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

EVIDENCE FOR RETROENDOCYTOSIS*

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Apolipoprotein E (apoE) is an important determinant for the uptake of triglyceride-rich lipoproteins and emulsions by the liver, but the intracellular pathway of apoE following particle internalization is poorly defined. In the present study, we investigated whether retroendocytosis is a unique feature of apoE as compared with apoB by studying the intracellular fate of very low density lipoprotein-sized apoE-containing triglyceride-rich emulsion particles and LDL after LDLr-mediated uptake. Incubation of HepG2 cells with [³H]cholesteryl oleate-labeled particles at 37 °C led to a rapid release of [³H]cholesterol within 30 min for both LDL and emulsion particles. In contrast, emulsion-derived ¹²⁵I-apoE was more resistant to degradation (≥120 min) than LDL-derived ¹²⁵I-apoB (30 min). Incubation at 18 °C, which allows endosomal uptake but prevents lysosomal degradation, with subsequent incubation at 37 °C resulted in a time-dependent release of intact apoE from the cells (up to 14% of the endocytosed apoE at 4 h). The release of apoE was accelerated by the presence of protein-free emulsion (20%) or high density lipoprotein (26%). Retroendocytosis of intact particles could be excluded since little intact [³H]cholesteryl oleate was released (<3%). In contrast, the degradation of LDL was complete with virtually no secretion of intact apoB into the medium. The intracellular stability of apoE was also demonstrated after hepatic uptake in C57Bl/6 mice. Intravenous injection of ¹²⁵I-apoE and [³H]cholesteryl oleate-labeled emulsions resulted in efficient LDLr-mediated uptake of both components by the liver (45–50% of the injected dose after 20 min). At 1 h after injection, only 15–20% of the hepatic ¹²⁵I-apoE was degraded, whereas 75% of the [³H]cholesteryl oleate was hydrolyzed. From these data we conclude that following LDLr-mediated internalization by liver cells, apoE can escape degradation and can be resecreted. This sequence of events may allow apoE to participate in its hypothesized intracellular functions such as mediator of the post-lysosomal trafficking of lipids and very low density lipoprotein assembly.

Apolipoprotein E (apoE)[1] 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-recognizing 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 radio-iodinated apoE emulsions and LDL suggest that, in contrast to

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[1] The abbreviations used are: apoE, apolipoprotein E; bLf, bovine lactoferrin; BSA, bovine serum albumin; CO, cholesteryl 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; EYPC, egg yolk phosphatidylcholine; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

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the efficient degradation of apoB, 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 resecreted 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 resecreted 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 the *in vivo* 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% NaHCO$_3$ (42). ApoE was dissolved in phosphate-buffered saline (PBS), pH 7.4 (2.0 mg/ml), and stored under argon at −80 °C. Bovine lactoferrin (bLf) was generously provided by Dr. Patrick van Berkel, Pharming Technologies BV, Leiden, The Netherlands. [1α,2α-^{3}H]Cholesteryl oleate ([^{3}H]CO) and ^{125}I (carrier-free) in NaOH were purchased from Amersham Pharmacia Biotech. Triolein (99% pure) and egg yolk phosphatidylcholine (EYPC; 98%) were from Fluka, Buchs, Switzerland. L-α-Lysophosphatidylcholine (99%), cholesterol (>99%), bovine serum albumin (BSA, fraction V), ethyl mercurithiosalicylate, monensin, and heparinase I (EC 4.2.2.7) from *Flavobacterium heparinum* were obtained from Sigma. Cholesteryl oleate (CO; 97%) was from Janssen, Beersse, Belgium. Peroxidase type II (200 units/mg), Precipath® L, and EDTA were from Roche Molecular Biochemicals. HEPES was from Merck, and heparin (5000 units/ml) was from Leo Pharmaceutical Products 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 (43) and dialyzed at 4 °C against PBS containing 1 mM EDTA, pH 7.4, with repeated 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 [^{3}H]CO by incubation with donor [^{3}H]CO-containing liposomes, in the presence of human lipoprotein-deficient serum. In short, liposomes were created by sonication of 25 mg of EYPC and 200 μCi of [^{3}H]CO using a Soniprep

150 (MSE Scientific Instruments, Crawley, UK) for 15 min at 18 μm output, and concentrated by density gradient ultracentrifugation. Subsequently, LDL was incubated with the liposomes (protein:liposomal 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 ^{3}H activity was ~15 dpm/ng protein. [^{3}H]CO-labeled LDL, apoE, and bLf were radioiodinated at pH 10.0 with carrier-free ^{125}I according to the ICl method (46). Free ^{125}I was removed by Sephadex G-50 medium gel filtration. More than 97, 98.5, and 99.5% of the radiolabels in LDL, apoE, and bLf, respectively, were 10% trichloroacetic acid-precipitable. The specific ^{125}I-activities of LDL, apoE, and bLf were 114–301, 329–499, and 675 dpm/ng of protein, respectively.

Preparation of (ApoE-Containing) Emulsions—Emulsions were prepared according to the sonication and ultracentrifugation procedure of Redgrave and Maranhao (47) from 100 mg of total lipid at a weight ratio triolein:EYPC:lysophosphatidylcholine:CO:cholesterol of 70:22.7:2.3:3.0:2.0, using a Soniprep 150 (MSE Scientific Instruments, UK) (4). For synthesis of radiolabeled emulsions, 100–400 μCi of [^{3}H]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 = 6) as 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 Molecular Biochemicals enzymatic kit for triacylglycerols. Association of apoE with the particles occurred by incubation of emulsions with (^{125}I-)apoE at TG: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 reisolated 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 molecules 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 Kaleidoscope prestained molecular weight standards as reference proteins. Resulting gels were stained for protein with Coomassie Blue R-250 or assayed for ^{125}I activity by exposure of dried gels to Kodak X-Omat films. In addition, the homodimer content and the aggregation state of (emulsion-bound) ^{125}I-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 NaP$_i$, 0.15 M 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% CO$_2$, 95% air atmosphere in 25-cm^2 flasks containing DMEM supplemented with 10% (v/v) heat-inactivated FCS, 20 mM HEPES, 10 mM NaHCO$_3$, 100 units/ml penicillin, and 100 μg/ml streptomycin. At 6–7 days prior to each experiment, cells were seeded into 2-cm^2 12-well dishes. At 24 h before the assays, cells were washed with 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 (^{3}H or ^{125}I), 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 ^{3}H or ^{125}I radioactivity, and aliquots of the cell lysates were used for protein determination. Degradation of ^{125}I-protein in the medium was determined by separating ^{125}I-protein from degradation products by 10% trichloroacetic acid precipitation as described (19). To determine the intracellular hydrolysis of [^{3}H]CO into [^{3}H]cholesterol, total lipid was extracted according to Bligh and Dyer (50) and separated using thin layer chromatography (heptane:diethyl ether:acetic acid = 60:40:1, v/v). CO (R_f 0.85) and cholesterol (R_f 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 ^{3}H radioactivity appeared as [^{3}H]CO.

Intracellular Processing and Retroendocytosis—Cells were preincubated in the presence of ^{125}I-apoE-containing emulsions (60 μg 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 apoE 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 apoE-deficient emulsion (0.50 mg of TG/ml). After incubation, the media and cells were treated as described above. In addition, 500-μl 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 × 0.5 ml) from top to bottom using a Multiprobe 104DT Robotic System from Packard Instrument Co., and fractions were counted for ^{125}I activity. Subsequently, 400-μl fraction aliquots were subjected to 10% trichloroacetic acid precipitation to separate ^{125}I and ^{125}I-tyrosine from intact ^{125}I-apoE. To identify the radioactivity within the emulsion and HDL-containing fractions as intact apoE 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), Thalamonal (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. [3H]CO or ^{125}I-apoE-labeled apoE-containing emulsions (reisolated after incubation at a ratio TG:apoE = 50:3.0, w/w) were injected (150 μg of TG, corresponding with 5 μg of apoE) via the inferior vena cava. At the indicated times, blood samples (<50 μl) and liver lobules were taken and processed as described in detail (18). Radioactivity in duplicate serum samples of 10 μl was counted either directly (^{125}I) or in 2.5 ml of Emulsifier Safe (Packard Instrument Co.) ([3H]). The total serum volumes of C57Bl/6KH mice were 1.068 ± 0.066 ml, as previously determined (18). Radioactivity in liver samples was also counted directly (^{125}I) or in 15 ml of Hionic Fluor (Packard Instrument Co.) after solubilization in 0.5 ml of Soluene®-350 (Packard) for 5 h at 65 °C ([3H]). Radioactivity values are corrected for the serum radioactivity (84.7 μl/g wet weight) present at the time of sampling (18). Since liver lobules were partly used for determination of particle degradation, total liver weights were estimated from the following equation: liver mass (g) = −0.204 + (0.0560 × body mass (g)).

Intrahepatic Processing of Emulsions in Mice—To determine the intrahepatic degradation of ^{125}I-apoE, aliquots of liver lobules were immediately frozen in liquid N_2, homogenized in ice-cold PBS, pH 7.4, and subjected to 10% trichloroacetic acid precipitation. The intrahepatic conversion of [3H]CO into [3H]cholesterol was assayed after similar freezing and homogenization of liver aliquots. Lipids were extracted and separated as described above.

RESULTS

Purity of Lipid-free and Emulsion-associated ApoE—The (radiochemical) purity of apoE was assessed by 10% SDS-PAGE under non-reducing conditions (Fig. 1A). As expected, apoE 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 apoE (48). Accordingly, this band was not detected after reduction with β-mercaptoethanol (not shown). After radiolabeling of apoE, 96% of the ^{125}I-activity could be recovered at the position of monomeric apoE (34 kDa). Elution of radiolabeled apoE on a Superdex® 200 column showed mainly radioiodinated tetramers (eluting in 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}I-apoE using SDS-containing eluent confirmed the presence of a small portion of ^{125}I-labeled homodimers (eluting at a position close to that of BSA) (Fig. 1C) that disappeared upon reduction with
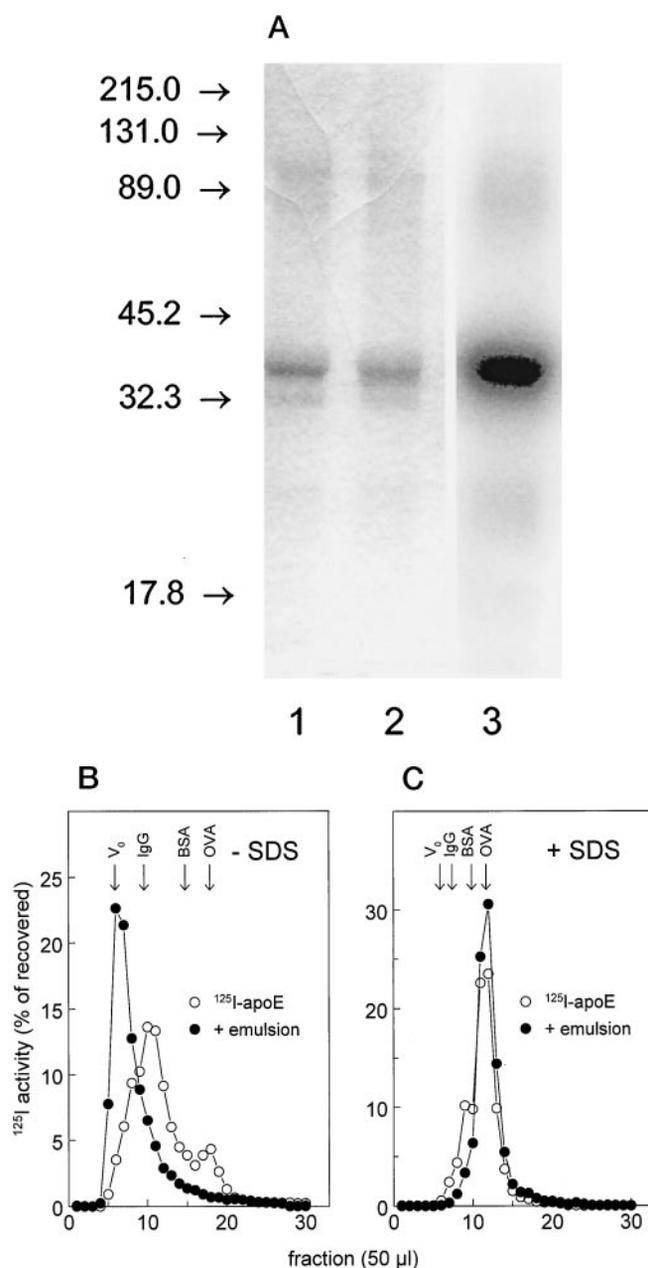
FIG. 1. **Electrophoresis and chromatography of (radiolabeled) apoE.** *A,* unlabeled (*lane 1*) and ^{125}I-labeled (*lanes 2* and *3*) apoE (4 μg) were added to SDS incubation buffer without β-mercaptoethanol (non-reducing conditions) and subjected to 10% SDS-PAGE. After electrophoresis, proteins were stained with Coomassie Blue R-250 (*lanes 1* and *2*), and ^{125}I activity was detected by autoradiography (*3*). *B* and *C,* lipid-free (○) and emulsion-bound (●) ^{125}I-apoE (1.6 μg) were eluted on a Superdex® 200 column using 50 mM NaP$_i$, 0.15 M NaCl, pH 7.4, in the absence (*B*) or presence (*C*) of 0.5% SDS, and fractions were analyzed for radioactivity. *Arrows* indicate the void volume (V_0) and the elution positions of mouse IgG (M_r 155,000), BSA (M_r 66,000), and ovalbumin (M_r 45,000).

β-mercaptoethanol (not shown). In contrast, emulsion-bound ^{125}I-apoE was only monomeric, which confirms earlier observations that the interaction of apoE 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.
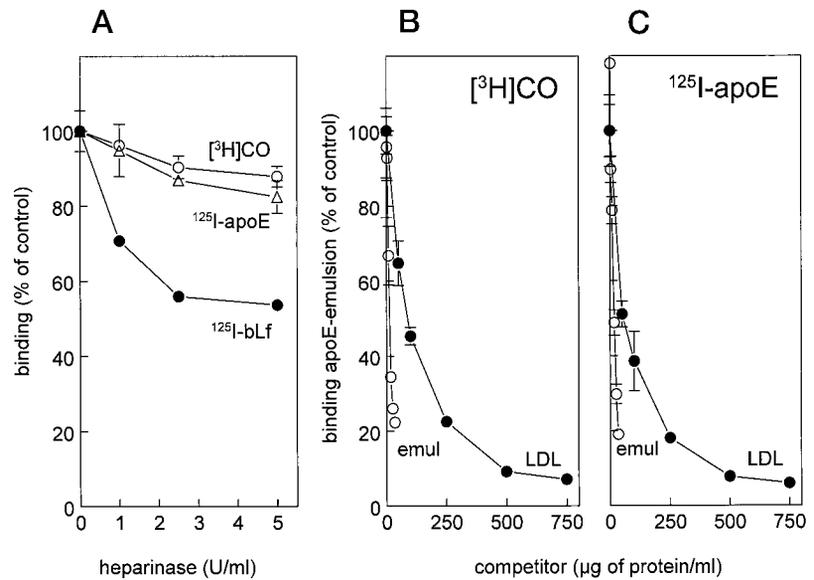
a 45% reduction of the binding of bLf. In contrast, only a minor effect of heparinase treatment (12–17% reduction) was observed on the binding of both the [³H]CO-labeled and ¹²⁵I-labeled apoE-enriched emulsions (incubated at a TG:apoE = 50:3 weight ratio). It is thus evident that HSPG play only a minor role in the binding of the apoE-enriched emulsion (Fig. 2*A*). 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. 2, *B* and *C*). LDL also efficiently inhibited the binding of the radiolabeled apoE emulsions for at least 93–94% (Fig. 2, *B* and *C*). Taking these data together, it is evident that the applied apoE-emulsion particles are also almost exclusively recognized by the LDLr 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-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 LDLr results in complete lysosomal degradation of both its lipid and protein components (19, 20). Incubation of HepG2 cells with [³H]CO or ¹²⁵I-apoB-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-
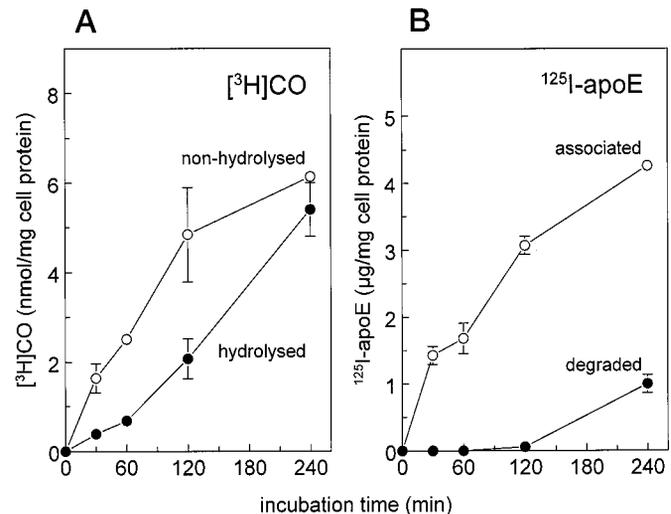
FIG. 3. **Intracellular processing and apoE emulsions in HepG2 cells.** Cells were incubated at 37 °C with [³H]CO-labeled (*A*) or ¹²⁵I-labeled (*B*) apoE-enriched emulsions (previously incubated at TG:apoE = 50:3.0; 60 μg of TG/ml). At the indicated times, the cells were washed and lysed, and cell protein was determined. Subsequently, total lipids were extracted, and non-hydrolyzed (○) and hydrolyzed (●) [³H]CO were separated by thin layer chromatography. Alternatively, cellular associated ¹²⁵I-activity was measured (○), and ¹²⁵I-degradation products in the medium (●) were determined by 10% trichloroacetic acid precipitation. Values are means ± S.D. of triplicate incubations.

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 LDLr-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
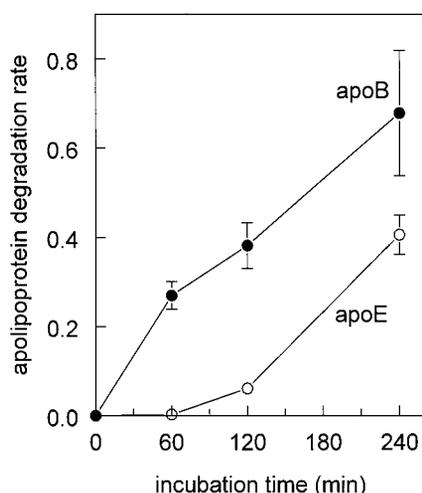
FIG. 4. **Degradation rates of apoE *versus* apoB in HepG2 cells.** Cells were incubated at 37 °C with [³H]CO- or ¹²⁵I-labeled apoE-enriched emulsions (60 μg of TG/ml) or LDL (20 μg of protein/ml). At the indicated times, non-hydrolyzed and hydrolyzed [³H]CO as well as cell-associated and degraded ¹²⁵I-apolipoprotein were determined as described in the legend to Fig. 1. The apolipoprotein degradation rates (fraction-degraded ¹²⁵I-apolipoprotein/fraction-hydrolyzed [³H]CO) were calculated. Values are means ± S.D. of triplicate incubations.

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 $52 \pm 4\%$ of the radioactivity still associated with the cells after 60 min of incubation. At this time point, the cell association was reduced to $45 \pm 1\%$ (Student's t test; $p < 0.05$) and $38 \pm 3\%$ ($p < 0.01$) 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 $18.6 \pm 0.8\%$ 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 $29.8 \pm 1.9\%$ ($p < 0.001$) 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 radiolabel 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-apoB 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. 6*A*), 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 C57Bl/6KH mice results in the monophasic 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 ($t_{1/2} < 10$ 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 ($43.7 \pm 2.3\%$ of the dose at 20 min after injection) was not coupled to rapid degradation, as $36.3 \pm 2.1\%$ 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 $8.8 \pm 0.2\%$ 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

Fig. 5. **Intracellular processing of endocytosed apoE in HepG2 cells.** Cells were incubated for 3 h at 18 °C with ^{125}I-apoE emulsions (previously incubated at TG:apoE = 50:3.0; 60 μg of TG/ml), extensively washed in the presence of 770 units/ml heparin, and further incubated in the absence (*A*) and presence of emulsion (0.50 mg of TG/ml) (*B*) or HDL (0.35 mg of protein/ml) (*C*) starting at $t = 0$. At the indicated times, the amount of cell-associated ^{125}I radioactivity (●), ^{125}I-degradation products in the medium (○), and trichloroacetic acid-precipitable ^{125}I-protein (▲) in the medium were determined. Data are expressed as percentage of initially cell-associated ^{125}I activity (41.2 ± 4.4 ng of apoE per mg of cell protein). Values are means ± S.D. of triplicate incubations.
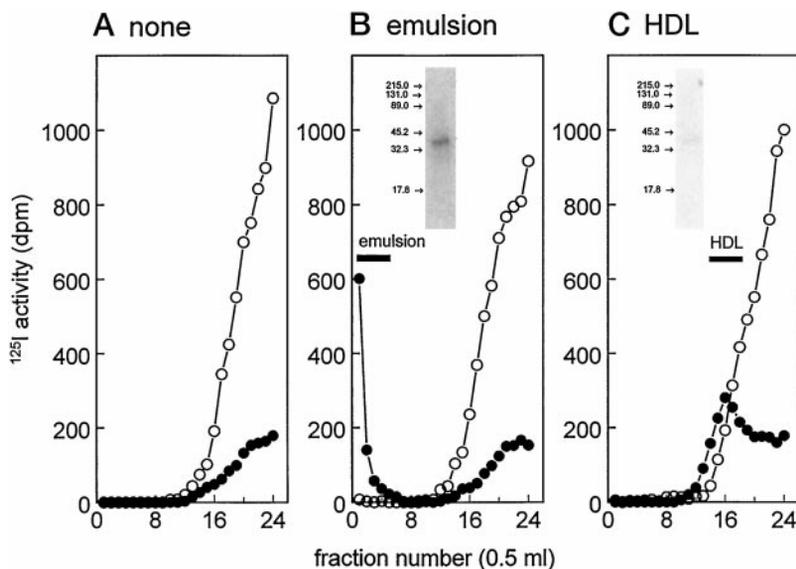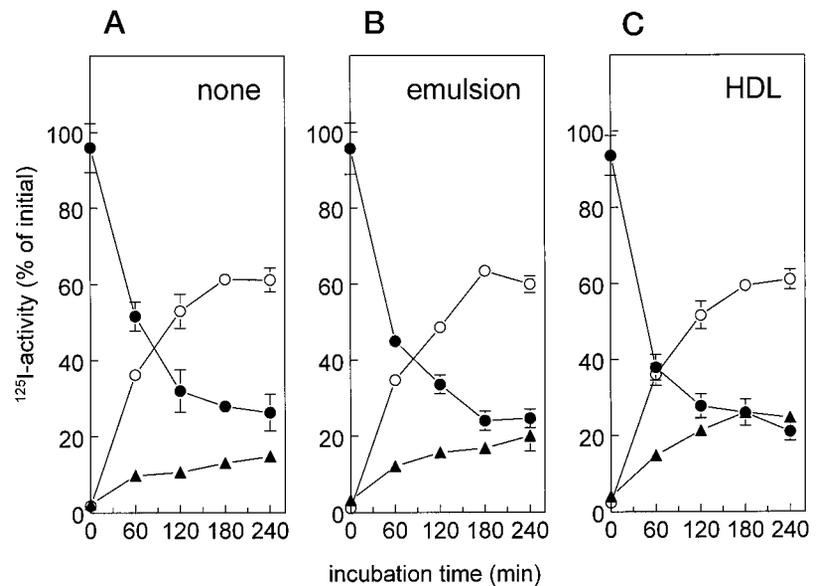
Fig. 6. **Density gradient ultracentrifugation profiles of intact and degraded ^{125}I-apoE in media of HepG2 cells.** Cells were incubated with ^{125}I-apoE emulsions in the absence (*A*) or presence of emulsion (*B*) or HDL (*C*) as described in the legend to Fig. 5. At 180 min, media were collected and subjected to density gradient ultracentrifugation. The gradients were subdivided from *top* (*fraction 1*) to *bottom* (*fraction 24*), and 10% trichloroacetic acid precipitation on 400-μl aliquots was performed to discriminate between degraded (○) and intact (●) protein. *Insets,* emulsion-containing fractions (*B*, *fractions* 1 and 2) and HDL-containing fractions (*C*, *fractions* 13–17) were dialyzed and subjected to non-reducing 4–20% SDS-PAGE, and radioactivity was visualized by imaging.

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 β-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 ± 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 ± 2 at a 50:3 weight ratio), which is similar to the apoE content of rat VLDL (29 μg/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 °C, and subsequent incubation at 37 °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

FIG. 7. **Liver uptake and serum decay of apoE emulsions in mice.** [³H]CO-labeled (○) or ¹²⁵I-apoE-labeled (●) apoE emulsions (150 µg of TG, corresponding to 5 µg of apoE) or lipid-free ¹²⁵I-apoE (5 µg) (▲), were injected into anesthetized mice. At the indicated times, the liver uptake (*left*) and serum decay (*right*) were determined. Liver values are corrected for serum radioactivity. Values are means ± variation of two experiments.
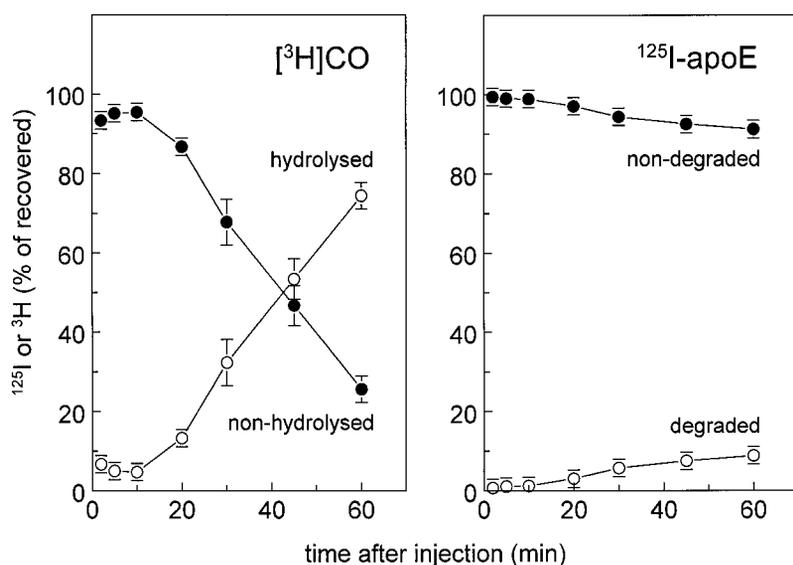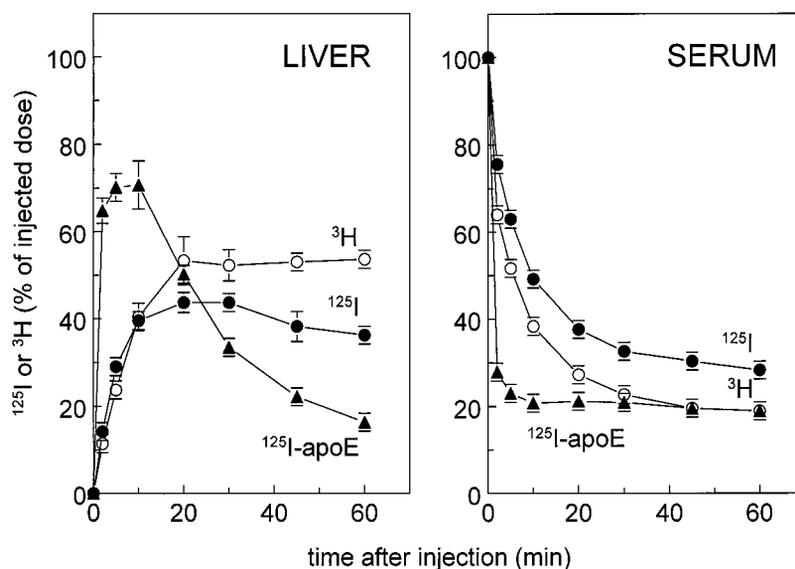
FIG. 8. **Intrahepatic degradation of apoE emulsions in mice.** [³H]CO-labeled (*left*) or ¹²⁵I-apoE-labeled (*right*) apoE emulsions (150 µg of TG) were injected into anesthetized mice. At the indicated times, liver lobules were taken, immediately frozen in liquid N₂, and homogenized in ice-cold PBS. After total lipid extraction, intact (●) and hydrolyzed (○) [³H]CO were separated by thin layer chromatography. Alternatively, intact ¹²⁵I-apoE (●) was separated from degradation products (○) by 10% trichloroacetic acid precipitation. Values are means ± variation of two experiments.

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

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 extrahepatic cells such as macrophages, our data may also implicate a role of retroendocytosed apoE in reverse cholesterol transport and regression of atherosclerosis.

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Apolipoprotein E Is Resistant to Intracellular Degradation *in Vitro* and *in Vivo* :
EVIDENCE FOR RETROENDOCYTOSIS

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