Regulated turnover of a cell surface-associated pool of newly synthesized apolipoprotein E in HepG2 cells

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Abstract **Newly synthesized apolipoprotein E (apoE) is incompletely secreted and partially degraded by hepatocytes. To evaluate current models concerning apoE's role in remnant lipoprotein clearance by the liver, we performed a detailed and quantitative pulse-chase analysis of apoE secretion, subcellular distribution, and proteolytic degradation by the human hepatoma cell line HepG2. Only about 35% of newly synthesized apoE were found to be secreted to the culture medium. As determined by a protease-protection assay, a substantial amount of newly synthesized apoE was transported to the cell surface, constituting more than half of the cellular apoE under steady-state conditions. A subpopulation representing almost 40% of newly secreted apoE was rapidly rebound to the cell surface, indicating a dynamic equilibrium between cell surface and secreted apoE. These pools of newly synthesized apoE were subject to proteolytic turnover that occurred in lysosomes, presumably after re-endocytosis. We found that the proteolytic turnover of cell surface and secreted apoE was regulated by the availability of apoE ligands, being almost completely suppressed by the presence of lipoprotein-containing human serum or isolated lipoproteins, namely LDL and HDL. The characteristics of regulated degradation of cell surface apoE shed new light on potential physiological functions of this pool of apoE. Our results provide evidence that hepatocytes are capable of forming a large pool of cell surface-associated apoE, thereby supporting the previously proposed secretion-recapture model for apoE.—**Schmitt, M., and T. Grand-Perret. **Regulated turnover of a cell surface-associated pool of newly synthesized apolipoprotein E in HepG2 cells.** ***J. Lipid Res.*** **1999.** 40: **39–49.**

Supplementary key words apolipoprotein E • secretion • cell surface • proteolysis • HepG2 cells • secretion-recapture model

Apolipoprotein E (apoE) is a component of several classes of lipoproteins, such as HDL, VLDL, and chylomicrons (1). Clearance of some of these particles from the circulation is mediated, at least in part, by apoE, which serves as a ligand for receptors of the LDL receptor family (2). ApoE interacts with the LDL receptor itself, the LDL receptor-related protein (LRP), as well as with other receptors such as the VLDL receptor (3–7). While apoE mRNA and protein have been detected in a large variety of tissues and cell types, the liver is responsible for about 70–80% of apoE synthesis (8–10).

The secretion of apoE has been studied in some detail, mostly in the human hepatoma cell line HepG2 and in macrophages (11–16). A considerable amount of newly synthesized apoE is not secreted to the culture medium, but instead remains associated with cells (15–17), forming a cell-associated pool of apoE. Cellular apoE has been detected at different locations (17–29), some of which are controversially discussed (19, 21, 27, 28). Several groups localized apoE on the cell surface of hepatocytes and macrophages (17, 19, 20, 23, 25, 28), where it is associated with heparan sulfate proteoglycans (HSPG; 17, 23, 28). In addition, intracellular apoE was reported to be localized in Golgi stacks (19, 21), endosomes (19, 21, 26, 29), lysosomes (26), peroxisomes (19), and a specialized part of the ER, the so-called mitochondria-associated membranes (24). In neurons, apoE has even been localized to the cytosol (18, 22). It is speculated that intracellular pools of apoE might participate in intracellular lipid metabolism and transport (19, 21, 30, 31).

Proteolytic degradation of newly synthesized apoE has been reported by several groups (15, 16, 26, 32–34). Still, the degradation pathway remains a matter of debate. A participation of cytosolic calpains in apoE degradation in HepG2 cells (33), as well as proteasomal degradation of apoE in macrophages (34) were reported. At present, most evidence points to lysosomal degradation of apoE, which seems to occur in both HepG2 cells and macrophages (26, 33). In this case, the route apoE might take on its way to lysosomes is not known. The discussed pathways, direct transport to lysosomes or transit via the cell surface, are not mutually exclusive (16, 26, 27, 33).

The secretion–recapture model (19, 35–39) assigns a key function in remnant lipoprotein clearance to hepatocyte apoE. According to this model, lipid-free apoE is

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; LRP, LDL receptor-related protein; HSPG, heparan sulfate proteoglycans.

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secreted to hepatocyte cell surface microvilli, where it is bound to HSPG (17, 19). Soluble apoE, also thought to be of hepatic origin, is enriched in the sinusoidal space of Disse (19). These two pools of apoE associate with apoE-poor remnant lipoproteins and facilitate their binding to the cell surface, followed by transfer to apoE-specific receptors (23, 25, 40). Subsequently, remnant lipoproteins are internalized via endocytosis. Although the LDL receptor is able to mediate remnant endocytosis (41), the LRP is thought to be the main remnant receptor (23, 40). At present, it is not clear whether HSPG and the LRP work independently, as a complex, or in a stepwise process (23, 37, 40). The secretion–recapture model is based on results obtained with experimental systems where either purified apoE was added in excess or the protein was strongly overexpressed (23, 25, 40, 42). Thus, the hepatic origin of cell surface apoE necessary for the recapture of remnant lipoproteins remains to be validated. A recent study challenges the supposed origin of apoE involved in the secretion–recapture process, as apoE synthesized by macrophages was shown to mediate remnant lipoprotein clearance by hepatocytes in bone marrow-transplanted apoE knock-out mice (43). The purpose of the present study was to analyze the secretion, localization, and degradation of endogenous apoE synthesized by the well-characterized human hepatoma cell line HepG2 in order to assess the physiological relevance of these processes with regard to the secretion–recapture model.

MATERIALS AND METHODS

Materials

[^{35}S]methionine was purchased from NEN Life Science Products. Cell culture media and reagents were purchased from Gibco BRL Life Technologies, with the exception of methionine-free RPMI 1640 (Eurobio) and fetal bovine serum (HyClone). T75 flasks and 6-well dishes were from TPP. Polyclonal antisera against human apoA-I, apoB, apoE, and human albumin were purchased from The Binding Site, Sigma Diagnostics, WBAG Resources, and Biosys, respectively. Isolated HDL and LDL were purchased from Biomedical Technologies INC. The ECL detection system was obtained from Amersham. Sulfo-NHS-biotin was obtained from Pierce. Aprotinin was purchased from Calbiochem. All other reagents were from Sigma.

Cell culture

The human hepatoma cell line HepG2 (44) was obtained from the American Type Culture Collection. Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in T75 flasks containing Eagle's basal medium (BME) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids (100 μm each), sodium pyruvate (1 mm), l-glutamine (2 mm), penicillin G (10 U/ml), and streptomycin (10 μg/ml). For pulse-chase experiments, cells were treated with 0.05% trypsin in 0.2% EDTA when they had reached 85–90% confluency. After resuspension in BME-medium supplemented as indicated above, cells were seeded in 6-well culture dishes (0.75–1 × 10^6 cells/4 ml) medium and grown for 3–4 days until they reached about 90% confluency.

Pulse-chase experiments and immunoprecipitations

Prior to metabolic labeling, HepG2 cells were washed twice with sterile Dulbecco's PBS. Cells were labeled for 10 or 15 min at 37°C with 1 ml methionine-free RPMI 1640 medium containing 0.25 mCi/ml [^{35}S]methionine. After removal of the [^{35}S]methionine-containing medium, the cells were washed twice with Dulbecco's PBS. Chase reactions were performed at 37°C in 1 ml RPMI 1640 medium containing unlabeled methionine for various periods of time. When indicated in the figure legends, chase reactions were performed in the presence of 10% human serum, 100 μg/ml isolated human LDL, or 10 μg/ml isolated human HDL. After the chase reaction, the cell culture medium was removed and the cells were lysed by addition of 200 μl 0.5% SDS in the presence of 100 μg/ml leupeptin, 100 μg/ml pepstatin A, 1 mm AEBSF, and 100 KIU/ml aprotinin. Culture media were adjusted to the same concentrations of SDS and protease inhibitors. For immunoprecipitation, cell lysates containing identical amounts of protein were diluted to 1 ml with water. Immunoprecipitation buffer was added to the diluted cell lysates and to the culture media so that final concentrations of 1% deoxycholate, 1% Triton X-100, 20 mm NaCl, 0.5 mm EDTA, 20 mm Tris/HCl, pH 7.2, were reached. The samples were precleared with Protein G-Sepharose beads in the absence of antibodies for 2 h. Immunoprecipitations of apoE, apoA-I, apoB, and albumin were performed with the respective polyclonal antibodies. The immunocomplexes were precipitated with Protein G-Sepharose beads. Samples were subjected to SDS-PAGE (45) and subsequently analyzed and quantified by phosphorimager analysis using a Storm 860 (Molecular Dynamics).

Trypsin treatment to quantify cell surface apoE

To determine the ratio of cell surface-associated versus intracellular apoE, HepG2 cells were incubated on ice with 50 or 200 μg/ml trypsin for 30 min. The treatment was stopped by addition of soybean trypsin inhibitor to a final concentration of 4 mg/ml. Samples were analyzed by immunoprecipitation, SDS-PAGE, and phosphorimager quantification as described above.

Several controls were performed to prove the reliability of this method; the trypsin-concentration used was in the optimal range, as higher concentrations did not lead to further apoE degradation. Secreted apoE was totally degraded under these conditions, indicating that the used concentration was high enough to completely degrade accessible apoE. As judged by Western blot analysis, no loss of several intracellular proteins occurred, indicating that cells remained physically intact during trypsin treatment. Cells were not detached from the culture dishes by the trypsin treatment. Before trypsin treatment, total cell surface proteins were biotinylated for 15 min on ice using 1 mg/ml Sulfo-NHS-biotin. Sulfo-NHS-biotin was removed by washing the cells three times with Dulbecco's PBS. Detection of biotinylated proteins was performed after SDS-PAGE and transfer to nitrocellulose membranes using a streptavidin–horseradish peroxidase conjugate and the ECL detection system (Amersham). By this method, the efficiency of the trypsin treatment to remove total biotinylated cell surface proteins was estimated to be at least 90%.

Determination of binding and degradation of newly synthesized apoE by HepG2 cells

To collect newly synthesized, radiolabeled apoE, HepG2 cells were metabolically labeled with [^{35}S]methionine for 15 min as described above. After removing the labeling medium

and washing, cells were incubated further with 650 μl RPMI 1640 containing unlabeled methionine to allow secretion of apoE. When indicated in the figure legends, 10% human serum, 100 μg/ml isolated human LDL, or 10 μg/ml isolated human HDL were present during this incubation. After 2 h, culture media of several wells containing secreted, radiolabeled proteins were pooled and centrifuged (5 min, 3000 *g*) to pellet possibly contaminating cells. This apoE-containing culture medium was added to non-radiolabeled HepG2 cells (1 ml/well). Except for the radiolabeling, these HepG2 cells had been treated identically as the cells that were used to collect the secreted radiolabeled apoE. The different volumes of culture medium used allowed a certain concentration of apoE in comparison to the number of cells per well. Cells were incubated on ice or at 37°C for different times to allow apoE binding and uptake. When indicated in the figure legends, the binding reactions were performed in the presence of 100 μm chloroquine, 1 mm NH_4Cl, 10 μg/ml heparin, 10% human serum, 100 μg/ml isolated human LDL, or 10 μg/ml isolated human HDL. Samples were analyzed by immunoprecipitation, SDS-PAGE, and phosphorimager quantification as described above.

RESULTS

ApoE is incompletely secreted from HepG2 cells, giving rise to a cell-associated pool that is proteolytically degraded

In order to assess the secretion of newly synthesized apoE by HepG2 cells quantitatively, we performed a detailed pulse-chase analysis (**Fig. 1A**, **B**). Cells were radiolabeled with [^{35}S]methionine for 10 min, followed by a chase in the absence of [^{35}S]methionine for different lengths of time to allow secretion to proceed. The short pulse period was chosen to facilitate interpretation of the

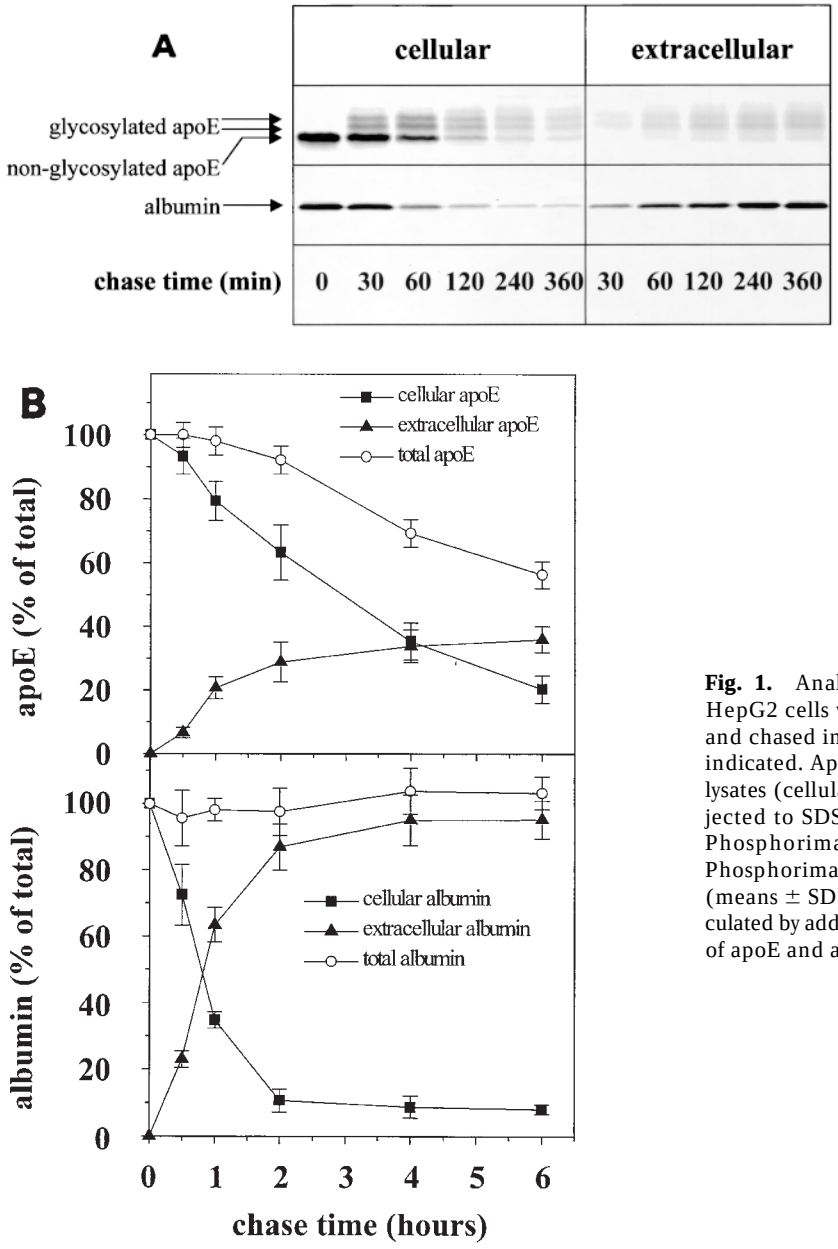

Fig. 1. Analysis of apoE and albumin secretion by HepG2 cells. HepG2 cells were radiolabeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for the times indicated. ApoE and albumin were immunoprecipitated from cell lysates (cellular) and culture supernatants (extracellular) and subjected to SDS-PAGE as described in Materials and Methods. (A) Phosphorimager analysis of representative SDS-PAGE gels. (B) Phosphorimager quantification of four independent experiments (means ± SD). All apoE bands were quantified. The totals were calculated by addition of cellular and extracellular material. The amounts of apoE and albumin after the pulse were set to 100%.

results, as no transport of newly synthesized apoE out of the endoplasmic reticulum was observed during the pulse period. This was indicated by the apparent absence of the O-glycosylated forms of apoE after the pulse period (Fig. 1A), which are generated in the Golgi apparatus during the chase reaction (13, 14).

Newly synthesized apoE was incompletely secreted to the culture medium, reaching 36% ± 4.1% of starting material after 6 h of chase (Fig. 1A, B). The efficiency of apoE secretion clearly differed from that of albumin, a typical secretory protein, which was almost completely secreted during the first 2 h of the chase period (Fig. 1A, B). Thus, our pulse-chase analysis confirmed previous findings that apoE is incompletely secreted from HepG2 cells, giving rise to a cell-associated pool of apoE (16, 17). The cell-associated pool of apoE was subject to proteolytic degradation, as indicated by the loss of 43.7% ± 4.1% of total radiolabeled apoE during the chase period (Fig. 1A, B), which was not observed when the chase was performed in the presence of a cocktail of protease inhibitors (data not shown). Neither significant cellular pools nor any loss of radiolabeled material were observed for albumin (Fig. 1A, B). Interestingly, the degradation of apoE seemed to occur only after a significant lag-phase of about 2 h (Fig. 1A, B). This lag-phase was not observed previously, most likely because of the longer pulse periods used (13, 16). There are two possible explanations for our observations: on the one hand, after 2 h of chase, non-secreted apoE might have reached a cellular localization from where almost no net secretion is possible. ApoE degradation might take place either directly at this localization or after transport to a proteolytic compartment. On the other hand, it is also possible that apoE secretion to the culture medium is a continuing process during the whole chase period, which is counterbalanced by re-internalization of apoE that begins after a lag-phase of about 2 h. In both cases, it is likely that it is the cell-associated apoE that is degraded, as no apoE degradation was observed in the culture media in the absence of cells (data not shown).

Newly synthesized apoE is transported to the cell surface before degradation

We investigated the localization of the cellular pool of apoE to gain insights into its potential role in apoE degradation. Several studies qualitatively demonstrated the presence of apoE on the cell surface of hepatocytes by different techniques (17, 19, 20, 23, 25). As we wanted to confirm these results in a quantitative manner, we developed an assay that allowed us to determine the ratio of cell surface versus intracellular apoE. HepG2 cells were incubated on ice with trypsin to determine how much of the cellular apoE was accessible to an externally added protease under conditions where the plasma membrane remained intact and the cells remained attached to the culture dish (see Materials and Methods). A detailed pulse-chase analysis revealed the transient accumulation of trypsin-accessible, cell-associated apoE, indicating cell surface association (**Fig. 2**). After 2 h of chase, a maximum level of 38.5% ± 1.4% of starting material was found to be trypsin-accessible (Fig. 2). During longer chase periods, the amounts of both radiolabeled cell surface and intracellular apoE decreased (Fig. 2), while again only a slight increase in apoE secreted to the culture medium was observed during the last 4 h of chase (data not shown; compare Fig. 1A, B). Thus, the proteolytic degradation of apoE was preceded by the accumulation of cell surface-associated apoE. The importance of the cell surface-associated pool of apoE is underlined by the analysis of steady-state apoE. As judged by immunoprecipitation and quantification, 54.1% ± 1.9% of steady state apoE in HepG2 cells radiolabeled with [^{35}S]methionine for 48 h was accessible to externally added trypsin at a concentration of 200 μg/ml, indicating a cell surface localization (**Fig. 3**). The presence of a trypsin-accessible pool of apoE was confirmed by Western blot analysis of the mass of cellular apoE (data not shown).

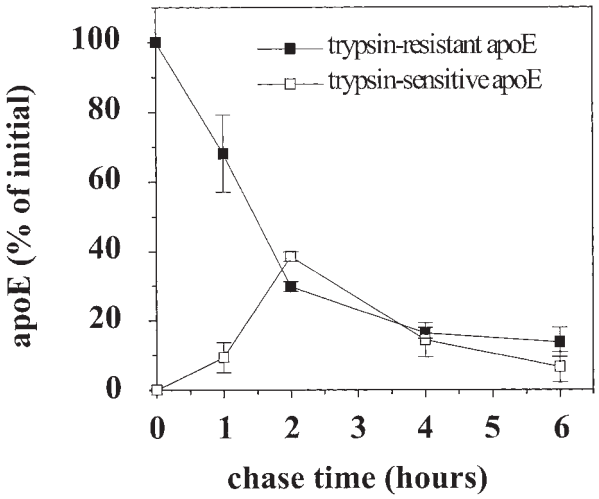

Fig. 2. Transient accumulation of newly synthesized apoE on the cell surface. HepG2 cells were radiolabeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for the times indicated. After the chase, cells were biotinylated and treated with 200 μg/ml trypsin as described in Materials and Methods. ApoE was immunoprecipitated from cell lysates of trypsin-treated and non-trypsin-treated cells as described in Materials and Methods. Samples were subjected to SDS-PAGE and quantified by phosphorimager analysis. The figure shows means ± SD of three independent experiments. Trypsin-sensitive apoE was calculated as the difference between apoE immunoprecipitations of trypsin-treated and non-trypsin-treated cells. The amount of apoE after the pulse was set to 100%.

These results demonstrate that a high proportion of cell-associated apoE is localized on the cell surface, although a significant amount of intracellular apoE also exists. The observation that degradation of apoE started only after a large amount of the protein had reached the cell surface (Figs. 1, 2) supports the hypothesis that apoE degradation takes place after transient localization at the cell surface and subsequent endocytosis. Still, it cannot be excluded that part of the intracellular apoE is directly degraded inside the cell.

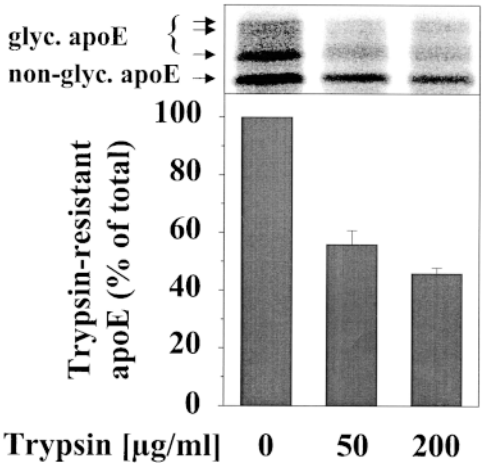

Fig. 3. Cell-associated apoE is localized predominantly on the cell surface. HepG2 cells were radiolabeled with [^{35}S]methionine for 48 h in RPMI-medium in the absence of serum. Afterwards, cells were treated with the indicated concentrations of trypsin as described in Materials and Methods. Cells were lysed and apoE was immunoprecipitated as described in Materials and Methods. The figure shows the phosphorimager analysis of a representative gel (upper panel) and the phosphorimager quantification of duplicate samples (means ± SD; lower panel). Similar results were obtained when cells were radiolabeled in the presence of 10% fetal bovine serum.

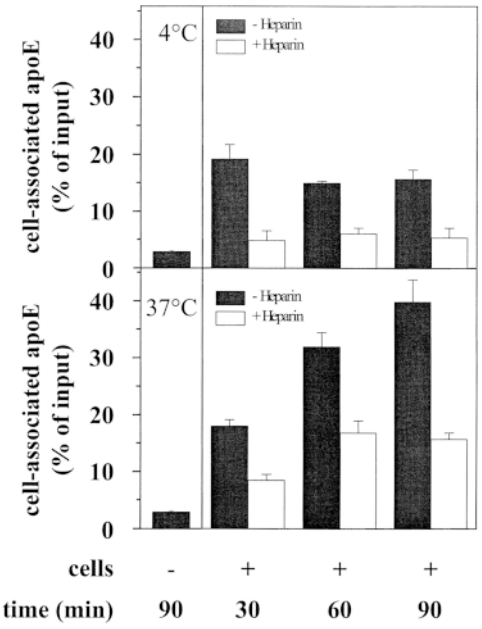

Fig. 4. Heparin-sensitive binding of apoE to HepG2 cells. HepG2 cells were radiolabeled with [^{35}S]methionine for 15 min and chased in the presence of unlabeled methionine for 2 h. Chase media containing radiolabeled secreted proteins were incubated with unlabeled HepG2 cells (+cells) in the presence or absence of heparin (10 μg/ml) as described in Materials and Methods. In parallel, chase media containing radiolabeled secreted proteins were incubated in empty wells of the culture dishes to assay non-specific binding (−cells). Incubations were performed either at 4°C (upper panel) or at 37°C (lower panel) for the times indicated. Afterwards, apoE was immunoprecipitated from cell lysates and culture supernatants and subjected to SDS-PAGE as described in Materials and Methods. Samples were quantified by phosphorimager analysis (means ± SD of three independent experiments). The amount of radiolabeled apoE applied to each well of unlabeled cells was set to 100%.

Secreted apoE is bound, internalized, and degraded by HepG2 cells

To further validate the hypothesis that apoE degradation takes place after transport to the cell surface, we wanted to know whether apoE, once secreted, could re-bind to HepG2 cells and whether the re-bound protein would be proteolytically degraded. Medium containing [^{35}S] methionine-labeled apoE, collected from HepG2 cells after a 2-h chase, was incubated with unlabeled HepG2 cells to monitor apoE binding. As shown in **Fig. 4**, almost 20% of radiolabeled apoE secreted by HepG2 cells was able to rebind in a heparin-sensitive manner to the surface of identically treated, unlabeled cells when the incubation was performed on ice. At 37°C, increasing amounts of cell-associated apoE were detected during a time course, reaching 39.9% ± 3.9% of input after 90 min (Fig. 4). Also at this temperature, the cell surface-association was inhibited by heparin (Fig. 4). A longer incubation did not lead to further apoE binding to HepG2 cells (**Fig. 5**). Only a negligible amount of apoE bound non-specifically to the culture dishes at both temperatures (Fig. 4). The specificity of the binding reaction is highlighted by the observation that apoA-I and apoB, the major apolipoproteins secreted by HepG2 cells, were not significantly re-bound by HepG2 cells, although they were present in comparable quantities (**Fig. 6**).

To analyze the fate of the cell-associated apoE, longer incubations were performed. Figure 5 shows that already during the binding period, a deglycosylation of apoE took place, as indicated by the appearance of high amounts of non-glycosylated apoE in the cell-associated fraction. This effect cannot be explained by more efficient binding of non-glycosylated apoE to the cell surface, as during the binding reaction, the total amount of non-glycosylated apoE increased to more than 300% of starting material (Fig. 5). When the cells were further incubated for up to 2 h, the cell-associated apoE was almost completely degraded in a specific manner, as apoA-I and apoB, which did not significantly re-bind to HepG2 cells, were not degraded (Figs. 5, 6). The non-bound fraction of apoE remained constant during the last 2 h of the reaction, with no further binding occurring after 90 min (Fig. 5).

Several explanations for the observed degradation of apoE can be envisaged. A possible degradation by secreted proteases after the release of apoE from the cells into the extracellular medium is highly unlikely because apoE was completely stable in HepG2-conditioned media in the absence of cells even during prolonged incubations at 37°C (data not shown). Thus, apoE degradation is likely to occur while the protein is cell-associated. The most probable mechanism for a cell-associated degradation of an extracellular protein is endocytosis followed by proteolysis in lysosomes. To test this possibility, apoE degradation was analyzed in the presence of the lysosomotropic agents chloroquine or NH_4Cl, which inhibit lysosomal proteases by perturbing the acidification of the endosomal/lysoso-

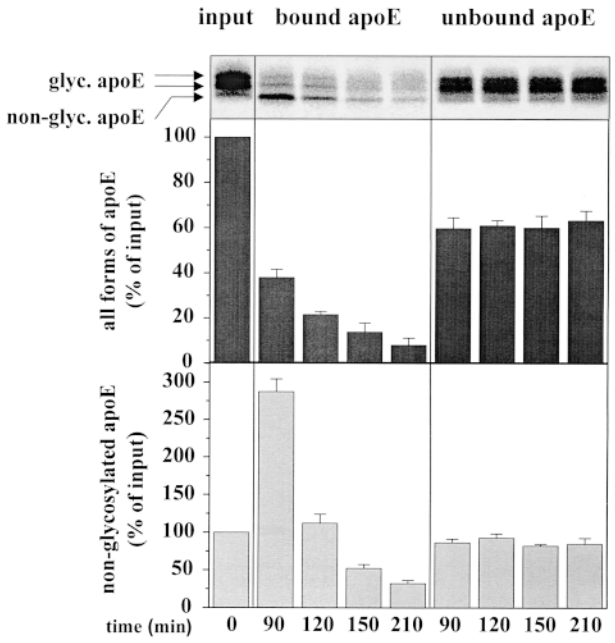

Fig. 5. Binding, deglycosylation, and degradation of apoE by HepG2 cells. HepG2 cells were radiolabeled with [^{35}S]methionine for 15 min and chased in the presence of unlabeled methionine for 2 h. Chase media containing radiolabeled secreted proteins were incubated with unlabeled HepG2 cells as described in Materials and Methods. Incubations were performed at 37°C for the times indicated. Afterwards, apoE was immunoprecipitated from cell lysates (bound apoE) and culture supernatants (unbound apoE) and subjected to SDS-PAGE as described in Materials and Methods. The upper panel shows the phosphorimager analysis of a representative SDS-PAGE gel. The middle panel shows the phosphorimager quantification of all bands of apoE, whereas the lower panel shows a quantification of the non-glycosylated, fastest migrating band of apoE (means ± SD of three independent experiments). The amount of radiolabeled apoE applied to each well of unlabeled cells (left lane, input) was set to 100%.

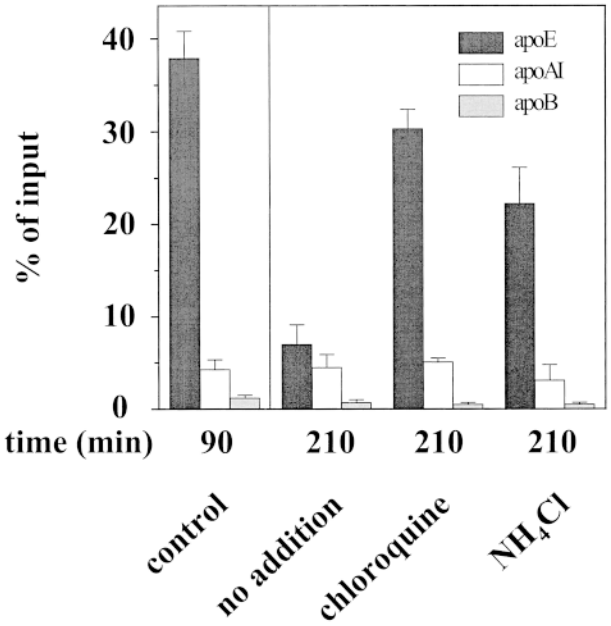

Fig. 6. Inhibition of apoE degradation by lysosomotropic agents. HepG2 cells were radiolabeled with [^{35}S]methionine for 15 min and chased in the presence of unlabeled methionine for 2 h. Chase media containing radiolabeled secreted proteins were incubated with unlabeled HepG2 cells as described in Materials and Methods. Incubations were performed at 37°C for the times indicated. After 90 min of incubation, either 100 μm chloroquine or 1 mm NH_4Cl was added to the samples as indicated. ApoE, apoA-I, and apoB were immunoprecipitated from cell lysates and culture supernatants and subjected to SDS-PAGE as described in Materials and Methods. The figure shows the phosphorimager quantification of two independent experiments (means ± SD). All apoE bands were quantified. The amount of radiolabeled apoE applied to each well of unlabeled cells was set to 100%.

mal compartment. Chloroquine and NH_4Cl indeed showed a pronounced effect in stabilizing cell-associated apoE, inhibiting apoE degradation by 75.5% and 49.4%, respectively (Fig. 6).

Thus, a subpopulation of about 40% of secreted apoE is specifically bound by HepG2 cells. The binding is mediated, at least in part, by HSPG, as suggested by the partial inhibition of the binding reaction by heparin (Fig. 4). Subsequently, the protein is internalized, deglycosylated, and degraded. The site of degradation, and most likely also deglycosylation, is the endosomal/lysosomal compartment. These results provide direct proof that cell surface apoE, which constitutes a substantial amount of apoE (Figs. 2, 3), is subject to proteolysis.

Ratio between pools of secreted, cell-associated, and degraded apoE is influenced by apoE ligands in the extracellular medium

All pulse-chase studies showing apoE degradation (15, 16, 26, 33), including ours (Fig. 1), were performed in serum-free chase medium. Thus, the only known potential ligands for apoE present were the few lipoproteins synthesized by the cells themselves. Under these conditions of rather poor ligand availability, the cell surface-associated and secreted pools of apoE are likely to exist in a lipid-free state (19, 46–49). Indeed, apoE was selectively re-bound by HepG2 cells, while the major constituents of HDL- and LDL/VLDL-like particles secreted by HepG2 cells, apoA-I, and apoB showed no significant binding (Fig. 6). According to the secretion-recapture model, the cell surface-associated and secreted pools of apoE are thought to be available for ligand binding and sequestration in the space of Disse under physiological conditions (38). Under conditions of poor ligand-availability, this pool might be subject to proteolytic turnover by re-internalization and degradation to avoid apoE accumulation at the cell surface.

To test this hypothesis, we performed a pulse-chase analysis of apoE secretion and degradation in the presence or absence of human serum, thereby supplying the chase medium with lipoproteins, i.e., potential apoE ligands. In the presence of 10% human serum, apoE secretion to the culture medium was strongly enhanced, reaching about 60% of starting material after 2 h of chase, while no further net apoE secretion was observed during longer chase reactions (**Fig. 7**). The cell-associated pool of apoE that accumulated during the first 2 h of chase was

much smaller in the presence of serum (Fig. 7). While this pool of apoE, which consists predominantly of cell surface apoE (Fig. 2), is prone to degradation in the absence of serum (Figs. 1, 7), only a very small amount of apoE was degraded in the presence of serum (Fig. 7). BSA in a concentration comparable to the protein concentration of serum did not significantly influence apoE secretion and degradation (data not shown). These results demonstrate that during the first 2 h of chase, ligand availability determines the fate of a subfraction of about 30% of newly synthesized apoE. In the absence of serum, this pool of apoE accumulates at the cell surface, from where it is transported to lysosomes for proteolytic degradation. In the presence of serum, apoE is secreted to the extracellular medium instead of accumulating at the cell surface, thereby being protected from degradation.

Our hypothesis predicts that the enhanced secretion in the presence of serum is due to the binding of cell surface-associated apoE to lipoproteins present in the serum, preventing endocytosis and degradation of apoE. A direct effect of human serum on apoE release from the cell surface into the culture medium is shown by the results presented in **Fig. 8A**. After a 2-h chase to accumulate radiolabeled apoE on the cell surface, a part of the apoE was released into the culture medium by 10% human serum during a subsequent incubation on ice, in the apparent absence of exocytosis (Fig. 8A). At 37°C, the effect was even more pronounced, although under these conditions it cannot be excluded that serum lipoproteins might stimulate apoE secretion indirectly (Fig. 8A). Further validation for our hypothesis is presented in Fig. 8B. Radiolabeled apoE secreted from HepG2 cells in the presence or absence of human serum was incubated with unlabeled cells to analyze binding and degradation (compare Figs. 4–6). ApoE that was secreted from HepG2 cells in the presence of 10% of human serum showed strongly reduced binding to HepG2 cells in comparison to apoE that was secreted in the absence of serum (Fig. 8B). A similar effect was observed when serum was only present during the binding reaction (Fig. 8B).

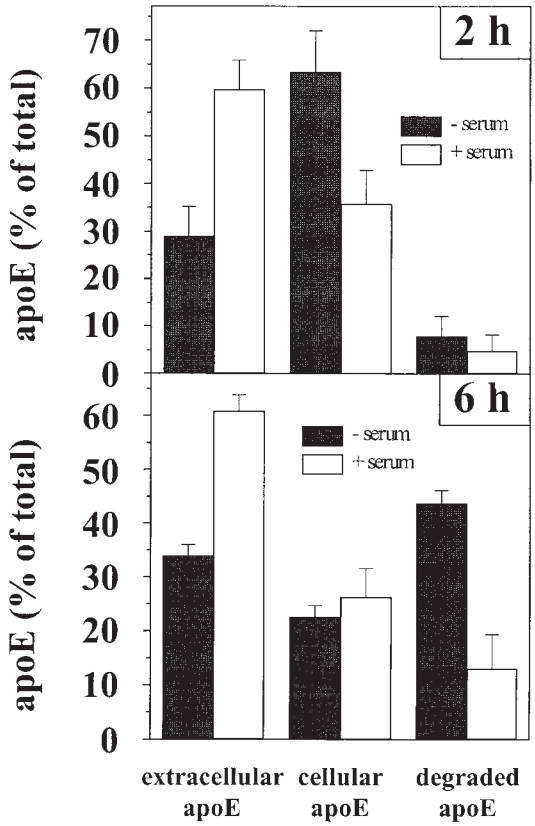

Fig. 7. Enhanced secretion and reduced degradation of apoE in the presence of human serum. HepG2 cells were radiolabeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for 2 h (upper panel) or 6 h (lower panel). ApoE was immunoprecipitated from cell lysates (cellular apoE) and culture supernatants (extracellular apoE) and subjected to SDS-PAGE as described in Materials and Methods. The figure shows the phosphorimager quantification of duplicate samples of a representative experiment (means ± SD). All apoE bands were quantified. The degraded material (degraded apoE) represents the difference between the sum of cellular and secreted apoE after the chase and the amount of apoE after the pulse. The amount of apoE after the pulse was set to 100%.

To further specify the serum effects on apoE secretion, re-binding, and degradation, we asked which class of serum lipoproteins might mediate the observed effects. ApoE3, the apoE isoform synthesized by HepG2 cells (12), is able to associate with all types of plasma lipoproteins, although a certain preference for HDL was reported (50). We found that apoE newly secreted by HepG2 cells bound preferentially to LDL particles present in the fasted serum used, but we also observed strong binding to HDL (data not shown). Like complete serum, isolated LDL or HDL enhanced apoE secretion, although to a somewhat lesser extent in the case of HDL (**Fig. 9A**). When LDL- or HDL-containing preconditioned medium was incubated with HepG2 cells, binding of apoE was reduced to the same extent as compared to complete serum (Fig. 9B).

These results indicate that the ratio between secretion and degradation of apoE can be dramatically changed by addition of physiological apoE ligands, such as LDL or HDL, to the system. These extracellular ligands seem to trigger the release of apoE from the cell surface, thereby inhibiting the re-internalization and subsequent degradation of apoE that remains attached to the cell surface.

DISCUSSION

The aim of the present study was to re-evaluate the characteristics of apoE secretion, cellular retention, and proteolytic degradation with regard to current models of apoE function. As reported previously (13, 16, 17), we found that newly synthesized apoE is incompletely secreted and partially degraded by HepG2 cells (Fig. 1). Thirty six percent of radiolabeled apoE reached the culture medium during a 6-h chase after a 10-min pulse, while 43.7% were degraded (Fig. 1). Taking into account that the experimental protocols were not identical, these results are in good agreement with some reports (13, 16)

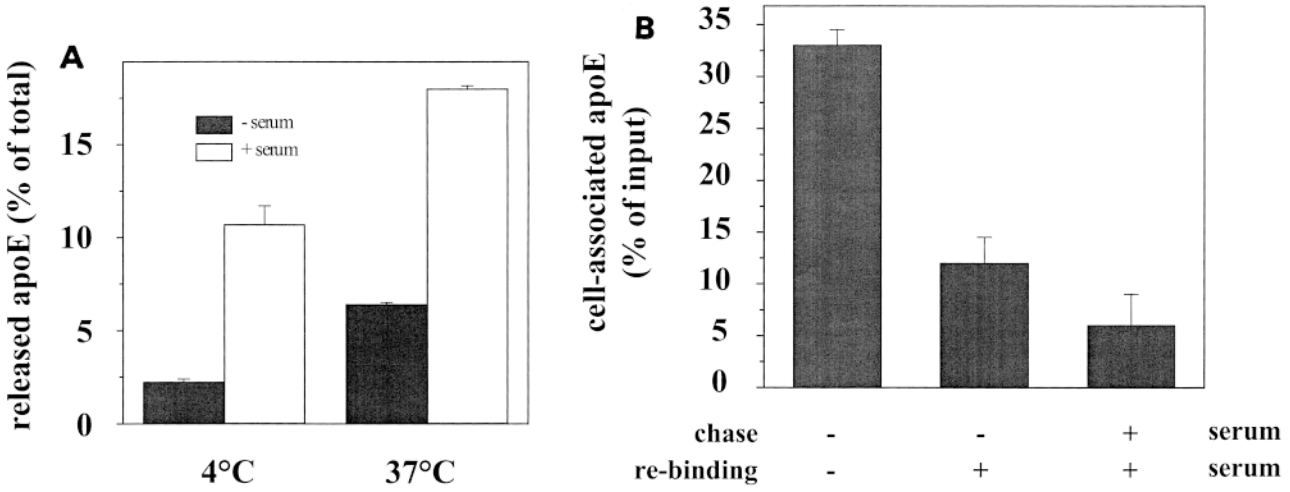

Fig. 8. Influence of human serum on apoE interaction with the cell surface. (A) HepG2 cells were radiolabeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for 2 h. Chase medium was discarded and cells were washed with Dulbecco's PBS. Subsequently, cells were chased for another 2 h in RPMI medium in the presence or absence of 10% human serum either at 4°C or 37°C. ApoE was immunoprecipitated from culture supernatants and subjected to SDS-PAGE as described in Materials and Methods. The figure shows the phosphorimager quantification of two independent experiments (means ± SD). All apoE bands were quantified. The amount of apoE after the pulse was set to 100%. (B) HepG2 cells were radiolabeled with [^{35}S]methionine for 15 min and chased in the presence of unlabeled methionine for 2 h (chase), either in the presence or absence of 10% human serum. Chase media containing radiolabeled secreted proteins were incubated for 90 min with unlabeled HepG2 cells as described in Materials and Methods (re-binding). The second chase was performed either in the presence or absence of 10% human serum. ApoE immunoprecipitation and detection were performed as described in (A). The amount of radiolabeled apoE applied to each well of unlabeled cells was set to 100%.

and do not contradict others (51). The analysis of intermediate time-points of the chase reaction revealed interesting details. Net secretion of apoE to the culture medium was not constant during the chase period, but ceased almost completely after 2 h (Fig. 1). At this time, a pool of newly synthesized apoE had accumulated at the cell surface (Figs. 2, 3), whereas degradation of apoE just started at this time-point (Fig. 1). Thus, cell surface accu-

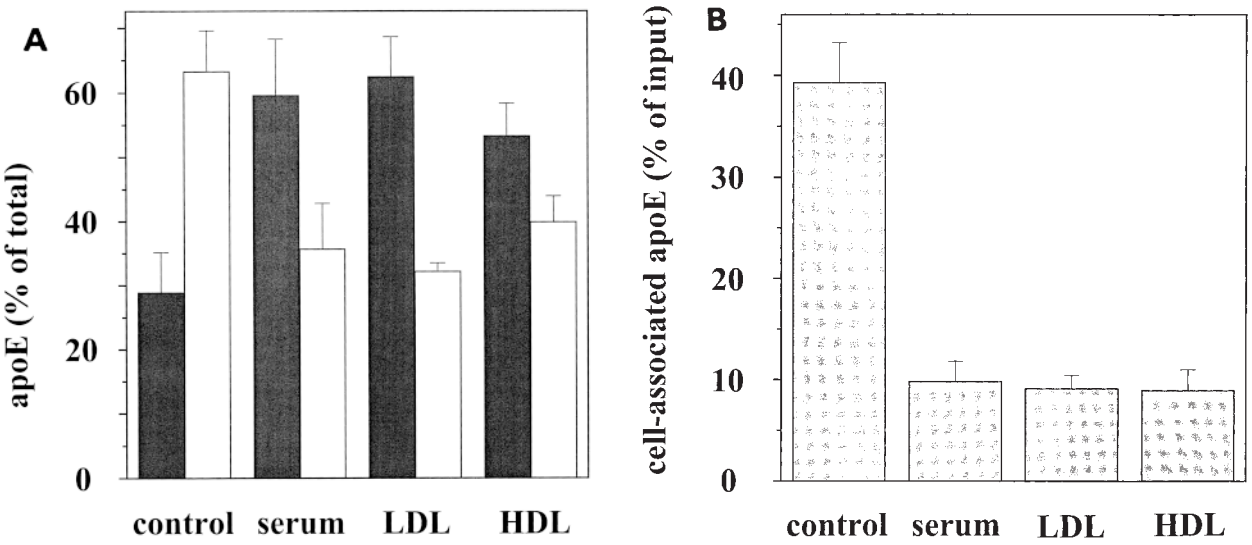

Fig. 9. The effect of isolated human LDL and HDL on apoE secretion and binding to HepG2 cells. (A) HepG2 cells were radiolabeled with [^{35}S]methionine for 10 min and chased in the presence of unlabeled methionine for 2 h in the presence or absence of either 10% human serum, 100 μg/ml isolated human LDL, or 10 μg/ml isolated human HDL. ApoE was immunoprecipitated from cell lysates (white bars) and culture supernatants (black bars) and subjected to SDS-PAGE as described in Materials and Methods. The figure shows the phosphorimager quantification of two independent experiments (means ± SD). All apoE bands were quantified. The amount of apoE after the pulse was set to 100%. (B) HepG2 cells were radiolabeled with [^{35}S]methionine for 15 min and chased in the presence of unlabeled methionine for 2 h in the presence or absence of 10% human serum, 100 μg/ml isolated human LDL, or 10 μg/ml isolated human HDL. The obtained chase media containing radiolabeled secreted proteins were incubated for 90 min with unlabeled HepG2 cells as described in Materials and Methods. ApoE immunoprecipitation and detection were performed as described in (A). The amount of radiolabeled apoE applied to each well of unlabeled cells was set to 100%.

mulation of apoE precedes proteolytic degradation of the protein, suggesting that the two processes might be coupled.

The degradation of apoE takes place in lysosomes (16; Fig. 6) after transport to the cell surface and re-internalization (Figs. 4 and 5). ApoE has been observed on the cell surface of various types of hepatocytes by different techniques (17, 19, 20, 23). Our protease-protection studies confirm these results while providing a quantitative analysis of cell surface apoE. Under steady state conditions, 54.1% of cellular apoE were found to be accessible to externally added trypsin, indicating cell surface localization (Fig. 3). The kinetic analysis of newly secreted cell surface apoE demonstrated that a large amount of apoE molecules were at least transiently localized to the cell surface. In our assay, apoE molecules that were on their way to and from the cell surface were part of the trypsin-inaccessible pool. This suggests that most likely more than the 38.5% of starting apoE, observed at the 2-h time-point, passed the cell surface before reaching the degradation pathway. This would be in accordance with a previous report that localized hepatic intracellular apoE mostly to Golgi stack saccular distensions and endosomes, that is, exocytic and endocytic compartments (21). Our findings strongly underline the functional importance of the cell surface-associated pool of apoE for the degradation of the protein.

To be degraded in lysosomes, cell surface apoE must re-enter the cell. It is well established that apoE is a ligand for cell surface receptors of the LDL receptor family (3–7), mediating the internalization of remnants of VLDL and chylomicrons (38, 39). Despite that, it was not clear up to now whether apoE secreted by HepG2 cells could be immediately re-internalized via endocytosis (16, 17). In our binding experiments, a subpopulation of secreted apoE, constituting almost 40% of total secreted material, was bound, internalized, deglycosylated, and degraded by HepG2 cells (Figs. 4–6). It remains to be established whether this subpopulation of apoE is part of lipoprotein particles secreted by HepG2 cells or represents free apoE, which is known to be secreted from lipoprotein-producing hepatocytes under certain conditions (46–49). We favor the latter possibility, as apoA-I and apoB, the major protein components of HDL and LDL/VLDL, respectively, were neither significantly bound nor degraded during the time course of our experiments (Fig. 6).

Our data provide evidence concerning the pathway of re-endocytosis of newly secreted apoE. ApoE binding and uptake were relatively slow (Fig. 4), which makes it unlikely that the LDL-receptor pathway, which proceeds during minutes via clathrin-coated pit-mediated internalization (52), is involved. The observed inhibition of apoE binding by heparin (Fig. 4), in combination with the slow kinetics of apoE internalization (Fig. 4), rather favors a mechanism analogous to the proposed interaction of apoE-containing lipoproteins with HSPG and the LRP (35, 36). Recent findings also demonstrate that HSPG, such as syndecans, are able to act directly as high capacity/low affinity receptors for relatively slow endocytosis of lipoproteins, possibly internalizing ligands via a caveolae-mediated mechanism (53, 54).

ApoE degradation in HepG2 cells occurs mainly in lysosomes. Lysosomotropic agents inhibited apoE degradation both in pulse-chase studies (data not shown; 33) and in our binding–degradation assay (Fig. 6). Still, other proteolytic systems might contribute to apoE degradation. The report that cytosolic calpains contribute to apoE degradation in HepG2 cells (33) remains to be validated, as the observed inhibition of apoE degradation might be an indirect effect of either ALLN, a non-specific protease inhibitor (55, 56) or Ca^{2+} depletion (57). We found that the specific proteasome inhibitor lactacystine did not affect apoE degradation in HepG2 cells (data not shown), whereas in macrophages, apoE might be degraded by the proteasome in a Golgi-like compartment (34). Macrophage apoE degradation was also reported to occur in lysosomes (26). It remains thus to be clarified to what degree the apoE degradation pathways in hepatocytes and macrophages are similar.

The secretion of apoB, the major apolipoprotein component of VLDL, is regulated by proteasomal degradation, which occurs during co-translational membrane translocation at the endoplasmic reticulum (58, 59). ApoE secretion is not affected by inhibition of microsomal triacylglycerol transfer protein (M. Schmitt, E. Nicodème, and T. Grand-Perret, unpublished results), conditions under which apoB secretion is inhibited and its degradation is enhanced (60). Moreover, both kinetic analysis (Fig. 1) and inhibitor studies (Fig. 6) indicate that the secretion of apoB and apoE is regulated by different proteolytic mechanisms.

What are the implications of our results for the physiological role of newly secreted, most likely free apoE? The cell surface and secreted pools of apoE are in a dynamic equilibrium. When HepG2 cells were incubated on ice, a small amount of cell surface apoE was released into the culture medium, an effect that was more pronounced in the presence of lipoproteins (Fig. 8). On the other hand, a subpopulation of secreted apoE was efficiently rebound by HepG2 cells (Fig. 4). Similar pools of at least partially free apoE were found to be enriched on the basolateral surface of rat hepatocytes and in the neighboring space of Disse (19, 25), although their hepatic origin is not proven. According to the secretion–recapture model, these pools of free apoE serve to sequester apoE-poor remnant lipoproteins in the space of Disse. There, the remnants are thought to be processed by hepatic lipase and to be further enriched in apoE, allowing their efficient binding to apoE-specific receptors (19, 35, 36). The origin of the apoE implicated in remnant lipoprotein clearance remains unclear. In apoE knock-out mice, in which macrophagic apoE expression was reconstituted by transplantation of wild-type bone marrow (61, 62), the macrophage-derived apoE mediated the hepatic clearance of remnant lipoproteins (43). Whereas these results leave the necessity of hepatic apoE for remnant clearance uncertain, our results indicate that hepatocytes are able to provide the necessary pools of apoE. While this article was being reviewed, a study further showing the importance of the hepatically synthesized pools of apoE was published (63). According to Linton et al. (63), macrophagic apoE is only

able to function via the LDL receptor-dependent pathway, whereas LRP-dependent remnant clearance absolutely requires the expression of hepatic apoE.

If the cell surface pool of apoE serves to sequester remnant lipoproteins, why then is this pool of apoE degraded? The data presented in Figs. 1–6 suggested that the proteolytic turnover of cell surface apoE might be caused by poor lipoprotein availability. We tested this hypothesis by monitoring apoE degradation in the presence of potential apoE ligands. Indeed, the presence of lipoproteins, either provided as total serum or as isolated lipoprotein fractions, almost completely inhibited apoE degradation, both in pulse-chase experiments (Fig. 7) and in our binding-degradation assay (Figs. 8, 9). Inhibition of apoE degradation was due to the accumulation of apoE in the extracellular medium, while the formation of the cell surface pool of apoE was diminished (Figs. 7, 9). Thus, potential apoE ligands, like LDL and HDL, are able to trigger the release of apoE from the cell surface.

In summary, apoE is transported to the hepatocyte cell surface (Figs. 1–3) where it is in a dynamic equilibrium with secreted apoE (Fig. 4). There, its fate depends on the types of lipoproteins present and on their concentrations. At low lipoprotein concentrations, apoE is re-internalized and degraded (Figs. 4–6). The physiological role of this phenomenon might be the regulation of local apoE levels. When higher concentrations of lipoproteins not readily taken up by hepatocytes in an apoE-dependent manner, like LDL or HDL, are present, apoE binds to those lipoproteins and is released from the cell surface (Figs. 7–9). When, in a similar experimental set-up, remnant lipoproteins are present in the sinusoidal space, apoE binds to them (23, 25, 40). This enrichment in apoE is thought to mediate the subsequent remnant uptake by endocytosis, as described by the secretion–recapture model (23, 25, 35–40). Our results further support this current model that links apoE secretion and function to remnant lipoprotein clearance.

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