Post-translational Regulation of Macrophage Apoprotein E Production*

(Received for publication, September 3, 1991)

Theodore Mazzone§, Lydia Pustelnikas, and Catherine A. Reardon
From the Departments of Medicine and Biochemistry, Rush Medical College and the Department of Pathology, University of Chicago, Chicago, Illinois 60612

We have transfected the murine macrophage cell line, J774, which does not express its endogenous apoE gene, with a constitutively expressed human apoE cDNA in order to study post-transcriptional and post-translational control of apoE production in macrophages. Loading cells with cholesterol using preincubations in acetylated low density lipoprotein, previously shown to enhance macropagene transcription and apoE synthesis, did not increase apoE synthesis or secretion in constitutively expressing transfected cells, suggesting that sterol control of macrophage apoE production occurs predominantly at a transcriptional locus. However, incubation in human high density lipoprotein (HDL₃) stimulated apoE secretion and appeared to inhibit degradation of newly synthesized apoE. This effect could be entirely reproduced by incubation with phosphatidylcholine vesicles which increased apoE accumulation in the medium by 2–6-fold. Pulse-chase experiments indicated that the effect of HDL₃ or phospholipid vesicles was very rapid (occurring within 15 min) and was independent of changes in apoE synthesis. Furthermore, the increased apoE which accumulated in the medium in the presence of phospholipid vesicles or HDL₃ was not due to altered rates of reuptake of labeled apoE since this difference was completely preserved in the absence of extracellular calcium. These results indicate that alteration of sterol content does not regulate macrophage apoE production at a translational or post-translational locus but that incubation with HDL₃ or phospholipid vesicles can enhance apoprotein E production independent of changes in apoE gene transcription or apoE synthesis. The nature of the signal generated by the phospholipid vesicles which leads to inhibition of intracellular apoE degradation and enhanced apoE secretion will require further investigation.

The regulation of apolipoprotein E (apoE) production has been extensively studied in several cell types including macrophages, steroidogenic cells, and preadipocytes (1–5). In the latter cell type, expression of apoE mRNA is regulated independently of expression of lipoprotein lipase but is positively correlated with differentiation from preadipocytes to adipocytes and with cellular lipid content, particularly free cholesterol content (5). In rat ovarian granulosa cells, apoE production has been shown to be responsive to stimulation by follicle-stimulating hormone, cAMP agonists, and phorbol esters. This observation led the authors to hypothesize a potential post-transcriptional locus of control for apoE production in this model system. In macrophage-type cells, apoE production is modulated as a function of differentiation of monocytes to a macrophage-like phenotype (6, 7), exposure to bacterial endotoxin or other activating agents (8, 9), and cellular sterol balance (1, 2, 10–12). In particular, macrophage-free cholesterol has been shown to positively regulate apoE gene expression (10, 11).

ApoE gene transcription rate is increased 8-fold after cholesterol loading of macrophages (11); apoE mRNA levels are decreased by induction of negative cholesterol balance in cholesterol-loaded macrophages; and apoE and low density lipoprotein receptor mRNA abundance vary inversely over an identical range of macrophage cholesterol content (11, 12). All of the above observations emphasize the importance of apoE gene response to perturbations of macrophage cholesterol homeostasis but do not address potential post-transcriptional loci of sterol control. Such loci have been well documented for other sterol-responsive pathways (13) (for example hydroxymethylglutaryl-CoA reductase) and have been suggested for apoE in the steroidogenic cell model (3, 4) (see above) and by recent data in macrophages suggesting that HDL₃ interaction with a plasma membrane receptor could modulate apoE secretion (14). In this series of studies, we have further examined the question of potential post-transcriptional control of apoE in macrophages using the mouse J774 cell model system (which produces no endogenous apoE) transfected with a constitutively expressed human apoE cDNA. Such a model precludes regulation at the level of gene transcription or subsequent processing of nascent apoE transcripts. Our results suggest that alterations in macrophage sterol content do not regulate macrophage apoE production at translational or post-translational loci (i.e. sterol regulation is transcriptional) but that HDL₃ can stimulate apoE secretion independent of changes in gene transcription. The effect of HDL₃ is independent of net cholesterol efflux and can be reproduced by incubations in phospholipid vesicles suggesting that neither HDL₃ apoproteins nor interaction with an apo-protein receptor are necessary for post-transcriptional regulation of apoE production by macrophages.

*This work was supported by Grants HL38653 and HL15062 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§Established Investigator of the American Heart Association. To whom correspondence should be addressed: Rush-Presbyterian St. Luke's Medical Center, 1653 W. Congress Parkway, Chicago, IL 60612. Tel.: 312-942-8231; Fax: 312-666-5114.

1The abbreviations used are: apo, apolipoprotein; HDL, high density lipoprotein; ALDL, acetylated low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; EGTA, (ethylenebis(oxyethylenenitrilo)tetracetic acid.
**EXPERIMENTAL PROCEDURES**

**MATERIALS**—[35S]Methionine (800 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL. A cell culture medium, DMEM, and Immunoprecipitin were obtained from Gibco/BRL. Egg phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Bioflour scintillant was obtained from Du Pont-New England Nuclear. S58035 was obtained from Sandoz Pharmaceuticals. Cyclic AMP was used as 8-(4-chlorophenylthio) derivative and was obtained from Sigma.

**Cell Culture**—J774 macrophages were transfected with a constitutively expressing human apoE cDNA. A cDNA expression vector containing the human metallothionine IIa promoter and SV40 16S splice junction and polyadenylation signal was prepared. The 840-base pair HindIII fragment of pSHS1 (15) (obtained from Michael Karin, University of California, San Diego) containing the human metallothionine IIa promoter was ligated to the XhoI-PstI fragment of pL1 (16) containing the 16S splice junction and subcloned into the HindIII-PstI site of pUC19. The HindIII-PstI fragment was then subcloned into the HindIII-Xhol-digested pDV1 vector (16). The AatII-Hin1I fragment of the human apoE cDNA pE368 (17) was subcloned into the unique Xbal site downstream from the promoter after generating blunt ends and the addition of Xbal linkers to the apoE fragment.

Stably transfected J774 cells were prepared by cotransfection with the human apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Five × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

Cultures were maintained at 37 °C in a humidified incubator (5% CO₂) in 75-cm² flasks containing 12 ml of growth medium consisting of Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 200 μg/ml G418. G418 was removed from the growth medium 2 weeks prior to initiation of experiments. Cells were removed from the culture flask by scraping.

**Lipoproteins and Phospholipid Vesicles**—Human HDL₃ (d = 1.21-1.210 g/ml) was prepared by ultracentrifugation in KBr as previously described (10). Phosphatidylcholine vesicles were prepared as described by Batzri and Korn (19). Vesicles were dialyzed overnight containing the human metallothionine IIA promoter and SV40 16S enhancer. Cells were electroporated with the apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Fifty × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

**Cell Culture**—J774 macrophages were transfected with a constitutively expressing human apoE cDNA. A cDNA expression vector containing the human metallothionine IIa promoter and SV40 16S splice junction and polyadenylation signal was prepared. The 840-base pair HindIII fragment of pSHS1 (15) (obtained from Michael Karin, University of California, San Diego) containing the human metallothionine IIa promoter was ligated to the XhoI-PstI fragment of pL1 (16) containing the 16S splice junction and subcloned into the HindIII-PstI site of pUC19. The HindIII-PstI fragment was then subcloned into the HindIII-Xhol-digested pDV1 vector (16). The AatII-Hin1I fragment of the human apoE cDNA pE368 (17) was subcloned into the unique Xbal site downstream from the promoter after generating blunt ends and the addition of Xbal linkers to the apoE fragment.

Stably transfected J774 cells were prepared by cotransfection with the human apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Fifty × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

Cultures were maintained at 37 °C in a humidified incubator (5% CO₂) in 75-cm² flasks containing 12 ml of growth medium consisting of Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 200 μg/ml G418. G418 was removed from the growth medium 2 weeks prior to initiation of experiments. Cells were removed from the culture flask by scraping.

**Lipoproteins and Phospholipid Vesicles**—Human HDL₃ (d = 1.21-1.210 g/ml) was prepared by ultracentrifugation in KBr as previously described (10). Phosphatidylcholine vesicles were prepared as described by Batzri and Korn (19). Vesicles were dialyzed overnight containing the human metallothionine IIA promoter. Cells were electroporated with the apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Fifty × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

**Cell Culture**—J774 macrophages were transfected with a constitutively expressing human apoE cDNA. A cDNA expression vector containing the human metallothionine IIa promoter and SV40 16S splice junction and polyadenylation signal was prepared. The 840-base pair HindIII fragment of pSHS1 (15) (obtained from Michael Karin, University of California, San Diego) containing the human metallothionine IIa promoter was ligated to the XhoI-PstI fragment of pL1 (16) containing the 16S splice junction and subcloned into the HindIII-PstI site of pUC19. The HindIII-PstI fragment was then subcloned into the HindIII-Xhol-digested pDV1 vector (16). The AatII-Hin1I fragment of the human apoE cDNA pE368 (17) was subcloned into the unique Xbal site downstream from the promoter after generating blunt ends and the addition of Xbal linkers to the apoE fragment.

Stably transfected J774 cells were prepared by cotransfection with the human apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Fifty × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

Cultures were maintained at 37 °C in a humidified incubator (5% CO₂) in 75-cm² flasks containing 12 ml of growth medium consisting of Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 200 μg/ml G418. G418 was removed from the growth medium 2 weeks prior to initiation of experiments. Cells were removed from the culture flask by scraping.

**Lipoproteins and Phospholipid Vesicles**—Human HDL₃ (d = 1.21-1.210 g/ml) was prepared by ultracentrifugation in KBr as previously described (10). Phosphatidylcholine vesicles were prepared as described by Batzri and Korn (19). Vesicles were dialyzed overnight containing the human metallothionine IIA promoter. Cells were electroporated with the apoE cDNA expression vector and pSV2-neo by calcium phosphate coprecipitation (18). Fifty × 10⁶ cells were treated with 25 μg of linearized apoE expression vector and 25 ng of linearized pSV2-neo for 4 h in the presence of 10 mM ammonium chloride. Transfected cells were selected with 400 μg/ml G418 and grown to confluency, and G418-resistant single clones were used for these studies. Cells transfected with pSV2-neo without apoE did not synthesize or secrete detectable apoE.

Cultures were maintained at 37 °C in a humidified incubator (5% CO₂) in 75-cm² flasks containing 12 ml of growth medium consisting of Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, and 200 μg/ml G418. G418 was removed from the growth medium 2 weeks prior to initiation of experiments. Cells were removed from the culture flask by scraping.
apoE secretion and may have slightly decreased its synthesis as indicated by lysate counts in the cells labeled for 6 h. The ALDL incubations were effective in increasing cellular free cholesterol (18.0 ± 2.2 μg versus 24.9 ± 1.6 μg of cholesterol/mg of cell protein) and esterified cholesterol (0 versus 35.2 ± 6.0 μg/mg). These data argue against translational or post-translational regulation in contributing to the well established increase in apoE secretion in cholesterol-loaded macrophages.

It has been previously suggested that HDL₃ can induce apoE secretion in mouse peritoneal macrophages and that this effect was post-translational and dependent on interaction between HDL₃ and a plasma membrane HDL₃ receptor (14). Fig. 2 shows that addition of HDL₃ (400 μg/ml) to the J774 cells during the 6-h labeling period approximately doubles the amount of apoE secreted into the culture medium. HDL₃ incubation with many cell types, including mouse peritoneal macrophages, produces net cholesterol efflux (10, 11, 23, 24). This effect can be detected either by a fall in total cholesterol content or, more sensitively, by an increase in endogenous cellular cholesterol synthesis. J774 cells, however, unlike mouse peritoneal macrophages, do not respond to HDL₃ with net cholesterol efflux (23). This "defect" in efflux has been well studied by Glick and colleagues, who have shown that HDL₃ can promote net cholesterol efflux from J774 cells only in the presence of cAMP, which also stimulated cholesterol ester hydrolysis (25). In Fig. 3, we show the results of an experiment utilizing cAMP and HDL₃ to determine what effect these have on apoE secretion. The upper panel illustrates the results of cell cholesterol mass measurements which show that in the absence of cAMP there is no effect of HDL₃ on the free cholesterol or cholesterol ester content of sterol-loaded macrophages. When cAMP is added in the absence of HDL₃, there is a fall in cholesterol ester but a net rise in cellular free cholesterol resulting in little change in cell total cholesterol. When HDL₃ is added in the presence of cAMP, cholesterol ester content remains depressed reflecting the net hydrolysis of cholesterol ester produced by cAMP; however,
the incremental change in free cholesterol is abolished indicating net cholesterol efflux from cells in the presence of both additives. These results are consistent with the findings of Glick and colleagues (25), suggesting that cAMP can produce net hydrolysis of cholesterol ester in J774 cells. In the absence of HDL₃, this free cholesterol remains within the cell but in the presence of HDL₃ is available to participate in HDL₃-mediated net cholesterol efflux. In the bottom panel is shown the results of these incubations on apoE secretion. The data indicate that HDL₃ approximately doubles the amount of apoE secreted during the 7-h incubation, whether or not J774 cells undergo net cholesterol efflux.

HDL₃ is a complex particle made up of several species of apolipoproteins, phospholipid, cholesterol, and a small amount of triglyceride. The data in Fig. 4 indicate that a single component of the HDL₃ particle, i.e. the phospholipid component, is capable of reproducing the effect of HDL₃ on macrophage apoE secretion. In this experiment, cells were pulsed with labeled methionine for 2 h with a 6-h chase with or without phospholipid vesicles or for 6 h with or without vesicles. The addition of vesicles produced an approximate 2-3-fold increase in apoE secretion, similar to what we observed using HDL₃. Furthermore, it can be seen in Fig. 4 that the vesicles produced similar effects whether they were added during the synthesis of labeled apoE (6-h pulse) or when they are added only during a 6-h chase period (after a 2-h pulse). This observation suggests that the addition of phospholipid exerts its effect on apoE at a post-translational regulatory locus. The effects of HDL₃ and phospholipid vesicles on apoE secretion in J774 cells are further compared and analyzed, in detail, using pulse-chase experiments. For the experiments shown in Fig. 5, cells were pulse-labeled using labeled methionine for 30 min and chased with 500 µM cold methionine for the periods of time indicated in the figure. Phospholipid vesicles or HDL₃ were added at the start of the chase period. The top panel shows disintegrations/min in total secreted apoE in the presence or absence of HDL₃; the bottom panel shows the same in the presence or absence of phospholipid vesicles. It can be seen from this figure that the effects of HDL₃ or vesicles are very rapid and are already evident within 15 min. The results in Fig. 5 show total labeled apoE secreted into the media over the chase period indicated; however, the results are essentially similar when the amount of apoE secreted is expressed on the basis of total labeled protein secreted. In Table I, it can be seen that in the presence of phospholipid vesicles, J774 cells secrete 3-6-fold more apoE per dpm of labeled secreted protein, establishing that phospholipid effects on apoE secretion are not a reflection of changes in total secreted protein.

Because the cell model utilized in these studies precludes transcriptional loci of regulation, it is likely that the differences observed in the experiments described above are due to differences in the cellular fate and/or stability of newly synthesized apoprotein E (i.e. post-translational regulation). This issue is further examined in data presented in Table II which utilizes phospholipid vesicles to enhance apoE production.

**Fig. 4.** The effect of phosphatidylcholine vesicles on apoE secretion. J774 cells were plated as described in the legend to Fig. 1. After 48 h, the cells were washed with methionine-free DMEM and incubated with a medium with labeled methionine for 30 min. At that time, some cultures were harvested (zero time chase), and the rest were washed and placed in DMEM with 500 µM cold methionine ± HDL₃ (---) (upper panel) or ± phospholipid vesicles (---) (lower panel) for the indicated chase times. After harvesting, media were analyzed for the total number of disintegrations/min in secreted apoE. Values shown are mean ± S.D. from triplicate wells. For data points where error bars are not visible, they are contained within the indicated data points. ---, control.

**Fig. 5.** Pulse-chase analysis of the effect of HDL₃ and phosphatidylcholine vesicles on apoE secretion. Cells were plated as described in the legend to Fig. 1. After 48 h, the cultures were washed with methionine-free DMEM and incubated in this medium with labeled methionine for 30 min. At that time, some cultures were harvested (zero time chase), and the rest were washed and placed in DMEM with 500 µM cold methionine ± HDL₃ (---) (upper panel) or ± phospholipid vesicles (---) (lower panel) for the indicated chase times. After harvesting, media were analyzed for the total number of disintegrations/min in secreted apoE. Values shown are mean ± S.D. from triplicate wells. For data points where error bars are not visible, they are contained within the indicated data points. ---, control.

**Table I.**

| Chase time | Increase with vesicles |
|------------|-----------------------|
| min        | -fold                 |
| 0          |                      |
| 15         | 2.2 ± 0.5             |
| 30         | 6.4 ± 0.9             |
| 90         | 4.1 ± 0.5             |
| 150        | 3.5 ± 0.7             |
Immediately after the 30-min pulse time (zero time chase period) total counts (shown on the right) and cell lysate counts (shown on the left) are essentially the same, indicating that a negligible amount of labeled apoE was secreted by this time point. By 15 min into the chase period, there is a fall in the number of lysate apoE counts which is larger in the presence of phospholipid vesicles, but total apoE counts remain the same in the presence or absence of vesicles. The same changes are observed at the 30-min time point, that is, total counts remained the same; however, lysate counts again fall more substantially in vesicle-incubated cells than in control cells. By 90 min, however, the results are quite different. Lysate counts at this time period are identical in vesicle-treated and control cells and show a substantial fall in both circumstances. However, in the presence of vesicles, total counts have remained essentially stable between 30 and 90 min indicating that the large fall in cell lysate counts between 30 and 90 min was due to net secretion of labeled apoE. On the other hand, in control cells total apoE counts fall substantially between 30 and 90 min indicating net degradation of labeled apoE. These data then indicate that the incubation with phospholipid vesicles increases the secretion and enhances the stability of newly synthesized apoE.

Macrophages have high affinity sites which bind and internalize apoprotein E, and the ability of apoE to be recognized by these sites may be influenced by its secondary structure which in turn may be influenced by its association with lipid. It is therefore conceivable that the effects detected above are due to the ability of HDL₃ or phospholipid vesicles to inhibit rapid reuptake of apoE and thereby preserve it against intracellular degradation after it has been secreted. To address this issue, cells were labeled for 30 min with high specific activity methionine and chased in the absence of calcium. High affinity apoE uptake in macrophages is absolutely dependent on calcium (26, 27), and therefore this protocol should eliminate observed differences if they were due to rapid reuptake and degradation of newly secreted apoE in control cells. To eliminate calcium from the chase medium, we employed two separate approaches. In one set of experiments, cells were chased in the presence of DMEM containing EGTA at a concentration adequate to chelate total medium calcium and magnesium. In the second experiment, cells were chased in the presence of calcium- and magnesium-free Hanks’ balanced salt solution. The results are shown in Table III. In the presence of EGTA, phospholipid vesicles and HDL₃ produce a 5.9- and 9.1-fold increase in apoE secretion, respectively, during a 15-min chase. In the presence of Hanks’ balanced salt solution the increases are 4.5- and 6.9-fold, respectively, during a 30-min chase. The data indicate that the apoE degraded in control cells (i.e. in the absence of phospholipid vesicles or HDL₃) was not degraded as a result of secretion and subsequent reuptake and degradation in secondary lysosomes but was, most likely, degraded prior to its secretion from cells.

**DISCUSSION**

The regulation of apoE production by macrophages has been widely studied. Differentiation state, exposure to endotoxin, and cellular cholesterol balance have all been shown to alter the amount of apoE secreted by macrophages (1, 2, 6-12). The stimulatory effect of cholesterol loading, in particular, has been studied in mouse peritoneal macrophages, primary cultures of human monocyte-derived macrophages, and in the human monocyte-macrophage cell line THP1 (1, 2, 10-12, 22). The measurement of apoE mRNA abundance and apoE gene nuclear run-off transcription in human and mouse macrophages has given clear evidence that the apoE gene transcription rate contributes to increased apoE secretion in sterol-loaded cells (11). The experiment shown in Fig. 1 was designed to address the importance of post-transcriptional control of macrophage apoE secretion in the presence of an expanded cellular sterol pool. There are well studied precedents for such regulatory loci in controlling the expression of other sterol-responsive proteins (13) (e.g. hydroxyethylglutaryl-CoA reductase) as well as other apolipoproteins (28, 29) (e.g. apoB-100). As shown in Fig. 1, J774 macrophages, transfected to constitutively express a human apoE cDNA, do not synthesize or secrete more apoE after a 48-h incubation in ALDL in spite of a substantial increment in cellular cholesterol content. These results strongly suggest, therefore, that regulation of apoE gene transcription is the primary locus for sterol modulation of macrophage apoE secretion.

The data in Figs. 2-5, however, indicate that like hepatocyte apoB secretion (28, 29), the amount of apoE secreted by macrophage is, in fact, subject to post-transcriptional and post-translational control. Incubation with human HDL₃ substantially increases the amount of apoE secreted by cells transfected to constitutively express a heterologous apoE cDNA (Fig. 2). This result is consistent with Dory’s observations in mouse peritoneal macrophages expressing their native apoE gene and protein (14). The effect of HDL₉ on apoE secretion can be definitively separated from its effect on net cholesterol efflux in J774 cells, since this cell line does not respond to HDL₉ with net cholesterol efflux. Furthermore, the addition of cAMP which activates cholesterol flux from

**TABLE II**

| Chase time | Lysate apoE dpm | Total apoE dpm |
|------------|----------------|----------------|
| min       | Control        | Vesicles      | Control | Vesicles |
| 0         | 34,384 ± 3791  | 34,572 ± 3,861|
| 15        | 26,482 ± 924   | 21,982 ± 1,892|
| 30        | 23,765 ± 2,525 | 18,296 ± 2,127|
| 90        | 8,947 ± 537    | 7,886 ± 703   |

**TABLE III**

|                | Control | HDL₃ | Vesicles |
|----------------|---------|------|----------|
| apoE disintegrations/min over control | 929 ± 204 | 5,670 ± 926 | 3,432 ± 122 |
| apoE disintegrations/min over control | 1,427 ± 245 | 9,987 ± 1,451 | 6,391 ± 863 |

The effect of removing extracellular calcium on HDL₃ and phospholipid enhancement of apoE accumulation in the medium

Cells were prepared and pulse-labeled for 30 min as described in the legend to Fig. 5. At that time, some of the cells were washed and chased in DMEM containing 1.5 mM EGTA ± HDL₃ or phospholipid vesicles for 15 min. The rest of the cells were washed with and placed in magnesium- and calcium-free Hanks’ balanced salt solution (HBSS) ± HDL₃ or phospholipid vesicles and chased for 30 min. At the end of each chase period, medium was harvested for analysis of apoE disintegrations/min. Values shown are means ± S.D. from triplicate wells.
J774 cells to HDL3 does not further augment the apoE response to HDL3. However, this experimental result does not eliminate the possibility that the addition of phospholipid vesicles or HDL3 could lead to some rearrangement of cholesterol distributed in subcellular pools and thereby influence apoE production.

In studies using mouse peritoneal macrophages it has been suggested that HDL3 must interact with a macrophage plasma membrane receptor in order to modulate apoE secretion (14). This interaction, and a primary role for HDL in apoE proteins in this interaction, was suggested by the observation that tetranitromethane modification of HDL3 apoE proteins abolished HDL3 stimulation of macrophage apoE secretion. In our system, the effect of HDL3 on apoE secretion could be entirely reproduced by incubation with phosphatidylcholine vesicles. While these observations do not establish that HDL3 effects are mediated by its phospholipid content, they demonstrate that the phospholipid component of HDL3 could completely account for the effect of HDL3 on macrophage apoE secretion in our cell model.

Because the cell model we have utilized constitutively expresses the human apoE cDNA, the changes noted in apoE protein in Figs. 2-4 must be due to changes in apoE synthesis (i.e., translation rates), apoE stability, or apoE secretion. The data from the pulse-chase experiments in Fig. 5 indicate that changes in apoE protein translation rates do not account for our observations. In these experiments, HDL3 or phospholipid vesicles are added at the time the labeled methionine pulse is completed, and the decay of labeled apoE is followed for variable chase periods. As can be seen from the data in Table II, there is a substantial fall in the amount of labeled apoE detectable in cell lysates between 30 and 90 min of chase in both control cells and in cells incubated with phospholipid vesicles. However, as can be seen from the preservation of total apoE counts over this same period in the presence of vesicles, the fall in lysate apoE is due to net degradation of labeled apoE in control cells, while it is due to net apoE secretion in cultures incubated with vesicles. Therefore, the addition of phospholipid vesicles at the beginning of the chase period preserves a substantial portion of the apoE synthesized during the prior 30 min from degradation. Whether the vesicles inhibit apoE degradation as a secondary effect of enhancing secretion (e.g., by promoting translocation from a pool where it is susceptible to degradation) or act primarily by inhibiting degradation will require additional studies. The direction of such investigations will need to take account of several important conclusions one can draw from the data in Fig. 5 and Tables I and III.

First, it can be seen (Fig. 5) that the response to HDL3 or phospholipid vesicles is extremely rapid, occurring within 15 min of their addition. Second, the effect of phospholipid vesicles on apoE secretion does not reflect a generalized effect on macrophage protein secretion (Table I), and third, the effect of HDL3 or phospholipid vesicles on apoE accumulation in the medium is not due to altered high affinity uptake of secreted apoE since the effect is preserved even when high affinity degradation of apoE is abolished (26, 27) by removing extracellular calcium (Table III).

Human HDL3 is a complex particle made up of several species of apoproteins, cholesterol, and phospholipid. Dissecting the mechanism by which HDL3 produces its effects on macrophage apoE secretion and degradation could be correspondingly complex. It could be expected that mechanistic dissection of the effect of single species phospholipid vesicles would be more straightforward. These vesicles may fuse with the plasma membrane of cells and thereby direct intracellular apoE to this location where secretion may be favored over intracellular degradation. Phosphatidylcholine vesicles have a reasonably high affinity for apoE as demonstrated by their ability to acquire apoE from β-very low density lipoprotein when they are co-incubated in vitro (30). Alternatively, phospholipid vesicles could be internalized and become associated with intracellular apoE and thereby reduce its susceptibility to degradation either directly or by altering its subcellular localization. Interestingly, in cryothin sections of hepatocytes, apoE can be detected by immunogold labeling in peroxisomes and in areas between multivesicular bodies and bile canalculi (31). These observations suggest that apoE may function as an intracellular as well as a secreted protein. It is therefore possible that the presence of extracellular HDL or phospholipid modulates the distribution of intracellular apoE between a pool with a primarily intracellular function (and therefore destined for eventual intracellular degradation) and a pool targeted for eventual secretion.

Modulating the degradation of newly synthesized protein also appears to be important for determining the eventual secretion rate of apoB from hepatocytes. In HepG2 cells, up to 60% of newly synthesized apoB-100 is degraded (32). This degradation appears to occur in a pre-Golgi compartment and is insensitive to inhibition by chloroquine, leupeptin, pepstatin, or chymostatin, i.e., this degradation appears to occur in a non-lysosomal compartment closely related to the endoplasmic reticulum (29). The fraction of apoB-100 which is degraded intracellularly by HepG2 cells can be altered by the addition of exogenous oleate, which inhibits degradation and enhances secretion, or by insulin, which enhances intracellular degradation (28, 33). The mechanism for the regulatory effects of oleate and insulin is unknown, but recently a great deal of information has become available regarding the details of the intracellular processing of apoB in hepatocytes (34–36). Much less is known about the intracellular processes and secretion of apoE in macrophages beyond the basic observation that apoE is secreted from macrophages in association with lipid, primarily phospholipid (1, 37). It is now clear, however, that a more careful dissection of this process will be required in order to examine the mechanisms and implications of the multiple loci of control for macrophage apoE production.

Acknowledgments—We thank Dr. Godfrey S. Getz for helpful discussions and for critical review of the manuscript; and Gladys Lee for preparing the manuscript.

REFERENCES

1. Basu, S. K., Ho, Y. K., Brown, M. S., Bilheimer, D. W., Anderson, R. G. W., and Goldstein, J. L. (1982) J. Biol. Chem. 257, 9788–9795
2. Basu, S. K., Brown, M. S., Ho, Y. K., Havel, R. J., and Goldstein, J. L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7545–7549
3. Driscoll, D. M., Schreiber, J. R., Schmit, V. M., and Getz, G. S. (1985) J. Biol. Chem. 260, 9031–9038
4. Wyne, K. L., Schreiber, J. R., Larsen, A. L., and Getz, G. S. (1989) J. Biol. Chem. 264, 981–989
5. Zechner, R., Moser, R., Newman, T. C., Fried, S. K., and Breslow, J. L. (1991) J. Biol. Chem. 266, 10583–10588
6. Amsden, J. H., Deeb, S., Brunzell, J. D., Peng, R., and Chait, A. (1988) Biochemistry 27, 2651–2655
7. Werb, Z., and Chin, J. R. (1983) J. Cell Biol. 97, 1113–1118
8. Werb, Z., and Chin, J. R. (1983) J. Biol. Chem. 258, 10642–10648
9. Werb, Z., and Chin, J. R. (1983) J. Exp. Med. 158, 1272–1283
10. Mazzone, T., Gump, P., and Getz, G. S. (1987) J. Biol. Chem. 262, 11557–11562
11. Mazzone, T., Basheeruddin, K., and Poulos, C. (1989) J. Lipid Res. 30, 1055–1064
Post-translational Regulation of ApoE

12. Mazzone, T., and Basheeruddin, K. (1991) J. Lipid Res. 32, 507–514
13. Goldstein, J. L., and Brown, M. S. (1990) Nature 343, 425–530
14. Dory, L. (1991) J. Lipid Res. 32, 783–792
15. Karin, M., and Richards, R. I. (1982) Nature 299, 797–802
16. Okayama, H., and Berg, P. (1983) Mol. Cell. Biol. 3, 280–289
17. Zannis, V. I., McPherson, J., Goldberger, G., Karathanasis, S. K., and Breslow, J. L. (1984) J. Biol. Chem. 259, 5495–5499
18. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S., and Axel, R. (1979) Cell 16, 777–785
19. Batari, S., and Korn, E. D. (1973) Biochim. Biophys. Acta 298, 3015–3019
20. Chamberlain, V. P. (1979) Anal. Biochem. 98, 132–135
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
22. Roux, M., Nigon, F., Eggerman, T. L., Brewer, H. B., Jr., and Chapman, M. J. (1989) Eur. J. Biochem. 189, 447–453
23. Bernard, D. W., Rodriguez, A., Rothblat, G. H., and Glick, J. M. (1989) Arterioscler. Thromb. 9, 135–144
24. Oran, J. F., Albers, J. J., Cheung, M. C., and Bierman, E. L. (1981) J. Biol. Chem. 256, 8348–8356
25. Bernard, D. W., Rodriguez, A., Rothblat, G. H., and Glick, J. M. (1991) J. Biol. Chem. 266, 719–716
26. Koo, C., Wernette-Hammond, M. E., and Innerarity, T. L. (1986) J. Biol. Chem. 261, 11194–11201
27. Van Lenten, B. J., Fogelman, A. M., Hokom, M. M., Benson, L., Haberland, M. E., and Edwards, P. A. (1983) J. Biol. Chem. 258, 5151–5157
28. Dixon, J. L., Furukawa, S., and Ginsberg, H. N. (1991) J. Biol. Chem. 266, 5080–5086
29. Sato, R., Imanaka, T., Takatsuki, A., and Takano, T. (1990) J. Biol. Chem. 265, 11880–11884
30. Williams, K. J., Tall, A. R., Binaider, C., and Brocia, R. (1987) J. Clin. Invest. 79, 1466–1472
31. Hamilton, R. L., Wong, J. S., Guo, L. S. S., Krisans, S., and Havel R. J. (1990) J. Lipid Res. 31, 1589–1603
32. Borchardt, R. A., and Davis, R. A. (1987) J. Biol. Chem. 262, 16494–16492
33. Sparks, J. D., and Sparks, C. E. (1990) J. Biol. Chem. 265, 8535–8542
34. Davis, R. A., Prewett, A. B., Chan, D. C. F., Thompson, J. J., Borchardt, R. A., and Gallaher, W. R. (1989) J. Lipid Res. 30, 1185–1196
35. Davis, R. A., Thrift, R. N., Wu, C. C., and Howell, K. E. (1990) J. Biol. Chem. 265, 10005–10011
36. Bamberger, M. J., and Lane, M. D. (1988) J. Biol. Chem. 263, 11868–11878
37. Dory, L. (1989) J. Lipid Res. 30, 809–816