Regulation of apolipoprotein E secretion by high density lipoprotein$_3$ in mouse macrophages

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Abstract Recent reports from this laboratory indicate that exposure of cholesterol-loaded macrophages to high density lipoprotein$_3$ (HDL_3) stimulates not only cholesterol efflux, but also results in a two- to threefold increase in apoE accumulation in the media (Dory, L., 1989. *J. Lipid Res.* **30:** 809–816). The present experiments demonstrate that the effect of HDL_3, and to a lesser extent HDL_2, on apoE secretion is specific, concentration-dependent, and may require interaction with the HDL receptor. Very low density lipoproteins (VLDL) and low-density lipoproteins (LDL) fail to specifically stimulate apoE secretion by cholesterol-loaded macrophages. The effect of HDL_3 is maximal at 25–50 μg/ml (0.26–0.52 μM) and can be totally abolished by mild nitrosylation (with 3 mM tetranitromethane (TNM)). Data are also presented to indicate that the increased rate of apoE secretion in the presence of HDL_3 is not due to a "protective" effect of this lipoprotein on possible proteolytic degradation or cellular reuptake of apoE secreted into the media. The stimulatory effect of HDL on apoE secretion can be clearly dissociated from cholesterol efflux; HDL stimulates apoE secretion from oxysterol-treated cells in the absence of measurable cholesterol efflux, while TNM-HDL promotes substantial cholesterol efflux from cholesterol-loaded cells but has no effect on apoE secretion. The kinetics of apoE synthesis and secretion, determined in short-term labeling studies, demonstrate that under all experimental conditions examined a substantial portion of cellular apoE is not secreted. Furthermore, in cholesterol-loaded cells HDL_3 increases apoE secretion essentially by diversion of a greater portion of cellular apoE pool for secretion. While HDL_3 has no effect on the rate of apoE synthesis, cellular apoE turns over two-fold faster in cells incubated in the presence of HDL_3 than in its absence ($t_{1/2}$ = 11 ± 2 and 22 ± 4 min, respectively), an observation corresponding well with the changes in the rates of apoE secretion under similar conditions. The HDL_3-mediated increase in apoE secretion by cholesterol-loaded macrophages suggests another mechanism by which HDL exerts a protective effect in the development of atherosclerosis; increased contribution to the metabolic pool of apoE by peripheral tissues may lead to a more effective clearance of peripheral cholesterol by the liver (reverse cholesterol transport). —**Dory, L.** Regulation of apolipoprotein E secretion by high density lipoprotein$_3$ in mouse macrophages. *J. Lipid Res.* 1991. **32:** 783–792.

Supplementary key words VLDL • LDL • HDL_2 • HDL_3 • 25-hydroxycholesterol • reverse cholesterol transport

Apolipoprotein (apo) E is a component of a number of circulating plasma lipoproteins, including VLDL, HDL, and chylomicron remnants (1). Its primary function appears to be that of a recognition ligand for the receptor-specific removal of cholesteryl ester-rich lipoproteins from the circulation (2–6). It also plays a role in local transport of cholesterol, as seen in nerve tissue injury repair (7, 8), and has been implicated in mediating immune responses and cell proliferation, processes that may not be related to its association with lipid (for review, see ref. 9).

Although the liver is the major site of apoE synthesis (10), a number of nonhepatic tissues have been shown to contain apoE mRNA or immunoreactive apoE (11–16). ApoE synthesis by peripheral tissues has also been demonstrated under in vivo conditions (17). Recent studies indicate that as much as 40% of the total apoE message may be located extrahepatically, suggesting a significant contribution of these tissues to the apoE metabolic pool (18). Mouse peritoneal and human monocyte-derived macrophages were the first nonhepatic tissues shown to synthesize and secrete apoE (19, 20). The relevance of this model for studies of the regulation of apoE synthesis and secretion is emphasized by the fact that foam cells, the hallmark of the atherosclerotic plaques, are primarily derived from macrophages that accumulate excessive lipoprotein-derived cholesterol (21) through the scavenger receptor-mediated uptake of cholesterol-rich, modified lipoproteins (22).

A number of factors is known to regulate apoE synthesis in macrophages. Its synthesis in immature, bone marrow-derived mononuclear phagocytes or in the mono-

Abbreviations: HDL, high density lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins; acLDL, acetylated low density lipoproteins; apo, apolipoprotein; DPBS, Dulbecco's phosphate-buffered saline; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNM, tetranitromethane; ACAT, acylCoA:cholesterol acyltransferase; 25-OHC, 25-hydroxycholesterol.

cytic THP-1 cells is turned on by differentiation to macrophages (19, 23–26). Activation of macrophages by pyran co-polymer, tuberculin vaccine, or endotoxin, on the other hand, is accompanied by a nearly complete inhibition of apoE synthesis (23). Numerous macrophage-like cell lines, despite expressing the phenotype that resembles various stages of macrophage differentiation, do not synthesize apoE (24). Induction of apoE synthesis in macrophages can also be achieved by sterol loading or exposure to oxysterols (19, 27). All of these regulatory steps appear to be mediated primarily at the transcriptional level: changes in apoE synthesis follow changes in apoE mRNA levels. It should be pointed out that in most studies the extent of apoE synthesis by cultured cells has been inferred from the extent of apoE secretion. Since it is generally assumed that apoE is a secretory protein, this may have been a reasonable assumption and provides a qualitative estimate of the extent of apoE synthesis. A systematic comparison between the rates of apoE synthesis and secretion under the various conditions studied is not available.

Recent studies reported from this laboratory suggest that apoE synthesis and secretion by mouse thioglycolate-elicited macrophages can be regulated by HDL_3 (28). Overnight loading of macrophages with human acLDL induces an increase in apoE mRNA levels and a fivefold increase in apoE accumulation in the media over 5 h. The rate of [^{35}S]apoE secretion is further increased up to threefold, when sterol-loaded cells are incubated in the presence of HDL_3. This increase is accompanied by a 20% reduction in cellular cholesterol content over a 5-h period. In the presence of HDL_3 and an ACAT inhibitor (Sandoz 58.035), an additional 50% increase in the rate of [^{35}S]apoE secretion is observed, accompanied by a 35% loss of cellular cholesterol. Significantly, in the presence of the ACAT inhibitor alone, cholesterol-loaded macrophages secrete less apoE than in its absence, despite the fact that the free cholesterol content of the cells is increased by 70%. HDL_3 or the ACAT inhibitor have no effect on apoE mRNA levels. These observations suggested the possibility of regulation of apoE secretion by HDL_3 at a posttranscriptional locus (28).

The present studies were therefore undertaken to further characterize the effect of HDL_3 on the extent and kinetics of apoE synthesis, secretion, and cellular degradation by macrophages, and to examine the relationship between cholesterol efflux and apoE secretion under these conditions.

METHODS

Materials

L-[^{35}S]methionine (sp act > 1100 Ci/mmol) was obtained from ICN Biomedicals, Inc. Culture media, including Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle media, methionine-free media, and fetal bovine serum were obtained from Gibco. All other reagents were obtained from Sigma or Baxter (Scientific Products Division.

Cell culture

Mouse peritoneal macrophages were collected from male Swiss-Webster mice (18–20 g), obtained from Harlan, 4 days after an intraperitoneal injection of 1.5 ml of sterile 4% thioglycolate broth. All subsequent procedures were carried out under sterile conditions and were previously described in detail (28). After an overnight incubation with DMEM/10% FBS, cells were loaded with cholesterol by an overnight incubation with human acLDL at 100 μg/ml. In some experiments, adherent cells were treated with trace amounts of 25-hydroxycholesterol (0.05–1 μg/ml), dissolved in ethanol. Long-term labeling studies (up to 4.5 h) were performed in cholesterol-loaded cells, washed 3 times in methionine-free DMEM, by incubations with the labeling media (methionine-free DMEM, containing 50 μCi/ml [^{35}S]methionine) in the presence or absence of HDL_3 or other plasma lipoproteins. Short-term, pulse-label studies were carried out in a similar manner; incubations were stopped at 15- to 30-min intervals, up to 2 h. In pulse-chase studies, cells were labeled in the presence or absence of HDL_3 for 60 min, and chased in regular DMEM in the presence or absence of HDL_3 for up to 80 min. All incubations were carried out at 37°C in a humidified atmosphere containing 5% CO_2.

Total protein and apoE synthesis

Total secreted (media) or cellular protein synthesis was determined by trichloroacetic acid precipitation of an aliquot of media or cell suspension. Incorporation of [^{35}S]methionine into cellular and media apoE was determined by immunoprecipitation with a goat monospecific polyclonal antibody, as previously described (28). Addition of increasing amounts of human apoE (in the form of VLDL or purified apoE) had no effect on the extent of mouse [^{35}S]apoE immunoprecipitation. The extent of macrophage-derived [^{35}S]apoE immunoprecipitation was also unaffected by the addition of HDL_3 to the media or cell lysate immediately prior to immunoprecipitation. The immunoprecipitates were resolved by SDS-PAGE (29), fluorographed, and the apoE bands were cut out, solubilized overnight in 30% peroxide and 70% perchloric acid (17), and counted by liquid scintillation spectrometry. The results were calculated in terms of corrected cpm/mg cell protein. Lipoprotein and cellular protein contents were determined by the method of Lowry et al. (30).

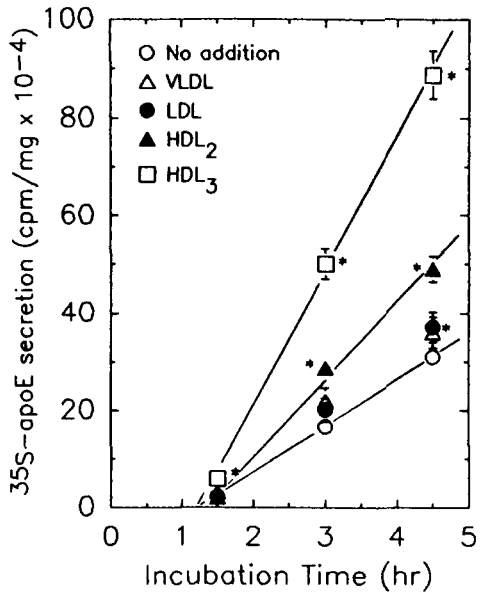

Fig. 1. Rates of [^{35}S]apoE secretion by cholesterol-loaded macrophages in the presence of human VLDL, LDL, HDL_2, and HDL_3, at 50 μg/ml. Mouse macrophages were obtained and cultured as described in Methods. On the second day of culture, cells were washed and incubated with acLDL (100 μg/ml of DMEM, 2 ml/well) for 24 h. On the third day, cells were washed three times with and briefly (10 min) incubated in methionine-free DMEM. After this treatment they were switched to the labeling media (methionine-free DMEM containing 50 μCi/ml [^{35}S]methionine) containing the various lipoproteins. Media apoE was immunoprecipitated at indicated time points, as described in Methods. Each point represents an average ± SEM of five wells; *, significantly different from the corresponding "no addition" values (at $P < 0.05$).

Lipoprotein isolation

Various plasma lipoproteins (VLDL, LDL, HDL_2, and HDL_3) were isolated by sequential ultracentrifugation at d < 1.006, d 1.03–1.063, d 1.063–1.125, and d 1.125–1.21 g/ml, respectively (31). An apoE-free HDL fraction was obtained by heparin-Sepharose affinity chromatography of the total HDL fraction (d 1.063–1.21 g/ml) (32). The ultracentrifugally prepared HDL_3 or the apoE-free HDL had no detectable apoE when examined by SDS-PAGE.

Human LDL was acetylated by repeated additions of acetic anhydride (22). Tetranitromethane modification of human HDL_3 was performed as described by Brinton et al. (33).

Cellular cholesterol determination

At the end of the various incubations the cells were washed three times with ice-cold PBS, scraped off with a rubber policeman, and their lipid was extracted as previously described (28). Free and total cholesterol mass were determined by fluorometric enzymatic assay (34), and the cholesteryl ester mass was calculated.

RESULTS

Specificity of HDL in stimulating increased rates of apoE secretion by cholesterol-loaded macrophages

Rates of apoE secretion by cholesterol-loaded macrophages were examined in the presence of various human plasma lipoproteins. Cholesterol-loaded macrophages were incubated in the labeling media (consisting of met-free DMEM, containing 50 μCi of [^{35}S]methionine and the various plasma lipoproteins at 50 μg/ml), and the accumulation of immunoprecipitable [^{35}S]apoE was followed for up to 4.5 h. The results are shown in **Fig. 1**. ApoE secretion by the cholesterol-loaded macrophages remained linear for at least 4.5 h in the absence or presence of various lipoproteins. Moreover, the rate of apoE secretion in the presence of HDL_3 was threefold higher than in cells incubated in the absence of lipoproteins. ApoE secretion was also significantly increased in the presence of HDL_2 (a 1.6-fold increase). Only slight (20%) increases in apoE secretion were observed in the presence of VLDL or LDL at this concentration. Although not shown, apoE-free HDL, prepared by heparin-Sepharose affinity chromatography, had an effect identical to that of HDL_3.

Equal protein concentrations of the various lipoproteins tested (50 μg/ml) are equivalent to widely different particle numbers. The stimulatory effect of HDL_3, and to a lesser extent of HDL_2, may thus be a reflection of the significantly higher number of lipoproteins when compared to VLDL or LDL. It is estimated that up to 20-, 6-, and 1.5-fold as many HDL_3 particles are present as VLDL, LDL and HDL_2, respectively, under these conditions. In order to investigate the possibility that the increase in apoE secretion in the presence of HDL is related to the number of lipoprotein particles present in the incubation mixture, the effect of the various lipoproteins was determined over a range of overlapping concentrations, expressed in μmol/l. Lipoprotein concentrations were calculated on the basis of average molecular weights of 10×10^6, 2.38×10^6, 0.36×10^6, and 0.175×10^6 Da for VLDL, LDL, HDL_2, and HDL_3, respectively (35). As shown in **Fig. 2**, HDL_3 is most effective in stimulating apoE secretion by cholesterol-loaded macrophages: a 2.5- to 3-fold increase in apoE secretion is observed at relatively low concentrations (25–50 μg/ml or 0.26–0.52 μM). At similar concentrations, HDL_2 stimulates a 1.5- to 2-fold increase, LDL a 1.4-fold increase, and VLDL no increase in apoE secretion. The specificity of HDL is further emphasized when apoE secretion is expressed as percent of total protein secretion. ApoE represents 11% of the total secreted protein in the absence of lipoproteins, but increases to 26.8% at 0.5 μM HDL_3, and to 18% at 1.36 μM of HDL_2. On the other hand, apoE represents only 12% and 9% of total secreted pro-

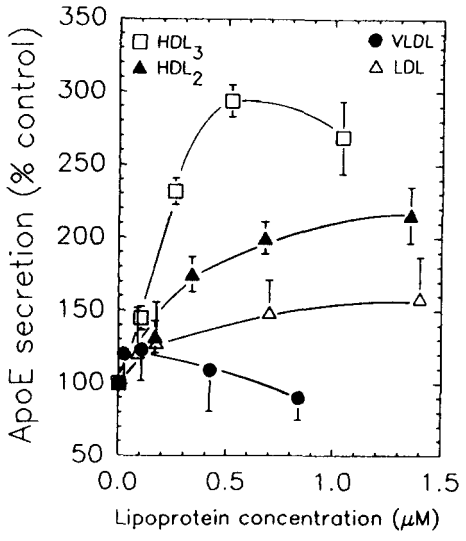

Fig. 2. Rates of [^{35}S]apoE secretion by cholesterol-loaded macrophages in the presence of human VLDL, LDL, HDL_2, and HDL_3 present at indicated concentrations. Lipoprotein concentrations were calculated on the basis of the following molecular weights: VLDL, 10×10^6; LDL, 2.38×10^6; HDL_2, 0.36×10^6; HDL_3, 0.175×10^6 (35). The experimental design was identical to that described in Fig. 1 except that apoE secretion was quantified after 3-h incubations, and the results are expressed as percent of [^{35}S]apoE secretion observed in the absence of lipoproteins. Each point represents an average ± SEM of three experiments of two wells each. The extent of total ^{35}S-labeled protein secretion was determined by TCA precipitation of an aliquot of media, and the results are discussed in the text.

tein in the presence of LDL (1.4 μM) and VLDL (0.84 μM), respectively, demonstrating a lack of specific stimulation of apoE secretion in the presence of these lipoproteins. These results suggest that the stimulation of apoE secretion by HDL_3 and HDL_2 is specific and concentration-dependent. At equimolar concentrations HDL_3 is much more effective than HDL_2.

The possibility that the increased rate of apoE secretion in the presence of HDL_3 is due to a "protective" effect of HDL_3 on apoE degradation within the media or reuptake by the cells was also investigated. Macrophage-conditioned media, containing [^{35}S]apoE, obtained in the absence of HDL_3 over a 3-h labeling period, were *1*) removed from the cells and further incubated at 37°C for 120 min in the presence or absence of HDL_3, or *2*) removed from the cells, exhaustively dialyzed to remove free [^{35}S]methionine, and reincubated with unlabeled, cholesterol-loaded macrophages in the presence or absence of HDL_3 for 120 min. The results are shown in **Figs. 3 A and B**. Under both experimental conditions the extent of [^{35}S]apoE loss over 120 min does not exceed 20%. Furthermore, the presence of HDL_3 has no significant effect on the extent of [^{35}S]apoE loss.

Effect of HDL_3 and TNM-HDL_3 on apoE secretion in the absence of cholesterol efflux

Incubation of cholesterol-loaded macrophages with HDL_3 results in significant cholesterol efflux (28). Based on this observation and on the findings that cellular cholesterol is an important regulator of apoE synthesis, it is reasonable to suggest that the increase in apoE secretion under these conditions is dependent on cholesterol efflux from the cells. It was therefore necessary to examine this possibility.

Advantage was taken of the observation that treatment of macrophages with 25-hydroxycholesterol (25-OHC) results in an induction of apoE mRNA and apoE secretion, in a manner analogous to treatment with acLDL. As shown in **Fig. 4**, treatment of macrophages with 25-OHC results in an over twofold increase in both apoE mRNA and apoE secretion. Maximal effect is seen at trace levels of 25-OHC (0.5 μg/ml). As shown in **Fig. 5**, overnight treatment of macrophages with 0.5 μg/ml of 25-OHC does not significantly alter cellular cholesterol mass when compared to control, untreated cells. Overnight loading with acLDL, on the other hand, results in an over threefold increase in cell cholesterol, mostly in esterified form. Fig. 5 also shows changes in cell cholesterol mass in the various groups after a 3-h incubation with HDL_3 (or TNM-HDL_3, HDL_3 modified with 3 mM tetrani-

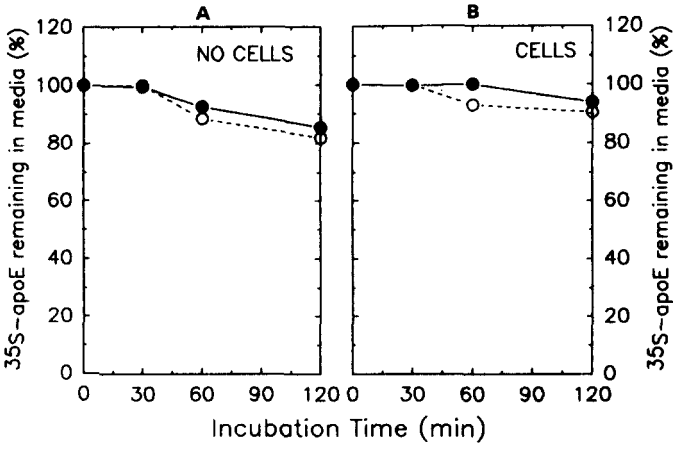

Fig. 3. The effect of HDL_3 (50 μg/ml) on the extent of [^{35}S]apoE degradation in the absence (A) or presence (B) of cholesterol-loaded macrophages. Media, containing macrophage-derived [^{35}S]apoE, obtained after a 3-h labeling period of cholesterol-loaded macrophages in the absence of HDL_3 were removed from the cells, and (A) further incubated in the absence of cells at 37°C for 120 min in the absence (○) or presence (●) of HDL_3, or (B) extensively dialyzed to remove free [^{35}S]methionine, and re-incubated with unlabeled cholesterol-loaded macrophages in the absence (○) or presence (●) of HDL_3 at 37°C for 120 min. The remaining [^{35}S]apoE was immunoprecipitated at indicated time points as described in Methods. Each value represents an average of two experiments of two wells each.

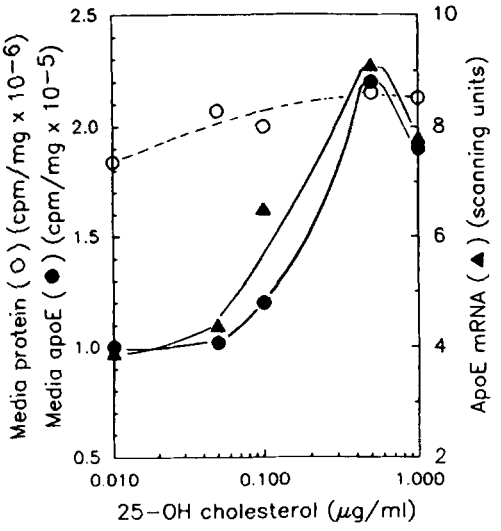

Fig. 4. The effect of 25-hydroxycholesterol on apoE mRNA (▲), apoE secretion (●), and total protein secretion (○). Cells were obtained and cultured as described. On the second day, cells were treated with indicated amounts of 25-hydroxycholesterol (added in ethanol solution to DMEM) for 24 h. After this treatment, some cells were extracted for total RNA, and apoE mRNA content was determined by Northern blot analysis, as previously described (28). Other cells were incubated in the labeling media for 3 h, and the extent of [^{35}S]apoE or total protein secretion was determined as described in Methods.

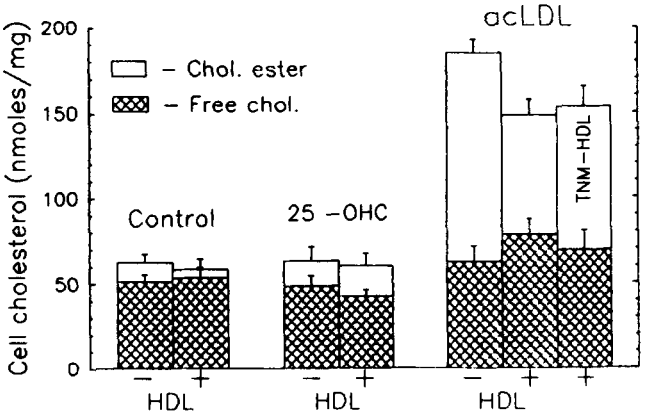

Fig. 5. Cholesterol content and mass distribution in control, 25-hydroxycholesterol-, and acLDL-treated cells before and after a 3-h exposure to HDL_3 or TNM-HDL_3. Macrophages were incubated overnight in DMEM alone (control), or DMEM containing 0.5 μg/ml 25-hydroxycholesterol (25-OHC), or DMEM containing 100 μg/ml acLDL (acLDL). After the overnight incubations, the cells were further incubated in the absence (−) or presence (100 μg/ml) (+) of HDL_3 or TNM-HDL_3 (modified with 3 mM TNM) for 3 h. At the end of the incubations, cells were scraped, washed, and an aliquot was extracted for the fluorometric determination of total and unesterified cholesterol, as previously described (28). Each column represents an average ± SEM of three experiments of three wells each.

tromethane). As expected, exposure of control or 25-OHC-treated cells to HDL_3 for 3 h does not lead to a significant loss of cholesterol. On the other hand, both HDL_3 and TNM-HDL_3 promote a significant, 20% loss of total cell cholesterol, and a 30–40% loss of cellular cholesteryl ester, from acLDL-loaded cells. The similarity in the extent of cholesterol efflux in the presence of HDL_3 or TNM-HDL_3 suggests that receptor-HDL interaction is not essential for cholesterol efflux.

The extent of apoE secretion during incubations of these cells in the presence or absence of HDL_3, as described above, is shown in **Fig. 6**. It is clear that HDL_3 stimulates a significant increase in apoE secretion in all groups of cells. A small, but significant increase (40%) is observed even in control cells. A much greater effect is seen, however, in sterol-induced cells: over twofold increases in apoE secretion from both acLDL-loaded and 25-OHC-treated cells are seen, despite the fact that measurable cholesterol efflux did not take place in the latter group. Furthermore, while TNM-HDL_3 is an efficient promoter of cholesterol efflux from acLDL-loaded cells, it does not stimulate apoE secretion.

A mild modification of HDL_3 with TNM has been shown to inhibit HDL binding to its receptor (33). Significantly, while such a modification was shown not to affect its ability to promote cholesterol efflux (Fig. 5), it abolished the ability of HDL_3 to stimulate apoE secretion (Fig. 6). In mixing experiments, using purified, iodinated human apoE, TNM-HDL, and plasma, it was found that TNM-HDL retains its ability to accept trace amounts of ^{125}I-labeled apoE (data not shown). The lack of a stimulatory effect of TNM-HDL_3 is thus not likely due to its inability to accept apoE, but perhaps more likely due to its inability to bind to the HDL receptor.

These observations establish that the HDL_3-mediated increase in apoE secretion is independent of cholesterol efflux. Furthermore, these experiments provide support for a receptor-mediated stimulation of apoE secretion by macrophages.

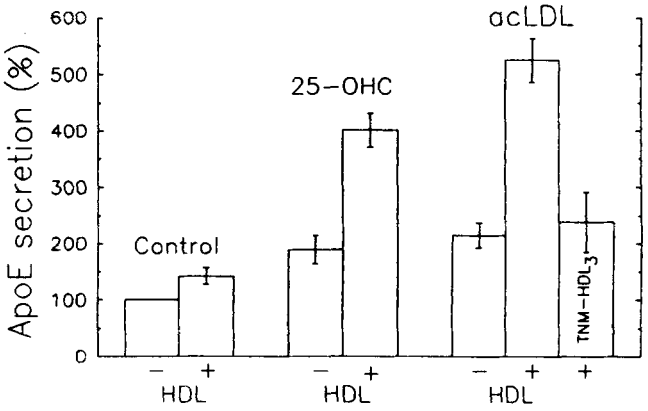

Fig. 6. [^{35}S]apoE secretion, over a 3-h period, by control, 25-hydroxycholesterol-, and acLDL-treated cells in the absence (−) or presence (+) of HDL_3 or TNM-HDL_3 (100 μg/ml). The experimental conditions are identical to those described in Fig. 5 except that the last 3-h incubation was in the presence of the labeling media, and apoE secretion was determined by immunoprecipitation. Each column represents an average ± SEM of three experiments of three wells each.

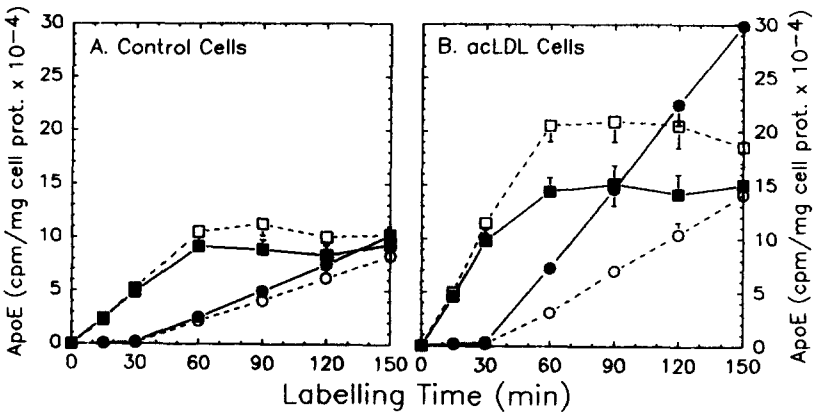

Fig. 7. The kinetics of [^{35}S]apoE synthesis and secretion in control (A) or acLDL-loaded macrophages (B) in the absence (open symbols) or presence (closed symbols) of HDL_3 (50 μg/ml). Cells were obtained and cultured as described in Methods; acLDL-loaded cells were incubated overnight with acLDL (100 μg/ml, 2 ml/well), while control cells were exposed to DMEM alone. On the following day all cells were washed and incubated briefly (10 min) in methionine-free DMEM, after which they were incubated in the labeling media with or without HDL_3. Cellular (squares) and media (circles) [^{35}S]apoE was immunoprecipitated at indicated times, as described in Methods. The calculation of the rates of apoE synthesis and secretion is described in the test. A typical experiment is shown. Each point represents an average ± SEM of four wells.

Effect of cholesterol loading and exposure to HDL_3 on the kinetics of apoE synthesis and secretion by macrophages

Work by others (19, 27) as well as previous studies in this laboratory (28), have demonstrated the induction of apoE mRNA and apoE secretion by macrophages loaded with acLDL. Rates of apoE synthesis have not been determined previously, and were thus not compared to the observed secretory rates. Furthermore, previous work in this laboratory has demonstrated that the increased rate of apoE secretion in the presence of HDL_3 is not accompanied by changes in apoE mRNA levels in the macrophages (28). The effect of cholesterol-loading and subsequent treatment by HDL_3 on apoE synthesis and secretion in macrophages was therefore further investigated. The kinetics of apoE synthesis and secretion were examined in control and cholesterol-loaded macrophages, in the presence or absence of HDL_3, by short-term labeling studies. The results are shown in **Figs. 7 A and B**. Rates of apoE synthesis were determined from the slope of the linear portion of the curve representing incorporation of [^{35}S]methionine into cellular apoE (open and closed squares in the absence or presence of HDL_3, respectively). The plateau reflects cellular apoE pool size; at this time the specific activity of the newly synthesized apoE is equal to that of the cellular apoE pool. The extent of secretion, in terms of radioactivity, can thus be directly compared to rates of synthesis, since equal radioactivity represents equal mass. Rates of apoE secretion were determined from the slope of the line representing the appearance of [^{35}S]apoE in the media (open and closed circles, in the absence or presence of HDL_3, respectively).

As would be expected, rates of apoE synthesis are twofold higher in cholesterol-loaded cells (Fig. 7 B) when compared to control cells (Fig. 7 A): $3.5 \pm 0.3 \times 10^3$ versus $1.6 \pm 0.2 \times 10^3$ cpm/min per mg cell protein. The presence of HDL_3 in the incubation mixture has no effect on the rates of apoE synthesis in either group of cells, in agreement with the previously observed lack of effect on apoE mRNA (28). On the other hand, presence of HDL_3 in the incubation mixture has a significant effect on the rates of apoE secretion in control, and especially in cholesterol-loaded cells. A particularly significant stimulation of apoE secretion (over twofold) is observed in cholesterol-loaded cells: $2.5 \pm 0.2 \times 10^3$ versus $1.17 \pm 0.15 \times 10^3$ cpm/min per mg protein in the presence or absence of HDL_3, respectively (Fig. 7 B). A similar lack of effect of HDL_3 on apoE synthesis, but a significant effect on apoE secretion, was also observed, when the cells were pre-incubated with HDL_3 (1 h) prior to the addition of the labeling media.

One of the important findings in these experiments is the apparent dissociation of the secretory rates from the synthetic rates. In control cells, in the absence of HDL_3, the rate of apoE secretion is only 44% of the measured rate of apoE synthesis. In the presence of HDL_3, the rate of apoE secretion is 56% of the apoE synthetic rate. Differences between the synthetic and secretory rates of apoE and their dependence on the presence of HDL_3 are even more prominent in cholesterol-loaded cells. In the absence of HDL_3, the rate of apoE synthesis is $3.5 \pm 0.3 \times 10^3$ cpm/min, while the rate of the apoE secretion is $1.17 \pm 0.15 \times 10^3$ cpm/min (or 33% of the secretory rate). In the presence of HDL_3 the differences in the synthetic and secretory rates are diminished: $3.5 \pm 0.3 \times 10^3$ versus $2.5 \pm 0.2 \times 10^3$ cpm/min, respectively (or the secretory rate is 71% of the synthetic rate). For a constitutively secreted protein one would expect that essentially all, or at least a constant fraction of the protein made, be secreted. Clearly, under all of the experimental conditions examined, a significant proportion of apoE remains within the cells and is presumably degraded. The differences, depending on the presence or absence of HDL_3, in the proportion of the apoE that is secreted versus apoE synthesized may reflect the extent of apoE degradation within the cells; the data suggest that in the absence of HDL_3 approximately twice as much

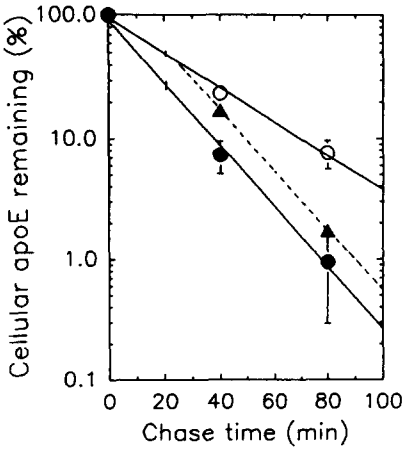

Fig. 8. Cellular turnover of [^{35}S]apoE in cholesterol-loaded macrophages in the absence or presence of HDL_3 (50 μg/ml). Cells were grown and loaded with acLDL as described in Methods. On the third day, after washing and brief incubation in methionine-free DMEM, cells were pulsed in the labeling media in the absence (○, ▲) or presence (●) of HDL_3. After a 60-min pulse period, cells were chased in regular DMEM in the absence (○) or presence (●, ▲) of HDL_3, and cell-associated [^{35}S]apoE at indicated time points was determined by immunoprecipitation. Circles (open or closed) represent an average ± SEM of three experiments of two wells each. Closed triangles represent an average ± SEM of two experiments of two wells each.

apoE is degraded (since synthetic rates are unaffected, but secretory rates are less than one half of these seen in the presence of HDL_3).

ApoE turnover in cholesterol-loaded macrophages

Pulse-chase experiments, shown in **Fig. 8 and Fig. 9**, provide additional support for the notion that HDL_3 accelerates apoE secretion in cholesterol-loaded cells and diminishes cellular degradation. Cholesterol-loaded macrophages were pulsed and chased in the presence or absence of HDL_3, and the cellular apoE was immunoprecipitated at 40 and 80 min after the initiation of the chase. Due to HDL_3-dependent differences in apoE pool size after a 60-min pulse period (see labeling kinetics in Fig. 7 B), the results were expressed as percent of [^{35}S]apoE remaining in the cells (Fig. 8). In the absence of HDL_3, after a 60-min pulse with the labeling media, cellular [^{35}S]apoE $t_{1/2}$ was 22 ± 4 min. When HDL_3 was present during the pulse and chase period, apoE $t_{1/2}$ was reduced to 11 ± 2 min. The effect of HDL_3 on cellular apoE turnover was similar even when HDL_3 was absent during the pulse period and present only during the chase (dashed line in Fig. 8): after a short lag period of 25 min, the slope of apoE disappearance from the cells was identical to that seen when the cells were pulsed and chased in the presence of HDL_3.

In similar experiments, cholesterol-loaded cells were pulsed in the presence or absence of HDL_3, and chased under identical conditions. Cellular [^{35}S]apoE was immunoprecipitated at 0 time and at 80 min of the chase period. Media[^{35}S]apoE was also immunoprecipitated after 80 min of chase and total [^{35}S]apoE recoveries were calculated. The results are shown in Fig. 9. It is clear that a significantly greater portion of apoE is recovered when HDL_3 is present in the incubation mixture (69 ± 10% vs. 31 ± 6% in the absence of HDL_3). In the presence of HDL_3, apoE is virtually absent in the cells after 80 min (<2% of that found at 0 time). In the absence of HDL_3, by 80 min, most of the apoE is also absent from the cells (8% of that found at 0 time), but a much smaller portion is recovered in the media (23%). These data provide further support for the earlier observation that in the absence of HDL_3 a significant portion of apoE (more than twice as much) is degraded within the cells.

DISCUSSION

The presented studies expand on the earlier observations that the rate of apoE secretion by cholesterol-loaded macrophages is enhanced by the presence of HDL_3 in the media (28). Several lines of evidence are provided to support the notion that the HDL_3-mediated effect on apoE secretion is specific. HDL_3, and to a lesser extent HDL_2, stimulate apoE secretion in a dose-dependent fashion. At equimolar concentrations HDL_3 is much more effective than HDL_2. VLDL and LDL fail to specifically stimulate apoE secretion. The presented data indicate that sterol-mediated induction of apoE synthesis facilitates the effectiveness of HDL in stimulating apoE secretion: the effect of HDL is much more pronounced in cholesterol-loaded

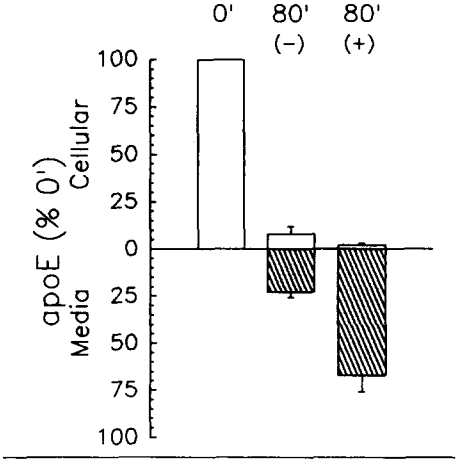

Fig. 9. Fate of cellular [^{35}S]apoE (open bar), obtained after a 60-min pulse label of acLDL-loaded cells in the presence (+) or absence (−) of HDL_3 (50 μg/ml), chased in regular DMEM under identical conditions. Cell-associated (open bar) and media apoE (hatched bar) were immunoprecipitated at indicated time points. Recoveries are expressed as percent of cell-associated apoE at the beginning of the chase period. Each bar represents an average ± SEM of two experiments of two wells each.

or sterol-treated macrophages than in control cells. This may be related to the increased rates of apoE synthesis or the the increased binding of HDL to sterol-treated cells (36, and unpublished observations in this laboratory, Dory, L.). In agreement with previous findings on apoE mRNA (28), HDL_3 has no effect on the rate of apoE synthesis in either control or cholesterol-loaded cells, but acts exclusively at some site distal to translation, possibly at the site of sorting.

An attempt was made to eliminate the possibility that the HDL_3-mediated effect is due to a protective action of this lipoprotein on the subsequent degradation of apoE in the media or re-uptake by the macrophages. The provided evidence greatly reduces this possibility: the extent of apoE degradation in the media or re-uptake by the macrophages is minimal, and the presence of HDL_3 has no effect on these processes. The HDL-stimulated fraction of secreted apoE represents over 50% of the total apoE in the media (28); it is unlikely that such a substantial fraction would be removed so rapidly as to be absent at the time of media isolation for the subsequent incubation studies shown in Fig. 3 A and B. Furthermore, if such a "protective" mechanism was operative, other plasma lipoproteins that have a capacity to bind apoE, such as VLDL, should have also stimulated an apparent increase in apoE secretion. As shown, VLDL had no effect on apoE secretion by macrophages. The apoE content of the various lipoprotein fractions tested did not correlate with the ability of lipoproteins to stimulate apoE secretion either. Both HDL_3 and LDL had no detectable apoE (by SDS-PAGE), yet HDL_3 stimulated apoE secretion, while LDL did not. On the other hand, both VLDL and HDL_2 had substantial amounts of apoE, as determined by SDS-PAGE, but only HDL_2 stimulated apoE secretion.

The effect of HDL_3 is not dependent on cholesterol efflux from the cells, which normally occurs under such circumstances. Efficient stimulation of apoE secretion by HDL_3 is also observed in oxysterol-induced cells, when no measurable cholesterol efflux takes place. It should be noted that the experiments with the oxysterol-treated cells have not totally excluded the possibility that apoE secretion is accompanied by an HDL-receptor-mediated translocation of small amounts of intracellular cholesterol to the plasma membrane, followed by cholesterol efflux (37). Such a receptor-mediated cholesterol efflux, if present, represents a small fraction of cellular cholesterol under these conditions, since no changes in the cellular sterol content are detected by the fluorometric assay technique used. Conversely, even a modest modification of HDL_3 with TNM (3 mM) completely abolishes its ability to stimulate apoE secretion, but not its ability to promote cholesterol efflux in cholesterol-loaded cells. These observations not only support the lack of relationship between mass cholesterol efflux and apoE secretion, but provide additional circumstantial evidence for a receptor-mediated effect of HDL on apoE secretion. Modification of HDL by TNM has been previously shown to completely abolish binding of HDL to its receptor, but not to inhibit cholesterol efflux (33). The independence of apoE secretion and cholesterol efflux from macrophages has been previously demonstrated, under different experimental conditions, by a selective use of monensin and cholesterol acceptors in the macrophage culture media (38).

Although none of these observations provide unequivocal evidence for an HDL-receptor-mediated mechanism, taken together they make a strong case for such a mechanism. Purification of the receptor and antibody availability in the future may provide a more definitive answer.

A potentially important finding of the present work is the large difference in the rates of apoE synthesis and apoE secretion observed in control as well as cholesterol-loaded cells and the ability of HDL_3 to promote apoE secretion without affecting its synthesis. In control cells, the rate of apoE secretion is only 44% and 56% of the synthetic rates in the absence or presence of HDL_3, respectively. In cholesterol-loaded cells incubated in the absence of HDL_3, only 33% of synthesized apoE is secreted. In the presence of HDL_3, on the other hand, 71% of apoE is secreted. The stimulatory effect of HDL_3 on apoE secretion is not mediated by increased synthesis. Rather, HDL_3 appears to shift a significant portion of cellular apoE from degradation to a regulated secretory pathway.

The accelerated rate of apoE secretion by cholesterol-loaded macrophages treated with HDL_3, observed in long- and short-term labeling studies, is also supported by the data obtained in pulse-chase experiments. The fractional turnover of cellular apoE is two-fold increased in HDL_3-treated cells; the $t_{1/2}$ of cellular apoE decreases from 22 to 11 min. While nearly 70% of the cellular apoE is recovered after 80 min of chase in the presence of HDL_3, only 30% of apoE is recovered in the absence of HDL_3. Since experiments described earlier demonstrate little if any degradation of apoE in the media, and no additional effect of HDL on apoE once it is secreted, these results indicate a substantially higher rate of intracellular rate of apoE degradation in the absence of HDL.

The mechanism by which HDL_3 stimulates increased rates of apoE secretion has yet to be elucidated. The characteristics of this action suggest several possibilities. The HDL_3-receptor interaction at the cell surface might generate a signal that mediates a diversion of cellular apoE for secretion. HDL has been shown to promote mitogenic activity in vascular endothelial cells, mediated by phosphorylation of a 27 kDa protein, an action that could be mimicked by phorbol ester and synthetic di-

acylglycerols (39). This observation suggests an involvement of second messengers generated by the HDL-receptor interaction.

In light of recent studies, it is also possible that an HDL-receptor interaction, followed by internalization of HDL_3, may be required for the stimulation of increased rates of apoE secretion. There is considerable morphological evidence for internalization of gold- or ferritin-labeled HDL by macrophages, followed by retroendocytosis (36, 40), in a manner analogous to the fate of the transferrin-receptor complex (41). Such a pathway could bring the HDL-receptor complex, within the endocytic vesicles, to close physical proximity with the cholesterol-rich *trans*-Golgi vesicles, containing the newly synthesized apoE. A fusion of Golgi-derived vesicles containing apoE and endocytic vesicles, containing the HDL, may promote the intracellular association of apoE and HDL and target the resulting complex for secretion. Indeed, some evidence of apoE enrichment of retroendocytosed HDL was recently presented (42). Although direct evidence for such a mechanism has yet to be obtained, energy-dependent fusion events between the various vesicles in macrophages have been demonstrated (43). It should also be noted that morphological studies using colloidal gold (and possibly other large labels) must be interpreted with caution, as they may modify the intracellular pathway of the ligand (40).

The possibility also exists that apoE is secreted in such a manner that a fraction of it remains "anchored" in the plasma membrane, in a manner analogous to the secretion of lipoprotein lipase by cultured mouse adipocytes (44), and continues to recycle into the cells. HDL_3 may be able, specifically, to partition the membrane-associated apoE into its surface. Much work is needed to determine which, if any, of these mechanisms are responsible for the observations reported here.

The present studies provide evidence that apoE production by the various cells expressing the apoE gene may also be regulated by the external milieu surrounding the cell. Specifically, external stimuli may influence the extent of apoE secretion and degradation within the cells. These findings may be taken as preliminary evidence for a regulated pathway of apoE secretion, in addition to the constitutive pathway of apoE secretion by macrophages and possibly other cell types. A similar constitutive and regulated pathway for the secretion of lipoprotein lipase has recently been proposed (44).

The protective effect of HDL in the development of atherosclerosis has been demonstrated by epidemiological studies (45). Biochemical substantiation of these observations thus far has been limited to its demonstrated ability to promote cholesterol efflux from various cells (46, 47) and thus participate in reverse cholesterol transport. The present studies identify another, potentially equally important component of the protective effect of HDL_3: its ability to stimulate increased rates of apoE secretion in macrophages, and potentially other nonhepatic tissues, under in vivo conditions. The increased availability of apoE, mediated by the action of HDL_3, may accelerate the rate of receptor-mediated removal of the cell-derived cholesterol by the liver.

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