed in the AcLDL control separation ($t_{ret} = 49$ min) represented 67% of total protein and 72% after incubation with EM. The highest peak observed in the OxLDL control separation ($t_{ret} = 48$ min) represented 31% of total protein and 22% after incubation with EM. These results show that EM binds to native and modified LDL.

Since many cell receptors internalize modified lipoproteins, we studied the effect of increasing concentrations of TC within modified LDL preparations relative to the TC concentration in the emulsion on the uptake rate of chylomicron-like TAG-rich emulsion particles (EM) labeled with [^{3}H]-COE by macrophages (Fig. 2). Freshly collected cells drawn from mice on different days were used in each experimental set. Cell uptake rates were significantly different for all modified LDL groups when LDL-TC concentration was five times higher than EM-TC concentration. Moreover, at all tenfold LDL-TC concentration sets, [^{3}H]-COE-EM uptake rates were not greater than those observed at fivefold LDL-TC concentrations. Therefore, the fivefold LDL-TC concentration was used in all subsequent experiments.

Confirming its partial occurrence through the LDL receptor, the uptake rate by macrophages of the [^{3}H]-COE-EM co-incubated with NaLDL in the presence of EDTA was lower than that in the absence of EDTA (Fig. 3).

The uptake rate of [^{3}H]-COE-EM co-incubated with OxLDL by macrophages was negative and significantly different from the positive rates obtained with co-incubation with NaLDL, AgLDL or AcLDL, that did not differ from each other (Fig. 4). Thus, except for OxLDL, that inhibited the [^{3}H]-COE-EM uptake, the other co-incubations favored the uptake, indicating that the macrophage receptors for native, aggregated, and acetylated LDL are involved.

The importance of the scavenger receptor in the uptake of [^{3}H]-COE-EM co-incubated with LDL was analyzed by using fucoidan (Fig. 5). In its presence, the uptake rate of [^{3}H]-COE-EM was inhibited to the same extent as when the compound was co-incubated with NaLDL or AcLDL, although fucoidan did not alter the negative uptake rate seen with OxLDL. This set of experiments showed that the scavenger receptor is an important pathway for EM uptake in the presence of LDL. However, considering that this receptor plays a role in the uptake of modified LDL, it may be asked whether macrophages could have modified LDL by an oxidative process. Thus, probucol was used to inhibit oxidation [Parthasarathy et al., 1986]. The percent uptake of [^{3}H]-COE-EM co-incubated with NaLDL decreased significantly in the presence of fucoidan, probucol,

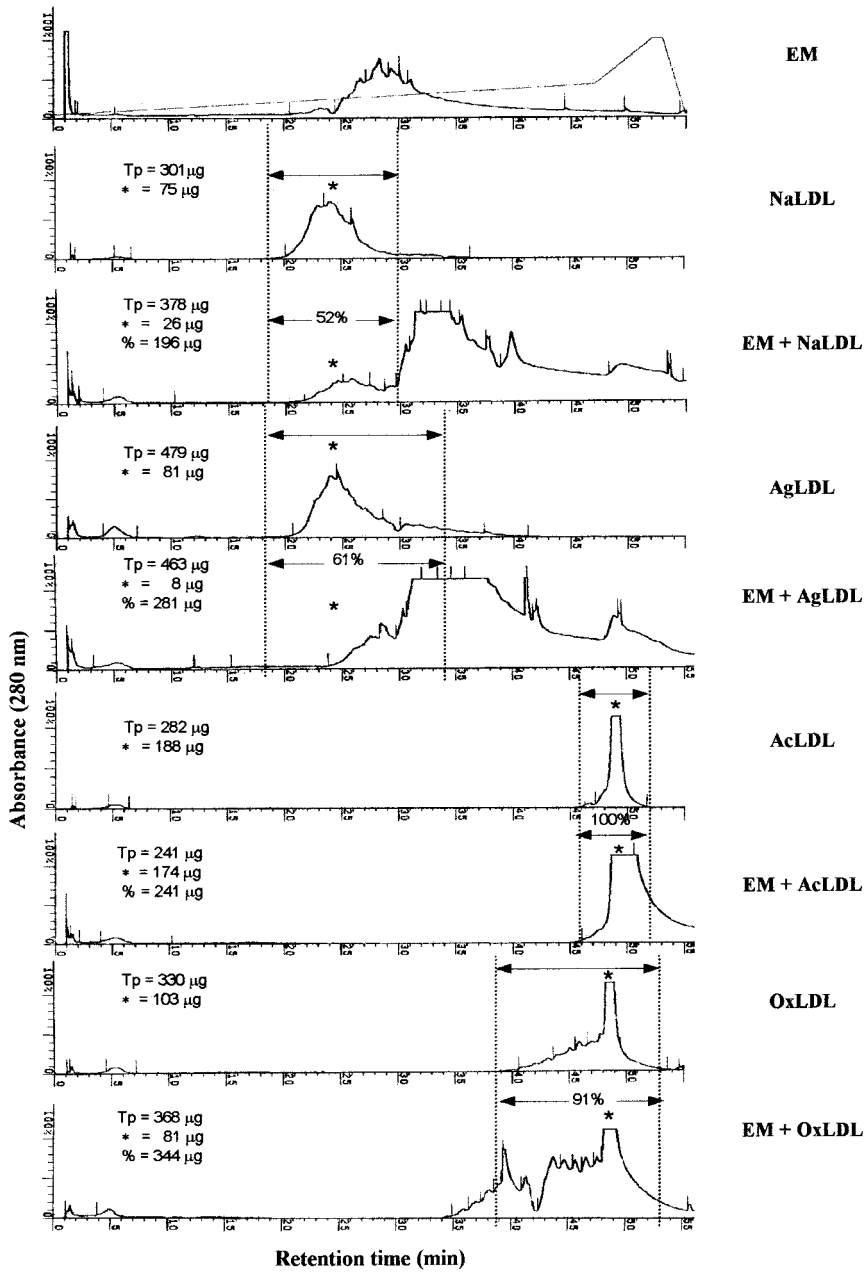

Fig. 1. FPLC separation of protein-free EM (1.5 mg/ml TAG and 0.12 mg/ml TC) and LDL (0.6 mg/ml protein and 0.6 mg/ml TC, corresponding to the horizontal arrow). EM and native (Na), aggregated (Ag), acetylated (Ac), or copper oxidized (Ox) LDL were incubated alone (control) or co-incubated as described in Materials and Methods. The chromatograms represent total protein (Tp in µg) belonging to LDL and protein concentrations (µg) corresponding to the largest chromatogram peak (*). Percent and protein concentration values correspond to the total area of retention of LDL alone (controls) as compared to those found in the co-incubations.

and probucol plus fucoidan (Table III). Consequently, NaLDL must have been oxidized by macrophages, thereby facilitating [^{3}H]-COE-EM uptake by the scavenger receptor. This conclusion led us to ask whether the oxidative process might have been involved in the uptake of [^{3}H]-COE-EM alone (Table IV). [^{3}H]-COE-EM was then incubated in the presence of fucoidan, probucol, and fucoidan plus probucol. The percent uptake of [^{3}H]-COE-EM in all of these experimental settings was similar to that observed in the control group, leading us to conclude that no oxidation of EM alone occurred.

Since, phagocytosis might also be involved in this uptake process, we then analyzed the

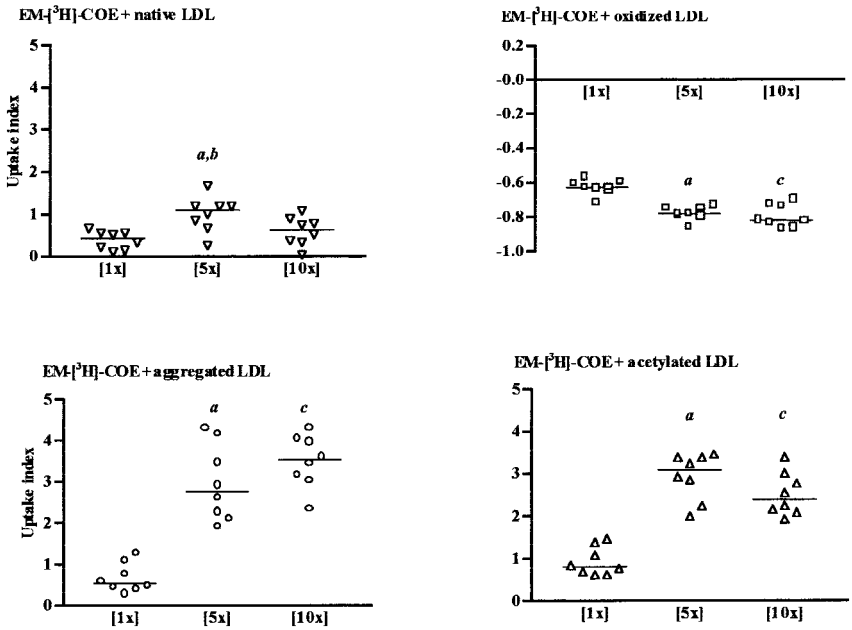

Fig. 2. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion together with increasing concentrations of total cholesterol (TC) of native (Na), oxidized (Ox), aggregated (Ag), or acetylated (Ac) LDL relative to the TC of the [^{3}H]-COE-EM. The horizontal bar represents the median value ($n = 8$). Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$). (**a**) EM + LDL [5 ×] vs. EM + LDL [1 ×]; (**b**) EM + LDL [5 ×] vs. EM + LDL [10 ×]; (**c**) EM + LDL [10 ×] vs. EM + LDL [1 ×].

influence of cytochalasin B. As shown in Figure 6, except for OxLDL, cytochalasin B impaired the uptake of [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL or AcLDL. Nonetheless, in the presence of OxLDL, either with or without cytochalasin B, the uptake rate was negative. These results indicate that EM co-incubated with NaLDL, AgLDL or AcLDL, but not with OxLDL, is taken up by means of a phagocytic mechanism.

Macrophage lipoprotein lipase might also have influenced the uptake of the EM since it contains 69% TAG. The uptake of [^{3}H]-COE-EM co-incubated with NaLDL and with all modified LDL preparations was then investigated in the presence of LPS, a well-known inhibitor of that enzyme (Fig. 7). LPS brought about a significant decrease in the uptake of [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL, or AcLDL. Again, the uptake values of [^{3}H]-COE-EM

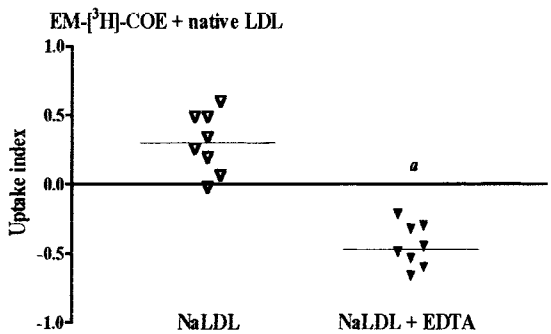

Fig. 3. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion co-incubated with native LDL (NaLDL) in the presence or absence of 20 mM EDTA to hinder the LDL receptor. The horizontal bar represents the median value ($n = 8$). Mann-Whitney test with $P < 0.05$. (**a**) EM + NaLDL vs. EM + NaLDL + EDTA.

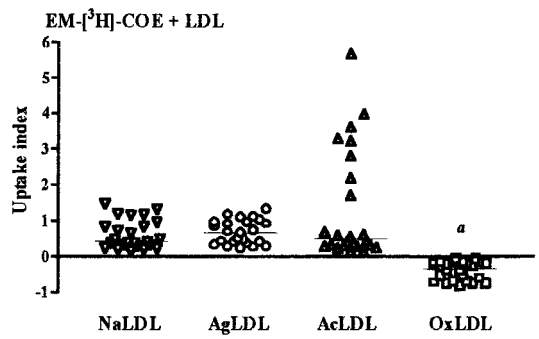

Fig. 4. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion co-incubated with native LDL (NaLDL), aggregated LDL (AgLDL), acetylated LDL (AcLDL), or oxidized LDL (OxLDL). The horizontal bar represents the median value ($n = 24$). Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$). (**a**) EM + OxLDL vs. EM + NaLDL, EM + AgLDL or EM + AcLDL.

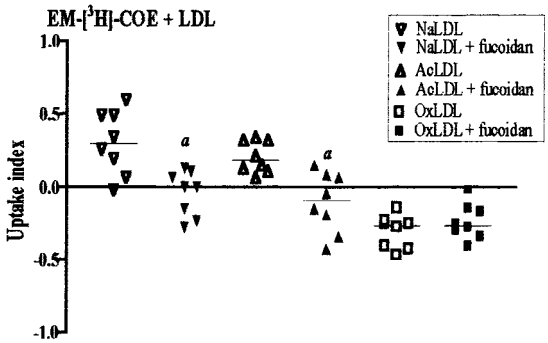

Fig. 5. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion co-incubated with native LDL (NaLDL), acetylated LDL (AcLDL), or oxidized LDL (OxLDL) in the presence or absence of fucoidan (50 mg/ml), a scavenger receptor inhibitor. The horizontal bar represents the median value ($n = 8$). Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$). (**a**) EM + LDL vs. EM + LDL + fucoidan.

co-incubated with OxLDL were not modified by LPS. Therefore, lipoprotein lipase plays a role in the uptake of the EM co-incubated with NaLDL, AgLDL, or AcLDL.

In order to investigate whether the inverse situation might also have taken place, namely, EM influencing LDL cell uptake, LDL labeled with [^{14}C]-CO was co-incubated with [^{3}H]-COE-EM (Table V). A significant decrease was then seen in the percent uptake of [^{14}C]-CO when NaLDL and AgLDL were co-incubated with [^{3}H]-COE-EM as compared with NaLDL or AgLDL alone. On the other hand, the percent uptake values of [^{14}C]-CO-AcLDL or [^{14}C]-CO-OxLDL alone or co-incubated with [^{3}H]-COE-EM were similar. As compared to [^{3}H]-COE-EM

TABLE III. Macrophage Percent Uptake Over a Period of 4 h of the [^{3}H]-COE Labeled Triacylglycerol-Rich Emulsion (EM) Co-Incubated With Native LDL (NaLDL) With or Without Fucoidan (50 μg/ml), a Scavenger Receptor Inhibitor ($n = 8$)

EM + NaLDL	Without fucoidan	With fucoidan
Alone	3.97[a]	2.86
	3.19/4.31	2.48/3.81
With probucol*	2.32	2.34
	1.96/2.55	1.66/4.49

Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$).
*Pre-treatment for 24 h with 5 μM probucol, an inhibitor of LDL oxidation.
[a]EM + NaLDL alone vs. EM + NaLDL alone + fucoidan; EM + NaLDL with probucol; and EM + NaLDL with probucol + fucoidan.

TABLE IV. Macrophage Percent Uptake Over a Period of 4 h of the Triacylglycerol-Rich Emulsion (EM) Labeled [^{3}H]-COE, With or Without Fucoidan (50 μg/ml), a Scavenger Receptor Inhibitor ($n = 8$)

EM	Without fucoidan	With fucoidan
Alone	1.23	1.07
	1.20/1.50	0.82/1.30
With probucol*	1.22	1.07
	1.14/1.54	1.03/1.17

Kruskal-Wallis test: not significant.
*Pre-treatment for 24 h with 5 μM probucol, an inhibitor of LDL oxidation.

alone, a significant increase was found in its percent uptake when co-incubated with NaLDL, AgLDL, or AcLDL. Nevertheless, there was inhibition of the uptake of [^{3}H]-COE-EM co-incubated with OxLDL. On the other hand, [^{14}C]-CO uptake as AcLDL or OxLDL was greater than as NaLDL. Therefore, macrophages take up EM using receptors that recognize native, aggregated, and acetylated LDL. Moreover, the percent uptake of [^{14}C]-CO as AgLDL was greater than as NaLDL, AcLDL, or OxLDL.

DISCUSSION

TAG plays a controversial role in atherosclerosis [Grundy, 1998], and several mechanisms are potentially involved. Macrophages take up large amounts of lipids using two general mechanisms that may be involved in atherosclerosis [Kruth, 1997] and in the

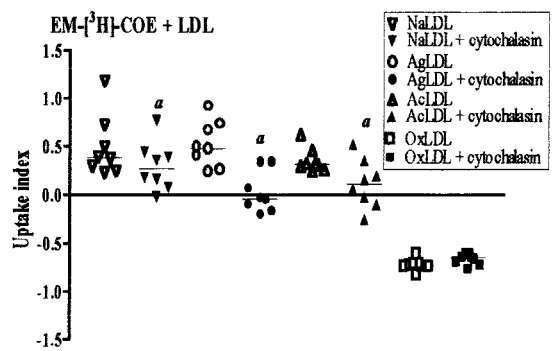

Fig. 6. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion co-incubated with native LDL (NaLDL), aggregated LDL (AgLDL), acetylated LDL (AcLDL), or oxidized LDL (OxLDL) in the presence or absence of cytochalasin (10 μg/ml), an inhibitor of phagocytosis. The horizontal bar represents the median value ($n = 8$). Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$). (**a**) EM + LDL vs. EM + LDL + cytochalasin.

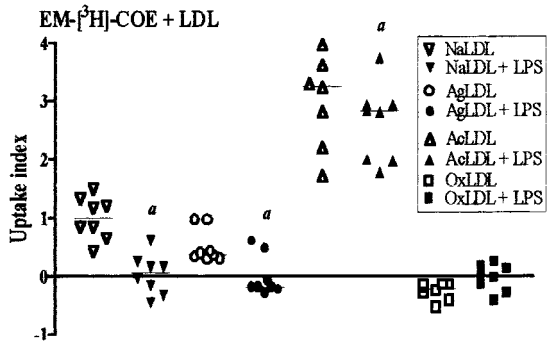

Fig. 7. Macrophage uptake index over a period of 4 h of the [^{3}H]-COE labeled triacylglycerol-rich emulsion co-incubated with native LDL (NaLDL), aggregated LDL (AgLDL), acetylated LDL (AcLDL), or oxidized LDL (OxLDL) in the presence or absence of LPS (500 ng/ml), a lipoprotein lipase inhibitor. The horizontal bar represents the median value ($n = 8$). Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$). (**a**) EM + LDL vs. EM + LDL + LPS.

formation of eruptive xanthomata of primary hyperchylomicronemia [Benlian et al., 1996]: (1) receptor-mediated endocytosis of plasma lipoproteins, either in solution or forming complexes with other tissue constituents [Brown and Goldstein, 1983], or (2) phagocytosis of whole cells, fragments of membranes containing cholesterol, or as aggregated LDL [Khoo et al., 1988]. Chylomicron remnants may be atherogenic due to their pro-inflammatory and lipid-loading inducing effect on macrophages [Mamo et al., 1997].

TABLE V. Macrophage Percent Uptake Over a Period of 4 h of the [^{3}H]-COE Labeled Triacylglycerol-Rich Emulsion (EM) Co-Incubated With Modified LDL Labeled With [^{14}C]-CO ($n = 8$)

	[^{14}C]-CO-LDL	[^{3}H]-COE-EM
EM	—	2.25
	—	2.10/2.30
NaLDL	13.10[a]	—
	11.75/13.89	—
EM + NaLDL	10.25	3.00[e]
	9.50/11.00	2.70/3.20
AgLDL	32.50[a,b]	—
	29.40/36.40	—
EM + AgLDL	27.95	4.60[e]
	24.70/29.15	4.25/4.85
AcLDL	22.65[c]	—
	20.85/25.40	—
EM + AcLDL	22.20	3.15[e]
	20.40/23.60	2.75/3.55
OxLDL	21.90[d]	—
	17.70/25.70	—
EM + OxLDL	20.65	1.80[e]
	17.80/22.25	1.60/2.00

Kruskal-Wallis test with the Student Newman-Keuls contrast post-test ($P < 0.05$).
[a]NaLDL vs. EM + NaLDL, and AgLDL vs. EM + AgLDL.
[b]AgLDL vs. NaLDL, AcLDL, or OxLDL.
[c]AcLDLac vs. NaLDL.
[d]OxLDL vs. NaLDL.
[e]EM vs. EM + LDL.

In the presence of EDTA, a metal chelator known to interact with the LDL receptor [Mamo et al., 1996; Verhoeye et al., 1996], the uptake of EM alone decreased significantly (Table I). Macrophages make and secrete apoE [Basu et al., 1982] that may have ambiguous functions regarding foam-cell formation, simultaneously facilitating lipoprotein uptake and cell cholesterol efflux [Mazzone and Reardon, 1994; Fazio et al., 1997]. Furthermore, animal models have been useful in the study of the genetics of atherosclerosis, such as the apoE-KO mouse [Nakashima et al., 1994] that, similarly to humans, develops hypercholesterolemia and atherosclerosis ascribed to lipoprotein remnants entering the liver less efficiently [Ross, 1999], because apoE is critical for chylomicron remnant clearance from plasma [van Lenten et al., 1985; St. Clair and Beisiegel, 1998], and for beta-VLDL uptake by mouse peritoneal macrophages [Perrey et al., 2001]. We then examined the effect of apoE secretion by macrophages on the cell uptake of EM alone. As compared to control mice, macrophages from apoE-deficient mice took up less EM and the addition of EDTA further impaired this activity (Table II). The latter may have occurred because EDTA impairs EM uptake by modifying an early endocytic step, namely, the pinching off of coated vesicles. This interpretation stems from the fact that, besides the LDL receptor, other cation-dependent mechanisms must be involved in EM uptake since cations are required for initial ligand-binding activity [Dickeson et al., 1998]. In this regard, EDTA inhibits pinocytosis [Bonzon et al., 1998], which is an endocytic cycle dependent on coated pits [Naito and Wisse, 1978; Suh et al., 1998]. Thus, the present study indicates that, besides pinocytosis, the uptake of EM alone is facilitated by apoE made by the macrophage. This result agrees with a previous study indicating that apoE associated with emulsion particles is crucial for the removal of the particles from plasma [Rensen et al., 1997; Saito et al., 2001].

The present study also shows that the rate of macrophage [^{3}H]-COE-EM uptake increased progressively when a fixed concentration of EM-TC was co-incubated with increasing concentrations of TC as NaLDL, AgLDL, or AcLDL

(Fig. 2). A similar finding in experimental atherosclerosis in rabbits was ascribed to the serum levels of the TAG-rich lipoproteins and was seemingly dependent on the serum cholesterol content [Minnich and Zilversmit, 1989]. Furthermore, by using the clathrin-coated pit pathway different receptors, such as the LDL receptor [Norman et al., 1999], are known to play a role in the endocytosis of a wide range of molecules. As shown in Figure 3, the uptake by macrophages of [^{3}H]-COE-EM co-incubated with NaLDL was also inhibited by EDTA. This result agrees with the fact that the LDL receptor participates in the uptake of natural chylomicron remnants [Yu et al., 1999]. Nonetheless, we have also shown an impairment of [^{3}H]-COE-EM uptake in co-incubations with increasing quantities of OxLDL-TC (Figs. 2 and 4; Table V). This finding should not be related to an impaired efficiency of LDL in interacting with EM because in all preparations LDL bound to EM (Fig. 1). In addition, acetylation or oxidation of LDL undoubtedly modifies the LDL retention time on FPLC analysis. Accordingly, the degree of LDL oxidation is proportional to its retention time in FPLC [Vedie et al., 1991]. Conceivably, the surprising influence of the copper-oxidized LDL on EM uptake stemmed from an inhibition of the macrophage lipoprotein lipase activity which does not occur with moderately oxidized LDL [Makoveichuk et al., 1998].

The ability to bind to the scavenger receptor is a property that distinguishes acetylated or oxidized LDL from native LDL [Steinbrecher, 1999]. In order to determine whether the scavenger receptor is involved in the uptake of EM in the presence of these modified forms of LDL, we investigated the effect of the polysaccharide fucoidan, a known inhibitor of the modified LDL binding to the scavenger receptor A (SR-A) [Yokota et al., 1998]. Fucoidan did not influence the cell uptake of [^{3}H]-COE-EM alone (Table I) but impaired this process in co-incubations with NaLDL or AcLDL, although not with OxLDL (Fig. 5). These results support the following interpretations regarding the mechanisms of EM uptake: (1) SR-A is involved when NaLDL or AcLDL is present, and (2) a mild oxidation of NaLDL by the macrophages occurred. In this regard, LDL is known to be minimally oxidized in vitro by arterial wall cells such as endothelial cells [Steinbrecher et al., 1984], smooth muscle cells [Heinecke et al., 1984], and macrophages [Parthasarathy, 1992]. Hodis et al. [1994] observed that, under appropriate conditions, formation of oxidized LDL may occur in plasma. The LDL oxidation process is believed to be atherogenic as macrophages lead to the formation of foam cells [Brown and Goldstein, 1983; Steinbrecher et al., 1984]. Probucol has been proposed to protect LDL from being oxidized in vitro [Parthasarathy, 1992]. Our data (Table III) show that probucol simultaneously inhibits the cell-mediated oxidative modification of LDL and the uptake of [^{3}H]-COE-EM co-incubated with NaLDL. This effect of probucol persists in the presence of fucoidan. In contrast, cell-mediated oxidation of EM alone might also have taken place. In this regard, a mild degree of in vitro LDL oxidation is required to convert LDL into an atherogenic particle [Tertov et al., 1998]. Nonetheless, as shown in Table IV, macrophages did not mediate the oxidation of [^{3}H]-COE-EM alone because its uptake by the cell was not modified by probucol and/or fucoidan. Consequently, macrophages did not oxidize EM alone, and its uptake via the scavenger receptor should be ascribed to a mild oxidation of NaLDL alone.

The uptake of lipoproteins by macrophages occurs through receptor-mediated endocytosis [Brown and Goldstein, 1983; Yen et al., 1994]. However, native LDL is taken up at a slow rate by macrophages because they express few receptors for this particle [Goldstein et al., 1979]. On the other hand, these cells display abundant receptors for lipoproteins that have been modified or are complexed with other molecules [Steinbrecher, 1999]. Aggregation is known to enhance the macrophage uptake of native LDL through a LDL receptor-dependent mechanism, suggesting that the phagocytotic pathway is involved [Khoo et al., 1988]. In this regard, cytochalasin B, a known inhibitor of microfilament function and phagocytosis [Cooper, 1987], inhibited the uptake of EM in the presence of NaLDL, AgLDL, or AcLDL, but not in the presence of OxLDL (Fig. 6). Cooper [1987] ascribed the uptake of aggregated lipoproteins to phagocytosis, which is actin-dependent. However, actin dependence does not necessarily mean phagocytosis alone since other endocytic pathways also occur in macrophages, such as macropinocytosis and the actin-dependent system known as patocytosis [Zhang et al., 1997; Kruth et al., 1999a,b].

Thus, the greater uptake of [^{3}H]-COE-EM, when co-incubated with NaLDL, AgLDL, and AcLDL, seems to be related to an actin-dependent mechanism. In fact, cytochalasin did not influence the uptake of EM particles alone (Table I), indicating that this process is operated by a mechanism independent of actin.

The role of triacylglycerols in atherosclerosis is controversial [Grundy, 1998]. Lipoprotein lipase (LPL) is the rate-limiting enzyme that hydrolyzes TAG from chylomicrons and VLDL [Jansen et al., 1998]. In addition, LPL enhances the binding of these lipoproteins to the extracellular matrix and their uptake by cell-specific receptors through other mechanisms that are not lipolysis-dependent [Olivecrona and Olivecrona, 1995]. Patients with deficiency of the LPL gene or of its cofactor, apoCII, usually display eruptive xanthomatosis and hyperchylomicronemia early in life [Benlian et al., 1996].

Chylomicrons may cause or exacerbate atherosclerosis due to the formation of remnants [Proctor and Mamo, 1996]. Furthermore, macrophages are able to secrete lipoprotein lipase and, unlike other phagocytic leukocytes, also metabolize TAG [Hill et al., 1995; Yin et al., 1997]. LPS inhibited the expression of LPL and also reduced the uptake of [^{3}H]-COE-EM when co-incubated with NaLDL, AgLDL, or AcLDL, but not with OxLDL (Fig. 7). However, LPS did not modify the uptake of [^{3}H]-COE-EM alone, quite likely due to the absence of apolipoproteins in the emulsion (Table I). On the other hand, LPS is known to reduce the activity of actin [Hill et al., 1995], a fact that must have been responsible for the slow uptake of [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL, or AcLDL. Moreover, LPS binds to SR-A [van Lenten and Fogelman, 1992; van Oosten et al., 1998]. Thus, the lower uptake rate of [^{3}H]-COE-EM co-incubated with NaLDL, AgLDL, or AcLDL in the presence of LPS may be due in part to the binding of LPS to the scavenger receptor, although other receptors might also be involved [Viktorov et al., 1989].

The mechanisms suggested in the present study whereby the modified LDL particles facilitate the EM internalization are in agreement with the fact that acetylated LDL cooperates with native LDL in its uptake via macropinocytosis [Jones et al., 2000].

The macrophage uptake of [^{14}C]-CO-NaLDL or of [^{14}C]-CO-AgLDL was slowed down upon their co-incubation with [^{3}H]-COE-EM (Table V), and yet both LDL forms facilitated the uptake of EM. These findings lead to the conclusion that EM competes with both NaLDL and AgLDL for the LDL receptor. In the present study, the uptake rates of [^{14}C]-CO-AcLDL or of OxLDL were higher than that of NaLDL, as expected, and were not modified by co-incubation with EM. These two forms of LDL are also known to lead to massive cell cholesterol accumulation [Goldstein et al., 1979; Steinbrecher et al., 1984; Sparrow et al., 1989]. Moreover, macrophages markedly take up AgLDL [Maor et al., 1997; Maor and Aviram, 1999], and we have now shown that this is a more efficient process than the uptake of AcLDL and OxLDL.

In summary, we have shown that EM alone is taken up by a coated-pit-dependent mechanism. Interestingly, it has been shown that macrophages take up cholesterol crystals [Kruth et al., 1995] without the presence of apolipoproteins. However, macrophages secrete apoE which, as shown here, facilitates EM uptake. Furthermore, macrophage EM uptake is facilitated by native and by modified LDL. Thus, apolipoproteins inherent to natural chylomicrons and VLDL might be less critical for atherogenesis than the existence of the apolipoprotein produced by macrophages or the presence of native and of modified forms of LDL. Therefore, further investigations are needed in identical experimental settings utilizing remnants of natural chylomicrons, in addition to monoclonal antibodies neutralizing specific cell receptors, and also macrophages from LDL receptor-deficient mice. Furthermore, the biology of macrophages displays similarities [Gianturco et al., 1988; Kruth et al., 1995] and differences [Traber et al., 1981] between mice and humans that also should be taken into account in future experiments.

Native, aggregated, acetylated, and moderately macrophage-oxidized LDL interact with EM stimulating its uptake through: (1) an actin-dependent pathway; (2) scavenger receptors; and (3) lipolysis mediated by lipoprotein lipase. On the other hand, the cell EM uptake is impaired by copper-oxidized LDL possibly due to its lower affinity towards lipoprotein lipase [Makoveichuk et al., 1998]. We have also shown that macrophages take up the aggregated form better than the acetylated or oxidized form of LDL. These mechanisms may be involved in the process of atherosclerosis in hyperchylomicronemia.

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