Apolipoprotein E Is Resistant to Intracellular Degradation in Vitro and in Vivo

EVIDENCE FOR RETROENDOCYTOSIS

(Received for publication, April 16, 1999, and in revised form, December 14, 1999)

Patrick C. N. Rensen‡,§, Miek C. Jong‡,§, Leonie C. van Vark‡, Hans van der Boom‡, Wendy L. Hendriks‡, Theo J. C. van Berkel‡, Erik A. L. Biessen‡, and Louis M. Havekes††

From the ‡Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, University of Leiden, Sylvius Laboratory, P. O. Box 9503, 2300 RA Leiden, ¶TNL-Prevention and Health, Gaubius Laboratory, P. O. Box 2215, 2301 CE Leiden, and the Departments of Cardiology and General Internal Medicine, Leiden University Medical Center, P. O. Box 9600, 2300 RC Leiden, The Netherlands

Apolipoprotein E (apoE) plays a key role in the hepatic metabolism of triglyceride (TG)-rich lipoproteins such as chylomicrons and very low density lipoproteins (VLDL) (1–3) and TG-rich emulsions (4–5). In the blood, these particles are converted into TG-rich remnants through the hydrolysis of core TG by lipoprotein lipase (LPL) and the concomitant enrichment with apoE. Various apoE-recognition systems have been proposed to participate in remnant removal, including the low density receptor (LDLr) (6–8), a distinct specific apoE receptor (1, 9), the LDLr-related protein (8, 10, 11), and heparan sulfate proteoglycans (HSPG) alone (12, 13) or in concert with LDLr-related protein (5, 14). However, the LDLr pathway plays a substantially greater role in the overall clearance of TG-rich lipoprotein remnants in mice than the non-LDLr pathway (15, 16). It has been shown that the affinity of TG-rich lipoproteins and emulsions for the LDLr pathway and non-LDLr pathway is governed by their particle size. Whereas large particles are mainly processed via the liver-specific non-LDLr recognition site, small particles (<50 nm) are almost exclusively recognized and taken up by the LDLr (17, 18).

The intracellular metabolism of TG-rich remnants may be far more complex than for LDL, of which both the protein and lipid components are completely degraded within perinuclear lysosomes while the receptor recycles back to the cell surface (19, 20). Whereas LDL shows a single interaction of apoB with the LDLr, four molecules of apoE on TG-containing lipoproteins can bind a single LDLr (21). In addition, apoE-containing lipoproteins can interact with multiple LDLrs (22). In either case, when lipoproteins or emulsions are provided with at least four molecules of apoE, their affinity for the LDLr is 20–25-fold higher than for LDL (21–23). The multivalent binding of apoE-containing particles leading to the cross-linking of LDLrs may explain the delayed perinuclear lysosomal targeting of β-VLDL, VLDL, and (LPL-treated) VLDL as compared with LDL after equally rapid endocytosis by macrophages (24–26), human fibroblasts (27), and HepG2 cells (28), respectively. Another difference between LDL and TG-rich lipoproteins may be that their apolipoprotein components differ in their susceptibility to intracellular degradation. Preliminary data on radioiodinated apoE emulsions and LDL suggest that, in contrast to

* This work was supported by the Netherlands Heart Foundation Grants 95128 and 97067. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Division of Biopharmaceutics, Leiden/Amsterdam Center for Drug Research, University of Leiden, Sylvius Laboratory, P.O. Box 9503, 2300 RA Leiden, The Netherlands. Tel.: 31 71 5276051; Fax: 31 71 5276032; E-mail: p.rensen@lacdr.leidenuniv.nl.

†† The abbreviations used are: apoE, apolipoprotein E; bLf, bovine lactoferrin; BSA, bovine serum albumin; CO, cholesterol oleate; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; HSPG, heparan sulfate proteoglycans; LDLr, low density lipoprotein receptor; LPL, lipoprotein lipase; PBS, phosphate-buffered saline; TG, triglyceride; PAGE, polyacrylamide gel electrophoresis; EYPG, egg yolk phosphatidylcholine; VLDL, very low density lipoprotein; HDL, high density lipoprotein.
the efficient degradation of apoE, the degradation of apoE may be retarded, whereas fluorescently labeled lipids in TG-rich particles and LDL followed a similar intracellular route toward lysosomes (29, 30).

Besides its involvement in the cellular recognition and uptake of TG-rich lipoprotein remnants and emulsions, apoE is also hypothesized to play a role in the intracellular trafficking of lipids (31–36). Initial data suggested that apoE affects the transport and metabolism of free fatty acids and free cholesterol released from the lysosomes (33). In addition, apoE appeared to have a physiological role in the assembly and secretion of VLDL (34–36). Although de novo synthesized apoE in hepatocytes (37) may be used for VLDL assembly, lipoprotein-derived apoE that is taken up by the cells may also serve this purpose since apoE from radiolabeled VLDL remnants could be recovered in hepatic Golgi fractions (exocytotic compartments) after intravenous injection into mice (38).

The aim of the present study was to investigate whether apoE that is internalized by hepatocytes can escape from lysosomal degradation and can be resorbed through retroendocytosis. For this purpose, we utilized small apoE-containing TG-rich emulsions that mimic the metabolic fate of TG-rich lipoproteins in the blood (4, 39) and are exclusively taken up via the LDLr in mice (18). The data show that apoE is indeed relatively resistant to intracellular degradation as compared with the cholesteryl oleate (CO) moiety of the emulsion both in hepatoma cells in vitro (HepG2) and in hepatocytes in vivo (C57BL/6KH mice) and that apoE is resorbed at least in vitro. Since these emulsions mimic TG-rich lipoproteins, we anticipate that apoE, which has been delivered to the hepatocyte as a constituent of chylomicron remnants, can possibly be re-used for intracellular lipid trafficking, such as the assembly and secretion of VLDL, or may participate in lipoprotein remnant internalization via the “secretion-recapture” pathway (40, 41).

**MATERIALS AND METHODS**

**Animals**—10–12-Week-old male C57Bl/6KH mice weighing 23–24 g (from Broekman Instituut BV, Someren, The Netherlands) fed ad libitum with regular chow were used for all experiments.

**Chemicals**—Recombinant human apoE (isoform E3) was a generous gift from Dr. Tikva Vogel, Bio-Technology General Ltd., Rehovot, Israel, and was supplied as a lyophilized powder containing 76% apoE, 11.7% L-cysteine, and 12.0% NaHCO3 (42). ApoE was dissolved in phosphate-buffered saline (PBS), pH 7.4 (2.0 mg/ml), and stored at −80 °C. Bovine lactoferrin (bLf) was generously provided by Dr. Patrick et al. Lowry Pharmaceuticals B.V., Weesp, The Netherlands. Multiwell cell culture dishes were from Costar, Cambridge, MA. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Flow Laboratories, Irvine, UK. Human LDL (1.019–1.057 g/ml) was isolated from plasma of normal human donors by differential ultracentrifugation as described (43) and incubation with donor [3H]CO-containing liposomes, in the presence of 120 °C. Bovine lactoferrin (bLf) was generously provided by Dr. Patrick et al. Lowry Pharmaceuticals B.V., Weesp, The Netherlands. Multiwell cell culture dishes were from Costar, Cambridge, MA. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Flow Laboratories, Irvine, UK. All other chemicals were of analytical grade.

**Isolation of Lipoproteins**—Human LDL (1.019 < d < 1.063 g/ml) and HDL (1.063 < d < 1.21 g/ml) were isolated from the blood of healthy volunteers by differential ultracentrifugation as described (44) and dialyzed at 4 °C against PBS containing 1 mM EDTA, pH 7.4, with several changes of buffer.

**Protein Assay**—Protein concentrations were determined according to Lowry et al. (44) using BSA as a standard.

**Radiolabeling of (Lipo)proteins**—LDL was labeled with [3H]CO by incubation with donor [3H]CO-containing liposomes, in the presence of phospholipid (-1.8, w/w) for 8 h at 37 °C under argon in the presence of 20 mM ethyl mercurithiosalicylate (45). LDL was purified by density gradient ultracentrifugation and Superose 6® (Amersham Pharmacia Biotech) gel filtration. The specific [3H] activity was ~15 dpm/μg protein. [3H]CO-labeled LDL, apoE, and bLf were radiolabeled at pH 10.0 with carrier-free 125I according to the ICI method (46). Free 125I was removed by Sephadex G-50 medium gel filtration. More than 97, 98.3, and 99.9% of the radiolabels in LDL, apoE, and bLf, respectively, were recovered by phase-precipitation and Superose 6® gel filtration. The specific [3H] activities of radiolabeled LDL, apoE, and bLf were 114–301, 329–499, and 675 dpm/μg of protein, respectively.

**Preparation of (ApoE-Containing) Emulsions**—Emulsions were prepared according to the sonication and ultracentrifugation procedure of Redgrave and Marahan (47) from 100 mg of total lipid at a weight ratio triolein: EYPC:lysophosphatidylcholine:CO:cholesterol of 70:22:7:2:3:5:0.2:0.0, using a Soniprep 150 (MSE Scientific Instruments, UK) (4). For synthesis of radiolabeled emulsions, 100–400 μCi of [3H]CO (67.3 mCi/mg) was added. An emulsion fraction containing VLDL-sized particles was obtained by consecutive density gradient ultracentrifugation steps exactly as described (18). The emulsions were homogeneous with respect to size (low polydispersity of 0.23–0.31) and the mean particle diameter was 44.3 ± 2.6 nm (mean ± S.D.; n = 3 experiments). The emulsions were determined by photon correlation spectroscopy using a Malvern 4700 C system (Malvern Instruments, Malvern, UK) (18). The TG content of the emulsion fractions was determined with the Roche Biochemicals enzymatic kit for triacylglycerols. Association of apoE with the particles occurred by incubation of emulsions with 125I-apoE at TA:apoE = 50:0.3 (low) and 50:3.0 (high) weight ratios for 30 min at 37 °C. Emulsion-bound apoE was separated from free apoE using density gradient ultracentrifugation as described (39). The apoE contents of the resuspended emulsions were 4.6 ± 1.1 μg/mg TG (50:0.3) and 34.2 ± 2.6 μg/mg TG (50:3.0), which corresponds to 4 ± 1 and 27 ± 2 μg of apoE per particle. Emulsions were stored at 4 °C under argon and used for characterization and metabolic studies within 7 days following preparation.

**Characterization of (Radiolabeled) ApoE**—The (radiochemical) purity of apoE as well as the presence of disulfide-linked apoE homodimers (48) were checked using 10% SDS-PAGE under non-reducing conditions, using Kaleidopect prestained molecular weight standards as reference proteins. Resulting gels were stained for protein with Coomassie Blue R-250 or assayed for 125I activity by exposure of dried gels to Kodak X-Omat films. In addition, the homodimer content and the aggregation state of (emulsion-bound) 125I-apoE was examined by fast protein liquid chromatography (SMART system; Amersham Pharmacia Biotech), using a Superdex 200 column at a flow rate of 50 μl/min and with 50 mM NaF, 0.1% BSA, 0.1% or NaCl, pH 7.4, in the absence or presence of 0.5% SDS as eluent.

**Culture of HepG2 Cells**—HepG2 cells were cultured at 37 °C in a humidified 5% CO2, 95% air atmosphere in 25-cm2 flasks containing DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM HEPES, 10 mM NaHCO3, and 1% BSA. DMEM containing 1% BSA and were further incubated with DMEM containing 10% (v/v) lipoprotein-deficient serum instead of FCS.

**Cell Binding, Association, and Degradation Studies**—Binding, association, and degradation studies were performed essentially as described previously (28). For some experiments, cells were pretreated with heparinase I for 2 h at 37 °C (14). Cells were washed three times (DMEM + 1% BSA) and were incubated at 4 or 37 °C with 0.5 ml of the same medium with the indicated amounts of radiolabeled LDL or emulsions ([3H] or [125I]), in the absence or presence of an excess of unlabeled particles. After incubation, the cells were cooled to 0 °C, and the incubation media were removed. The cells were washed three times with PBS + 0.1% BSA, once with PBS, and were dissolved in 1 ml of 0.2 M NaOH. Aliquots of media and cell lysates were counted for [3H] or [125I] radioactivity, and aliquots of the cell lysates were used for protein determination. Degradation of 125I-protein in the medium was determined by separating [125I]-protein from degradation products by 10% trichloroacetic acid precipitation as described (19). To determine the intact protein content of the hydrolysate of [3H]CO into [3H] cholesterol, total lipid was extracted according to Bligh and Dyer (50) and separated using thin layer chromatography (heptane:ethyl ether:acetic acid = 60:40:1, v/v). CO, R (0.85), and cholesterol (R, 0.23) were visualized with iodine vapor, scraped off, and counted in 15 ml of Hionic Fluor (Packard Instrument Co.). Using this method, 99.6% of the emulsion-associated [3H] radioactivity appeared as [3H]CO.
Intracellular Processing and Retroendocytosis—Cells were preincubated in the presence of \(^{125}\text{I}\)-apopE-containing emulsions (60 \(\mu\)g of TG/ml) for 3 h at 18 °C, which does not impair binding and endocytosis, but blocks the fusion of endosomes with lysosomes. As a result, cell-associated emulsions accumulate in the early endosomal compartment, without being degraded (51, 52). Cells were washed with DMEM + 1% BSA to remove unbound ligand, and cell surface-bound apopE was released by a subsequent wash with heparin (770 units/ml in DMEM + 1% BSA) (53, 54). Cells were washed once with PBS + 0.1% BSA and further incubated at 37 °C with 0.5 ml of DMEM + 1% BSA in the absence or presence of HDL (0.35 mg of protein/ml) or apopE-deficient emulsion (0.56 mg TG/ml). After incubation, the media and cells were treated as described above. In addition, 500-\(\mu\)l aliquots of media, combined from triplicate samples, were subjected to density gradient ultracentrifugation at 40,000 rpm for 18 h at 4 °C as described (43). Tubes were fractionated (24 \(\times\) 0.5 ml) from top to bottom using a Multiprobe 104DT Robotic System from Packard Instrument Co., and fractions were counted for \(^{125}\text{I}\) activity. Subsequently, 400-\(\mu\)l fraction aliquots were subjected to 10% trichloroacetic acid precipitation to separate \(^{125}\text{I}\) and \(^{125}\text{I}\)-tyrosine from intact \(^{125}\text{I}\)-apopE. To identify the radioactivity within the emulsion and HDL-containing fractions as intact apopE or degradation products, the fractions were desalted by dialysis against 100-fold diluted PBS, freeze-dried, and subjected to 4–20% gradient SDS-PAGE under non-reducing conditions. The radioactivity on the gel was visualized by imaging using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA).

Liver Uptake and Serum Decay of Emulsions in Mice—Mice were anesthetized by subcutaneous injection of a mixture of ketamine (120 mg/kg body weight), Thalonal (0.03 mg/kg fentanyl and 1.7 mg/kg droperidol), and Hypnorm (1.2 mg/kg fluanisone and 0.04 mg/kg fentanyl citrate), and the abdomens were opened. \(^{3}\text{H}\)CO or \(^{125}\text{I}\)-apopE-containing emulsions (reisolated after incubation at a ratio TG:apoE = 50:3.0, w/w) were injected (150 \(\mu\)l aliquots of media, combined from triplicate samples) at 18 °C, which does not impair binding and endocytosis, but blocks fusion of endosomes with lysosomes (51, 52). Cells were washed with DMEM + 1% BSA to remove unbound ligand, and cell surface-bound apopE was released by a subsequent wash with heparin (770 units/ml in DMEM + 1% BSA) (53, 54). Cells were washed once with PBS + 0.1% BSA and further incubated at 37 °C with 0.5 ml of DMEM + 1% BSA in the absence or presence of HDL (0.35 mg of protein/ml) or apopE-deficient emulsion (0.56 mg TG/ml). After incubation, the media and cells were treated as described above. In addition, 500-\(\mu\)l aliquots of media, combined from triplicate samples, were subjected to density gradient ultracentrifugation at 40,000 rpm for 18 h at 4 °C as described (43). Tubes were fractionated (24 \(\times\) 0.5 ml) from top to bottom using a Multiprobe 104DT Robotic System from Packard Instrument Co., and fractions were counted for \(^{125}\text{I}\) activity. Subsequently, 400-\(\mu\)l fraction aliquots were subjected to 10% trichloroacetic acid precipitation to separate \(^{125}\text{I}\) and \(^{125}\text{I}\)-tyrosine from intact \(^{125}\text{I}\)-apopE. To identify the radioactivity within the emulsion and HDL-containing fractions as intact apopE or degradation products, the fractions were desalted by dialysis against 100-fold diluted PBS, freeze-dried, and subjected to 4–20% gradient SDS-PAGE under non-reducing conditions. The radioactivity on the gel was visualized by imaging using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA).

Purity of Lipid-free and Emulsion-associated ApoE—The (radiochemical) purity of apopE was assessed by 10% SDS-PAGE under non-reducing conditions (Fig. 1A). As expected, apopE appeared to be present mainly as a 34-kDa protein as determined by staining with Coomassie Blue R-250. In addition, a minor protein band with an apparent mass of ~100 kDa was observed, which has previously been shown to represent the disulfide-linked dimer of apopE (48). Accordingly, this band was not detected after reduction with \(\beta\)-mercaptoethanol (not shown). After radiolabeling of apopE, 96% of the \(^{125}\text{I}\)-activity could be recovered at the position of monomeric apopE (34 kDa). Elution of radiolabeled apopE on a Superdex® 200 column showed mainly radiolabeled tetramers (eluting at between mouse IgG and BSA) in addition to monomers (eluting similarly as ovalbumin) and a small amount of higher aggregates (eluting before IgG) (Fig. 1B). Gel filtration of \(^{125}\text{I}\)-apopE using SDS-containing eluent confirmed the presence of a small portion of \(^{125}\text{I}\)-labeled homodimers (eluting at a position close to that of BSA) (Fig. 1C) that disappeared upon reduction with \(\beta\)-mercaptoethanol (not shown). In contrast, emulsion-bound \(^{125}\text{I}\)-apopE was only monomeric, which confirms earlier observations that the interaction of apopE homodimers with lipidic particles does not withstand ultracentrifugation conditions (48).

Binding of ApoE-containing Emulsions to HepG2 Cells—To establish the contribution of HSPG and the LDLr to the recognition of apoE emulsions by HepG2 cells, binding experiments were conducted at 4 °C (Fig. 2). Removal of HSPG from the cell surface by treatment with heparinase (2.5 units/ml) resulted in...
FIG. 2. Binding of apoE emulsions to HepG2 cells. A, cells were treated (2 h at 37 °C) with heparinase (0–5 units/ml), washed, and incubated (3 h at 4 °C) with [3H]CO-labeled or 125I-labeled apoE-enriched emulsions (previously incubated at TG:apoE = 50:3.0; 60 μg of TG/ml or 125I-bLf (2 μg/ml); B and C, alternatively, non-pretreated cells were incubated (3 h at 4 °C) with [3H]CO-labeled or 125I-labeled apoE-enriched emulsions (60 μg of TG/ml) in the presence of increasing concentrations of unlabeled apoE-enriched emulsions or LDL. A–C, after incubation, cells were washed, lysed, and cell protein and cell-associated radioactivities were determined. Data are expressed as percentage of binding in the absence of treatment or competitor (55.3 ± 3.4 and 51.5 ± 3.6 ng of protein per mg of cell protein for [3H]CO-labeled and 125I-labeled emulsions, respectively). Values are means ± S.D. of triplicate incubations.

FIG. 3. Intracellular processing and apoE emulsions in HepG2 cells. Cells were incubated at 37 °C with [3H]CO-labeled (A) or 125I-labeled (B) apoE-enriched emulsions (previously incubated at TG:apoE = 50:3.0; 60 μg of TG/ml). At the indicated times, the cells were washed and lysed, and cell protein was determined. Subsequently, total lipids were extracted, and non-hydrolyzed (C) and hydrolyzed (D) [3H]CO were separated by thin layer chromatography. Alternatively, cellular associated 125I-activity was measured (O), and 125I-degradation products in the medium (●) were determined by 10% trichloroacetic acid precipitation. Values are means ± S.D. of triplicate incubations.

a 45% reduction of the binding of bLf. In contrast, only a minor effect of heparinase treatment (12–17% reduction) was observed on the binding of both the [3H]CO-labeled and 125I-labeled apoE emulsions (incubated at a TG:apoE = 50:3 weight ratio). It is thus evident that HSPG play only a minor role in the binding of the apoE-enriched emulsion (Fig. 2A). The binding of both the [3H]CO-labeled and 125I-labeled apoE-enriched emulsions was dose-dependently inhibited by an excess of unlabeled particles (Fig. 2B and C). LDL also efficiently inhibited the binding of the radiolabeled apoE emulsions for at least 93–94% (Fig. 2B and C). Taking these data together, it is evident that the applied apoE-emulsion particles are also almost exclusively recognized by the LDL receptor on HepG2 cells in vitro, whereas HSPG play only a minor role in particle recognition.

Association and Degradation of ApoE-containing Emulsions by HepG2 Cells—To evaluate whether apoE is relatively resistant to intracellular degradation, the metabolic fate of the [3H]CO and 125I-labeled apoE moiety of apoE emulsions (incubated at a TG:apoE = 50:3 weight ratio) in HepG2 cells during incubation at 37 °C was determined (Fig. 3). The CO and apoE components showed a similar time-dependent cellular uptake. However, whereas the hydrolysis of CO started within 30 min after incubation and was very effective (47% of the total uptake at 4 h), the degradation of the apoE moiety started slowly (≥120 min) and was to a much lower extent (19% at 4 h). The emulsion particles were taken up as unity since the total uptake of both the CO and apoE moieties (11.6 nmol of CO and 5.3 μg of apoE per mg of cell protein at 4 h) is proportional to the ratio of these components in the emulsion (1.9 nmol of CO per μg of apoE). The involvement of apoE in the cellular uptake of the emulsion was confirmed by a 5-fold increase in the total CO uptake as compared with the apoE-free emulsion (not shown).

Previous studies have shown that internalization of LDL by the LDL receptor results in complete lysosomal degradation of both its lipid and protein components (19, 20). Incubation of HepG2 cells with [3H]CO- or 125I-labeled LDL (20 μg of protein/ml), which resulted in a similar rate of particle uptake as compared with apoE emulsions (∼1012 particles per mg of cell protein at 4 h), led to the rapid onset of both [3H]CO hydrolysis and 125I-apoB degradation (both within 30 min) (not shown). In Fig. 4 the relative apolipoprotein degradation rates, as calculated from the fraction of degraded apolipoprotein (degraded/total uptake 125I-protein) divided by the fraction of hydrolyzed CO (hydrolyzed/total uptake [3H]CO), are shown for emulsion-associated apoE and LDL-associated apoB. It appears that apoE is far more resistant to intracellular degradation as compared with apoB. These data thus indicate that apoE emulsions, after LDL receptor-mediated uptake, may have a different intracellular fate as compared with LDL, as a result of which apoE may (partially) escape from lysosomal degradation.

Intracellular Processing and Retroendocytosis of ApoE by HepG2 Cells—Since internalized apoE was shown to be resistant to intracellular degradation, it was reasoned that intact apoE may be recovered in the medium after cellular uptake through retroendocytosis. To evaluate this hypothesis, apoE emulsions were incubated with cells for 3 h at 18 °C (51, 52). Unbound particles were removed by extensive washing with DMEM/BSA. Residual cell surface-associated 125I-activity was released by 770 units/ml heparin (53, 54), which led to a reduction in total cell association of ∼30%. The cells were further incubated at 37 °C in the absence or presence of protein-free inhibitors.
emulsion or HDL in the medium as potential acceptors of secreted apoE (55–57) (Fig. 5). A time-dependent decrease in the cellular association of apoE was observed in the absence of acceptor, with 52 ± 4% of the radioactivity still associated with the cells after 60 min of incubation. At this time point, the cell association was reduced to 45 ± 1% (Student’s t test; p < 0.05) and 38 ± 3% (p < 0.01) in the presence of the protein-free emulsion or HDL, respectively. The presence of these acceptors had no effect on the degradation rate of 125I-apoE but did result in a significantly increased secretion rate of trichloroacetic acid-precipitable radioactivity (representing intact protein) as compared with the absence of acceptor (repeated measures analysis of variance, p < 0.01 and p < 0.001, respectively). More specifically, whereas 18.6 ± 0.8% of the radioactivity secreted into the medium was recovered as precipitable protein after 4 h of incubation in the absence of acceptor, 23.0 ± 0.7 (Student’s t test; p < 0.01) and 29.8 ± 1.9% (p < 0.001) of the radioactivity could be precipitated from the medium in the presence of emulsion and HDL, respectively (Fig. 5).

Loading the cells with [3H]CO-labeled apoE emulsions at 18 °C led to the hydrolysis of the vast majority of radiolabeled into [3H]cholesterol (>90%) after 4 h of incubation at 37 °C, whereas hardly any intact [3H]CO could be detected in the medium (<3% of the initially cell-associated radiolabel) (not shown). In the same experimental set up, loading of HepG2 cells with 125I-LDL (10 μg/ml) resulted in a decrease of cellular 125I-apoE radioactivity with a half-life of ~2 h. In contrast to apoE, virtually all radioactivity released into the medium was trichloroacetic acid-soluble (>95%) (data not shown), which is in full accordance with our previous observations (28).

To evaluate whether the secreted apoE is still functional in that it recombines with lipids, aliquots of the media were harvested after 3 h of incubation and subjected to density gradient ultracentrifugation (Fig. 6). Whereas both intact and degraded apoE were detected in the bottom fractions of the tubes from media without acceptor (Fig. 6A), 45 and 50–60% of the trichloroacetic acid-precipitable radioactivity were recovered in the emulsion and HDL fractions when the respective acceptors were present in the media (Fig. 6, B and C). The integrity of apoE on these particles was confirmed by the detection of a radiolabeled 34-kDa protein in these fractions after protein separation by non-reducing 4–20% gradient SDS-PAGE, followed by imaging (insets in Fig. 6, B and C). Apparently, both emulsion particles and HDL can function as acceptors of secreted apoE, albeit that HDL is more effective than emulsion particles in stimulation of the total release of apoE under the given conditions (Fig. 5).

Intrahepatic Processing of ApoE Emulsions in Mice—Subsequently, we investigated whether the finding that apoE can escape intracellular degradation upon entry of the lysosomal route is relevant for the intrahepatic metabolism of apoE emulsions in the intact animal. We have previously shown that intravenous injection of the protein-free [3H]CO-labeled emulsion (150 μg of TG) into C57BI/6KH mice results in the monophase elimination of radiolabel from the serum with a half-life of ~45 min. Concomitantly, a progressively increasing LDLr-dependent liver uptake reaching ~45% of the injected dose at 45 min after injection was observed (18). As shown in Fig. 7, preassociation of apoE with the [3H]CO-labeled emulsion accelerated the serum clearance (t1/2 < 10 min) and liver uptake (~50% at 20 min) of the emulsion. The initial rate of serum clearance and liver uptake of the protein and lipid components of the emulsion were essentially similar (Fig. 7). In contrast, injection of an equal dose of 125I-apoE in a lipid-free state led to the rapid elimination of 80% of the injected dose from the serum within 2 min, with a high uptake by the liver (70% at 5 min after injection).

In general, the intrahepatic degradation of endocytosed apolipoproteins leads to rapid elimination of radiolabel from the liver and nonspecific distribution over the body (58), which also appears to occur with lipid-free apoE (Fig. 7). The hepatic uptake of lipid-associated apoE (43.7 ± 2.3% of the dose at 20 min after injection) was not coupled to rapid degradation, as 36.3 ± 2.1% was still present within the liver at 60 min after injection. The stability of lipid-associated apoE was also confirmed by a low level of degradation products in the liver, reaching only 8.8 ± 0.2% of the recovered radioactivity at 60 min after injection (Fig. 8). In contrast, the intrahepatic hydrolysis of [3H]CO was rapid and efficient, with 50% hydrolysis achieved at 45 min after injection.

**DISCUSSION**

Radioiodination of TG-rich lipoproteins results in the labeling of various apolipoproteins (especially apoCs) that may all have a different susceptibility to proteolysis, which hampers the interpretation of the metabolic fate of apoE. In order to chase the intracellular fate of apoE only, we thus decided to utilize VLDL-sized TG-rich emulsions (44 ± 3 nm) that can be enriched with radiolabeled apoE (4, 18). The metabolic behavior of these particles in vivo is completely dependent on the presence of the LDLr, as determined by kinetic studies on [3H]CO-labeled emulsions in wild-type versus LDLr-deficient mice (18). As a reference for LDLr-mediated processing of substrates, we used LDL, which only contains a single copy of apoB that is labeled upon radioiodination. Whereas radioiodinated apoE did contain some disulfide-linked apoE homodimers, analysis of emulsion-bound 125I-apoE confirmed that the radioactivity was associated with 34-kDa apoE only. It can thus be excluded that the results are confounded by the presence of apoE homodimers (48).

The total binding values of the emulsions, calculated from the specific radioactivities of either the [3H]CO-labeled and 125I-labeled emulsions, were similar (55.3 ± 3.4 and 51.5 ± 3.6 ng of protein/mg of cell protein, respectively), which indicates that the apoE-emulsion particle binds to the cell as a unity, without preferential binding of either the lipid or protein moiety (Fig. 2). The binding of the emulsion to HepG2 cells appeared to be largely mediated by the LDLr (~90% of the total
binding), whereas HSPG contributed for only a low extent (\(-10\%\)) as determined after treatment of the cells with heparinase. In accordance with previous observations by Ji and Mahley (59), heparinase treatment inhibited the binding of bLf to HepG2 cells by 45%, which demonstrates that HSPG had been effectively removed. The finding that HSPG are hardly involved in the binding of apoE emulsion particles is in agreement with our previous observations that, as opposed to LPL, apoE is not essential for the binding of \(b\)-VLDL to HSPG (60).

The association and degradation of the emulsion by HepG2 cells appeared to be dependent on the presence of apoE. The apoE-deficient emulsion showed a low cellular association and degradation, which was not substantially increased by the addition of 4 \(\times\) 1 molecules of apoE per emulsion particle, obtained at a TG:apoE = 50:0.3 weight ratio (not shown). In contrast, the addition of a physiologically relevant number of apoE molecules per particle (27 \(\pm\) 2 at a 50:3 weight ratio), which is similar to the apoE content of rat VLDL (29 \(\mu\)g/mg of TG, corresponding to 26 molecules per particle) (61), resulted in a 5-fold increased cellular association of the emulsion CO core. These findings correspond well with the previously reported stimulatory effect of apoE on LDLr-mediated uptake of TG-rich emulsions by J774 macrophages (33).

After cellular uptake of LDL, both its protein and lipid constituents follow the same lysosomal pathway (19, 20), although the initial rate of CO hydrolysis appears to be faster than apoB degradation (Fig. 4). This may be explained by the fact that full protein degradation (leading to release of \(^{125}\)I-Tyr) requires multiple enzymatic steps, whereas CO hydrolysis is achieved by a single enzymatic cut. In addition, the optimal conditions for efficient hydrolysis by cholesteryl esterases may be reached at an earlier stage in the endosomal pathway than for proteases. Emulsion-derived apoE is much more resistant to intracellular degradation as compared with LDL-derived apoB, as evident from a later onset of protein degradation (120 versus 30 min) and a much lower degradation rate (Fig. 4). These data are in full agreement with the observed relative intracellular stability of apoE as compared with apoB after uptake by human fibroblasts and mouse J774 macrophages (30).

After pulse labeling of HepG2 cells with apoE emulsions at 18 \(^\circ\)C, and subsequent incubation at 37 \(^\circ\)C, a time-dependent release of intact apoE into the medium (14% of the initially endocytosed apoE) could be detected. The release of apoE was increased to up to 20 and 26% in the presence of the protein-free emulsion or HDL in the medium, respectively. The gradual reappearance of intact \(^{125}\)I-apoE in the medium cannot be
explained by a slow release of apoE that was incompletely washed from the cell surface after incubation at 18 °C, since lipid-free 125I-apoE that was bound to the cell surface after incubation for 3 h at 4 °C was effectively removed by heparin (770 units/ml).

Previous pulse-chase experiments with radioiodinated VLDL also resulted in the appearance of intact protein in the medium (28). Our present data rule out that secretion of intact particles occurs upon endocytosis but rather show that apoE can selectively undergo retroendocytosis. By contrast, most LDL-derived apoB was recovered in the medium in a degraded state (>95%), which confirms previous observations (28, 62). It is thus clear that retroendocytosis is a unique feature of apoE as compared with apoB. In this study, we have not examined the intracellular fate of the other apolipoprotein constituents of TG-rich lipoproteins. A recent paper suggests that apoCs may also be released from cells upon internalization (54).

We observed that apoE, which is released from cells by retroendocytosis, can recombine with both the protein-deficient emulsion and HDL. Both the presence of the emulsion and HDL in the medium stimulated retroendocytosis of apoE, without an effect on apoE degradation. In all cases, the rate of apoE retroendocytosis may even be underestimated, since it is known that a considerable amount of newly synthesized apoE remains associated with cells instead of being secreted into the medium (56, 63). In addition, newly synthesized apoE that binds to the cell surface may partially be proteolytically degraded upon re-entry into the cell (57). It has been shown before that the presence of serum or isolated lipoproteins may prevent the degradation of re-endocytosed apoE by triggering the release of apoE from HepG2 cells (57) and macrophages (55), but it is not clear from these studies and our observations whether the applied concentration of HDL in the medium can extract all of the secreted apoE from the cell surface, especially since radiolabeled apoE must compete with HepG2-derived apoE for the binding to HDL.

In an attempt to examine the physiological relevance of our in vitro findings for the situation in vivo, we also determined the intrahepatic handling of the apoE-enriched emulsion after intravenous injection into mice. Indeed, it appeared that after simultaneous hepatic uptake of both the particle core (reflected by the CO moiety) and the preassociated apoE (45–50% of the injected dose at 20 min after injection), only 10–20% of the apoE is degraded in contrast to as much as 75% of the CO moiety at 1 h after injection (Figs. 7 and 8). It is tempting to assume that in vivo, intrahepatic apoE may also undergo retroendocytosis, resulting in the release of apoE from hepatocytes with subsequent attachment to HSPG in the space of cords.
Disse or circulating lipoproteins such as HDL. However, this hypothesis is hard to establish conclusively under the present experimental conditions.

The mechanism of apoE retroendocytosis remains an intriguing issue. In theory, apoE may be shuttled through CURL as is the case for transferrin, which returns to the cell membrane after having delivered its iron load (64, 65). The fact that the recycling of transferrin in HepG2 cells occurs with a half-time of less than 10 min (64), whereas apoE is slowly and gradually released from these cells after pulse labeling, suggests that other mechanisms should account for escaping degradation. A recent paper (54) indeed showed by microscopic analysis that incubation of fibroblasts with TG-rich lipoproteins and transferrin results in the appearance of apoE and transferrin in distinct endosomal vesicles. Alternatively, apoE may escape degradation via reversible aggregation into multimeric complexes at low pH, as suggested by Chen et al. (66), but this hypothesis evidently requires further investigation.

In conclusion, we have shown that apoE is relatively resistant to degradation after cellular uptake by hepatoma cells in vitro and hepatocytes in vivo and that retroendocytosis of apoE occurs at least in vitro. It is already known that newly synthesized apoE that is secreted and bound to cell surface HSPG can be re-endocytosed upon the binding of lipoproteins (so called secretion-recapture pathway) (40, 41, 57). Taking these data together, it can be envisioned that apoE, after synthesis and secretion by the hepatocyte, can be recycled by the cell several times until final degradation occurs. It may also be possible that endocytosed apoE is involved in the assembly and secretion of VLDL by hepatocytes (34, 35). Provided that the stability of endocytosed apoE can also be demonstrated in extracellular systems such as macrophages, our data may also implicate a role of retroendocytosed apoE in reverse cholesterol transport and regression of atherosclerosis.

Acknowledgments—We thank Dr. Tikva Vogel (Bio-Technology General, Ltd., Rehovot, Israel) for generously supplying human recombinant apoE. We thank Patrick H. C. van Berkel (Pharming Technologies BV, Leiden, The Netherlands) for providing bovine lactoferrin and Dr. Hendrik N. J. Schifferstein (Department of Marketing and Marketing Research, Agricultural University, Wageningen, The Netherlands) for statistical analysis.

REFERENCES

1. Mahley, R. W. (1988) Science 240, 622–630.
2. Weigtl, K. H. (1994) Adv. Protein Chem. 45, 249–302.
3. Mahley, R. W., and Ji, Z.-S. (1999) J. Biol. Chem. 274, 1589–1603.
4. Mortimer, B. C., Beveridge, D. J., Martins, I. J., and Redgrave, T. G. (1995) J. Biol. Chem. 270, 29767–29776.
5. Gianturco, S. H., Gotto, A. M., Jr., Hwang, S. L., Karlin, J. B., Lin, A. H., Prasad, S. C., and Bradley, W. A. (1983) Science 218, 15804–15811.
6. Gianturco, S. H., Gotto, A. M., Jr., Hwang, S. L., Karlin, J. B., Lin, A. H., Prasad, S. C., and Bradley, W. A. (1983) Science 218, 15804–15811.
7. Innerarity, T. L., Friedlander, E. J., Rall, S. C., Jr., Weisgraber, K. H., and Shinto, L. H. (1991) J. Biol. Chem. 266, 10485–10491.
8. Willnow, T. E., Sheng, Z., Ishibashi, S., and Herz, J. (1994) J. Biol. Chem. 269, 12766–12772.
9. Van Dijk, M. C. M., Ziere, G. J., Boers, W., Linthorst, C., Bijsterbosch, M. K., Dunn, W. A., Hubbard, A. L., and Aronson, N. N., Jr. (1980) J. Lipid Res. 21, 1471–1474.
10. Dunn, W. A., Hubbard, A. L., and Aronson, N. N. Jr., (1980) J. Lipid Res. 21, 1471–1474.
11. Beisiegel, U., Weber, W., and Hilpert, J. (1995) Circulation 92, suppl. I-491.
12. Hamilton, R. L., Wong, J. S., Guo, L., Srisawa, S., and Havel, R. J. (1990) J. Biol. Chem. 265, 1589–1603.
13. Dahan, S., Aghvamia, J. P., Linton, M. F., Hasty, A. H., and Swift, L. L. (1999) J. Biol. Chem. 274, 8696–8700.
14. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
16. Ishibashi, S., Perrey, S., Chen, Z., Osuga, J., Shimada, M., and Ohashi, K. (1993) J. Clin. Invest. 92, 258–266.
17. Lombardi, P., Mulder, M., Van der Boom, H., Frants, R. R., and Havekes, L. M. (1993) J. Biol. Chem. 268, 26113–26119.
18. Rensen, P. C. N., and Van Berkel, T. J. C. (1996) J. Biol. Chem. 271, 12766–12772.
19. Goldstein, J. L., and Brown, M. S. (1974) J. Biol. Chem. 249, 15153–15162.
Apolipoprotein E Is Resistant to Intracellular Degradation in Vitro and in Vivo: EVIDENCE FOR RETROENDOCYTOSIS
Patrick C. N. Rensen, Miek C. Jong, Leonie C. van Vark, Hans van der Boom, Wendy L. Hendriks, Theo J. C. van Berkel, Erik A. L. Biessen and Louis M. Havekes

J. Biol. Chem. 2000, 275:8564-8571. doi: 10.1074/jbc.275.12.8564

Access the most updated version of this article at http://www.jbc.org/content/275/12/8564

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 47 of which can be accessed free at http://www.jbc.org/content/275/12/8564.full.html#ref-list-1