The recycling of apolipoprotein E in macrophages: influence of HDL and apolipoprotein A-I

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Abstract The ability of apolipoprotein E (apoE) to be spared degradation in lysosomes and to recycle to the cell surface has been demonstrated by our group and others, but its physiologic relevance is unknown. In this study, we characterized apoE recycling in primary murine macrophages and probed the effects of HDL and apoA-I on this process. In cells pulsed with $^{125}$I-apoE bound to VLDL, intact apoE was found in the chase medium for up to 24 h after the pulse. Approximately 27 ± 5% of the apoE internalized during the pulse was recycled after 4 h of chase. Addition of apoA-I and HDL increased apoE recycling to 45 ± 3% and 46 ± 3%, respectively, similar to the amount of apoE recycled after pulsing the cells with $^{125}$I-apoE-HDL. In addition, apoA-I-producing macrophages from transgenic mice showed increased apoE recycling at 4 h (38 ± 3%). Increased ABCA1 expression potentiated apoE recycling, suggesting that recycling occurs via ABCA1. Finally, in the presence of apoA-I, recycled apoE exited the cells on HDL-like particles. These results suggest that apoE recycling in macrophages may be part of a larger signaling loop activated by HDL and directed at maximizing cholesterol losses from the cell.—Hasty, A. H., M. R. Plummer, K. H. Weisgraber, M. F. Linton, S. Fazio, and L. L. Swift. The recycling of apolipoprotein E in macrophages: influence of HDL and apolipoprotein A-I. J. Lipid Res. 2005. 46: 1433–1439.

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Apolipoprotein E (apoE) is a 34 kDa glycoprotein found on all plasma lipoproteins except the smallest LDLS. The primary extracellular function of apoE is to serve as a ligand for receptor-mediated uptake of lipoproteins through the LDL receptor, the LDL receptor-related protein, and heparan sulfate proteoglycans (1, 2). ApoE also plays a key role in intracellular lipid metabolism, influencing processes such as the assembly and secretion of lipoproteins (3–6), intracellular routing of endocytosed remnant lipoproteins (7, 8), and cholesterol efflux to HDL (9, 10). In macrophages, the effect of apoE on cholesterol efflux, as well as its other pleiotropic effects (2, 11, 12), may be critical in protecting the artery wall from atherosclerotic lesion formation (13–16).

We and others have established that a portion of the apoE internalized by cells via lipoprotein receptors escapes lysosomal degradation and is recycled. This recycling is not cell specific, as it occurs in hepatocytes (17–20), fibroblasts (21, 22), hepatoma cells (21–25), and macrophages (19, 26, 27). The physiologic relevance of apoE recycling is unknown. Studies in our laboratory have shown that apoE recycling in hepatocytes is stimulated by apoA-I. Heeren et al. (22) showed that HDL increased the recycling of apoE as well as the efflux of cholesterol in fibroblasts using double-label experiments with $^{125}$I-labeled apoE and $^3$H-labeled cholesterol. It is also known that apoA-I stimulates the secretion of endogenous apoE by macrophages (28). Based on these observations, we speculated that apoE recycling is linked to reverse cholesterol transport both by serving as a signaling mechanism for HDL cholesterol entry into the cell and by increasing intracellular cholesterol efflux in response to HDL signaling (19). If apoE recycling is associated with cholesterol efflux and HDL metabolism, then apoE recycling in macrophages may have important implications for atherogenesis, as even small amounts of apoE produced in macrophages are atheroprotective (15, 16). Macrophages trapped in the arterial intima would benefit from the potential to recycle apoE to maintain adequate cholesterol efflux.

In this study, we investigated the recycling of apoE in

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primary cultures of peritoneal macrophages. We demonstrate that apoE recycling in macrophages follows the internalization of either VLDL or HDL, that the amount of apoE recycled can be increased in the presence of HDL or lipid-free apoA-I, and that apoE is resecreted as an HDL particle. Our data demonstrate that apoE sparing in macrophages is a bulk process regulated by HDL and suggest a novel mechanism for the atheroprotective role of apoE in the artery wall.

EXPERIMENTAL PROCEDURES

Macrophase collections
C57BL/6 mice were given 3 ml of a 2% thioglycollate solution by intraperitoneal injection. After 3 days, the peritoneal cavity was gavaged with sterile DMEM, and macrophages were collected. Cells were washed, counted, and plated at a density of 3 x 10^6 cells in six-well dishes (Costar). Fresh medium was added to cells at 4 h after plating. All experiments were performed within 72 h of cell collection.

Labeling of apoE
Purified recombinant human apoE3 used in all studies was produced as described (29). ^125I was purchased from Amersham (IMS-30). Iodination of apoE was performed using Iodogen tubes from Pierce (Rockford, IL) according to the manufacturer’s instructions. The integrity of labeled apoE was confirmed by SDS-PAGE followed by exposure to Kodak film. The specific activity for each ^125I-apoE preparation ranged from 12,000 to 16,000 cpm/ng.

Isolation of VLDL and HDL
Plasma VLDL (d < 1.019 g/ml) or HDL (d = 1.040–1.210 g/ml) for all studies was isolated from human plasma by ultracentrifugation from the same fasted donor as described previously (19). The protocol was approved by the Vanderbilt University Institutional Review Board, and informed consent was obtained before blood collection. The apolipoprotein composition of VLDL was determined by SDS-PAGE (19), and protein content was determined by the bicinchoninic acid method (Pierce), modified to eliminate interference by lipids (30).

Recycling experiments
The protocol used for all recycling experiments was the same as that described previously for hepatocytes (19). Briefly, radiolabeled apoE was combined with human VLDL at 37°C for 1 h. The ^125I-apoE-VLDL was incubated with primary macrophage cultures at a concentration of 2 x 10^6 cpm ^125I-apoE/20 µg VLDL protein/well for a 2 h pulse period. The ^125I-apoE-VLDL-containing medium was removed, and the cells were washed five times in PBS, fresh DMEM and 10% FBS were added for chase periods of 0 to 1,440 min. At the end of each experiment, medium and cells were collected. Radioactivity remaining in cells was determined using a γ counter (Packard). One-half of the medium samples were treated with 20% TCA (Sigma) to precipitate intact proteins. Radioactive counts in the pellet after TCA precipitations represented intact recycled apoE, and the nonprecipitable material represented degraded apoE. apoE in the remaining medium was extracted with Liposorb (Calbiochem) and separated by SDS-PAGE on 4–12% gels. The proteins were transferred to nitrocellulose membranes, and the signal was captured on a phosphorimaging screen. All studies were performed in duplicate wells, and each n represents a separate experiment with different cells and ^125I-apoE. Data are presented as percentages of total counts (cellular plus intact apoE in medium plus degraded apoE in medium) as means ± SEM.

ABCA1 upregulation
Cells were treated with 1 or 10 µM TO-901317 (Sigma) for 18 h before the initiation of recycling experiments. These concentrations have been proven to maximally upregulate ABCA1 (31, 32).

Fast-protein liquid chromatography
Free ^125I-apoE, ^125I-apoE-VLDL, and ^125I-apoE-VLDL after a 2 h pulse and after a 4 h chase were separated by gel filtration chromatography using a Superose 6 column (Pharmacia). Aliquots of medium (100 µl) were applied to the column, fractions (0.5 ml) were collected, and cpm/fraction was determined. Data for each fraction are expressed as percentages of total counts collected from the column.

Statistical analyses
Statistical analyses were performed using ANOVA with Bonferroni post hoc analysis.

RESULTS

ApoE recycling in mouse primary peritoneal macrophages
To determine whether human apoE recycles in macrophages, we performed pulse-chase experiments as described in Experimental Procedures. After a 2 h pulse with ^125I-apoE-VLDL, cells were washed extensively and fresh medium was added for chase periods of 0, 10, 30, 60, and 240 min and 24 h. ^125I-apoE in the cells decreased, whereas intact recycled apoE in the medium increased for up to 24 h. At 4 h, 27 ± 5% of the internalized apoE was recycled. The percentage of apoE recycled reached 35 ± 4% by 24 h (Fig. 1). The amount of completely degraded apoE at 4 and 24 h was 25 ± 3% and 37 ± 3%, respectively. There was no evidence of large proteolytic fragments on SDS-PAGE gels (data not shown).

Effects of HDL on apoE recycling in macrophages
To determine whether apoE recycling is exclusively the consequence of endocytosis of triglyceride-rich lipoproteins by the macrophage, pulse-chase experiments were carried out using radiolabeled apoE combined with HDL (^125I-apoE-HDL). Recycled apoE was found in the medium at all time points, with 42 ± 4% of the internalized apoE recovered in the medium at 4 h. In contrast, only 25 ± 1% of apoE had been degraded at the same time point (Fig. 2A).

To determine whether the presence of HDL stimulates recycling, HDL was added to both the pulse and the chase media using ^125I-apoE-VLDL as the apoE carrier (Fig. 2B). apoE recycling in the presence of HDL was increased at every time point, with 45 ± 3% of the internalizedapoE recycled and 22 ± 2% degraded at 4 h. We compared the times at which the relative amount of recycled apoE exceeded intracellular apoE (i.e., the point at which the curves intersect). In the absence of HDL, recycled apoE did not exceed intracellular apoE until nearly 24 h, whereas in the presence of HDL, this occurred in less than 4 h.
ApoA-I and HDL increase apoE recycling in macrophages

Effects of apoA-I on apoE recycling

To investigate whether the effect of HDL on apoE recycling was attributable to its main protein component, apoA-I, we added apoA-I to the pulse and chase media at concentrations of 10, 25, and 100 µg/ml in a 4 h chase experiment. Although all doses of apoA-I stimulated apoE recycling, we found maximal effects at 25 µg/ml (Fig. 3A), and this dose was used in the time-course studies described below.

ApoA-I addition to both the pulse and the chase media increased apoE recycling to 46 ± 3% at 4 h and to 48 ± 3% at 24 h (Fig. 3B). Addition of apoA-I to only the pulse medium increased apoE recycling to 36 ± 6% at 4 h (n = 5; data not shown), and addition of apoA-I only to the chase medium caused a similar increase in apoE recycling (34 ± 5%; n = 5; data not shown). As with the addition of HDL, addition of apoA-I reduced the amount of time required before relative amounts of recycled apoE exceeded those of intracellular apoE.

ApoE recycling was also studied in macrophages from mice harboring a macrophage-specific human apoA-I expression construct (33). These cells secrete ~65 ng/ml apoA-I in 4 h. Recycling of apoE at 4 h was 38 ± 5%, with degradation at 25 ± 3.2% (Fig. 4). Thus, the presence of apoA-I, whether exogenous or produced by the cells, increases the ability of apoE to recycle.

Lipoprotein association of recycled apoE

To determine whether apoA-I influenced the lipidation of recycled apoE, we performed fast-protein liquid chromatography (FPLC) of labeled apoE under four different conditions: 1) 125I-apoE; 2) 125I-apoE-VLDL; 3) 125I-apoE-VLDL after the 2 h pulse in the presence of apoA-I; and 4) 125I-apoE-VLDL after a 4 h chase in the presence of apoA-I. Data are shown in Fig. 5. Free 125I-apoE was detected primarily in fractions 54–56 of the FPLC, indicating that it was not lipidated. The 125I-apoE-VLDL was detected in fractions 15–17, indicating that the 125I-apoE was completely bound to VLDL. At the end of the pulse period, the apoE remained bound to VLDL-sized particles. After a 4 h chase in the presence of apoA-I, there was no evidence of 125I-apoE in the VLDL range or in the unlipidated range; rather, recycled apoE was detected on lipoprotein particles in fractions 32–36. This is in the range of small HDLs, indicating that recycled apoE exits macrophages in a remodeled, moderately lipidated form.
Role of ABCA1 in apoE recycling

To determine whether ABCA1 is involved in apoE recycling, macrophages were treated with the liver X receptor agonist TO-901317 (1 and 10 μM) for 18 h before the recycling experiment. Recycled apoE was determined after a 4 h chase. Cellular, degraded, and recycled apoE are indicated. Data are expressed as means ± SEM for two separate experiments in duplicate wells. B: Time-dependent effects of apoA-I on apoE recycling. ApoA-I (25 μg/ml) was added to the pulse and chase media. ApoE recycling was measured at 0, 10, 30, 60, and 240 min and 24 h after chase. ApoE in cells, degraded in media, and intact in media (recycled) are expressed as percentages of total counts and represent means ± SEM for five separate experiments for 0, 10, 30, 60, and 240 min and for three separate experiments for the 24 h time point in duplicate wells. Error bars are included for all data points, but in some cases they are not visible because of the small SEM. * P < 0.05 compared with apoE recycled under control conditions.

DISCUSSION

In the present study, we examined the effects of HDL and apoA-I on the recycling of apoE in mouse peritoneal macrophages. Using a pulse-chase protocol in which cells were incubated with 125I-apoE-VLDL and chased for various times after removal of the radiolabeled sample, we found intact apoE in cells and media for up to 24 h after the pulse. When 125I-apoE-HDL was used in the pulse, apoE recycled 60% more efficiently than when apoE entered on VLDL particles. Furthermore, in experiments using 125I-apoE-VLDL, the addition of HDL or apoA-I to either the pulse or chase medium increased apoE recycling. In the presence of apoA-I, the recycled apoE exited macrophages on a lipidated HDL-like particle. Finally, we demonstrated that ABCA1 may be involved in increasing the recycling of apoE, because the liver X receptor agonist TO-901317 increased recycling to the same extent as apoA-I. These data suggest that apoE recycling in macrophages is associated with HDL metabolism and support the overall contention that apoE recycling could have antiatherogenic effects.
Previously, we reported that apoE recycles in mouse peritoneal macrophages (19). Using macrophages from apoE−/− mice incubated with 125I-apoE-VLDL, we demonstrated apoE in the media and cells for up to 24 h. In the present study, we added radio-iodinated apoE to human VLDL and found that ~27% of the internalized apoE was recycled at 4 h after the pulse. In these experiments, the chase medium contained 10% FBS to act as an acceptor for the recycled apoE. Heeren and coworkers (22) reported that 10% human serum promotes apoE recycling in both fibroblasts and hepatoma cells to the same extent as human HDL3 does at 50 µg/ml. We found that the addition of HDL (100 µg/ml) or apoA-I (25 µg/ml) to the medium containing 10% FBS stimulated apoE recycling even further, suggesting that the biology of HDL- and apoA-I-mediated apoE recycling is different in macrophages than in other cell types. ApoA-I stimulates apoE recycling to nearly the same degree whether it was added exogenously to the pulse or chase or was produced by the macrophage (Figs. 3, 4), even though the apoA-I-producing macrophages accumulate much less apoA-I in the media (33). Taken together, these data are compatible with a mechanism whereby apoE is delivered as part of the HDL-unloading process, possibly as a signaling event to inform the cell of the change in cholesterol burden. Conversely, the cellular cholesterol redistribution induced by apoA-I or HDL might involve an enhanced apoE recycling pathway to maximize efflux.

Although studies are under way in our laboratories to address the possibility that the entry point determines the degree or route of apoE recycling, at present we do not know the exact receptors that mediate the uptake of apoE on VLDL into macrophages. We previously published data showing that recycling continues in the absence of the LDL receptor and that downregulation of LDL receptor-related protein does not affect recycling in hepatocytes (20). Our previous data on the recycling of HDL-apoE (19) also suggest that any delivery of apoE to the cell will activate the recycling pathway. It is possible that apoE recycles because it binds more tightly to any one of its receptors than it does to the lipoprotein surface at the point of pH decrease in the prelysosome.

Our studies also suggest that apoE recycling in macrophages is different from apoE recycling in other cell types. In hepatocytes and fibroblasts, apoE recycling peaks between 1 and 2 h after the removal of the radiolabeled protein (19, 20, 22–25). In macrophages, the percentage of apoE that recycles increases continually for up to 24 h. In addition, apoE is retained in macrophages for 24 h after the end of the pulse (Fig. 1), whereas in hepatocytes, very little apoE is present in media or cells at 24 h (19). These data suggest that apoE undergoes repeated cycles of internalization and resecretion in macrophages compared with fibroblasts and hepatocytes, providing evidence that recycling in macrophages may have a different physiologic function than in other cell types.

We propose that apoE recycling in macrophages protects cells against cholesterol accumulation, thereby preventing foam cell formation and atherogenesis. A large body of published data suggest an atheroprotective role of apoE secreted by macrophages (14, 15, 34–38). We have shown that reconstitution of apoE−/− mice with wild-type marrow results in a near-normalization of plasma cholesterol levels and a >50-fold reduction in atherosclerotic lesion area (35). We have also shown that reconstitution of C57BL/6 mice with apoE−/− marrow resulted in a 10-fold increase in lesion area (14). Additionally, we have used retroviral vectors to express either human or mouse apoE from macrophages of apoE−/− mice. In these animals, apoE levels in plasma were too low to result in changes in lipid levels, but they were sufficient to reduce atherosclerotic lesion area by 60% (15, 16). Thus, many lines of evidence have shown that the production of apoE by arterial macrophages is atheroprotective. Our current data suggest that recycling of apoE in macrophages could contribute to this important physiologic role of apoE in delaying atherosclerotic lesion formation.

It is well established that plasma levels of both HDL and apoA-I are inversely correlated with coronary heart disease in humans. A portion of the antiatherogenic effects of apoA-I may be attributable to its ability to upregulate the secretion of apoE in macrophages (28, 39). We now show that apoA-I, both endogenous and exogenous, can facilitate the recycling of apoE in macrophages. It is possible that when macrophages trapped within atherosclerotic lesions are exposed to incoming HDL and/or apoA-I, they are able to recycle apoE and more efficiently efflux excess cholesterol. In addition, our data indicate that recycled apoE exits macrophages on HDL-like particles, providing further evidence for a role of apoE recycling in cholesterol efflux. Our observation that increasing expression of ABCA1 stimulates apoE recycling provides further support for this concept. Thus, we propose that apoE recycling is an additional protective mechanism by which macrophages can eliminate excess cholesterol, making it available for reverse cholesterol transport pathways.
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