Lysosomal degradation and sorting of apolipoprotein E in macrophages

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Abstract We previously reported that a substantial amount of newly synthesized apoE in mouse macrophages is degraded prior to secretion; a portion of this pool of apoE can be rescued by the addition of HDL$_3$ to the incubation medium. In the present studies, the location and nature of the intracellular degradation of apoE were more closely examined. Inhibitors of protein trafficking (brefeldin A) as well as a number of protease inhibitors were used. The experiments using brefeldin A (5 μg/ml) clearly established that neither the endoplasmic reticulum nor the Golgi complex are the sites of apoE degradation. Using a pulse-chase design, [^{35}S]apoE cannot be chased out in the presence of brefeldin A and remains undegraded within the cell. The accumulated apoE lacks the sialic acid residues, indicating that this final stage of processing must occur in the *trans*-Golgi network or later. Lysosomotropic agents, ammonium chloride and chloroquine, on the other hand, inhibit apoE degradation by over 70 and 80%, respectively, while total cell protein degradation remains unaffected. Similarly, a cocktail consisting of four lysosomal protease inhibitors (pepstatin, E-64, chymostatin, and antipain), inhibits specifically apoE degradation by over 60%. In contrast, ALLN, an inhibitor of Ca^{2+}-dependent cysteine proteases, has a moderate effect on apoE degradation (30% inhibition) and a more pronounced effect on total protein degradation. These data suggest that the site of intracellular apoE degradation in the macrophage is the lysosome. These conclusions are supported by light and electron microscopy of macrophages, clearly showing the presence of immunoreactive apoE (along with cathepsin D) in the endosomal/lysosomal compartment of control and lysosomotropic agent-treated cells. In contrast, little or no labeling is seen in this compartment in brefeldin A-treated cells. At lower concentrations of the lysosomotropic agents, the extent of inhibition of apoE degradation is compensated for by its increased secretion, in a manner analogous to the effect of these agents on lysosomal enzymes. Higher concentrations of these agents, which lead to a profound inhibition of apoE degradation, also specifically block apoE secretion. The block in apoE secretion in the presence of high concentrations of chloroquine leads to undiminished or higher concentrations of immunoreactive apoE in the endosomal/lysosomal compartment, suggesting that apoE is targeted for lysosomal degradation directly, without prior secretion or surface association. These data strongly suggest pH-dependent sorting of apoE in macrophages to the degradative and secretory pathways and imply a protein–protein interaction in the process.—**Deng, J., V. Rudick, and L. Dory.** Lysosomal degradation and sorting of apolipoprotein E in macrophages. *J. Lipid Res.* 1995. **36:** 2129–2140

Supplementary key words protease inhibitors • lysosomotropic agents • protein degradation

Apolipoprotein (apo)E plays an important role in cholesterol transport. It is a key component of cholesterol-rich lipoproteins (1), where it serves as a ligand for the removal of these particles from the circulation through a specific receptor on the hepatocyte plasma membrane (2–6). Over-expression of circulating apoE in transgenic mice leads to decreased plasma cholesterol levels (7), while apoE-deficient mice generated by targeted gene disruption exhibit large increases in plasma cholesterol levels and develop premature atherosclerosis (8–11).

Although the liver is the major site of apoE synthesis and secretion (12–14), a number of other peripheral tissues have been shown to express this protein (13–18). Macrophages, especially cholesterol-loaded foam cells, express a significant amount of apoE (19–23). While these cells actively synthesize apoE, our previous work demonstrated that a significant portion of apoE is degraded prior to secretion (24). Furthermore, when HDL is present in the incubation media, it is able to "rescue" a substantial portion of the apoE destined for intracellular degradation and direct it to secretion (24). Recent studies on apoE metabolism in the HepG2 hepatoma

Abbreviations: apo, apolipoprotein; TGN, *trans*-Golgi network; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BFA, brefeldin A; AC, ammonium chloride; CQ, chloroquine; ALLN, N-acetyl-leucyl-leucyl-norleucinal; CHO, Chinese hamster ovary; FITC, fluorescein-5-isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ER, endoplasmic reticulum; acLDL, acetylated low density lipoprotein; HDL, high density lipoprotein.

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cell line also indicate a substantial intracellular degradation of this protein by a neutral, Ca^{2+}-dependent cysteine protease (25). Intracellular degradation of apoB in HepG2 cells (26, 27) and HMG-CoA reductase in CHO cells (28) by Ca^{2+}-dependent cysteine proteases has also been recently described. The intracellular degradation of these proteins appears to play a role in their post-translational regulation of expression. In light of these observations, we decided to examine intracellular metabolism of apoE in primary mouse peritoneal macrophages. Our results suggest that the lysosome is the compartment of apoE degradation in primary mouse macrophages. Furthermore, the present results suggest direct sorting of apoE into the endosomal/lysosomal compartment, bypassing the secretory pathway. These observations identify additional regulatory steps in apoE expression in peripheral tissues. The specific, pH-dependent secretion of apoE further points to the complexity of apoE sorting in macrophages. Our results also eliminate the endoplasmic reticulum and the Golgi complex as the sites of degradation and neutral cysteine proteases as the enzymes responsible for significant intracellular degradation of apoE in macrophages.

MATERIALS AND METHODS

Materials

Chloroquine, ammonium chloride, leupeptin, and brefeldin A were obtained from Sigma. ALLN (calpain inhibitor I), antipain, pepstatin, chymostatin, and E-64 were obtained from Boehringer Mannheim. Metabolic labeling of proteins was carried out using EXPRE^{35}S^{35}S protein labeling mixture (sp act > 1,100 Ci/mmol; NEN DuPont). Rabbit antiserum to cathepsin D was purchased from H.T.I. Bioproducts, Inc., and FITC and TRITC coupled secondary antibodies were from Biodesign International and Sigma, respectively. Gold-labeled rabbit anti-goat IgG (5 nm) was obtained from Amersham Life Sciences. All culture media, including DMEM, methionine-free DMEM, fetal bovine serum, and antibiotics were purchased from Gibco.

Cell culture

Mouse peritoneal macrophages were obtained from male Swiss-Webster mice (14–18 g; Harlan) 4 days after an intraperitoneal injection of 1.5 ml of sterile 4% thioglycolate broth. Cells were harvested and cultured, as previously described (23). On the second day of culture, the cells were routinely incubated with 25-hydroxycholesterol (0.8 μg/ml); this treatment did not change the cellular sterol content or distribution, as previously shown, but resulted in a 3-fold transcriptional induction of apoE expression (24). After the oxysterol treatment, cells were washed and incubated in DMEM for an additional 24-h period prior to the experiments.

Pulse-chase studies

Prior to the labeling, the cells were incubated briefly (10 min) in methionine-free DMEM. They were then pulsed for 60 min with [^{35}S]methionine (40 μCi/ml) in the presence or absence of individual protease inhibitors in methionine-free DMEM. After the pulse period, the cells were washed and then chased for up to 60 min in DMEM containing the appropriate inhibitor. At each time point of the chase period the cell-associated and media [^{35}S]apoE was quantitatively immunoprecipitated, as previously described (23). The sum of cell-associated and media apoE activity at each time point represents total recovery, and is expressed as a percentage of the cell-associated activity at the beginning of the chase period (0 time chase). The difference between the amounts of apoE recovered at 60 min of chase (cells and media) and that present in the cells at 0 time of chase is defined as the extent of degradation. Previous work and additional experiments for these studies established that the extent of [^{35}S]apoE degradation in the media is negligible (24). Parallel studies on the fate of total cellular protein were also performed. Incorporation of [^{35}S]methionine into cellular and media proteins was determined by trichloroacetic acid precipitation of an aliquot of cell lysate or media, respectively, on a Whatman #5 filter paper disc.

Immunofluorescent microscopy

Macrophages growing on sterilized glass coverslips were washed twice and incubated in DMEM ± 100 μM CQ for 2 h before fixation with 2% formaldehyde for 10 min at room temperature. Fixed cells were then washed twice with 5 ml DPBS and permeabilized by incubating for 10 min at RT in DPBS containing 0.1% Triton X-100 and 0.05% SDS. After three DPBS washes, intracellular apoE and cathepsin D were labeled by incubating the permeabilized macrophages with a mixture of purified goat anti-rat apoE IgG and DEAE purified rabbit antiserum to cathepsin D for 60 min at room temperature. Nonspecific binding was determined by an identical procedure, except that a mixture of non-immune goat IgG and non-immune rabbit antiserum was used in place of the specific antibodies. In addition, cells were incubated separately with either anti-apoE or anti-cathepsin D. After washing in DPBS containing 1% Triton X-100, coverslips were then incubated with either a mixture of rabbit anti-goat IgG conjugated with FITC (apoE labeling) and goat anti-rabbit IgG conjugated with TRITC (cathepsin D labeling) or the appropriate separate secondary antibody for single labeling for 60 min at room temperature. All coverslips were then washed three

times with DPBS before mounting in Mowiol containing 2.5% DABCO. All primary and secondary antibodies were diluted with DPBS containing 3% BSA. Macrophages were viewed with a Zeiss Photomicroscope II and photographed using Kodak TMZ p3200 film.

Electron microscopy and immunocytochemistry

Cryofixation for the preparation of frozen sections was accomplished as follows. Macrophages grown in 6-well plates were fixed with 0.2% glutaraldehyde/4% formaldehyde in 0.125 M PIPES buffer, pH 7.4, and then scraped immediately into conical centrifuge tubes. The centrifuged cell pellet was resuspended in 1% BSA, re-centrifuged, and the supernatant was removed. Fresh fixative was then placed on the cells for 2 h at room temperature. After centrifugation the cell pellets were infiltrated overnight at room temperature with 2.1 M sucrose in PIPES buffer with gentle agitation, placed on stubs, and plunged into liquid N_2. Sections of 80–100 nm thickness were cut using an Ultracut S microtome and cryoattachment (Leica Inc., Deerfield, IL) and collected on formvar carbon-coated nickel grids that had been negatively charged before use. For immunostaining, grids were washed 3 times for 5 min each with 0.1 M phosphate buffer, pH 7.4, then with 0.02 M glycine in the same buffer twice for 7 min each, and finally with the blocking agent (5% BSA, 0.85% NaCl, and 0.021% $MgCl_2 \cdot 6H_2O$ in the same buffer) for 15 min. Blocked grids were incubated with the appropriate concentration of anti-apoE antibody either for 20 h at 4°C or for 1 h at room temperature. After two washes in phosphate-buffered 0.1% BSA and three washes of distilled water, a 5-nm IgG-gold probe was placed onto the grid for 1 h. After washes, each grid was transferred onto a drop of 2% methyl cellulose containing 0.2% uranyl acetate for 10 min, then the methyl cellulose was removed and the grid was dried and viewed. Sections were observed with a Hitachi 600 electron microscope (Hitachi Instruments, San Jose, CA) and pictures were taken using Kodak SO-163 negative film. Appropriate controls, omitting either primary or secondary antibodies and substituting non-immune serum or non-related antibody in their place, were performed.

Analytical procedures

Protein determinations were carried out according to Lowry et al. (29) and SDS-PAGE by the method of Laemmli (30). ApoE immunoprecipitates were resolved in reduced, SDS-containing 10% polyacrylamide gels. The apoE bands, identified by the use of mol wt and apoE standards, were cut out, dissolved overnight in 30% hydrogen peroxide, and quantified by scintillation spectroscopy. The cell lysate and media ^{35}S-labeled protein precipitates on filter paper discs were washed extensively (2 × 12 h) in 10% trichloroacetic acid, 100% ethanol, and ether. The dry discs were placed in scintillation vials and counted after the addition of scintillation fluid.

RESULTS

ApoE turnover in 25-hydroxycholesterol-treated macrophages

Studies in other laboratories indicate that the extent of apoB degradation in the liver cell is regulated by the availability of triglyceride for VLDL assembly and secretion (26, 31). Although apoE secretion and cholesterol efflux from macrophage-foam cells are independent of each other (24, 32), it is possible that, in a situation analogous to what is found in the hepatocyte, the amount of unesterified cholesterol potentially available for efflux may determine the extent of intracellular apoE degradation. In our previous studies we examined the cellular apoE $t_{½}$ in acLDL-derived cholesterol-loaded macrophages in the presence or absence of HDL_3 (24). In the present studies we examine apoE turnover in cells exposed to trace amounts of 25-hydroxycholesterol (0.8 μg/ml), sufficient to up-regulate apoE expression 3-fold, without causing a measurable change in cellular cholesterol mass and distribution (24). The fate of metabolically labeled, cell-associated apoE during a chase period of up to 60 min in these cells is shown in **Fig. 1.** Cell-associated apoE activity decays steadily during the chase period, with an apparent $t_{½}$ of 22 ± 2 min, in a manner identical to that seen in cholesterol-loaded cells (24), accompanied by a steady increase in media apoE. The accumulation of apoE in the media is maximal at 60 min chase (additional times were examined but are not shown). It is clear, however, that as previously reported in cholesterol-loaded cells (24), a significant portion (>50%) of the cell-associated apoE lost during the 60-min chase period in these cells is not recovered in the media. It should be noted that we previously demonstrated that the extent of apoE degradation in the media or re-uptake by cholesterol-loaded macrophages is negligible (5%) during this period (24). Thus, as with cholesterol-loaded cells, a significant portion (>50%) of newly synthesized apoE in oxysterol-treated cells is most likely degraded intracellularly, prior to secretion. The identical $t_{½}$ and extent of intracellular degradation of apoE in oxysterol-treated cells, when compared to macrophages loaded with acLDL, suggest that cellular cholesterol and cholesteryl ester mass have no effect on the extent of intracellular apoE degradation or secretion.

Effect of brefeldin A on apoE turnover and processing

In order to localize the site of intracellular apoE degradation, we first examined the endoplasmic reticulum and the Golgi apparatus. We took advantage of the BFA-mediated blockage of the vectorial transport of newly synthesized proteins from the ER to the Golgi, and continuous recycling of the Golgi enzymes and membrane into the ER (33, 34). Consequently, if degradation of apoE took place in the ER or the Golgi compartments, BFA would not be expected to inhibit apoE degradation. The recovery of immunoprecipitable cellular apoE in the presence of BFA (5 μg/ml) at various times after chase initiation is shown in **Fig. 2,** and the disappearance of [^{35}S]apoE from BFA-treated cells is compared to that from control cells. Treatment of macrophages with BFA prevents apoE degradation completely (3.1 ± 1.8% vs. 53 ± 4% in control cells). It should be noted that, in control cells, the apoE immunoprecipitates at all time points contain multiple bands, a reflection of extensive sialiation (incubation with neuraminidase eliminates all but the lowest mol wt band; data not shown). In contrast, intracellular apoE in BFA-treated cells migrates as a single, neuraminidase-resistant band, indicating the lack of addition of the terminal sialic acid. As expected, BFA also completely inhibits total protein secretion. These experiments indicate that: 1) BFA prevents both intracellular degradation and secretion of [^{35}S]apoE, and 2) BFA completely prevents the addition of the terminal sialic acid residues, the final stage of apoE processing prior to secretion. Based on the known mechanism of BFA action, these results indicate that neither the endoplasmic reticulum nor the Golgi components are responsible for the intracellular degradation of apoE, and that the addition of sialic acid occurs at the TGN or later.

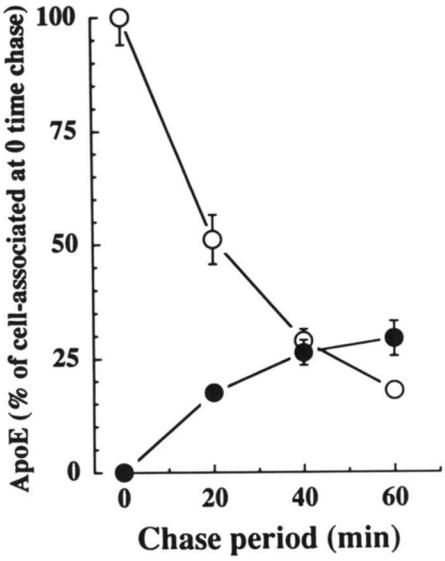

Fig. 1. [^{35}S]apoE turnover in oxysterol-treated mouse peritoneal macrophages. Cells were cultured and treated with 25-hydroxycholesterol as described in Materials and Methods. ApoE turnover was examined by a pulse-chase design: cells were pulsed for 60 min in methionine-free DMEM containing 40 μCi [^{35}S]-met/ml. After the pulse period, cells were chased for the indicated periods of time. At each time point of the chase both cell-associated (open circles) and media apoE (closed circles) were quantitatively immunoprecipitated and quantified, as described in Materials and Methods. Each point represents an average ± SEM of three separate experiments of three dishes per experiment.

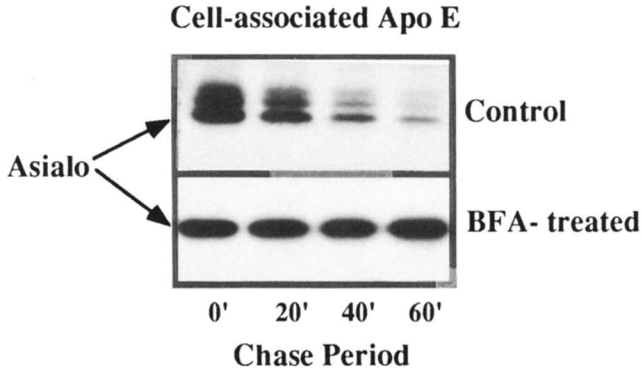

Fig. 2. Disappearance of [^{35}S]apoE from control and brefeldin A-treated cells. An experimental design similar to that described in the legend for Fig. 1 was used. BFA was used at 5 μg/ml and was present 0.5 h before and during the pulse as well as chase periods. Cell-associated [^{35}S]apoE at various chase times was compared by fluorography of the quantitative immunoprecipitates resolved by SDS-PAGE. The presence of the asialo form of apoE in BFA-treated cells was confirmed by neuraminidase treatment (results not shown).

Effect of lysosomotropic agents and protease inhibitors on apoE degradation

In this series of experiments we examined the role of lysosomal as well as neutral proteases in intracellular degradation of apoE. The lysosomotropic agents ammonium chloride (AC) and chloroquine (CQ) were used first. As shown in **Fig. 3** (A and B), both AC and CQ treatments result in a profound and concentration-dependent inhibition of apoE degradation; AC inhibits apoE degradation by >70% at 50 mM, while CQ inhibits apoE degradation by >80% at 100 μM. The role of lysosomal enzymes in apoE degradation is further indicated by the results obtained using a cocktail of four lysosomal protease inhibitors: antipain, E-64 (both inhibit lysosomal cysteine proteases, cathepsins A, B, and L), pepstatin (cathepsin D), and chymostatin (cathepsins A, B, and D). The mixture of these inhibitors (each at 100 μg/ml) inhibits apoE degradation by 63% (see **Fig. 4**). In light of the recent reports on the role of Ca^{2+}-dependent neutral cysteine proteases in the degradation of a number of proteins involved in lipid metabolism, including apoE (25), apoB (26) in HepG2 cells, and HMG-CoA reductase (28) in CHO cells, we also

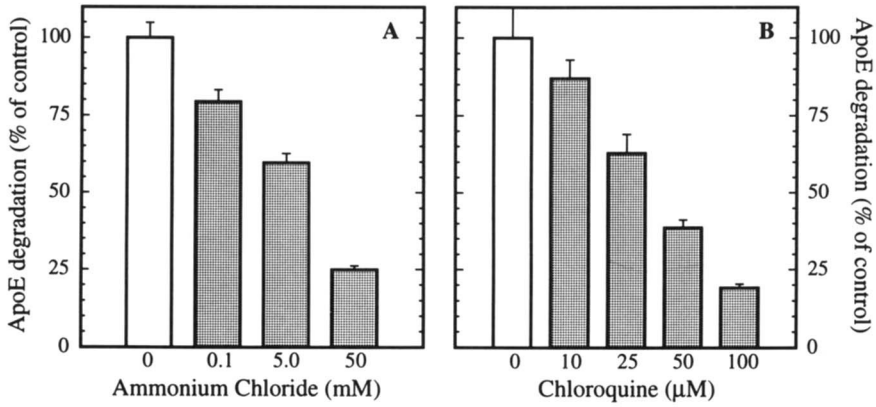

Fig. 3. Effect of ammonium chloride (A), and chloroquine (B) on apoE degradation. Cells were incubated with the indicated concentrations of lysosomotropic agents during the pulse (60 min) and chase periods. At the end of the chase period (60 min) both cell-associated and media [^{35}S]apoE were quantitatively immunoprecipitated and quantified. The extent of degradation was determined as the amount of cell-associated [^{35}S]apoE at 0 time chase minus the sum of media and cell-associated [^{35}S]apoE at 60 min of chase. Detailed procedures are described in Materials and Methods. The results are expressed as a percentage of cell-associated apoE at the end of the pulse period (0 time chase). Each bar represents an average of two experiments of three dishes each ± SEM. Each of the treatment points is statistically significantly different from the untreated value ($P < 0.05$).

examined the effect of ALLN and leupeptin, inhibitors of this protease (35). Leupeptin has no effect on the extent of apoE degradation (data not shown). Treatment of macrophages with ALLN at 50 and 100 μg/ml inhibits apoE degradation by 20 and 30%, respectively (Fig. 4). Compared to the lysosomotropic agents and lysosomal enzyme inhibitors, ALLN thus affords apoE relatively modest protection from degradation. As shown in **Fig. 5** (A–C), the inhibition of apoE degradation by CQ and AC is specific; these agents, as well as

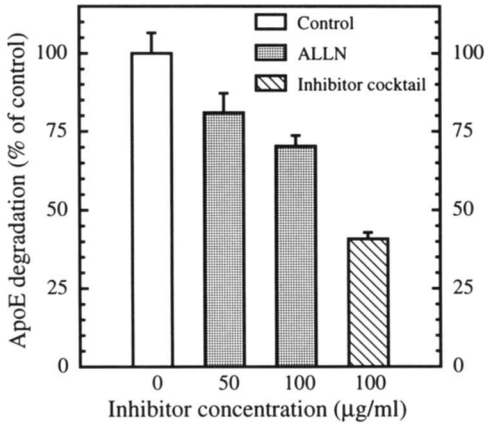

Fig. 4. Effect of ALLN and lysosomal protease inhibitor cocktail on apoE degradation. The experimental design was identical to that described for Fig. 3, except that ALLN or a cocktail of inhibitors was used at indicated concentrations. The cocktail contained antipain, E-64, pepstatin, and chymostatin dissolved in DMSO. The final concentration of DMSO was 0.1%. The ALLN data represent an average of two experiments of three dishes each ± SEM, while the inhibitor cocktail data was obtained from a single experiment of six dishes. Each of the treatment points is statistically significantly different from the untreated value ($P < 0.05$).

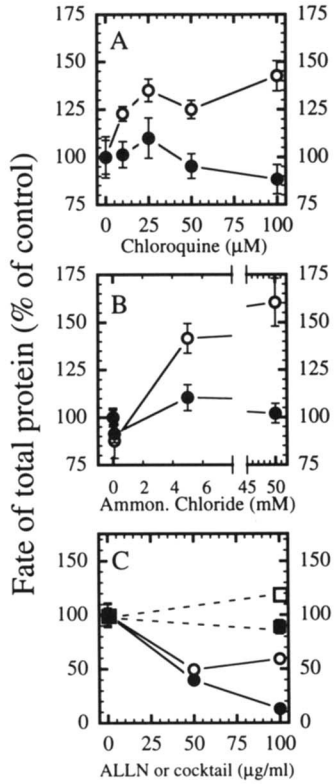

Fig. 5. Effect of chloroquine (A), and ammonium chloride (B) and ALLN and lysosomal protease inhibitors (C) on total cellular protein degradation and secretion. The experimental designs were identical to those described in the legend for Fig. 3 and 4, except that [^{35}S]methionine incorporation into trichloroacetic acid-precipitable proteins was determined as described in the Materials and Methods. The extent of degradation (closed circles and squares) and secretion (open circles and squares) in cells treated with chloroquine (A), ammonium chloride (B), and ALLN (circles) or the lysosomal protease inhibitor cocktail (squares), (described in the legend for Fig. 4) (C), were related to those measured in control (untreated) cells.

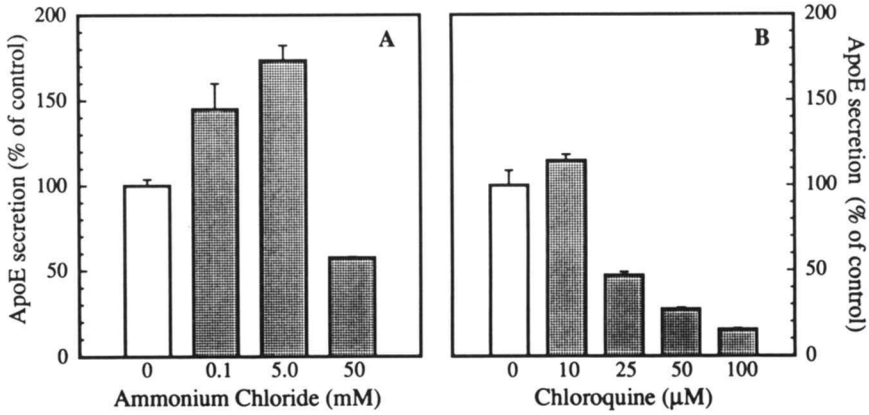

Fig. 6. Effect of ammonium chloride (A) and chloroquine (B) on apoE secretion. Experimental design is identical to that described in the legend for Fig. 3. The extent of [^{35}S]apoE secretion was quantified by quantitative immunoprecipitation, as described in the Materials and Methods, and expressed as a percentage of cell-associated apoE at the end of the pulse period (0 time chase). Each bar represents an average of two experiments of three dishes each ± SEM. Each of the treatment points is statistically significantly different from the untreated value ($P < 0.05$).

the lysosomal protease inhibitors, have no effect on total cellular protein degradation. In contrast, the modest effect of ALLN on apoE degradation is accompanied by a >80% inhibition of total cellular protein degradation, pointing out a rather nonspecific effect.

Effect of lysosomotropic agents on apoE secretion

Our initial hypothesis suggested that inhibition of apoE degradation would lead to a compensatory increase in apoE secretion. We examined this in the next series of experiments and the results are shown in **Fig. 6** (A and B). Both lysosomotropic agents exhibit a biphasic effect on apoE secretion. At lower concentrations (0.05–10 mM AC or 10 μM CQ), they inhibit apoE degradation and, as expected, stimulate apoE secretion. Higher concentrations of these agents (>10 mM AC or >10 μM CQ) not only prevent apoE degradation, but also inhibit apoE secretion by 50–90%. The inhibitory effects of these agents on apoE secretion appear to be specific, as both CQ and AC stimulate the secretion of total, trichloroacetic acid-precipitable proteins by macrophages in a dose-dependent manner (Fig. 5). Although we previously ruled out significant re-uptake of secreted apoE by macrophages (24), we examined the extent of apoE re-uptake by CQ-treated macrophages. Treatment of macrophages with 100 μM CQ has little additional effect on re-uptake; it essentially reduces re-uptake from a minimal value of ~5% to a negligible value of <3% (data not shown).

The inhibitory effect of CQ (100 μM) on apoE secretion in a pulse-chase experiment is shown in **Fig. 7.** It is apparent that 1) CQ treatment results in a block in apoE secretion, an effect analogous to that of BFA, and 2) in contrast to the effect of BFA, cellular apoE in the presence of CQ is partially sialiated, and the extent of sialiation increases with the time of incubation (Fig. 7B), suggesting that the block in apoE secretion is post-TGN.

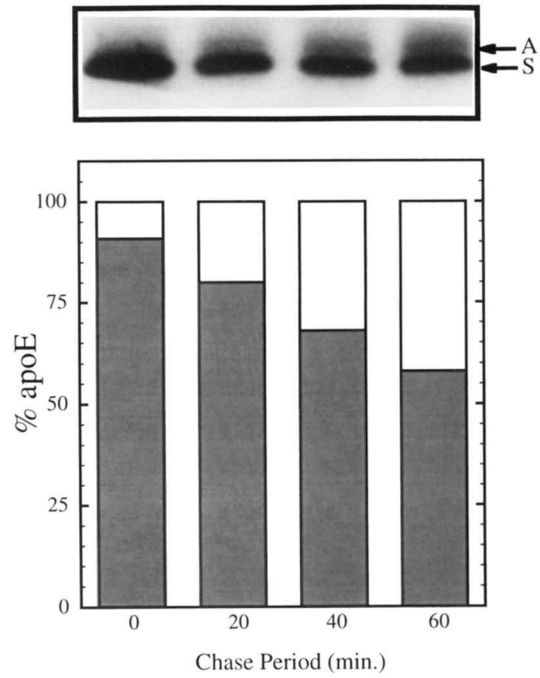

Fig. 7. Effect of chloroquine (100 μM) on apoE secretion and extent of sialiation. A pulse-chase experimental design described in Materials and Methods was used. CQ was present in all stages of the experiment. ApoE immunoprecipitates from cell lysates were resolved by SDS-PAGE and visualized by fluorography (top panel). The relative distribution of sialiated (open portion of the bars) and asialo (closed portion of the bars) forms of apoE was estimated by quantitative computerized scanning (PDI, Inc.), and results from a typical experiment (one of three) are shown (bottom panel).

Immunofluorescent light microscopy of macrophages

As high concentrations of CQ inhibit both apoE degradation and secretion, we compared control and CQ-treated immunostained macrophages at the light microscopic level in order to localize intracellular apoE. As can be seen in **Fig. 8** (A–D), addition of CQ to macrophages leads to a slightly enhanced overall staining of the cells for apoE and cathepsin D, a lysosomal enzyme active in both mature lysosomes and endosomes (36). Both of these proteins co-localize in intensely stained cytoplasmic vesicles predominantly near the cell surface and around the large vacuoles, characteristic of macrophages (37). Co-localization of apoE with cathepsin D provides further indication of apoE distribution in the endosomal/lysosomal compartment. The presence of these vesicles may be seen more clearly in magnified cells (Fig. 8, F) immunostained for both apoE and cathepsin D. In contrast, intensely stained cytoplasmic vesicles are not seen in control cells; both apoE and cathepsin D are distributed more evenly in smaller punctate bodies throughout the cytoplasm. Non-immune antibodies (Fig. 8, E) resulted in little immunolabeling.

Electron microscopy and immunolocalization of apoE

To further investigate the results obtained at the light microscopic level and to confirm our biochemical data, we also examined apoE distribution within the macrophage by immunogold labeling of thin frozen sections. As can be seen in **Fig. 9** (A–D), use of non-immune antibody followed by the secondary, gold-conjugated antibody results in little or no immunolabeling (Fig. 9A). In contrast, when the specific anti-apoE antibody is used on untreated macrophages, gold-labeled apoE can clearly be observed in endosomes and lysosomes (Fig. 9B). Treatment of macrophages with brefeldin A, which prevents both the degradation and secretion of newly synthesized apoE, results in almost complete loss of gold label from the endosomal/lysosomal compartment (Fig. 9C). In CQ-treated cells (100 μM), endosomal labeling remained unchanged or was even heavier (Fig. 9D), supporting the data obtained by fluorescent light microscopy and described above.

DISCUSSION

Our present studies establish that apoE turnover and the extent of its intracellular degradation are independent of the macrophage sterol content. The cellular apoE half-life of 22 ± 2 min in oxysterol-treated macrophages observed in these experiments is identical to that observed in macrophage-foam cells produced by loading with acLDL and reported earlier by this laboratory (24). The extent of apoE intracellular degradation (> 50%) is also indistinguishable from that observed in cholesterol-loaded cells.

Extensive studies in HepG2 cells provide convincing evidence for the continuous degradation of apoB within the ER compartment (38). Accordingly, we examined the role of this compartment in apoE degradation by the use of BFA, a compound that blocks the vectorial transport of newly synthesized proteins from the ER to the Golgi, but not the recycling of the Golgi components into the ER (33, 34). The apoE trapped in the ER under these conditions is not measurably degraded over the 60-min chase period, indicating that it is protected from degradation within this compartment. It should also be noted that the trapped apoE is incompletely processed, lacking the terminal sialic acid residues. This observation provides evidence that the sialiation of apoE, an O-linked glycoprotein, occurs in the TGN or later. The effectiveness of the BFA treatment in inhibiting apoE secretion was confirmed by quantitative immunoprecipitation of media apoE and by electron microscopy, where a nearly complete absence of gold labeling was noted in endosomes and lysosomes.

The lysosomotropic agents CQ and AC effectively inhibit the receptor–ligand dissociation and lysosomal protein degradation by increasing the lysosomal (39) and endosomal (40) pH. Treatment of macrophages with either of these agents results in a dose-dependent and nearly complete inhibition of apoE degradation with CQ being more effective in this action than AC. This is not surprising, as CQ is over 100-fold more effective (in terms of concentrations) in achieving an equivalent change in lysosomal pH in macrophages (41).

The role of lysosomal proteases in apoE degradation is further confirmed by the use of a cocktail consisting of four inhibitors of lysosomal proteases. Although these inhibitors were used at concentrations of 100 μg/ml, the extent of their penetration of intact cells is not known. The lack of complete inhibition of apoE degradation (or approaching that seen with AC or CQ) may be due to relatively low intracellular concentrations of these inhibitors or due to action of other lysosomal proteases that are not inhibited by these agents. Nevertheless, these agents are significantly more effective in protecting apoE from degradation than ALLN or leupeptin. The relatively small inhibition of apoE degradation by ALLN observed by us may be due to its well-documented effect on lysosomal cysteine proteases (cathepsins B and L) (42).

Immunolocalization of apoE followed by both light and electron microscopy provides additional evidence

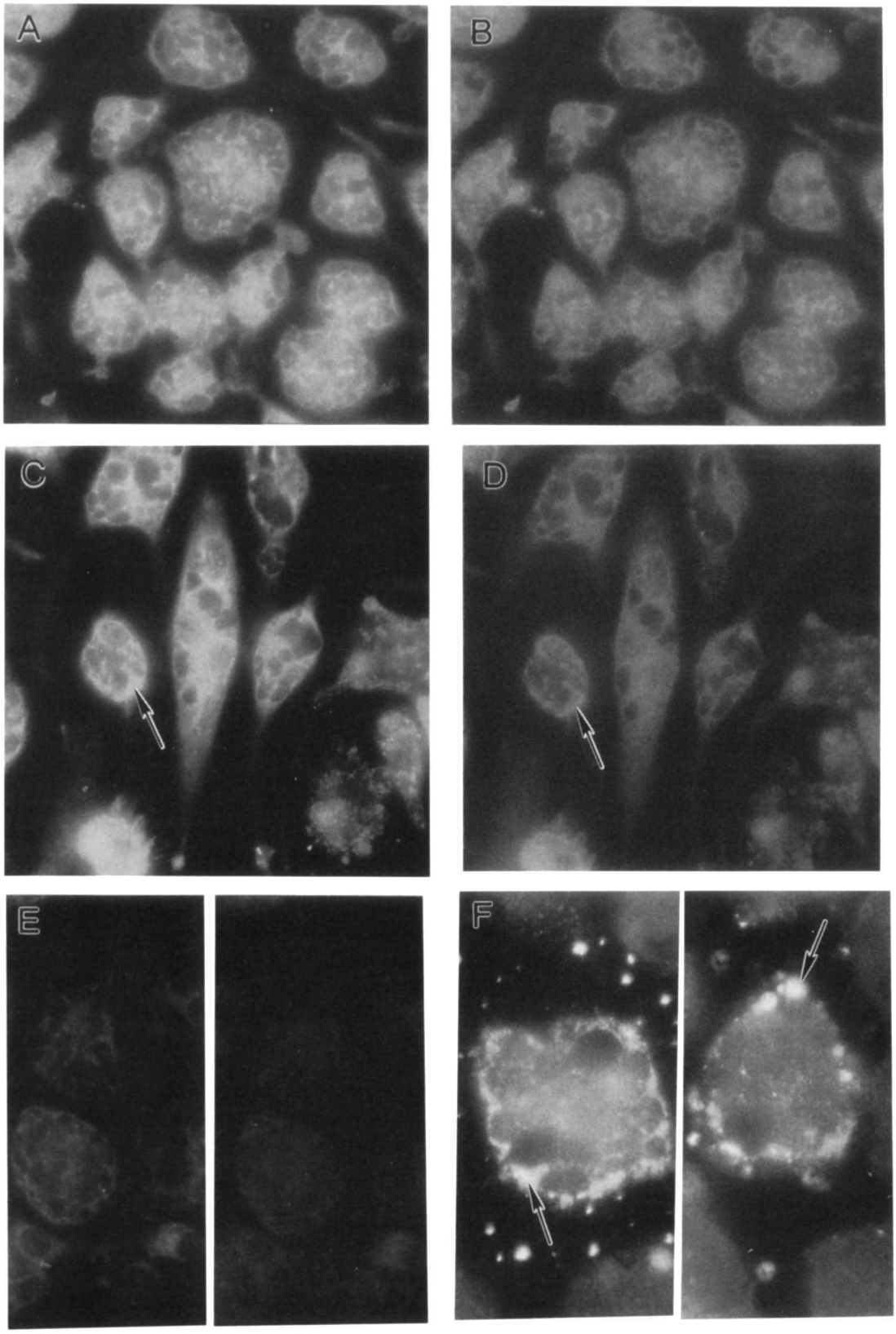

Fig. 8. Effect of chloroquine on the distribution of apoE and cathepsin D in macrophages. Confluent monolayers grown on coverslips were immunostained for apoE and cathepsin D as described in Materials and Methods. A–D are double labeled for apoE and cathepsin D. A and C show cathepsin D localization, while B and D show apoE localization; (A and B) untreated; no drug was present in the medium; final magnification is 745 ×. (C and D) chloroquine-treated cells; CQ was added to the medium to a final concentration of 100 μM. Note that apoE and cathepsin D co-localize in vesicles (arrows); final magnification is 745 ×. (E) control; non-immune goat IgG (left panel) or non-immune rabbit serum (right panel) was substituted for the primary antibodies; final magnification is 745 ×. (F) chloroquine-treated cell; CQ was added to the medium at a final concentration of 100 μM. Cells were immunostained for either apoE (right panel) or cathepsin D (left panel). Note the accumulation of apoE and cathepsin D in cytoplasmic vesicles (arrows); final magnification is 1340 ×.

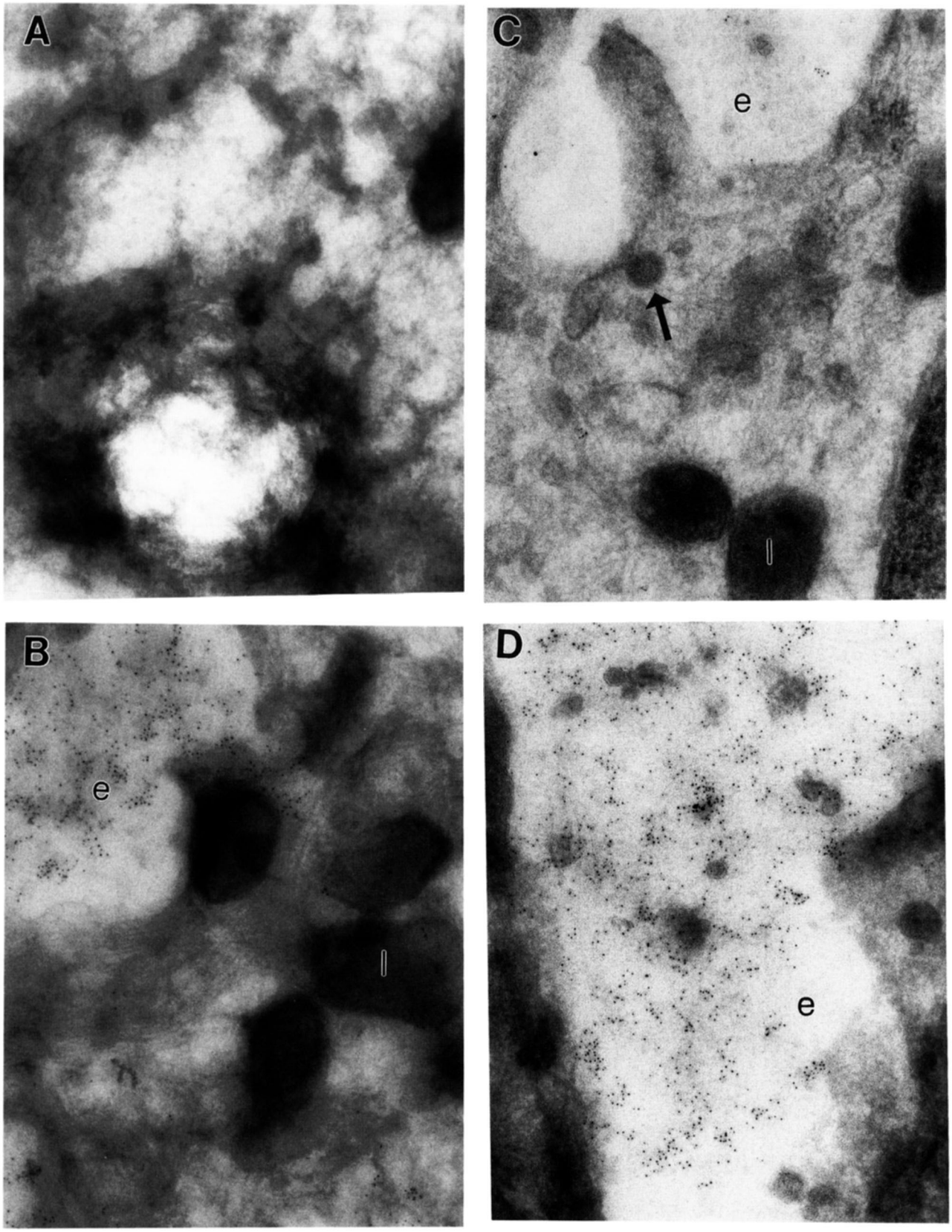

Fig. 9. Immunogold localization of apoE in mouse macrophages. Cryofixation for the preparation of frozen sections is described in Materials and Methods. Cells were then immunostained with a 1:1000 dilution of polyclonal goat anti-apoE, followed by incubation with a 1:50 dilution of 5 nm gold-conjugated anti-goat IgG. (A) control; non-immune goat IgG was substituted for the primary antibody. (B) untreated; no drugs were present in the media. (C) brefeldin A-treated cells; BFA was added to the media at 5 μg/ml; note low levels of gold labeling in endosomes and lysosomes when compared to (B). (D) chloroquine-treated cells; CQ was added to the media to a final concentration of 100 μM. l, mature lysosomes, e, endosomal compartment. The final magnification in each case is 58,000 ×.

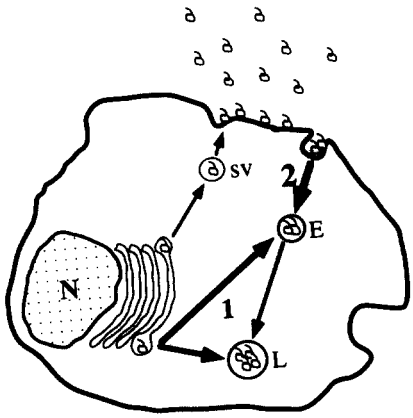

Fig. 10. Potential pathways of lysosomal targeting of apoE in mouse macrophages. Two potential pathways of apoE targeting to the lysosomal compartment can be envisioned: *1)* direct intracellular targeting of a fraction of apoE to the lysosomes, analogous to the targeting of lysosomal enzymes, or *2)* secretion of apoE, a fraction of which remains tightly bound to a component of the external side of plasma membrane, followed by endocytosis and delivery to the lysosome.

for lysosomal degradation of apoE in primary macrophages. This is in contrast to HepG2 cells, where both lysosomal and Ca^{2+}-dependent cytosolic cysteine proteases may be responsible for the intracellular degradation of apoE (25).

In contrast to the uniform, dose-dependent inhibition of apoE degradation by the lysosomotropic agents, their effect on apoE secretion is biphasic. At lower concentrations the inhibition of apoE degradation is accompanied by increased secretion, while at higher concentrations apoE secretion is also inhibited. The stimulation of apoE secretion with low doses of inhibitors is of particular interest. Both CQ and AC have been reported to interfere with protein trafficking in cells. In addition to their well-known disruption of the ligand–receptor uncoupling and consequent depletion of the receptor from the plasma membrane, shown for a number of receptor–ligand pairs (43–46), both AC and CQ stimulate the secretion of lysosomal enzymes due to the mannose-6-phosphate receptor sequestration (47). Our observations would thus imply that 1) sorting of apoE for lysosomal degradation is sensitive to very small changes in pH, and 2) an intracellular receptor for apoE targeting may be involved. In this respect, it should be noted that a number of intracellular apoE-binding proteins have been described, including a 59 kD protein residing in the ER (48). Greater changes in pH apparently inhibit, specifically, the transport of apoE through the secretory pathway as well, resulting in a nearly complete inhibition of secretion. The specific effect of pH on apoE may be explained by subtle, pH-dependent conformational changes in the tertiary structure of apoE which may directly or indirectly interfere with its transport through the secretory route. The specific inhibition of apoE secretion by CQ is not due to increased re-uptake by the macrophages; in fact, CQ essentially completely abolishes the already low levels of apoE reuptake.

These observations raise questions regarding the pathway apoE must take to arrive in the lysosome and beyond. Previously reported work from this laboratory (24) demonstrated that macrophages do not take up apoE from macrophage-conditioned media to a significant extent (5% over 120 min). In the present studies we extend this observation to CQ-treated cells as well. Two potential pathways, shown in **Fig. 10,** can thus be envisioned. First, direct intracellular targeting of a fraction of apoE to the lysosomes, analogous to the targeting of lysosomal enzymes, or 2) secretion of a fraction of apoE that remains tightly bound to a component of the external side of plasma membrane, followed by endocytosis and delivery to the lysosome. The observations of surface-bound cell-associated apoE in HepG2 cells (49) support the second hypothesis. Furthermore, Kruth et al. (50) have described a new endocytic mechanism in human monocyte-derived macrophages, in which apoE is localized within surface-connected compartments. It may be that the higher concentrations of CQ prevent the release of apoE from these sequestration sites. It should be pointed out, however, that Kruth's compartments did not contain cathepsin D, which suggests that the endosomes we observe are different. As CQ treatment blocks 90% of apoE secretion (and completely inhibits re-uptake), it is difficult to envision how a greatly diminished transport of apoE to the cell surface can lead to increased endosomal accumulation, unless apoE is targeted directly to this compartment from the TGN. Evidence for such delivery has recently been established; a proportion of newly synthesized lysosomal enzymes and class II major histocompatibility complex antigens (51) as well as transferrin receptors (52) have been shown to be carried directly to the endosomal compartment from the Golgi. Thus, the endosome as well as the TGN may play a critical role in protein sorting. Clearly, additional work is needed in this area.

In summary, it appears that pH gradients in the various compartments, beginning with and distal to the trans-Golgi compartment play an important role in the sorting and trafficking of apoE. A relatively small change in pH is sufficient to divert apoE destined for lysosomes to the secretory pathway. This part of the sorting process may involve an interaction with a specific apoE receptor/binding protein. Greater changes in pH may impair apoE transport to the cell surface. These observations further point to the complexity of apoE metabolism in macrophages.

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