Intracellular trafficking of recycling apolipoprotein E in Chinese hamster ovary cells

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Abstract **We have investigated apolipoprotein E (apoE) recycling in Chinese hamster ovary (CHO) cells, a peripheral cell that does not produce lipoproteins or express apoE. Using a pulse-chase protocol in which cells were pulsed with ^{125}I-apoE-VLDL and chased for different periods, ~30% of the apoE internalized during the pulse was resecreted within a 4 h chase in a relatively lipid-free state. The addition of lysosomotropic agents or brefeldin A had no effect on apoE recycling. Unlike previous results with hepatocytes and macrophages, neither apoA-I nor upregulation of ABCA1 stimulated apoE recycling. However, cyclodextrin, which extracts cholesterol from plasma membrane lipid rafts, increased recycling. Confocal studies revealed that apoE, internalized during a 1 h pulse, colocalizes with early endosomal antigen-1, Rab5, Rab11a, and lysobisphosphatidic acid but not with lysosomal-associated membrane protein-1. Colocalization of apoE and Rab11a persisted even after cells had been chased for 1 h, suggesting a pool of apoE within the endosomal recycling compartment (ERC). Our data suggest that apoE recycling in CHO cells is linked to cellular cholesterol removal via the ERC and phospholipid-containing acceptors in a pathway alternative to the ABCA1-apoA-I axis.**—Braun, N. A., P. J. Mohler, K. H. Weisgraber, A. H. Hasty, M. F. Linton, P. G. Yancey, Y. R. Su, S. Fazio, and L. L. Swift. **Intracellular trafficking of recycling apolipoprotein E in Chinese hamster ovary cells.** ***J. Lipid Res.*** **2006.** 47: **1176–1186.**

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Apolipoprotein E (apoE) is a 34 kDa protein that functions in plasma lipoprotein metabolism and intracellular lipid disposal. Extracellularly, apoE is a key mediator of the internalization of remnant lipoprotein particles by serving as a ligand for the LDL receptor (1, 2) and the low density lipoprotein receptor-related protein (LRP) (3, 4). ApoE is also internalized by binding heparan sulfate proteoglycans alone (5, 6) or via a mechanism involving both heparan sulfate proteoglycans and LRP (7, 8). Intracellularly, apoE modulates lipid metabolism (9, 10) and functions in the routing of internalized lipoprotein remnants (11, 12). In addition, it is involved in both the assembly (13, 14) and secretion (15, 16) of VLDL. ApoE also plays a critical role in cholesterol efflux from macrophages (17–20), a role that could be attributable to both intracellular and extracellular effects. Because apoE can easily transfer between lipoproteins and yet binds tightly to its receptors, we first hypothesized and later demonstrated that a substantial amount of internalized apoE escapes lysosomal degradation and is routed back (recycled) through the secretory pathway (21–23). We reported that apoE is found in the media of hepatocyte cultures from apoE-deficient mice transplanted with wild-type bone marrow (22), proof that systemic apoE can be retained by the liver cell and eventually resecreted. In addition, we reported that apoE was resecreted from apoE-deficient hepatocytes after incubation with apoE-containing VLDL (23). Furthermore, apoA-I stimulates apoE recycling in hepatocytes (23) and macrophages (24), suggesting a role for recycling in HDL metabolism. Finally, we have shown that apoE recycling occurs in the absence of the LDL receptor, under conditions of markedly reduced LRP expression, and in the absence of an intact Golgi apparatus (25).

We initially studied hepatocytes and macrophages because these cells face a tremendous cholesterol burden under physiological conditions and are equipped with specialized tools to reduce this burden (e.g., lipoprotein assembly and secretion in hepatocytes, and multiple

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Abbreviations: apoE, apolipoprotein E; BFA, brefeldin A; β-CD, 2-hydroxypropyl β-cyclodextrin; CHO, Chinese hamster ovary; EEA1, early endosome antigen-1; ERC, endosomal recycling compartment; F12K, Kaighn's modification of Ham's F-12 medium; LAMP-1, lysosomal-associated membrane protein-1; LBPA, lysobisphosphatidic acid; LPDS, lipoprotein-deficient serum; LRP, low density lipoprotein receptor-related protein; LXR, liver X receptor; SR-BI, scavenger receptor class B type I.

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cholesterol efflux mechanisms in macrophages). In addition, both cells produce high levels of apoE, which could have multiple effects on cholesterol routing and may diminish the importance of apoE recycling. Thus, we were interested in identifying a nonmacrophage peripheral cell line that did not produce apoE but did express other relevant molecules, such as the LDL receptor, LRP, and ABCA1. In this regard, Chinese hamster ovary (CHO) cells provide an excellent model (26, 27). Here, we demonstrate apoE recycling in CHO cells. ApoA-I had no effect on recycling, nor did treatment of cells with liver X receptor (LXR) agonist (TO-901317), which has been shown to upregulate ABCA1 expression. However, other conditions that increase cholesterol efflux, such as incubation with cyclodextrin, increased apoE recycling. Furthermore, confocal studies indicate that recycling apoE traffics through the early endosomal compartment and the endosomal recycling compartment (ERC). Our data suggest that neither apoA-I nor ABCA1 is directly involved in apoE recycling in CHO cells but that recycling is linked to cellular cholesterol removal via the ERC and phospholipid-containing acceptors.

EXPERIMENTAL PROCEDURES

Materials

CHO cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in six-well dishes (BD-Falcon, Bedford, MA) in Kaighn's modification of Ham's F-12 medium (F12K) containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Purified human apoA-I was purchased from Biodesign (Saco, ME). The LXR agonist TO-901317 was from Sigma (St. Louis, MO). Rabbit anti-human apoE antibody was purchased from Dako (DakoCytomation, Carpinteria, CA). Antibodies to Rab5, lysosomal-associated membrane protein-1 (LAMP-1), and early endosome antigen-1 (EEA1) were purchased from BD Biosciences (Transduction Laboratories, San Diego, CA). Antibody to Rab11a was a gift from Dr. James Goldenring (Vanderbilt University School of Medicine). Antibody to lysobisphosphatidic acid (LBPA) was a gift from Dr. W. Gray Jerome (Vanderbilt University School of Medicine). Polyclonal antibody to human ABCA1 was purchased from Novus Biologicals (Littleton, CO). Alexa 488- and 568-conjugated secondary antibodies were purchased from Molecular Probes (Eugene, OR). Brefeldin A (BFA), monensin, chloroquine, and 2-hydroxypropyl β-cyclodextrin (β-CD) were purchased from Sigma. Lipoprotein-deficient serum (LPDS) was prepared from FBS by ultracentrifugation.

Preparation of radiolabeled VLDL

Human VLDL was isolated at $d < 1.019$ g/ml from a healthy, fasted donor as described previously (23). The protocol was approved by the Vanderbilt Institutional Review Board, and informed consent was obtained before collecting blood. The apoprotein composition of VLDL was determined by SDS-PAGE (23). Purified recombinant human apoE3 was produced as described previously (28) and iodinated by the Iodogen method (Pierce Biochemicals, Rockford, IL) using ^{125}I (IMS-30) from Amersham Pharmacia Biosciences (Piscataway, NJ). Aliquots of labeled protein were analyzed by SDS-PAGE using the NuPAGE system (Invitrogen, Carlsbad, CA) followed by autoradiography. ^{125}I-apoE ($\sim 2 \times 10^6$ cpm) was incubated with VLDL (100 μg) for 60 min at 37°C. The association of labeled apoE with VLDL was confirmed by fast-protein liquid chromatographic analysis.

Recycling experiments

CHO cells were incubated (pulsed) with ^{125}I-apoE-VLDL (20 μg of protein and $\sim 4 \times 10^5$ cpm/well) for 2 h. At the end of the pulse, the medium was aspirated and cells were washed five times with ice-cold PBS, incubated with heparin (10 mg/ml in PBS) for 3 min at room temperature, and then washed five additional times with PBS. Fresh medium was added, and cells were incubated (chased) for various time periods. At the end of the chase period, the medium was collected, and cells were treated with heparin as described above. This heparin wash was added to the medium for analysis of resecreted apoE. After heparin treatment, cells were collected in 0.1 N NaOH. Recycling experiments used serum-containing, LPDS-containing, and serum-free media for the pulse and chase. In some experiments, purified human apoA-I was added to the pulse and chase media (25 μg/ml). Total cell protein was determined using the bicinchoninic acid assay method (Pierce Biochemicals) with BSA as the standard and modified to eliminate lipid interference (29). Degradation was defined as TCA-soluble radioactivity in the medium. Recycled apoE was defined as total TCA-precipitable label in the medium at the end of the chase period. Radioactivity (cpm) was normalized to cell protein, and data are expressed as percentage of total cpm/mg protein at each time point. Secretion of apoE was confirmed by immunoprecipitation of apoE from cell lysates and media, followed by SDS-PAGE and transfer to nitrocellulose membranes, as described previously (22). Membranes were exposed to a Cyclone SR Screen, and radiolabeled apoE was visualized using a Cyclone Storage Phosphor System with OptiQuant software (Packard Instrument Co., Meriden, CT).

Isolation of recycled apoE

To investigate the form of recycled apoE, recycling experiments were carried out as described above except that the chase medium was serum-free or contained LPDS. At the end of a 60 or 120 min chase, the medium was collected, the density was increased to 1.210 g/ml using solid KBr, and lipoproteins were floated using an Optima TLX Tabletop Ultracentrifuge (Beckman Coulter, Palo Alto, CA) with a TLA100.4 rotor. Total and TCA-precipitable radioactivity in the $d < 1.210$ and $d > 1.210$ g/ml fractions was determined.

Pharmacologic studies

The effects of various pharmacologic agents on apoE recycling in CHO cells were investigated using the recycling protocol described above. In general, the agents were added at various times before the pulse and were included in the medium throughout the experiment. BFA was added at 10 μg/ml; monensin was added at 10 μM; and chloroquine was added at 100 μM. For all agents, the solvent in which the agent was dissolved was added to control cells at the same concentration as in the experimental samples.

Cells were also treated with the LXR agonist TO-901317 (10 μM) for 18 h before the initiation of recycling experiments. This concentration of the agonist has been shown to increase ABCA1 mRNA expression in macrophages by 34-fold (30).

Immunoblotting for ABCA1

Cellular extracts from CHO cells treated with the LXR agonist were separated by 3–8% NuPAGE Tris-acetate gels (Invitrogen) and transferred to nitrocellulose membranes (31). The membranes were blocked, incubated with antibody to ABCA1, and visualized by ECL (Amersham Pharmacia Biotech) after incubation with secondary antibody conjugated with horseradish peroxidase.

Cholesterol depletion

β-CD was added during the chase (20 mM) to deplete cells of cholesterol. It was only added to medium containing LPDS or to serum-free medium and was not used in the presence of serum-containing medium.

Immunofluorescence

CHO cells were grown in eight-well chamber slides (Nunc Lab-Tek Chamber Slide System; Fisher Scientific, Norcross, GA) in F12K containing 10% FBS. The cells were then incubated with apoE-containing human VLDL for 1 h in F12K containing 10% LPDS. At the end of the incubation, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed three times with intracellular buffer (75 mM potassium acetate, 2.5 mM magnesium acetate, 1.8 mM calcium chloride, and 25 mM HEPES buffer, pH 7.2) at room temperature and permeabilized with 0.1% saponin in intracellular buffer with 0.4% BSA for 30 min at room temperature. All subsequent steps, including the washes, were performed in intracellular buffer containing 0.1% saponin and 0.4% BSA. The cells were incubated overnight with primary antibodies at 4°C and then rinsed three times for 10 min. The appropriate secondary antibodies were applied in the dark for 2 h at room temperature. After three washes of 10 min each, the cells were mounted with ProLong Antifade reagent (Molecular Probes). Primary antibodies were used at the following dilutions: anti-apoE, 1:10,000; anti-Rab5, 1:100; anti-EEA1, 1:500; anti-Rab11a, 1:100; anti-LBPA, 1:100; and anti-LAMP-1, 1:100. Fluorescent secondary antibodies were used at the following dilutions: Alexa 488-conjugated anti-rabbit IgG, 1:1,000; and Alexa 568-conjugated anti-mouse IgG, 1:1,000. In double-labeling experiments, the two primary antibodies were applied simultaneously, followed by a mixture of the two secondary antibodies. In control experiments, fluorescence was shown to be negligible in the absence of primary antibody. In addition, there was no cross-reactivity of the anti-mouse IgG or the anti-rabbit IgG secondary antibody with the polyclonal or monoclonal primary antibody, respectively. Finally, there was no bleed through of the Alexa 568 fluorophore at 488 nm or the Alexa 488 fluorophore at 568 nm.

Confocal microscopy

Images were collected and analyzed on a Zeiss LSM 510 confocal laser scanning inverted microscope using either a 40×/1.30 or a 63×/1.40 Plan-Apochromat objective (pinhole equals 1.0 Airy Disc) using Carl Zeiss imaging software. Both channels were collected on PMT3 using calibrations to confirm that fluorescence was collected in the linear range. For display, images were converted into TIFF format and processed using Adobe Photoshop software (version 7.0).

Statistical analyses

One-way ANOVA was performed using GraphPad InStat (version 3.00). Data are expressed as means ± SD.

RESULTS

ApoE recycling in CHO cells

CHO cells were incubated with ^{125}I-apoE-VLDL for 2 h and washed extensively, and the secretion of internalized, labeled apoE was monitored. The appearance of intact (TCA-precipitable) apoE in the medium was time-dependent, with ~25–30% of the internalized apolipoprotein being secreted in 240 min (**Fig. 1A**). Cellular apoE decreased by ~80% over the 4 h chase period. The amount

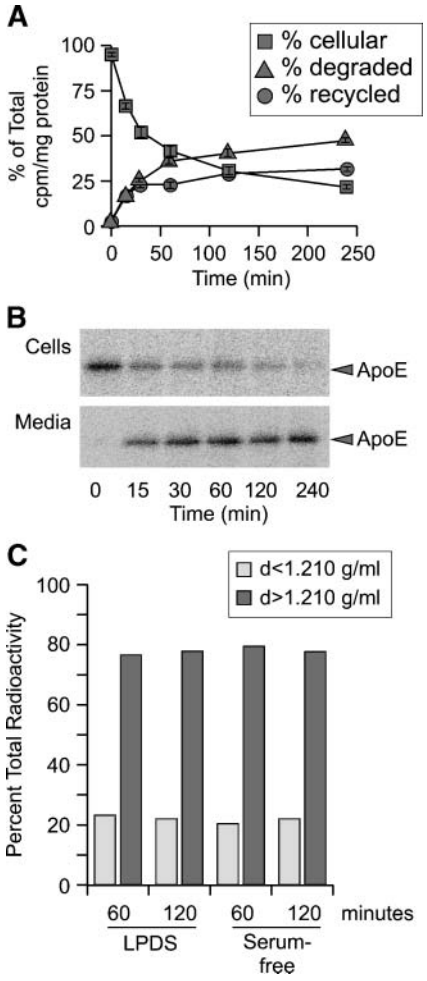

Fig. 1. Apolipoprotein E (apoE) recycling in Chinese hamster ovary (CHO) cells. A: Cells were pulsed with ^{125}I-apoE-VLDL for 2 h in serum-containing medium and washed extensively. Fresh serum-containing medium was added, and cells were incubated (chased) for different times. At the end of the experiment, medium was collected. Cells were solubilized in 0.1 N NaOH, and protein and cellular radioactivity were determined. Recycled apoE was defined as total TCA-precipitable label in the medium at the end of the chase period. Degradation was defined as TCA-soluble radioactivity in the medium. Radioactivity (cpm) was normalized to cell protein, and data are expressed as percentage total cpm/mg protein at each time point. Data represent the mean ± SD from five experiments. B: Secretion of apoE was confirmed by immunoprecipitation of apoE from cell lysates and medium followed by SDS-PAGE and transfer to nitrocellulose membranes. Membranes were exposed to a Cyclone SR Screen, and radiolabeled apoE was visualized using a Cyclone Storage Phosphor System with OptiQuant software. C: Ultracentrifugal distribution of resecreted apoE. Cells were pulsed in lipoprotein-deficient serum (LPDS)-containing medium and chased in either LPDS or serum-free medium, and the medium was fractionated into $d < 1.210$ and $d > 1.210$ g/ml fractions. TCA-precipitable radioactivity was determined in each fraction. The data from each time point represent the mean of duplicate fractionations from two separate experiments.

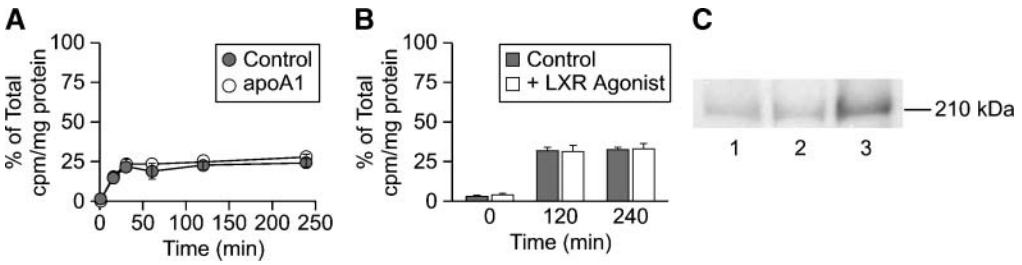

Fig. 2. Effects of apoA-I and a liver X receptor (LXR) agonist on apoE recycling in CHO cells. A: ApoE recycling experiments were carried out as described for Fig. 1 except that human apoA-I (25 μg/ml) was added to the chase. Only data for recycled apoE are shown. B: Cells were treated with the LXR agonist TO-901317 (10 μM) for 18 h before the initiation of recycling experiments. C: Immunoblot of ABCA1 in untreated CHO cells (lane 1) and cells treated with DMSO (lane 2) or the LXR agonist TO-901317 (10 μM) (lane 3) for 18 h. Data in A and B represent mean ± SD from three experiments.

of apoE degraded increased during the chase period, reaching ~50% of total apoE at the 240 min time point. Cellular and recycled apoE were also monitored via immunoprecipitation (Fig. 1B). Cellular apoE decreased over the course of the experiment, with the largest decrease occurring during the first 30 min of the chase period; however, cellular apoE was still visible after 240 min of chase. Recycled apoE was present in the medium at all time points, although the amount appeared to plateau between 30 and 60 min. Approximately 75–80% of the TCA-precipitable radioactivity in LPDS or serum-free chase medium was recovered in the $d > 1.210$ g/ml fraction, suggesting that the bulk of recycled apoE was either lipid-free or associated with limited amounts of lipid (Fig. 1C).

ApoE recycling was not affected by the addition of apoA-I (25 μg/ml) during the pulse and chase (**Fig. 2A**). Likewise, treatment of cells with the LXR agonist TO-901317 had no effect on apoE recycling (Fig. 2B), in spite of the threefold increase in expression of ABCA1 protein in the cells (Fig. 2C).

Pharmacologic interventions

Because nearly 50% of the apoE was degraded over the 4 h chase period, we tested the effects of lysosomotropic agents on apoE recycling to evaluate whether reduced degradation leads to increased resecretion. In the presence of chloroquine (100 μM), apoE degradation was clearly decreased; however, apoE recycling was unchanged (**Fig. 3**). Monensin (10 μM) produced a more modest decrease in the degradation of apoE than chloroquine (Fig. 3); however, apoE recycling was not altered. It is important to note that neither chloroquine nor monensin had any effect on the uptake of ^{125}I-apoE-VLDL.

To investigate the cellular routing of recycling apoE, CHO cells were pulsed with ^{125}I-apoE-VLDL and chased in

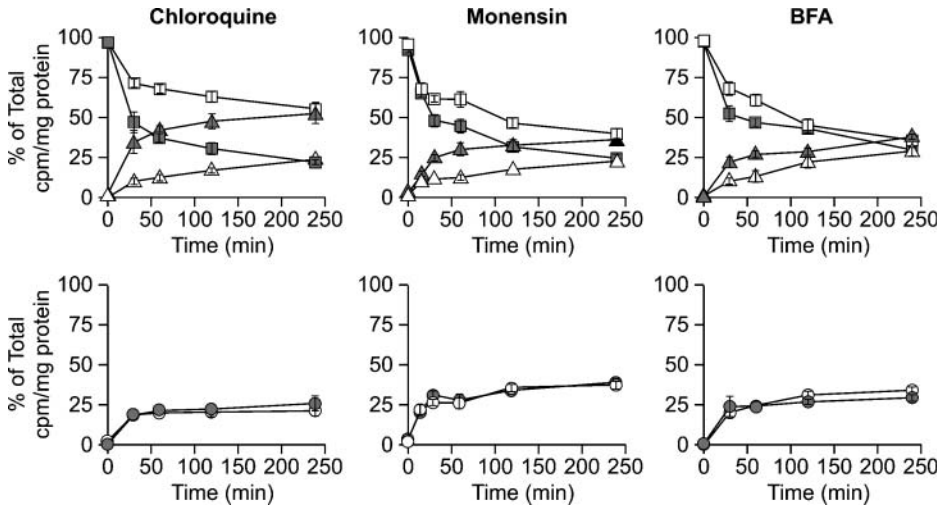

Fig. 3. Effects of chloroquine, monensin, and brefeldin A (BFA) on apoE recycling in CHO cells. Recycling experiments were performed as described for Fig. 1. Chloroquine (100 μM), monensin (10 μM), or BFA (10 μg/ml) was added during the pulse and chase. Cellular (squares), degraded (triangles), and recycled (circles) apoE are expressed as percentage total cpm/mg protein. Closed symbols, control; open symbols, treated. Data for each treatment represent mean ± SD from three experiments.

the presence of BFA (10 μg/ml). BFA is a fungal metabolite that blocks trafficking from the endoplasmic reticulum to the Golgi apparatus and ultimately leads to dissolution of the Golgi complex (32–34). In addition, BFA treatment blocks vesicular traffic from the ERC to the plasma membrane (35). ApoE recycling was not affected by the presence of BFA (Fig. 3).

ApoE recycling and cholesterol efflux

When CHO cells were pulsed in LPDS-containing medium, ^{125}I-apoE-VLDL uptake increased 2- to 3-fold compared with that in cells pulsed in serum-containing medium; however, the increased uptake did not result in increased recycling. In fact, the absolute amount of apoE that recycled was identical in cells pulsed and chased in serum- or LPDS-containing medium (data not shown). Consequently, the percentage of apoE recycled was significantly lower in cells pulsed and chased in LPDS compared with cells pulsed and chased in serum-containing medium (**Fig. 4A**). We hypothesized that the percentage of apoE recycled could be enhanced by including in the chase medium cholesterol acceptors that stimulate cholesterol efflux via mechanisms unrelated to ABCA1. To test this hypothesis, we used β-CD as an acceptor, because studies have shown that β-CD promotes the removal of cholesterol from the plasma membrane (36, 37) and the endocytic recycling compartment (38) via a mechanism independent of ABCA1 (39). When cells were pulsed in LPDS and chased in LPDS + β-CD, apoE recycling increased compared with that in cells pulsed and chased in LPDS alone (Fig. 4A). Likewise, β-CD increased apoE recycling when cells were pulsed and chased in the absence of cholesterol acceptors (Fig. 4B). The order of apoE recycling (Fig. 4C) suggests that the recycling pathway is linked to cholesterol efflux mechanisms that mobilize cholesterol to phospholipid-containing acceptors (40–42) rather than lipid-poor apoproteins (i.e., ABCA1-mediated efflux) (39, 42).

Confocal microscopic analysis of apoE trafficking

CHO cells were incubated with apoE-containing human VLDL for 1 h before fixation and preparation for confocal microscopy. ApoE was visualized within the cells in vesicular structures (**Fig. 5A, D, G, J**). The patterns varied between individual cells. In some cells, apoE fluorescence was observed throughout the cytoplasm; in other cells, apoE was found clustered in specific regions (e.g., under the plasma membrane, suggestive of an early endosomal location, or in a perinuclear region, suggestive of the ERC). Consistent with these observations, we found that apoE colocalized with EEA1 (Fig. 5A–C), Rab5 (Fig. 5D–F), Rab11a (Fig. 5G–I), and LBPA (Fig. 5J–L). In contrast, we found little colocalization of apoE with LAMP-1, a lysosomal marker. Colocalization with Rab11a was observed even after the cells were chased for 1 h, suggesting that apoE remains in the ERC for an extended period of time (**Fig. 6**).

As chloroquine and monensin decreased apoE degradation but did not alter recycling, we studied the location of apoE in cells pulsed in the presence of these two lysosomotropic agents. In cells treated with chloroquine, overall cellular apoE fluorescence was increased. Colocalization of apoE and EEA1 was very prominent in the perinuclear regions, as seen in control cells; however, there was increased EEA1 fluorescence and colocalization with apoE in the peripheral regions of the cell (**Fig. 7**). In monensin-treated cells, apoE fluorescence was concentrated in the perinuclear region of the cells; however, it was not homogenously distributed around the nucleus but seemed to be concentrated in specific areas. Nevertheless, much of this perinuclear staining colocalized with EEA1 (Fig. 7). Whereas we noted little colocalization of apoE with LAMP-1 in control cells, in chloroquine- and monensin-treated cells, we noted extensive colocalization of LAMP-1 with apoE (data not shown).

DISCUSSION

In this study, we examined apoE recycling in CHO cells using a pulse-chase protocol in which cells were incubated with ^{125}I-apoE-VLDL and chased for various times in the absence of the radiolabeled apoprotein. Greater than 50% of the internalized ^{125}I-apoE was degraded over a 4 h chase

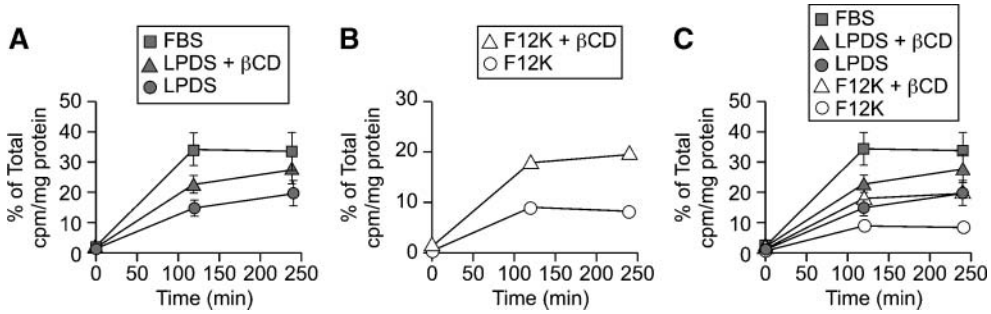

Fig. 4. ApoE recycling and cholesterol efflux in CHO cells. Recycling experiments were performed as described for Fig. 1. A: Cells were pulsed and chased in serum-containing medium or pulsed in the presence of LPDS and chased in LPDS with or without 2-hydroxypropyl β-cyclodextrin (β-CD; 20 mM). B: Cells were pulsed and chased in serum-free medium with or without β-CD (20 mM). C: Summary of apoE recycling under different pulse/chase conditions. Data represent mean ± SD. F12K, Kaighn's modification of Ham's F-12 medium.

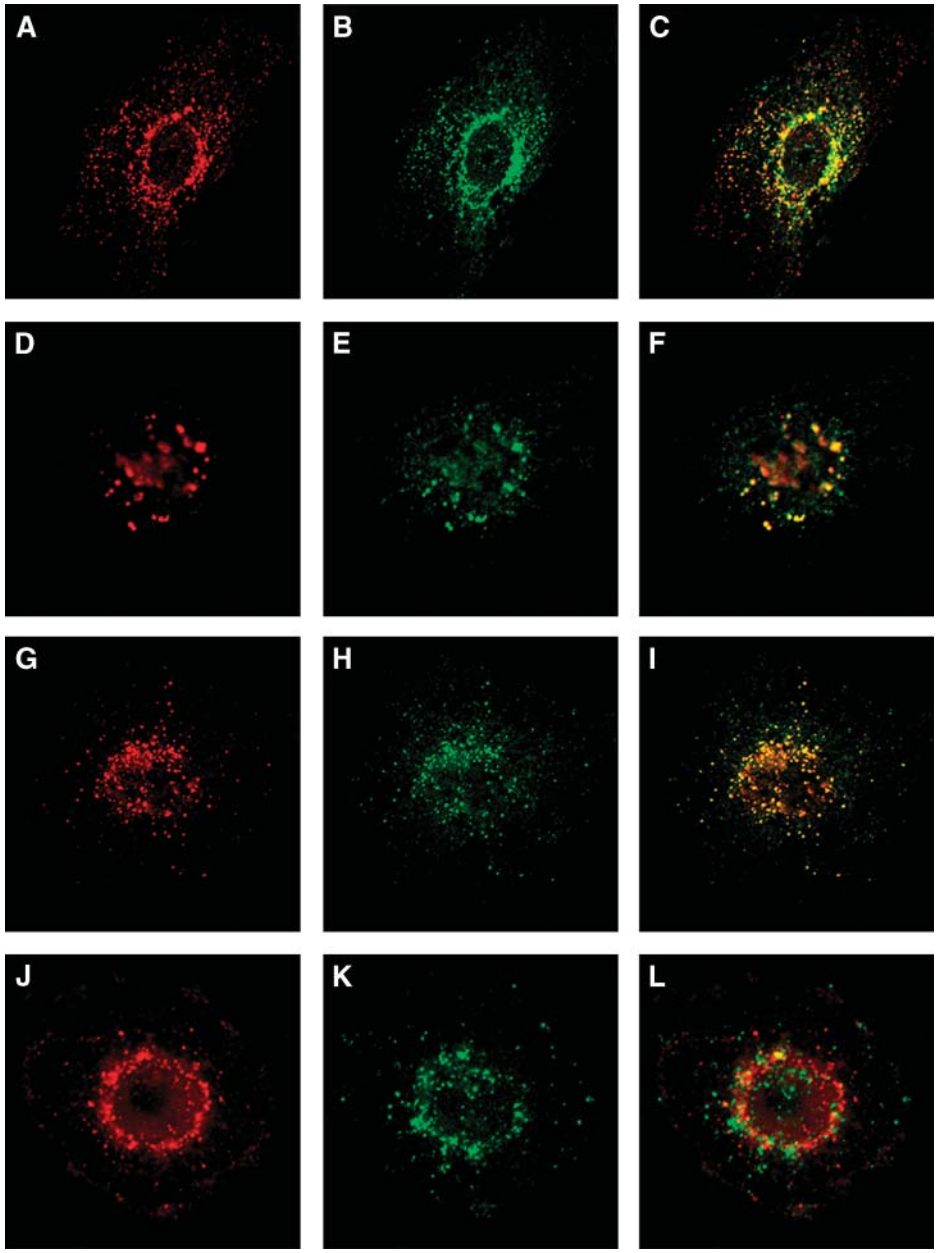

Fig. 5. Confocal microscopy of apoE recycling in CHO cells. CHO cells were pulsed with human VLDL for 1 h, washed, fixed, permeabilized, and incubated with antibodies, as described in Experimental Procedures. A, D, G, J: ApoE. B: Early endosome antigen-1 (EEA1). E: Rab5. H: Rab11a. K: Lysobisphosphatidic acid. C, F, I, L: Merge of apoE and the marker.

period; however, almost 30% of the internalized apoprotein escaped degradation and was resecreted. When the chase medium contained LPDS or was serum-free, the bulk of the recycled apoE was recovered in the $d > 1.210$ g/ml fraction, suggesting that it is lipid-free or associated with limited amounts of lipid. Inclusion of chloroquine in the pulse and chase media markedly decreased degradation of the apoprotein but had no effect on the amount of apoE recycled. Treatment of cells with monensin, a carboxylic ionophore that interrupts intracellular receptor recycling, produced a similar effect, in that apoE degradation was reduced but apoE recycling was unaffected. In addition, treatment of cells with BFA, a fungal metabolite that interrupts trafficking between the endoplasmic reticulum and the Golgi apparatus, leading to the dissolution of the Golgi complex, had no effect on apoE recycling. ApoA-I did not stimulate apoE recycling, nor did upregulation of ABCA1. However, apoE recycling and cellular cholesterol efflux appear to be linked, in that conditions that promote cholesterol efflux also promote apoE recycling. Confocal studies revealed colocalization of internalized apoE with the early endosomal compartment, the ERC, and the endolysosomal compartment. The colocalization of apoE with the ERC persisted even after cells were chased in the absence of apoE, suggesting an intracellular pool of apoE.

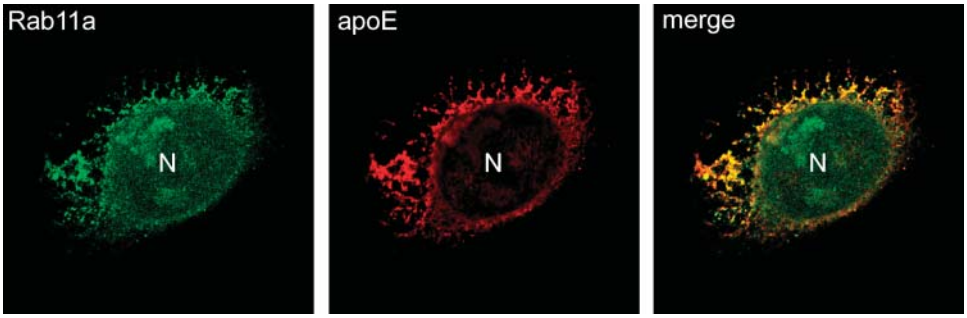

Fig. 6. Colocalization of apoE and Rab11a in CHO cells. Cells were pulsed with human VLDL (1 h) and chased for 1 h before fixation and processing for confocal microscopy, as described in Experimental Procedures. Colocalization of apoE with Rab11a persists after the 1 h chase. The nucleus is denoted by N.

Studies to investigate the intracellular routes for recycling apoE have been limited, but our previous studies with hepatocytes suggested that a portion of apoE is recycled through the Golgi apparatus (21, 22). In addition, we demonstrated apoE recycling in the absence of the LDL receptor (21, 25) and under conditions in which LRP is downregulated (25). Thus, we hypothesized that apoE recycles through multiple pathways. It is important to keep in mind that we previously established that endogenous production of apoE or active synthesis of lipoproteins does not affect the dynamic of apoE recirculation (21, 22). However, our studies to date have provided little insight

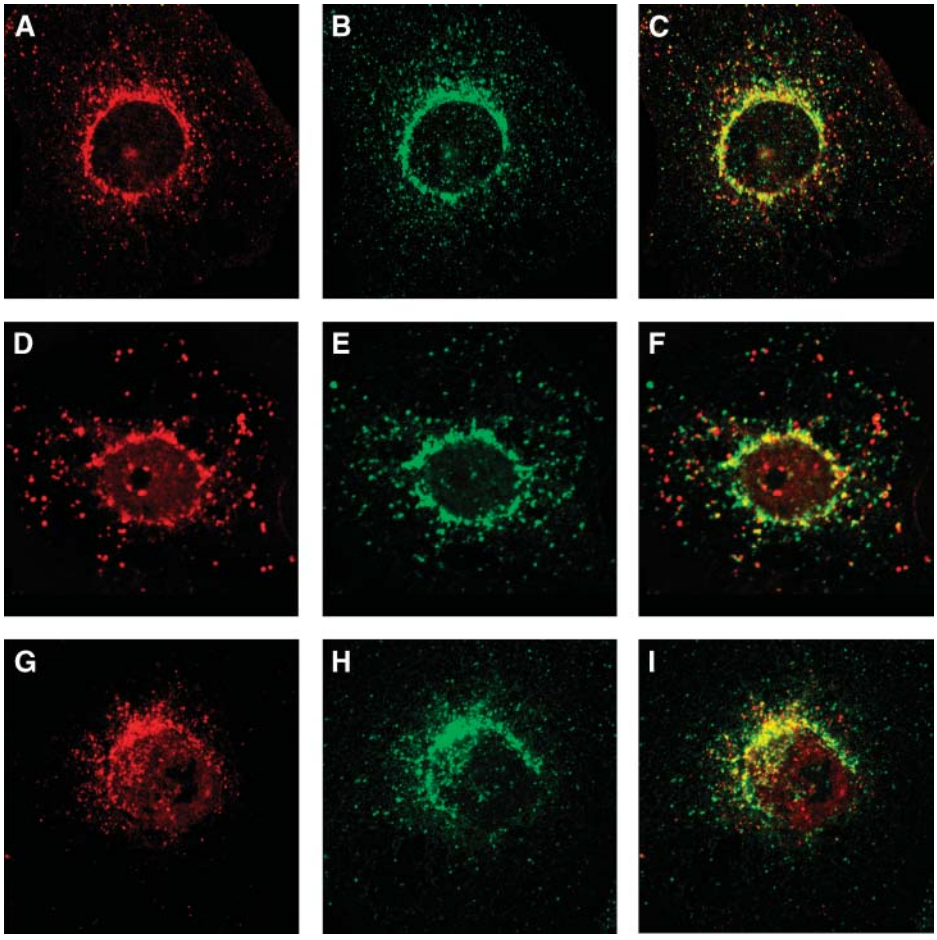

Fig. 7. Effect of chloroquine and monensin on the colocalization of apoE with EEA1. CHO cells were pulsed with human VLDL for 2 h in the presence of chloroquine (100 μM) or monensin (10 μM). The cells were washed, fixed, permeabilized, and incubated with antibodies, as described in Experimental Procedures. A, D, G: ApoE. B, E, H: EEA1. C, F, I: Merge of apoE and EEA1. A–C: Control. D–F: Chloroquine-treated. G–I: Monensin-treated.

into specific routes used by the recycling apoprotein. To investigate these routes, we wanted to use an established cell line that does not express apoE to represent the vast majority of body cells that confront the problem of cholesterol homeostasis without the help of lipoprotein secretion machinery or endogenous apoE. We opted to use CHO cells, which meet these criteria but do express lipoprotein receptors as well as ABCA1 (26, 27). Thus, CHO cells exhibit the characteristics we felt were necessary to probe the intracellular recycling pathway of apoE.

Our initial studies were designed to establish apoE recycling in CHO cells. Using the pulse-chase protocol, in which cells were incubated (pulsed) with ^{125}I-apoE-VLDL and chased for various times in the absence of the radioactive ligand, we found that intact ^{125}I-apoE appeared in the medium in a time-dependent manner (Fig. 1). Levels in the medium stabilized at 60 min with little increase at subsequent time points, whereas the cellular levels decreased over the 4 h chase, consistent with active degradation of the protein. One interpretation of these data is that recycled apoE is not internalized as efficiently as first-pass apoE. Alternatively, the resecreted pool of apoE could be internalized and recycled in a continual loop with little loss occurring during its trafficking in and out of the cell. After a 24 h chase, apoE is still detected in the medium, but very little apoE remains in the cell (data not shown). The fact that any apoE is detected in the cells after this period suggests that there is a pool of apoE that is either protected from degradation or degraded at a very slow rate. This is not unlike our previous results in studies with hepatocytes (22) and macrophages (24), which have demonstrated that internalized apoE remains within the cell for an extended period of time.

The amount of apoE degraded by CHO cells over a 4 h period was much greater (~50%) than that observed with either hepatocytes (<10%) (25) or macrophages (~25%) (24). To determine whether apoE recycling was in any way related to degradation, we treated cells with the lysosomotropic agents chloroquine and monensin to suppress degradation. Both agents effectively blocked lysosomal degradation but had no effect on apoE recycling (Fig. 3). Thus, protecting apoE from degradation is not sufficient to stimulate recycling, nor does it direct apoE into a recycling pathway. These results are consistent with our confocal studies showing little colocalization of apoE with LAMP-1 in control cells. In the presence of chloroquine, we did note significant colocalization of apoE with LAMP-1, suggesting that when lysosomal degradation is blocked, apoE accumulates in the lysosomal compartment. In contrast to our results with apoE, transferrin recycling is completely inhibited by monensin in K562 cells; however, it is not affected by chloroquine (43). Chloroquine treatment of fibroblasts was reported to increase retroendocytosis of LDL by 2- to 3-fold (44). The lack of an effect of either chloroquine or monensin on apoE recycling in CHO cells suggests that the recycling pathway(s) for apoE differ(s) from the recycling pathways for transferrin and the retroendocytic pathway for LDL.

ApoE recycling in CHO cells was not altered by BFA, similar to our results in hepatocytes (Fig. 3) (25). BFA not only induces a redistribution of Golgi components to the ER but also inhibits clathrin-coated vesicle formation, blocking recycling from the ERC (35). It has been shown to have only a moderate effect on the rate, and almost no effect on the extent, of transferrin recycling (45). In the presence of BFA, blocking recycling from the ERC, transferrin is believed to be shunted into the phosphatidylinositol-3-kinase dependent (fast) recycling pathway. Similarly, the lack of an effect of BFA on apoE recycling may be related to the shunting of apoE into a different recycling pathway when recycling from the ERC is blocked.

Our confocal studies demonstrate that the apoE recycling pathway uses the same compartments as other recycling proteins (Fig. 5). Namely, apoE colocalizes with EEA1, a marker for the early endosomal compartment. Our previous studies in hepatocytes have shown that recycling does not depend on entry site into the cell (21, 25); however, as far as we know, most of the apoE enters the cell via a receptor-mediated mechanism. Thus, we would expect apoE to be present within the early endosomes and to colocalize with a marker for this compartment. ApoE also colocalized with Rab5, a small GTPase that is localized to early endosomes as well as clathrin-coated vesicles and the plasma membrane (46). Rab5 is required for transport from the early endosomal compartment to the ERC (47, 48), and Rab11a is a marker for this compartment (47, 49). It is important to note that apoE colocalization with Rab11a persisted after a 1 h chase period (Fig. 6), suggesting that the pool of apoE observed in the pulse-chase experiments may be the ERC. The localization of apoE with LBPA-positive compartments is consistent with targeting of the apoprotein for degradation, as LBPA is considered a marker for the late endosomal compartment (50). Proteins targeted for degradation would be expected to traverse this compartment.

Previous studies from our laboratory on apoE recycling in hepatocytes and macrophages suggested a link between apoE recycling, HDL metabolism, and intracellular cholesterol trafficking. We reported that apoE recycling is upregulated by two fundamental components of the reverse cholesterol transport pathway, apoA-I and HDL (23, 24). In addition, we found potentiation of apoE recycling in macrophages when ABCA1 expression was increased via LXR stimulation (24). In contrast to macrophages, apoE recycling in CHO cells was unchanged by upregulation of ABCA1 with an LXR agonist (Fig. 2B). This indicates that apoE recycling in CHO cells is not directly linked to the ABCA1-mediated mobilization of cholesterol. Consistent with this concept are the observations that apoE recycling was minimally stimulated with acceptors that promote ABCA1 lipid efflux, including lipid-free apoA-I (Fig. 2A) and lipid-poor apoproteins (LPDS) (Fig. 4) (39, 42). In addition, apoE recycling was not affected by BFA or monensin, known inhibitors of ABCA1-mediated lipid efflux (39, 51). Our studies suggest that apoE recycling in CHO cells is most sensitive to cholesterol acceptors that induce efflux via mechanisms unrelated to ABCA1 (Fig. 4). For example, β-CD, which preferentially extracts cholesterol from plasma membrane lipid rafts (36, 37) via a mech-

anism independent of ABCA1 (39), markedly increased apoE recycling in CHO cells. Cyclodextrins stimulate the removal of cholesterol from the same cellular pools as do phospholipid-containing apoproteins (i.e., HDL) (39, 52–55). We believe that the apoE recycling pathway in CHO cells is linked to cellular cholesterol removal via phospholipid-containing acceptors and hence possibly via either scavenger receptor class B type I (SR-BI) or ABCG1 (40–42, 54). The extraction of membrane cholesterol should lead to a redistribution of newly synthesized and/or endosomal cholesterol (38) to the plasma membrane. Both SR-BI (56, 57) and ABCG1 (58, 59) have been localized to the ERC, a major intracellular cholesterol storage organelle (60). Because cholesterol is transported from the ERC to the plasma membrane via the same vesicles that carry recycling proteins (60), cholesterol efflux by β-CD or phospholipid-containing acceptors may enhance apoE recycling by stimulating cholesterol trafficking to the plasma membrane via the cholesterol-rich ERC. Consistent with this hypothesis is our observation that apoE colocalizes with Rab11a, a marker for the ERC (Figs. 5, 6).

Whereas our studies suggest that cholesterol efflux via ABCA1 does not play a direct role in apoE recycling, nascent HDL particles formed via ABCA1 lipidation of lipid-poor apoproteins could stimulate the recycling pathway by promoting cholesterol efflux via other mechanisms. Therefore, the different effects of lipid-free apoA-I (or LPDS) on apoE recycling in CHO cells and macrophages (24) may merely be a reflection of cellular differences in lipidation of the apoprotein via ABCA1. Macrophages form larger, free cholesterol-enriched nascent HDLs and lipidate more apoA-I via ABCA1 compared with fibroblasts (61). Our current studies show that the majority of apoE recycled by CHO cells is poorly lipidated (Fig. 1), whereas apoE recycled from macrophages is moderately lipidated and is on particles in the size range of HDL (24). This suggests differences between the two cell types in the degree of lipidation of recycled apoE via ABCA1. Thus, a physiologic function of the apoE recycling pathway may be to produce poorly lipidated apoE particles for use as cholesterol acceptors. The efficiency with which recycled apoE is used as an acceptor would depend on cellular cholesterol status and existing efflux mechanisms of the particular cell type.

In summary, our studies have shown that apoE recycling occurs in CHO cells. Furthermore, our studies suggest that apoE recycling in CHO cells is linked to cellular cholesterol removal via the ERC and phospholipid-containing acceptors and hence is likely associated with cholesterol efflux via SR-BI or ABCG1 rather than efflux to lipid-poor apoproteins via ABCA1. However, our studies also suggest that a physiologic function of the apoE recycling pathway is the production of poorly lipidated apoE as an acceptor for ABCA1. Thus, recycling of apoE in nonlipoprotein, non-apoE-producing peripheral cells may function as a "buoy" in the intracellular cholesterol tide, facilitating both the entry and the exit of lipid cargo through an uninterrupted loop.

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