Apoprotein E mediates the interaction of β-VLDL with macrophages

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Abstract β-Very low density lipoproteins (β-VLDL) isolated from cholesterol-fed rhesus monkeys stimulated cholesteryl ester synthesis and accumulation in mouse peritoneal macrophages. The apoprotein specificity and requirement for the cell surface uptake of β-VLDL was investigated by treating the β-VLDL with trypsin (β-VLDL (T)), incubating the β-VLDL (T) with other lipoproteins or apoproteins, reisolating the β-VLDL (T) and measuring its biological activity which, for this study, is defined as the ability of the lipoprotein to stimulate cholesterol esterification in the macrophages. Trypsin treatment of β-VLDL abolished its biological activity. Apoprotein analysis of the β-VLDL (T) demonstrated the absence of intact apoproteins B-100, B-48, and E. The J774 macrophage-like cell line and mouse peritoneal macrophages responded similarly with respect to cholesterol esterification following incubation with inactive and treated β-VLDL. The J774 macrophage-like cell line was used to establish the conditions necessary for the restoration of biologic activity to the trypsinized β-VLDL. The loss of biological activity of β-VLDL (T) could be reversed by restoring apoprotein E to the β-VLDL (T) through incubation with either apoprotein E-containing LDL from hyperlipemic monkeys or purified apoprotein E. Apoprotein A-I had no such effect. The restored biological activity of the β-VLDL (T) was proportional to the amount of apoprotein E acquired by the lipoprotein. β-VLDL particles composed of apoprotein E and either intact or degraded apoprotein B-100 had comparable biological activity. Thus, intact apoprotein E, without intact apoprotein B, is a sufficient mediator for the biological activity and metabolism of β-VLDL by macrophages and plays a major role in receptor-lipoprotein interaction.—**Bates, S. R., B. A. Coughlin, T. Mazzone, J. Borensztajn, and G. S. Getz.** Apoprotein E mediates the interaction of β-VLDL with macrophages. *J. Lipid Res.* 1987. **28:** 787–797.

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Several experimental animal species respond to dietary challenge with cholesterol and other fats by demonstrating an increase in plasma cholesterol levels and an alteration in lipoprotein profiles. Under these circumstances, the very low density lipoproteins (VLDL) become depleted of triglyceride and enriched in cholesteryl esters and, upon agarose electrophoresis, migrate with a beta-mobility (1). The β-VLDL promote cholesteryl ester synthesis in macrophages, a characteristic that is not shared by VLDL isolated from normolipemic animals (2, 3). The promotion of cholesteryl ester formation in macrophages is not a property of LDL or HDL, especially HDL lacking apoE (2). The β-VLDL are taken up into the macrophages via a specific cell-surface receptor and neither fucoidin nor acetylated LDL interferes with the metabolism of β-VLDL by the cells (2). The β-VLDL are degraded in lysosomes, and cholesteryl esters are synthesized and accumulate in the macrophage cytosol (2).

The apoprotein specificity of the macrophage cell-surface receptor responsible for the binding and internalization of β-VLDL has been the subject of several investigations. It is believed that the protein moiety of the particle is necessary for the lipoprotein–cell interaction since reductive methylation of the β-VLDL proteins abolishes their ability to stimulate cholesteryl ester formation in macrophages (4). It has also been shown that, while unlabeled β-VLDL is able to compete with radioiodinated β-VLDL for uptake and degradation by macrophages (2), a high concentration of LDL or HDL without E does not prevent the degradation of β-VLDL (2, 5). Such data indicate that apoprotein B-100, the predominant apoprotein of human LDL, on an LDL-sized particle, or apoproteins A and C on an HDL-sized particle do not compete with β-VLDL for interaction with the β-VLDL receptor on macrophages.

The present investigation was initiated in order to examine the question of the apoprotein required for the lipoprotein-macrophage surface receptor interaction. A recent

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; LDL, low density lipoproteins; β-VLDL (T), β-VLDL treated with trypsin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LPDP, lipoprotein-deficient plasma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; H-HDL, hyperlipemic HDL; H-LDL, hyperlipemic LDL.

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report involving the addition of untreated apolipoprotein E to reductively methylated β-VLDL (6), strongly implicated apoprotein E as the protein ligand responsible for the interaction of β-VLDL with the macrophage cell surface receptor. Our approach differed from this study in that the β-VLDL was treated with the proteolytic enzyme, trypsin, which resulted in partial depletion of apoproteins from the lipoprotein particle and loss of its biological activity. The biological activity of the trypsin-treated hyperlipemic VLDL could be restored by incubation with other lipoproteins, serving as a source of replacement apoproteins. The biological activity of treated and untreated VLDL particles was assessed by their ability to stimulate cholesterol esterification in macrophages. Our results, utilizing proteolytic degradation and selective restoration of apoproteins to the β-VLDL particles, agree with the previous report (6) that apoprotein E is the predominant apoprotein on β-VLDL recognized by macrophages from different species.

MATERIALS AND METHODS

Tissue culture

Unstimulated peritoneal macrophages were isolated from female Swiss-Webster mice according to the method of Cohn and Benson (7) as outlined previously (8). The isolated cells were suspended in Dulbecco's Modified Eagle's medium (DMEM) (Grand Island Biological Co., Grand Island, NY) plus 10% heat-inactivated fetal calf serum (FCS) (K. C. Laboratory Supply Company, Indianapolis, IN), plated to a density of approximately 3×10^6 cells/well (0.1 mg of cell protein/well) in Multiwell tissue culture plates (Costar, 12 or 24 wells/plate) and used 2 days after isolation. The macrophage-like cell line J774A.1 (J774) was obtained from the American Type Tissue Culture Collection, Bethesda, MD, and was maintained in DMEM containing 10% FCS (9). Prior to experiments, the J774 cells were seeded to achieve a final density of 0.2 to 0.5 mg of cell protein/well. Both cell types were incubated at 37°C in a 5% CO_2 atmosphere.

At the start of an experiment, the cells were washed twice with phosphate-buffered saline (PBS) pH 7.2, prior to addition of the experimental media. In the experiments involving measurement of cholesterol esterification, the cells were incubated with the experimental media for 8 hr with 0.04 μCi of [^{14}C]oleate-bovine serum albumin (BSA) (1.7 mg of BSA, oleate:BSA molar ratio = 6.8:1) added to each well during the last 5 hr. The [^{14}C]oleate-BSA complex was made according to the method of St. Clair, Smith, and Wood (10) using [^{14}C]oleic acid (60 mCi/mol) from Amersham and fatty acid-free BSA from Pentex. In experiments involving measurement of cholesteryl ester accumulation, the cells were incubated with the experimental media for 24 hr. Both types of these experiments used cells grown in 12-well plates.

The procedure followed for termination of experiments involved removing the media, washing the cells twice with PBS, and dissolving the cells in 0.1 N NaOH for 20 min. An aliquot was then taken for lipid extraction and neutralized immediately with concentrated acetic acid. Lipids were extracted using the method of Bligh and Dyer (11).

Lipoproteins

Lipoproteins were isolated from the plasma of fasted hyperlipemic rhesus monkeys that had been on a diet of Purina monkey chow supplemented with 2% cholesterol and 25% coconut oil for 2 years (mean serum total cholesterol, 950 mg/dl). Normal lipoproteins were isolated from the plasma of either fasted rhesus monkeys maintained on a standard chow diet (mean serum total cholesterol, 120 mg/dl) or a fasted normal human male (mean serum total cholesterol, 150 mg/dl). To inhibit serine proteases, 1 mM phenylmethylsulfonyl fluoride was added to all plasma samples before processing. The plasma was then centrifuged at 25,000 rpm for 20 min in an SW 27 rotor to remove chylomicrons. VLDL ($d < 1.006$ g/ml), low density lipoproteins (LDL) (d 1.019–1.050 g/ml), and high density lipoproteins (HDL) (d 1.063–1.21 g/ml) were isolated according to the method of Havel, Eder, and Bragdon (12) with modifications as described by Scanu and colleagues (13). All lipoproteins were washed by recentrifugation and checked for purity by agarose electrophoresis. Lipoprotein-deficient plasma (LPDP) was obtained from normal rhesus plasma by centrifugation at a density of 1.21 g/ml, dialyzed, heated at 56°C for 30 min, and filtered. All lipoproteins were used within 2 weeks of preparation. The VLDL from coconut oil-fed hyperlipemic monkeys (β-VLDL) were beta-migrating and cholesterol-rich with a composition by weight of 11.7% free cholesterol, 52.0% cholesteryl ester, 6.6% triglyceride, 5.5% protein, and 24.2% phospholipid.

Trypsin treatment of lipoproteins

Trypsinization of β-VLDL was typically performed as follows: 400 μg of trypsin (Worthington Diagnostics, 40 μl of a 10 mg/ml solution in PBS) was incubated with 150 μg of β-VLDL protein in 0.4 ml of PBS for a final trypsin concentration of 0.91 mg/ml, for 30 min at room temperature. Longer incubations, up to 24 hr, did not modify the results. Six hundred μg of soybean trypsin inhibitor (Worthington Diagnostics, 60 μl of 10 mg/ml solution in PBS) for a final concentration of 1.2 mg/ml was then added to the mixture for 5 min (β-VLDL (T)). For the untrypsinized control, the same amounts of trypsin and trypsin inhibitor used in the experiment were preincubated at room temperature for 5 min and added to 150 μg of β-VLDL for a 35-min incubation (β-VLDL(Ti)). After addition of inhibitor, some of the trypsinized samples were incubated with various lipoproteins or with isolated apoprotein E (apoE) for 1 hr at room temperature. The samples and control were transferred to centrifuge tubes and the β-VLDL were reisolated

at d 1.006 g/ml by a 16-hr centrifugation in a 30.2 rotor at 29,000 rpm. Aliquots of these VLDL were analyzed for protein content, and extracted for total cholesterol analysis before being added to macrophage cells in culture and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for apoprotein analysis.

Isolation of apoproteins E and A-I and antibody preparation

Purified apoprotein E (apoE) used in incubations with trypsin-treated β-VLDL was isolated from the d < 1.019 g/ml lipoprotein fraction of hyperlipemic rhesus plasma by heparin sepharose affinity column chromatography as described by Shelburne and Quarfordt (14). Apoprotein A-I (apoA-I) was prepared according to the method of Edelstein, Lim, and Scanu (15). The purity of apoE and apoA-I was verified by SDS-PAGE and by immunodecoration.

For antibody production purposes, rhesus apoE was purified from rhesus plasma VLDL by a combination of column chromatography and preparative gel electrophoresis as described previously (16). The purified apoE was then used to immunize a goat. To ensure that antiserum was free of reactivity with other soluble apoproteins, the immune goat plasma was passed over an HDL_3-Sepharose column (17). The resulting anti-apoE antibody was shown to be monospecific by double immunodiffusion and immunodecoration of separated apoproteins. Nonimmune serum from normal goats was used to prepare anti-goat IgG antibodies in rabbits. The antibodies were coupled with peroxidase for immunodecoration purposes.

Radioimmunoassays of apoB, apoE, and apoA-I were performed using antisera raised to these purified peptides according to previously established methods (18, 19, and Y. Piran, L. A. Jones, R. A. Biehler, G. S. Getz, personal communication).

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out using conditions established by Laemmli (20) as described by Jones et al. (16). The samples were separated by electrophoresis on a 5–22.5% gradient gel at 3 mA for 16 hr. The gel was stained using the silver staining technique of Merril et al. (21).

Assays

Gas–liquid chromatographic techniques were used to determine the total cholesterol content of the trypsinized VLDL samples and the free, esterified, and total cholesterol content of the cells (μg of esterified cholesterol = μg of total cholesterol minus μg of free cholesterol) (22). [^{14}C]oleate incorporation into cholesteryl ester was quantified by fractionation of the lipid extracts on silica gel G plastic thin-layer chromatography plates (J. T. Baker) developed with petroleum ether–ethyl ether–acetic acid 75:25:1. The separated lipids were cut out from the plate and placed in Econofluor scintillant for counting. The internal standard, [1,2-^{3}H]cholesterol in chloroform, was added during the extraction and the results were corrected for recovery (mean recovery = 80%).

Protein was determined according to the method of Lowry et al. (23) and phospholipids according to the method of Bartlett (24). The total cholesterol and triglyceride content of the VLDL was analyzed with a Technicon Autoanalyzer II (25).

RESULTS

Cholesterol esterification reflects cell surface interaction of β-VLDL with mouse peritoneal macrophages

VLDL isolated from rhesus monkeys fed a 2% cholesterol, 25% coconut oil diet are β-migrating and cholesteryl ester-enriched and are designated as β-VLDL or hyperlipemic VLDL (26, 27). Incubation of mouse peritoneal macrophages with this rhesus β-VLDL increased the intracellular cholesterol content and stimulated the esterification of cholesterol, resulting in an enrichment of the macrophages with cholesteryl ester (**Table 1**). As others have shown (3), VLDL isolated from normolipemic rhesus monkeys at the same total cholesterol concentration in the medium did not stimulate cholesterol esterification (data not shown). The stimulation of cholesterol esterification (**Fig. 1**) produced by increasing concentrations of β-VLDL added to the media had the characteristics of a process tending to saturate at a β-VLDL medium concentration of 10–20 μg protein/ml or 100–200 μg total cholesterol/ml. The ratio of total cholesterol to protein in the β-VLDL particle is approximately 10 to 1 (**Table 2**) so each 1 μg of β-VLDL

TABLE 1. Stimulation by β-VLDL of cholesterol content and cholesterol esterification in mouse peritoneal macrophages

Exp.	Parameter	Additions to Medium: Albumin	Additions to Medium: β-VLDL
A	Cholesterol content:	μg cholesterol/mg cell protein	
	Free	17.2	31.9
	Ester	0.0	30.8
	Total	17.2	62.7
B	Cholesterol esterification:	cpm × 10^{-3}/mg cell protein	
	[^{14}C]oleate in CE	2.0	35.2

Mouse peritoneal macrophages were incubated with albumin (10 mg/ml) or β-VLDL. In experiment A, the macrophages were exposed to albumin or β-VLDL (240 μg of total cholesterol/ml) in DMEM for 24 hr and the intracellular cholesterol content was measured. In experiment B, the macrophages were exposed to albumin or β-VLDL (80 μg of total cholesterol/ml) for 8 hr with [^{14}C]oleate added for the final 5 hr and the incorporation of [^{14}C]oleate into cholesteryl ester (CE) was measured. The results are averages of two to four determinations which did not differ by more than 10% and are representative of the three experiments performed.

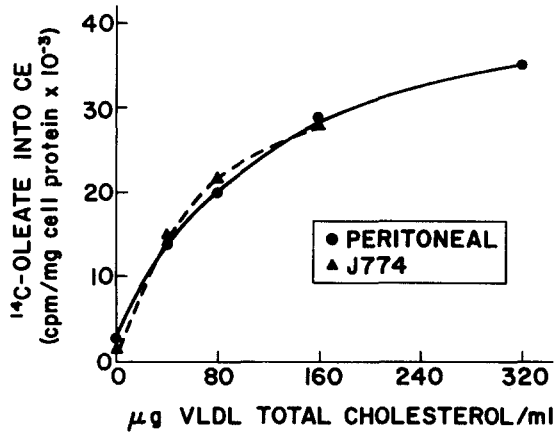

Fig. 1. Stimulation of cholesteryl ester synthesis in macrophages by β-VLDL. Increasing concentrations of β-VLDL were added to mouse peritoneal or J774 macrophages for 8 hr with [^{14}C]oleate added during the final 5 hr. CE, cholesteryl ester. The data are the means of two to six determinations and are representative of the five experiments performed.

protein/ml of medium carried 10 μg of total cholesterol/ml. The measurement of [^{14}C]oleate incorporation into cholesteryl ester appears to be a sensitive measure of the metabolism of the β-VLDL particle. A similar process was noted in J774 macrophages (Fig. 1).

Proteins of β-VLDL are required for its stimulation of macrophage cholesterol esterification

Experiments modifying the β-VLDL proteins by reductive methylation suggested that the protein moiety was important in the binding of β-VLDL to macrophage cell surface receptors (4). To examine the role of proteins in greater detail, the hyperlipemic VLDL were exposed to trypsin for 30 min. Soybean trypsin inhibitor was added to stop the action of the enzyme. The trypsin-treated β-VLDL were incubated for 1 hr with the d > 1.006 g/ml fraction of the serum from the same hyperlipemic monkey and then the β-VLDL were reisolated by centrifugation. The ability of these β-VLDL to interact with peritoneal macrophages as judged by their capacity to stimulate cholesterol esterification was measured and compared to that of untreated control β-VLDL or trypsin-treated β-VLDL which was not reincubated with d > 1.006 g/ml lipoproteins. The results of two independent experiments indicated that trypsin treatment, under the conditions employed, totally abolished the ability of β-VLDL to stimulate cholesteryl ester synthesis in macrophages. However, incubation of trypsinized β-VLDL with the other hyperlipemic lipoproteins restored approximately 70% (range 50–80%) of their capacity to promote cholesterol esterification.

A possible interpretation of these experiments is that treatment of β-VLDL with trypsin hydrolyzed the protein(s) responsible for β-VLDL binding to macrophages and that protein from the hyperlipemic d > 1.006 g/ml lipoproteins transferred to the trypsinized β-VLDL and partially restored their ability to promote cholesterol esterification. Thus, isolated lipoproteins were used to determine which of the hyperlipemic lipoproteins might be restoring the activity of the trypsinized β-VLDL and what, if any, proteins were being transferred. β-VLDL were trypsinized and incubated with hyperlipemic LDL or HDL, and reisolated. SDS polyacrylamide gel electrophoretic analysis of each of the lipoproteins revealed (**Fig. 2**) that β-VLDL have approximately equal amounts of apoproteins B-100, B-48, and a protein with the molecular weight of B-26 with appreciable quantities of apoprotein E and albumin. Apoprotein B-100, apoprotein B-48, albumin, and apoprotein E were identified using immunochemical techniques. Upon precipitation of β-VLDL with isopropanol (28), the majority of "B-26" precipitates along with B-100 and B-48 while apoprotein E and albumin remain in solution, indicating that the "B-26" probably represents an apoprotein B-like species. The albumin was firmly associated with the VLDL since it remained with the particle after three recentrifugations and the trypsin digestion. A similar protein migrating in the region of albumin in particles of d < 1.006 g/ml has also been described by Rudel, Shah, and Greene (27) in studies with rhesus monkeys fed 1 mg/Kcal of cholesterol and 45% of calories as lard.

Trypsin treatment failed to remove all of the apoproteins from the β-VLDL particle (Fig. 2). As shown in Table 2, the ratio of total cholesterol to protein of the β-VLDL increased little after trypsin digestion. This suggests that protein fragments generated by trypsin hydrolysis largely remain associated with the β-VLDL particle. SDS polyacrylamide gel bands representing intact apoproteins B-100,

TABLE 2. Cholesterol and apoprotein content of β-VLDL before and after trypsin digestion

β-VLDL Sample	Total Cholesterol ÷ protein Mean ± SD	(n)	% of Refrigerated Control ApoB	ApoE
Refrigerated	10.1 ± 2.5	(8)	100	100
(Ti)	9.4 ± 1.1	(8)	62	63
(T)	10.6 ± 1.7	(8)	52	3
(T) + H-LDL	9.6 ± 1.6	(8)	54	88
(T) + ApoE	8.6 ± 2.0	(5)	37	236

Hyperlipidemic VLDL were subjected to the various treatments indicated, then reisolated and analyzed for total cholesterol and protein content. These data are expressed as the mean ratio of μg of total cholesterol to μg of protein (± standard deviation) in each sample; (n) represents the number of samples analyzed. The β-VLDL were treated as follows: refrigerated, untreated and not recentrifuged; (Ti), trypsin and trypsin inhibitor were preincubated, then added to β-VLDL for 35 min; (T), trypsin was added to β-VLDL for 30 min, after which trypsin inhibitor was added for 5 min; (T) + H-LDL, after trypsin digestion of 100 μg of β-VLDL, 10 mg of hyperlipemic LDL was added for 1 hr; (T) + apoE, after trypsin digestion of 100 μg of β-VLDL, 100 μg of apoprotein E was added for 1 hr. The quantitation of apoB and apoE was performed by radioimmunoassay. The data are μg apoprotein/mg total cholesterol expressed as a % of the values for the refrigerated control β-VLDL sample. The apoprotein composition of β-VLDL was 85% apoB, 12% apoE, and 3% apoA-I. The 37–38% decrease in apoE and apoB in the (Ti) sample is due to recentrifugation.

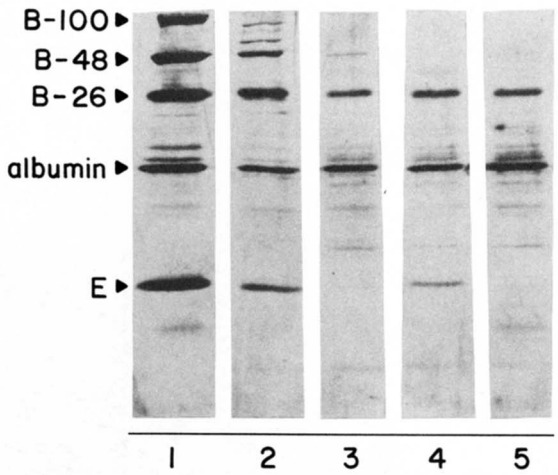

Fig. 2. The apoprotein composition of hyperlipemic VLDL before and after trypsin treatment. β-VLDL, treated and untreated as described in the Methods section, were examined using SDS polyacrylamide gel electrophoresis on a 5–22.5% polyacrylamide gradient gel and stained with silver stain. Lane 1, untreated β-VLDL; lane 2, β-VLDL + inactivated trypsin; lane 3, trypsinized β-VLDL = β-VLDL (T); lane 4, 129 μg of β-VLDL (T) protein + 1.3 mg of hyperlipemic LDL protein; lane 5, 129 μg of β-VLDL(T) protein + 1.3 mg of hyperlipemic HDL protein. All treated β-VLDL were reisolated by flotational centrifugation (d < 1.006 g/ml) after the incubations listed above. Two percent of the total protein content of hyperlipemic LDL is apoE.

B-48, and E were no longer detectable after trypsin digestion, but the "B-26" and albumin remained associated with the VLDL particle (Fig. 2). Apoprotein E was visually undetectable and present at very low levels as determined immunochemically (Fig. 2, lane 3; Table 2). Incubation of these particles with hyperlipemic LDL (Fig. 2, lane 4; Table 2), but not with HDL (Fig. 2, lane 5), restored most of the apoprotein E.

Use of J774 cells to explore the requirements for restoration of β-VLDL biological activity

In order to establish the conditions for the restoration of the maximum amount of biological activity to the trypsinized β-VLDL, the next series of experiments utilized the macrophage-like cell line, J774. J774 macrophages have been shown to resemble peritoneal macrophages in several respects (9, 29). Unlike the mouse peritoneal macrophage, however, the J774 macrophage has a binding site with a high affinity for LDL (30) as does the human monocyte macrophage (29, 31). However, the levels of LDL receptors on these cells are low unless they are induced by incubation in lipoprotein-deficient plasma (30). To study β-VLDL metabolism, these macrophages were exposed to the β-VLDL under conditions identical to those used with mouse peritoneal macrophages (Fig. 1). The stimulation of the rates of cholesteryl ester synthesis for the two types of macrophages was very similar (Fig. 1), enabling us to use J774 cells for further exploratory experiments.

The lipoproteins shown in Fig. 2 were tested for their capacity to stimulate [^{14}C]oleate incorporation into cholesteryl esters in J774 macrophages. As reported by others, unlike intact β-VLDL, hyperlipemic LDL, or HDL alone produced low levels of esterification (**Fig. 3**). Likewise, trypsinized β-VLDL had little effect. On the other hand, incubation of the trypsinized β-VLDL with hyperlipemic LDL restored 76–91% of the capacity of the β-VLDL to augment cholesteryl ester synthesis in the J774 macrophages, whereas incubation of the VLDL with hyperlipemic HDL did not restore activity.

The ability of hyperlipemic LDL to restore the biological activity of trypsin-treated β-VLDL was tested in many experiments and essentially identical results were obtained. Trypsin treatment of the β-VLDL abolished their ability to stimulate cholesteryl ester formation (10 ± 3% of untreated β-VLDL, n = 20). Incubation of the trypsin-treated β-VLDL with hyperlipemic LDL at a protein ratio of 150 μg of VLDL to 10 mg of LDL restored the ability of β-VLDL to stimulate cholesterol esterification up to 75 ± 20% of untreated β-VLDL activity (n = 20). It should be emphasized that, in all cases just described, the β-VLDL were reisolated by centrifugation and, therefore, separated from the lipoprotein with which they had been incubated.

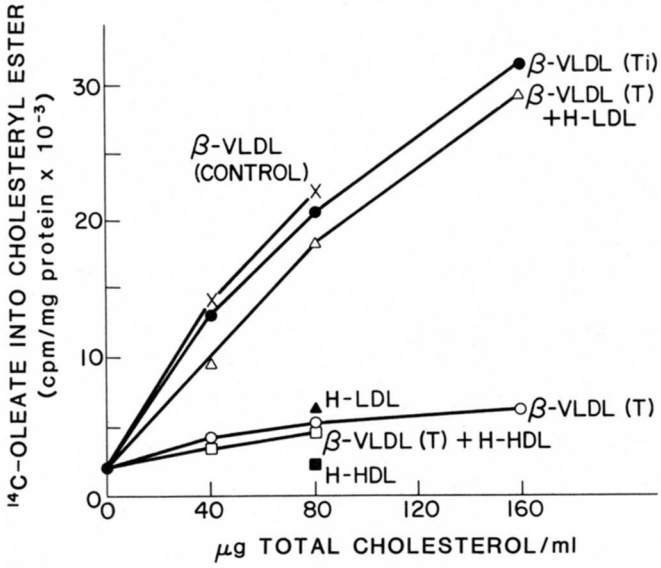

Fig. 3. Cholesteryl ester synthesis in macrophages exposed to various lipoproteins. J774 macrophages were incubated with the following lipoproteins at the concentrations shown for 8 hr as described in the Methods section: control β-VLDL, untreated hyperlipemic VLDL; β-VLDL (Ti), β-VLDL incubated with inactivated trypsin and reisolated; β-VLDL (T), β-VLDL treated with trypsin for 30 min, followed by addition of trypsin inhibitor and reisolation; β-VLDL (T) + H-LDL, 100 μg of trypsin-treated β-VLDL incubated with 10 mg of hyperlipemic LDL and reisolated; β-VLDL (T) + H-HDL, 100 μg of trypsin-treated β-VLDL incubated with 10 mg of hyperlipemic HDL and reisolated; H-LDL, hyperlipemic LDL; H-HDL, hyperlipemic HDL. All samples had 2.5 mg/ml albumin. Data points are single or duplicated determinations. A second experiment with J774 macrophages incubated in duplicate with the same lipoprotein preparations at 80 μg of total cholesterol/ml gave similar results.

The loss of apoB-100 did not affect the biological activity of β-VLDL

During the course of these experiments, it became clear that apoE could restore biological activity to trypsinized β-VLDL which did not contain intact apoB. Intact apoB always disappeared after trypsin treatment and was absent from all lipoproteins reconstituted by incubation with hyperlipidemic LDL, indicating that the reconstituted, reisolated VLDL was not contaminated with significant quantities of LDL. The VLDL reisolated after a room temperature incubation with or without inactivated trypsin resulted in partial (Fig. 2, lane 2), or almost complete loss (data not shown) of intact B-100 from the particles, but the ability to stimulate cellular cholesterol esterification in J774 macrophages was not affected (104 ± 2% compared to untreated β-VLDL controls, n = 3, two experiments). Loss of biological activity occurred only with trypsin treatment and the resultant removal of apoproteins B-100, B-48, and E (Fig. 2, lane 3).

Apoprotein E is an important component of the ligand for interaction of β-VLDL with the J774 cells

Incubation of trypsinized β-VLDL with various amounts of hyperlipemic LDL led to a concentration-dependent transfer of apoprotein E. **Fig. 4** shows the SDS polyacrylamide gels of 150 μg of trypsinized β-VLDL, reisolated after incubation with 0.75 to 15 mg of hyperlipemic LDL protein. Equal amounts of VLDL protein were loaded on the gels. Quantitation of such gels is difficult, but it appears that similar amounts of apoprotein E transferred from the 15 and 7.5 mg of hyperlipemic LDL to the VLDL while less apoprotein E transferred from 1.5 mg of hyperlipemic LDL

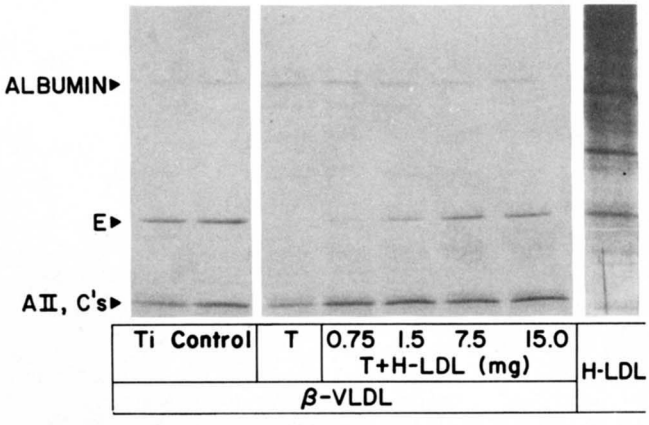

Fig. 4. SDS-PAGE gel patterns of trypsin-treated β-VLDL incubated with increasing concentrations of hyperlipemic LDL. Ti, trypsin was inactivated with trypsin inhibitor and then both were added to β-VLDL; Control, untreated β-VLDL; T, β-VLDL + trypsin for 30 min, + trypsin inhibitor; T + H-LDL, trypsinized β-VLDL incubated with increasing amounts of hyperlipemic LDL as shown. β-VLDL protein (150 μg) was used in each sample and all β-VLDL samples were reisolated by centrifugation before application to the gel. Ten μg of β-VLDL protein was applied to each lane, while 25 μg of H-LDL was applied to the last lane.

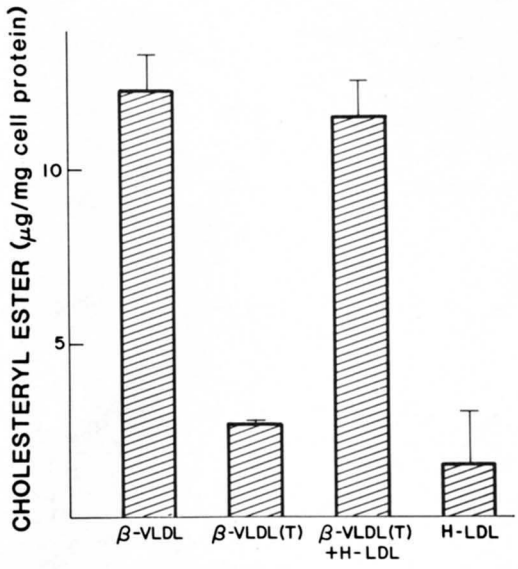

Fig. 5. Cholesteryl ester mass accumulation in macrophages exposed to various lipoproteins. J774 macrophages were incubated with the following lipoproteins for 24 hr: β-VLDL, untreated hyperlipemic VLDL; β-VLDL (T), β-VLDL treated with trypsin and reisolated; β-VLDL (T) + H-LDL, 150 μg of trypsin-treated β-VLDL incubated with 10 mg of hyperlipemic LDL and reisolated; H-LDL, hyperlipemic LDL. Lipoproteins were added to the cells at a final concentration of 40 μg of total cholesterol/ml. All samples contained 2.5 mg/ml albumin. The cholesterol accumulation caused by albumin alone (0.8 μg/mg cell protein) has been subtracted from all values shown. Data shown are the results of duplicate determinations.

and no apoprotein E was visible using 0.75 mg of LDL. The biological activity of these preparations and of preparations from several other experiments indicated that incubation of 150 μg of trypsinized β-VLDL with 5 to 7 mg of hyperlipemic LDL protein rendered the β-VLDL as active as the untreated particle. As the amount of apoprotein E on the β-VLDL particle decreased (Fig. 4), there was a concomitant reduction in the ability of the reconstituted particle to promote cholesterol esterification in J774 macrophages. The restored biological activity of the reisolated VLDL is unlikely to be attributable to contaminating hyperlipemic LDL. Since hyperlipemic LDL alone, even at 400 μg of total cholesterol/ml (200 μg of protein/ml), had little effect on cholesterol esterification, and judging from the data illustrated in Fig. 2, much more than 20% of the hyperlipemic LDL would have to contaminate the reisolated VLDL to raise the possibility of any effect on cholesterol esterification. At this level of contamination, intact apoprotein B-100 would have been detected among the apoproteins of the reconstituted VLDL and this was not seen.

The increase in cholesteryl ester synthesis was accompanied by an enrichment in the cellular cholesteryl ester content of the macrophages as illustrated in **Fig. 5.** J774 macrophages were exposed for 24 hr to hyperlipemic LDL or to hyperlipemic VLDL particles that had had no treatment (β-VLDL), had been trypsinized (β-VLDL (T)), or

had been trypsinized and then incubated with hyperlipemic LDL (β-VLDL (T) + H-LDL). The increase in the cholesteryl ester content of the cells was equivalent to the observed stimulation of [^{14}C]oleate incorporation into cholesteryl ester indicating that the changes in cholesteryl ester synthesis resulted in a net accumulation of cholesteryl ester.

In order to confirm our hypothesis that apoprotein E was responsible for the restoration of biological activity to the trypsinized β-VLDL particles, apoprotein E was isolated from the d < 1.019 g/ml lipoprotein fraction from hyperlipemic rhesus monkeys. This apoprotein E was incubated with the trypsin-treated β-VLDL which were then reisolated and tested for biological activity in J774 macrophages. As shown in **Fig. 6** and Table 2, incubation of the trypsinized VLDL with apoprotein E resulted in a net transfer of apoE to the β-VLDL particles and restoration of biological activity. The compilation of several experiments indicates that an incubation ratio of apoprotein E protein to trypsinized VLDL protein of 0.5 or greater fully restores the biological activity of the β-VLDL. Similar experiments using purified apoprotein A-I, while resulting in the association of A-I with the trypsinized β-VLDL particles, had no effect on their biological activity (data not shown).

The quantitative data on the amount of added apoE needed to reestablish the biological activity of trypsinized β-VLDL under the conditions used in this study is summarized by the following. Since 12% of the protein in β-VLDL is apoprotein E, for every 10 μg of β-VLDL there would be 1.2 μg of apoprotein E present. For every 10 μg of β-VLDL, 330 μg of hyperlipemic LDL was needed to restore activity and 2% of the LDL total protein content is apoE. Therefore, 6.6 μg of LDL apoE was needed for every 10 μg of β-VLDL (1.2 μg of apoE). In addition, 5 μg of the isolated apoE was required to fully reconstitute 10 μg of β-VLDL (1.2 μg of β-VLDL apoE). Thus, a fivefold excess of apoE was required to restore the normal metabolism of β-VLDL by macrophages whether the source of apoprotein E was hyperlipemic LDL or isolated apoprotein E.

Mouse peritoneal macrophages also respond to trypsinized β-VLDL reconstituted with apoprotein E with increased cholesterol esterification

The experiments shown in Figs. 3–6 defined the amount of hyperlipemic LDL or isolated apoprotein E needed to restore the biological activity of a protease-treated β-VLDL. The importance of apoprotein E for the metabolism of β-VLDL by mouse peritoneal macrophages was demonstrated in the next experiments. Trypsinized β-VLDL reisolated after incubation with hyperlipemic LDL or isolated apoprotein E, was tested for its ability to stimulate cholesteryl ester synthesis in peritoneal macrophages. **Fig. 7** shows that the elevated rate of cholesterol esterification promoted by β-VLDL was again lost as a result of protease treatment. Addition of apoprotein E to the inactive VLDL particle by incubation with hyperlipemic LDL or isolated apoprotein E restored the metabolism of β-VLDL by mouse peritoneal macrophages (Fig. 7).

The similarity of the metabolism of untreated and treated β-VLDL by J774 macrophages and by mouse peritoneal macrophages is emphasized in Fig. 7. Although both macrophages showed a relatively small response to hyperlipemic LDL, which contains small amounts of apoprotein

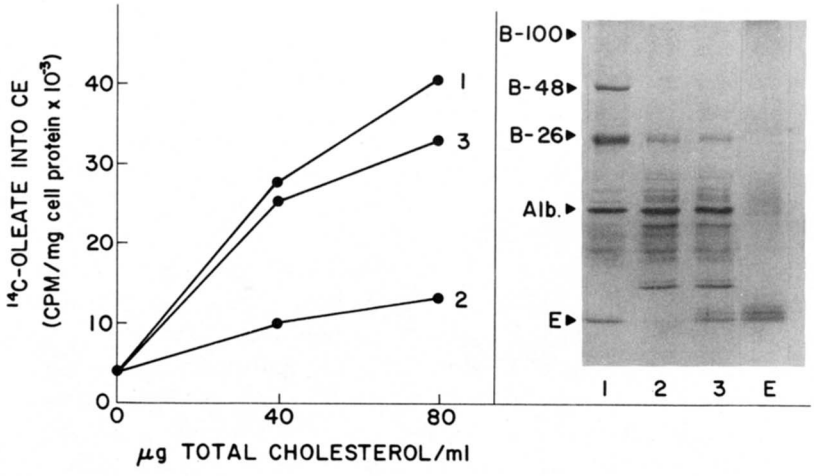

Fig. 6. Restoration of biologic activity to trypsin-treated hyperlipemic VLDL following incubation with isolated apoprotein E. The left side of the figure shows the effect of cholesteryl ester synthesis in J774 macrophages exposed to: *1)* untreated β-VLDL; *2)* β-VLDL treated with trypsin; *3)* β-VLDL treated with trypsin then incubated with apoprotein E isolated from rhesus monkey plasma. All samples contained 2.5 mg/ml albumin and all β-VLDL samples were reisolated after treatment. Data points are the average of duplicate determinations and this experiment is representative of eight performed. On the right side are the corresponding SDS-PAGE patterns for samples 1, 2, and 3 (5 μg each) and the isolated apoprotein E (2 μg).

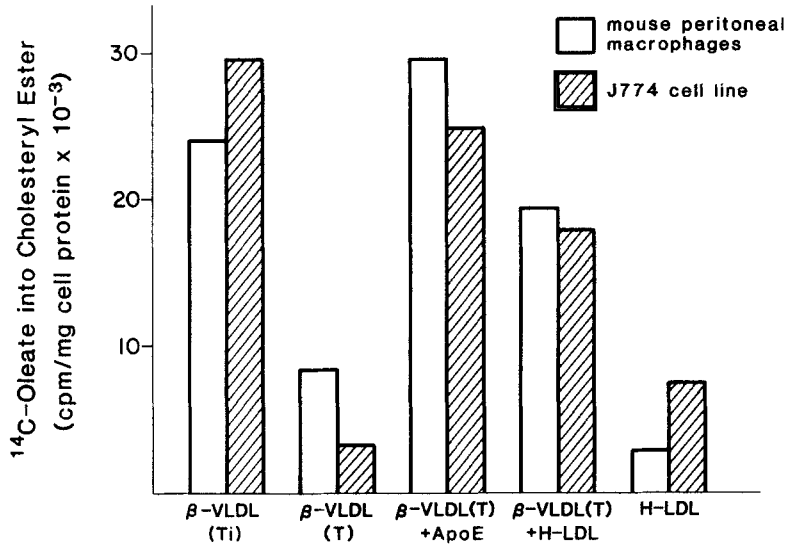

Fig. 7. Effect of the restoration of apoprotein E to a biologically inactive β-VLDL on its metabolism by the lipoprotein receptor on peritoneal macrophages. Mouse peritoneal macrophages (open bars) or J774 macrophages (hatched bars) were incubated with the following β-VLDL lipoproteins at 40 μg of total cholesterol/ml or with H-LDL at 400 μg of total cholesterol/ml. Incubation was for 8 hr with [^{14}C]oleate added during the final 5 hr: β-VLDL (Ti), VLDL incubated with inactivated trypsin; β-VLDL (T), β-VLDL + trypsin for 30 min, then trypsin inhibitor for 5 min; β-VLDL (T) + apoE, 100 μg of β-VLDL treated with trypsin, then incubated for 1 hr with 100 μg of rhesus apoprotein E; β-VLDL (T) + H-LDL, 100 μg of β-VLDL protein treated with trypsin, then incubated for 1 hr with 10 mg of hyperlipemic LDL protein; H-LDL, hyperlipemic LDL. All β-VLDL samples were reisolated at d 1.006 g/ml before incubation with cells. Data shown are the results of duplicate determinations and are representative of three experiments performed. All samples contained 2.5 mg/ml albumin. The cholesterol esterification caused by BSA alone (3968 cpm/mg of cell protein in mouse peritoneal macrophages and 2089 cpm/mg of cell protein in J774 cells) has been subtracted from each of the data points shown.

E (Table 2), the J774 macrophage was somewhat more responsive. Exposure to normal human LDL produced some cholesterol esterification in J774 macrophages with no effect in mouse peritoneal macrophages (data not shown) as has been reported by others (2, 29). However, parallel changes in rates of cholesteryl ester formation were noted in J774 and mouse peritoneal macrophages incubated with β-VLDL, trypsinized β-VLDL, and trypsinized β-VLDL reconstituted with apoprotein E from either H-LDL or isolated apoprotein E (Fig. 7).

DISCUSSION

Several types of natural lipoproteins are thought to be recognized by receptors on the surface of macrophages. These include VLDL from hypertriglyceridemic patients (32), and VLDL isolated from various animal species fed high-fat diets (3) including the β-VLDL from hyperlipemic rhesus monkeys used in the present study. Our results strongly suggest that apoprotein E is of major importance for the metabolism of β-VLDL by the macrophages, a conclusion that is based on several lines of evidence. First, trypsin treatment of β-VLDL removed visible and immunologically detectable apoprotein E from the lipoprotein particle and abolished its ability to stimulate cholesteryl ester synthesis and accumulation in macrophages. Secondly, the lost biological activity of the β-VLDL could be reversed by restoring apoprotein E to the β-VLDL particle through incubation with either hyperlipemic LDL or isolated apoprotein E. The addition of apoprotein A-I to the β-VLDL had no such effect. Finally, the biological activity of the trypsinized β-VLDL was proportional to the amount of apoprotein E replaced on the lipoprotein. This was the case whether the source of apoprotein E was hyperlipemic LDL or isolated apoprotein E. Our results like those of others (33, 34) emphasize the importance of apoprotein E for the interaction of lipoproteins with macrophages. In particular, Innerarity et al. (6) showed that apolipoprotein E was responsible for the metabolism of β-VLDL by mouse peritoneal macrophages.

The nature of the macrophage surface protein (or proteins) that mediates the uptake of β-VLDL is currently uncertain. LDL binds poorly to uninduced mouse peritoneal macrophages and is a relatively ineffective competitor for the saturable β-VLDL binding and degradation (2). Koo, Wernette-Hammond, and Innerarity (35), however, have indicated that the β-VLDL binding sites of mouse peritoneal macrophages share a number of properties with the classic LDL receptor of skin fibroblasts. J774 cells, like the human monocyte macrophages, bind and degrade both β-VLDL and LDL in a high affinity manner (29–31). Regard-

less of whether the recognition by J774 cells of these two ligands is mediated by the same or different receptor proteins, the data presented herein demonstrate the similarity of β-VLDL metabolism by J774 cells and mouse peritoneal macrophages, and indicate that apoE serves as ligand for β-VLDL metabolism by both cell types.

While the evidence clearly indicates that apoprotein E is a component of β-VLDL necessary for their interaction with the macrophage cell surface, our data do not exclude the possibility that apoE cooperates with a peptide originating from apoB and remaining on the particle surface after tryptic hydrolysis. Under the conditions here employed, most of the peptides generated by tryptic hydrolysis remain associated with the surface of the VLDL particle as indicated by the cholesterol/protein ratio of the isolated digested particle (Table 2). Although the "B-26" protein and albumin are the only proteins that remain intact after trypsin hydrolysis, there must be other peptide fragments remaining on the surface of the digested β-VLDL particle. Even though these fragments are not immunologically recognizable, they may, nevertheless, participate in conjunction with apoprotein E in the macrophage interactions. However, the results of Innerarity et al. (6) suggest that native apoE added to a β-VLDL particle inactivated by reductive methylation is sufficient by itself to restore reactivity with the macrophage cell surface.

Intact apoprotein B-100 or B-48 was not required for interaction with macrophages. β-VLDL particles containing apoprotein E and either intact or degraded apoprotein B-100 demonstrated comparable abilities to stimulate cholesterol esterification in macrophages. In addition, trypsin-degraded β-VLDL particles lacking intact apoprotein B-48 or B-100 required only apoprotein E for full restoration of biological activity. This lack of significant participation of intact apoprotein B has been noted in the receptor-mediated metabolism of other VLDL particles. Apoprotein B did not play a role in the apoprotein E-mediated uptake of HTG-$VLDL_1$ by human fibroblasts (36) nor in the interaction of dog liver membranes with β-VLDL from Type III patients (37). Apoprotein B-48 does not participate in the hepatic clearance of chylomicron remnants from rat plasma (38). Recently, it has been demonstrated that binding of both chylomicron remnants and β-VLDL to hepatic apoprotein E receptors and human LDL receptors occurred independently of apoprotein B-48 (39). However, it has also been reported that unlabeled thoracic duct lymph chylomicrons, which contain apoprotein B-48 and virtually no apoprotein E, will compete with ^{125}I-labeled β-VLDL for degradation by human monocyte macrophages (31). These last observations are not readily reconciled with our current results. However, the facts that human monocyte macrophages have LDL receptors and synthesize apoprotein E (40), together with the size differences between β-VLDL and lymph chylomicrons, are worthy of further consideration. In support of our data, human monocyte macrophages were recently demonstrated to take up and degrade normal human VLDL via an apoprotein E-dependent process distinct from the LDL receptor (41).

Partial or total proteolysis of lipoproteins has been previously employed to study the role of specific apoproteins in the interaction of lipoproteins with cell surface receptors or with lipid metabolizing enzymes (36, 38, 42, 43). Gianturco et al. (44) reported that the thrombin degradation of apoE on hypertriglyceridemic VLDL enhanced the binding of this lipoprotein to mouse macrophages. This appears to be in conflict with our observations. However, there are notable differences in the response of apoE on hypertriglyceridemic VLDL to thrombin treatment and on β-VLDL to the trypsin treatment employed here. Trypsin treatment of β-VLDL degrades essentially all apoE on these particles. Thrombin treatment of hypertriglyceridemic VLDL distinguishes two groups of apoE molecules by their conformation within these particles. Only one set of apoE molecules is susceptible to thrombin hydrolysis (36). The precise domains and conformation of apoprotein E that might be involved in the interaction of β-VLDL with the macrophage cell surface remain to be clarified.

Macrophages have been implicated in the development of atherosclerosis and may be involved in the deposition of cholesterol in the arterial wall lesions (45). The presence of atherosclerotic foam cell lesions has been correlated with the appearance of β-VLDL in the plasma of cholesterol-fed dogs, suggesting that β-VLDL may contribute to the initiation of the formation of atherosclerotic plaques in these animals (5, 46). The present results, together with those of others (6, 33), provide strong evidence that apoprotein E makes an important contribution to the metabolism of cholesteryl ester-rich, beta-migrating VLDL by macrophages. Convincing evidence is also accumulating that apoprotein E is important in the catabolism of normal triglyceride-rich VLDL (47). Though the precise role of apoprotein E in atherogeneis remains to be clarified, its pivotal function in the metabolism and clearance of both triglyceride-rich and cholesteryl ester-rich VLDL by hepatocytes and macrophages will surely prove to be important.

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