Regulation of macrophage apoE secretion and sterol efflux by the LDL receptor

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Abstract   Factors that regulate apolipoprotein E (apoE) secretion by macrophages will have important effects on vessel wall lipid flux and atherosclerosis. Macrophages express the LDL receptor, which binds apoE with high affinity and could thereby affect the net secretion of apoE from macrophages. In these studies, we demonstrate that treatment of J774 macrophages transfected to constitutively express a human apoE3 cDNA with simvastatin, to increase LDL receptor activity, reduces the secretion of apoE. To further examine the relationship between LDL receptor expression and apoE secretion from macrophages, mouse peritoneal macrophages (MPMs) were isolated from mice with constitutively high expression of human LDL receptor to increase overall LDL receptor expression by 2- to 3-fold. Cells with increased LDL receptor expression also showed reduced apoE secretion compared with MPMs with basal LDL receptor expression. The effect of changes in LDL receptor expression on apoE secretion was isoform-specific, with greater reduction of apoE4 compared with apoE3 secretion and no reduction of apoE2 secretion, paralleling the known affinity of each isoform for LDL receptor binding. The effect of the LDL receptor on apoE secretion for each isoform was further reflected in LDL receptor-dependent changes in apoE-mediated cholesterol efflux. These results establish a regulatory interaction between two branches of macrophage sterol homeostatic pathways that could facilitate a rapid response to changes in macrophage sterol content relative to need.—Lucic, D., Z. H. Huang, D. S. Gu, M. K. Altenburg, N. Maeda, and T. Mazzone. **Regulation of macrophage apoE secretion and sterol efflux by the LDL receptor.** *J. Lipid Res.* 2007. **48:** 366–372.

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Studies using multiple models of animal atherosclerosis have demonstrated that macrophage-derived apolipoprotein E (apoE) is important for maintaining normal vessel wall lipid homeostasis. For example, selective deletion of apoE expression in macrophages markedly accelerates atherosclerosis in mouse models of atherosclerosis (1–3). Factors that regulate macrophage apoE synthesis and secretion, therefore, are of interest for gaining insight into the pathophysiology of atherosclerosis. ApoE gene transcription in macrophages responds significantly to changes in macrophage sterol balance, and this response is mediated by the liver X receptor element located in a downstream enhancer (4–6). The macrophage apoE gene also responds to cytokines and macrophage differentiation state (7, 8). In addition to transcriptional regulation, there are important loci for posttranscriptional and posttranslational regulation of macrophage apoE expression (9). The importance of these regulatory loci is magnified because a large percentage of newly synthesized apoE in the macrophage is degraded before its secretion, and the fraction of apoE secreted versus that degraded is subject to regulation (7, 9). For example, we have shown previously that macrophage sterol balance modulates the stability and secretion of macrophage apoE at a posttranslational locus (10). The expression of apoE in macrophages produces sterol efflux from macrophages in an ABCA1-dependent and -independent manner, and the macrophage apoE response to sterols at transcriptional and posttranslational regulatory loci demonstrates its role in a homeostatic regulatory loop for defending macrophage sterol balance (11–13).

We reported previously that macrophage proteoglycans sequester newly synthesized apoE at the macrophage cell surface and thereby modulate apoE secretion (14, 15). Cell surface LDL receptors also sequester newly synthesized apoE (16). As yet, however, there is no information regarding the modulation of macrophage apoE secretion by changes in macrophage LDL receptor expression. The aim of the current studies was to address the role of

Abbreviations: apoE, apolipoprotein E; Ldlr, macrophages with increased low density lipoprotein receptor expression; MPM, mouse peritoneal macrophage; WT, macrophages with basal low density lipoprotein expression.
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macrophage LDL receptor expression for modulating apoE secretion from macrophages. Because of the well-known differences in human apoE isoform interaction with the LDL receptor (17, 18), we also addressed apoE isoform-specific effects. We used two models to investigate the role of the LDL receptor. In one, macrophage cholesterol synthesis was inhibited using simvastatin, thereby increasing LDL receptor expression. In the second model, we used mouse peritoneal macrophages (MPMs) from human apoE2, apoE3, and apoE4 knockin mice with basal or increased LDL receptor expression secondary to the expression of an LDL receptor minigene that produces an mRNA with increased half-life.

METHODS

Materials

Goat apoE antiserum was from International Immunology Corp. (Marietta, GA). [^{35}S]methionine was purchased from Amersham Biosciences Corp. (Piscataway, NJ). All other materials were from previously described sources (7–15).

Cell culture

Targeted replacement of the mouse apoE gene with three human apoE alleles (apoE2, apoE3, and apoE4) has been described by Maeda and coworkers (19). Mice homozygous for these apoE isoforms were bred with mice heterozygous for targeted replacement of the mouse LDL receptor gene with the human LDL receptor minigene (17). This LDL receptor minigene produces an mRNA with an increased half-life, leading to a 2- to 3-fold increase in LDL receptor transcript expression and increased LDL receptor activity (17, 20). Cells isolated from each of the human genotypes were named according to the apoE isoform expressed (E2, E3, and E4) and designated WT cells if they had basal, or Ldlr cells if they had increased, LDL receptor expression. Mice at 12–14 weeks of age were euthanized, and MPMs were harvested as described previously (21). The cells were plated on six-well plates and maintained in 10% FBS in DMEM. Cells were used for experiments 12–14 days after isolation.

J774 cells (which do not express an endogenous apoE gene) were stably transfected to express a human apoE3 cDNA in a constitutive manner under the control of the cytomegalovirus promoter. The apoE expression construct and the method for transfection have been described in detail (11).

For some experiments, cell surface proteoglycans were depleted as described previously in detail (14, 15). Briefly, cells were incubated in 4-methyl-umbelliferyl-β-D-xyloside at a final concentration of 1 mM for 72 h to inhibit cellular proteoglycan synthesis. Immediately after this incubation, cells were treated for 30 min at 37°C with heparinase at a final concentration of 3 U/ml. We have shown previously that these treatments reduce cell surface proteoglycans in macrophages by up to 80% (14, 15).

Quantitation of apoE synthesis and secretion

In some experiments, immunoblot analysis was performed on cell culture supernatants to measure steady-state levels of apoE released into the medium from macrophages. Immunoblot analysis was performed as described previously in detail, and the results were quantitated using Zero D-Scan software (Scanalytics, Inc., Fairfax, VA) (13). For the quantitative measurement of apoE synthesis and secretion rates, a pulse-chase experimental design was used as described previously in detail (9). Cells were pulse-labeled with [^{35}S]methionine for 30–45 min and chased for the times indicated in the figures. At the indicated chase times, cell media and lysates were used for quantitative immunoprecipitation of apoE as described previously (9). Quantitative immunoprecipitations are based on total TCA-precipitable radioactivity; therefore, they are already corrected for any differences in the synthesis or secretion of total protein. After immunoisolation, labeled apoE was resolved on SDS-polyacrylamide gels, and radioactivity present in cellular and secreted apoE was quantitated using an Amersham Biosciences phosphorimager and Image-Quant software. Results are expressed as scanning units. The percentage of apoE secreted was calculated by comparing radioactivity in apoE present in the medium at each indicated chase time with the total amount of radiolabeled apoE present in cells immediately after labeling.

Measurement of sterol efflux

Cellular sterol was radiolabeled to equilibrium using 48 h incubations in 1 μCi/ml [^{3}H]cholesterol as described previously (13, 15). Cells were placed in 0.1% BSA for measurement of sterol efflux as described previously in detail (1–3, 15). Sterol efflux is expressed as μg cholesterol released into the medium per mg cell protein by correcting medium [^{3}H]cholesterol (dpm) with cellular cholesterol specific activity.

Other assays

Total cellular cholesterol was measured enzymatically (Wako USA) in hexane-isopropanol extracts. Cell protein was measured using a DC protein kit (Bio-Rad). LDL receptor protein level was measured by immunoblot analysis as described previously (22). mRNA levels for LDL receptor were quantitated by RT-PCR using a probe and primer set for murine exon 1, which measures both human and murine mRNA species by a method described previously (17, 23).

Statistical analysis

The results of experiments representative of two to four additional experiments with similar results are presented and expressed as means ± SD of triplicate determinations of each group unless indicated otherwise. The significance of differences was analyzed by ANOVA using SPSS.

RESULTS

For the experiments shown in **Figs. 1**, **2**, simvastatin was used to inhibit cellular cholesterol synthesis and thereby increase LDL receptor expression (24, 25). After incubation with simvastatin (20–40 μM), LDL receptor protein levels were increased 2- to 4-fold as measured by immunoblot analysis (data not shown). The data in Fig. 1 show the results of an experiment evaluating the effect of simvastatin (over a range of doses) on the steady-state level of apoE released into the medium from J774-E cells, which constitutively express a human apoE3 cDNA. All doses of simvastatin reduced medium apoE, with maximal reduction observed at 20 μM simvastatin. The data in Fig. 2 show the effect of this dose of simvastatin on the percentage secretion and cellular retention of newly synthesized apoE in J774-E cells. After a 20 h incubation with or without 20 μM simvastatin, cells were pulse-labeled, and cells and medium were harvested at 60 and 120 min chase times.
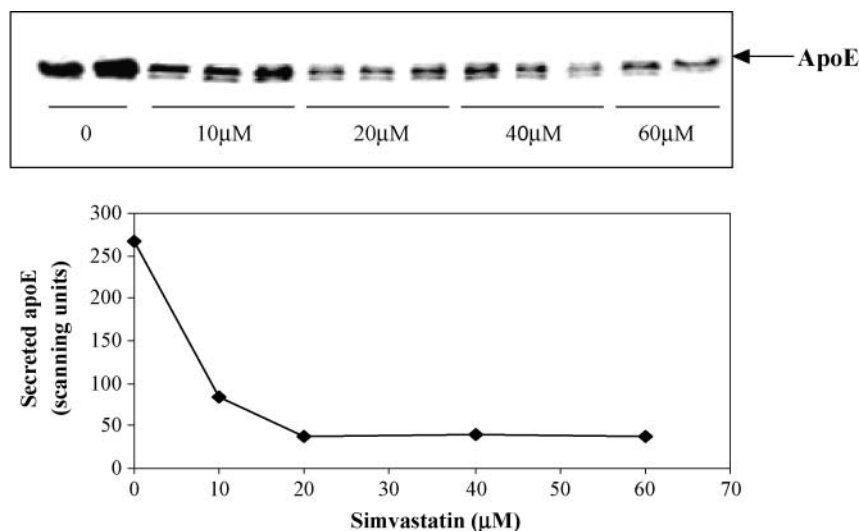
Fig. 1. Reduction of net release of apolipoprotein E (apoE) into the medium by J774-E cells during incubation with simvastatin. J774-E cells were incubated in 0.2% BSA alone or with simvastatin at the concentration indicated. At the end of 24 h, medium was harvested for apoE immunoblot analysis. Values shown are means of the duplicate determinations shown in the upper panel.

There was no statistically significant difference in cellular apoE at the end of the pulse (chase time 0) between cells incubated with or without simvastatin (data not shown). At each chase time, simvastatin significantly reduced apoE secretion, consistent with the results in Fig. 1. The amount of apoE retained in the cells at chase times 60 and 120 min was not different in control and simvastatin-treated cells. These results indicate that simvastatin treatment, with its attendant increase in LDL receptor expression, reduced the secretion of newly synthesized apoE3 in macrophages. The apoE not secreted was not retained within cells and therefore degraded; at 120 min, the amount of apoE degraded approximately doubled in cells incubated with simvastatin compared with control cells (from $32 \pm 5\%$ to

Fig. 2. Effect of simvastatin on the secretion and retention of newly synthesized apoE in J774-E cells. J774-E cells were pulse-labeled, and at 60 and 120 min chase times the percentage apoE secreted or retained in the cell was determined as described in Methods. Before labeling, cells were incubated with (Simva) or without (NA) 20 μM simvastatin for 20 h as indicated. Values shown are means ± SD of triplicate samples. * $P < 0.05$ for Simva versus NA at each chase time.

$60 \pm 8\%$). Furthermore, this is a posttranscriptional effect, as J774-E cells constitutively express a human apoE3 cDNA.

In addition to increasing LDL receptor expression, simvastatin may alter other cell pathways based on its inhibition of hydroxy methyl glutaryl-CoA reductase. Therefore, to more specifically assess the effect of the LDL receptor on apoE secretion from macrophages, we used MPMs from mice with basal or constitutively increased expression of the LDL receptor. Furthermore, MPMs from human apoE3, apoE4, and apoE2 knockin mice were evaluated to determine whether there were apoE isoform-specific effects of the LDL receptor. The generation and characterization of these mice has been reported previously in detail (17).

Figure 3 shows the results of a pulse-chase analysis of apoE secretion and cellular retention in WT and Ldlr cells for each of the apoE isoforms. As expected, LDL receptor mRNA levels were 2- to 4-fold higher in LDLr compared with WT cells (data not shown). For apoE3 (Fig. 3, upper panel) at cell 0 min (the end of the pulse-labeling incubation), labeled apoE was higher in Ldlr cells, indicating an apparent increased apoE synthesis. In spite of this increase in Ldlr cells at the start of the chase incubation, after 90 min of chase there was no difference in the amount of apoE secreted into the medium or retained within cells between Ldlr and WT cells. This result is consistent with the conclusion that increased LDL receptor expression reduced the secretion and enhanced the degradation of apoE, and with the results from experiments using J774-E3 cells in Fig. 2. The middle and lower panels of Fig. 3 show similar analyses for the apoE4 and apoE2 isoforms. In the case of apoE4, the amount of apoE present in cells at the end of the pulse incubation was reduced by 34% in Ldlr compared with WT cells. However, the rate of secretion was reduced by 80% and cell retention was reduced by 50%. In apoE2-expressing cells, Ldlr cells contained more labeled apoE after the pulse incubation and demonstrated
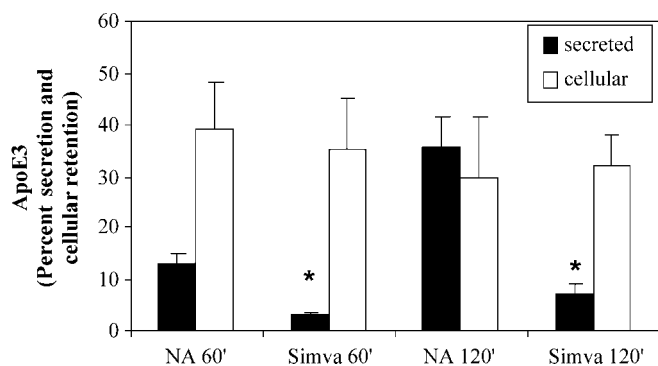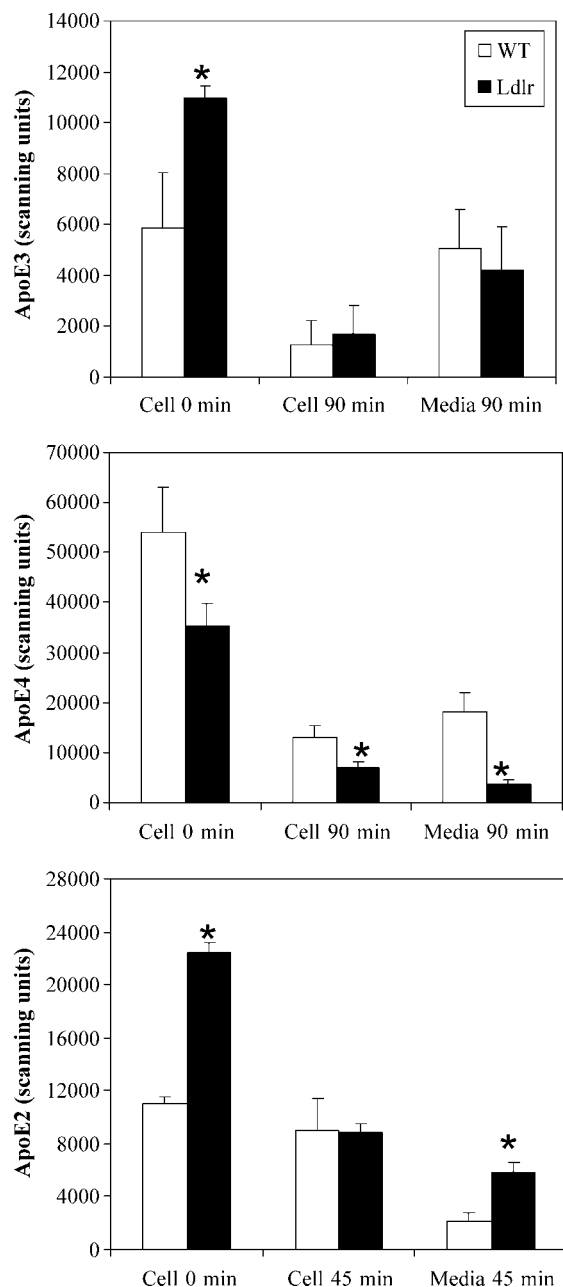
Fig. 3. Regulation of apoE secretion by the LDL receptor. Mouse peritoneal macrophages (MPMs) expressing human apoE3 (upper panel), apoE4 (middle panel), or apoE2 (lower panel) were harvested and cultured as described in Methods. The synthesis and secretion of apoE was measured using a pulse-chase analysis in cells with basal (WT) or increased (Ldlr) LDL receptor expression, as described in Methods. Values shown are means ± SD of triplicate samples. * $P < 0.05$ for WT versus Ldlr cells.

increased apoE secretion, with no change in cell retention of apoE. Because of the differences in labeled cellular apoE after the pulse incubation (at cell 0 min) between WT and Ldlr cells, we calculated the percentage secretion in WT and Ldlr cells for each isoform (**Table 1**). As shown here, in Ldlr cells there was a reduction in the secretion of newly synthesized apoE3, from 44% to 22%, with a trend toward statistical significance ($P = 0.08$). In apoE4 cells,

TABLE 1. Effects of LDL receptor expression on apoE2, apoE3, and apoE4 secretion in mouse peritoneal macrophages

Isoform	WT	Ldlr	P
E2	10 ± 3%	19 ± 3%	0.02
E3	44 ± 10%	22 ± 7%	0.08
E4	38 ± 9%	13 ± 4%	0.03

apoE, apolipoprotein E; Ldlr, macrophages with increased low density lipoprotein receptor expression; WT, macrophages with basal low density lipoprotein expression. The percentage of apoE secreted for each isoform was calculated as described in Methods.

the secretion of newly synthesized apoE was reduced significantly, from 38% to 13% ($P = 0.03$). For apoE2, the secretion of newly synthesized apoE increased in Ldlr cells compared with WT cells, from 10% to 19% ($P = 0.02$).

We have shown previously that cell surface proteoglycans can sequester newly synthesized apoE in macrophages (14, 15). Cell surface proteoglycans also have been shown to cooperate with apoE receptors to regulate lipoprotein metabolism in other cell types (26). Therefore, we evaluated whether modulation of apoE secretion from macrophages by the LDL receptor involved cellular proteoglycans. We chose the apoE4 isoform for evaluation, as the effect of the LDL receptor on the secretion of this isoform was greatest. ApoE4-expressing cells were depleted of proteoglycans as described previously (14, 15). We then evaluated the amount of apoE release into the medium during a 2 h labeling incubation (**Fig. 4**). Measurement of LDL receptor mRNA levels confirmed that a 3- to 4-fold increase was maintained in LDLr compared with WT cells after proteoglycan depletion (data not shown). In WT cells, depletion of proteoglycans approximately doubled the amount of apoE released into the medium, with no change or a slight increase in cellular apoE (Fig. 4, upper panel). In Ldlr cells, the depletion of proteoglycans led to an ~4-fold increase of apoE secreted in the medium, with no change in cellular apoE. The evaluation of proteoglycan depletion on apoE secretion was done in separate experiments for WT and Ldlr cells; therefore, it is not possible to compare WT and Ldlr cells directly. However, depletion of cellular proteoglycans in Ldlr cells led to a greater release of apoE from cells into the medium than from WT cells. These results are consistent with cooperation between cellular proteoglycans and the LDL receptor for modulating apoE secretion.

The results described above indicated that increased LDL receptor expression reduced the secretion of apoE3 and apoE4, but not apoE2, from macrophages. We previously established an important role for endogenous apoE in modulating sterol efflux from macrophages (11, 13, 15). Therefore, we next evaluated whether the influence of the LDL receptor on apoE secretion was reflected in differences in sterol efflux from macrophages and how this related to apoE isoform. **Figure 5** shows the results of sterol efflux experiments performed with WT and Ldlr cells expressing human apoE2, apoE3, or apoE4. There were no differences in total cell cholesterol mass at the start of the efflux incubations for any of the cell types
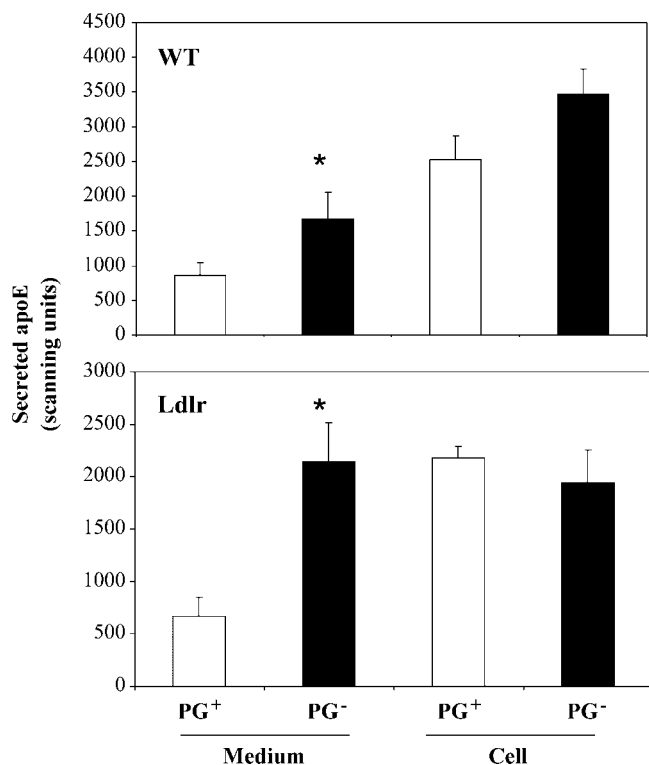
Fig. 4. Effect of proteoglycan depletion on LDL receptor modulation of apoE4 secretion. MPMs expressing human apoE4 with basal (WT) or increased (Ldlr) LDL receptor expression were treated with 1 mM 4-methyl-umbelliferyl-β-D-xyloside for 72 h in DMEM/10% FBS (PG⁻) or maintained in 10% FBS (PG⁺). Thirty minutes before incubation with [^{35}S]methionine, cells that were preincubated with 4-methyl-umbelliferyl-β-D-xyloside were also treated with 3 U/ml heparinase in 0.1% BSA/DMEM at 37°C. Control cells were placed in 0.1% BSA/DMEM only. Cells were then incubated with [^{35}S]methionine (50 μCi/ml) with 1 μM cold methionine at 37°C for 120 min. At that time, cell lysates and media were harvested for quantitative immunoprecipitation of apoE, as described in Methods. Values shown are means ± SD of triplicate samples. * $P < 0.05$ for PG⁺ versus PG⁻ cells.

compared. Increased expression of the LDL receptor reduced sterol efflux from macrophages expressing apoE3 and apoE4, similar to the effects of increased LDL receptor on apoE secretion. For the apoE2 isoform, the cells with increased LDL receptor expression showed slightly more efflux than WT cells, also consistent with changes in apoE2 secretion observed in Ldlr cells. Time points up to 120 min are presented in Fig. 5; however, differences between WT and Ldlr cells were maintained for up to 24 h data not shown).

DISCUSSION

Increased LDL receptor expression by the liver, such as that resulting from statin administration, leads to increased hepatic uptake and degradation of LDL particles with a subsequent decline in circulating LDL cholesterol level. Reducing LDL cholesterol level produces a significant atheroprotective effect in both animals and humans.

Relevant to our current observations, it has also been demonstrated that increased LDL receptor expression leads to the decreased secretion of apoB by hepatocytes (27). In this report, we provide evidence that the LDL receptor also regulates apoE secretion by macrophages.

The expression of apoE by macrophages is regulated by multiple pathways at transcriptional and posttranscriptional loci. Macrophage differentiation state, inflammatory cytokines, interaction with components of the extracellular matrix, and the availability of extracellular lipoproteins all modulate apoE synthesis and secretion by macrophages (4–9, 28). Importantly, macrophage free cholesterol content has large effects on macrophage apoE synthesis by working at both transcriptional and posttranslational loci (4–6, 10). Macrophage sterol content directly influences apoE gene transcription via a liver X receptor element found in downstream enhancer (4–6). Macrophage sterol content also stabilizes newly synthesized apoE, as demonstrated in cells with constitutive expression of an apoE cDNA (10). Endogenously expressed apoE in macrophages is also sequestered at the macrophage plasma membrane by cell surface proteoglycans (14, 15). We have shown previously that modulating the expression of cellular proteoglycans influences the secretion of newly synthesized apoE from the macrophage (14, 15). The LDL receptor at the cell surface also binds newly synthesized apoE, and apoE bound here can serve as an immediate precursor of secreted apoE (16). This observation raised the possibility that the level of LDL receptor expression would also influence the secretion of newly synthesized apoE.

Increasing LDL receptor expression using statins, or by expressing an LDL receptor mRNA species with increased stability, reduces apoE secretion from macrophages. This regulation of apoE secretion by the LDL receptor would allow for the rapid coordination of two homeostatic pathways involved in defending macrophage sterol balance. Changes in macrophage sterol content regulate the expression of the LDL receptor and apoE in opposite directions (29). With decreasing cholesterol content, or an increased need for cellular cholesterol, increased expression of the LDL receptor leads to increased internalization of cholesterol-containing lipoproteins. On the other hand, a net surplus of cell cholesterol leads to increased expression of apoE, which facilitates net sterol efflux from cells, and, therefore, to a net reduction in cellular sterol content. After migration of monocytes from the circulation to sites of tissue injury or inflammation, these cells likely undergo large variations in sterol content relative to need. The process of conversion to fully mature macrophages requires a dramatic expansion of the plasma membrane, which imposes a significant need for additional cellular cholesterol. Membrane expansion and turnover in fully mature macrophages will also be influenced by activation state and by phagocytic activity. On the other hand, the ingestion of apoptotic cells or modified lipoproteins can lead to substantial increases in macrophage cholesterol content. The ability of macrophages to rapidly coordinate the expression of two pathways with opposite effects on cellular sterol flux may be central to preserving cellular sterol balance.
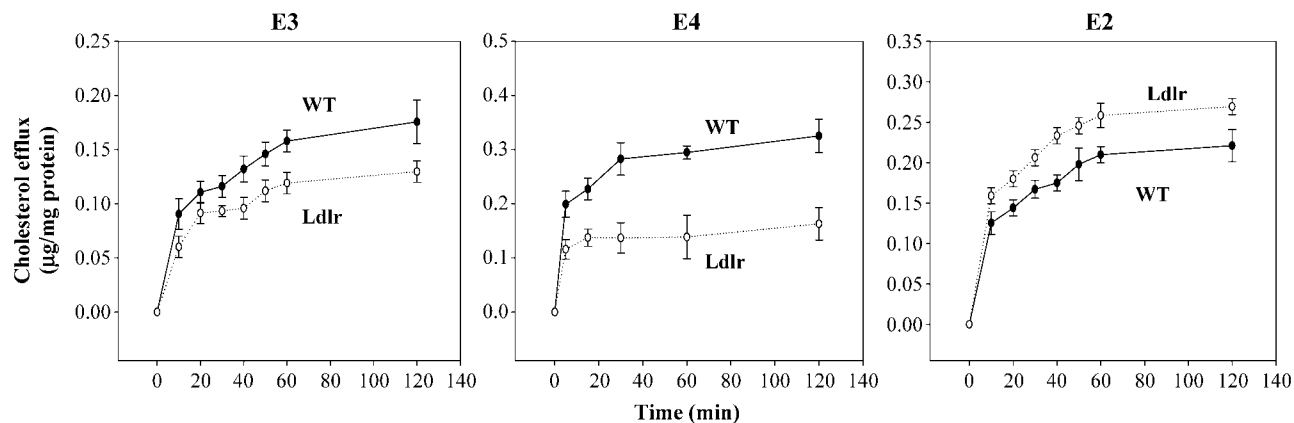
Fig. 5. LDL receptor expression regulates apoE-dependent sterol efflux in an apoE isoform-specific manner. Sterol efflux from MPMs expressing human apoE3, apoE4, or apoE2 with basal (WT) or increased (Ldlr) LDL receptor expression was measured as described in Methods. Each point shown represents the mean ± SD from triplicate wells of cells.

The influence of LDL receptor expression on apoE secretion that we observed was isoform-dependent, and the effect paralleled the established affinity of each apoE isoform for binding to the LDL receptor (17, 18). ApoE2 binds to the LDL receptor with much less affinity than apoE3 or apoE4, and increased LDL receptor expression did not decrease apoE2 secretion from macrophages. The increase in apoE secretion we measured in apoE2 Ldlr cells compared with apoE2 WT cells may be related to the much higher initial level of synthesis observed in Ldlr cells. ApoE4 binds to the LDL receptor with equal or higher affinity than apoE3. In our experiments, the reduction of apoE4 secretion with increased LDL receptor expression was the most easily detectable. The reduction of apoE3 secretion in Ldlr cells did not reach statistical significance, partly because initial apoE3 synthesis was also lower in apoE3 Ldlr cells. The results of experiments with J774-E cells, however, which constitutively express a human apoE3 cDNA (and therefore do not present the confounding problem of differences in initial apoE3 synthesis rates), showed that increased LDL receptor expression did decrease apoE3 secretion. The isoform dependence of the effect of the LDL receptor on apoE secretion strongly suggests that changes in apoE secretion require a direct interaction between apoE and the LDL receptor. This conclusion is also consistent with our previous observations that apoE is sequestered at the macrophage cell surface and can be displaced by the addition of monoclonal or polyclonal antisera to the LDL receptor at 4°C (16). Binding to cell surface proteoglycans also influences apoE secretion from macrophages (14, 15). Cullen et al. (30) previously reported that apoE4 has the highest affinity of the apoE isoforms for macrophage cell surface proteoglycans. Our results indicate that the effect of the LDL receptor on apoE4 secretion depends on the presence of an intact complement of cellular proteoglycans.

In our experiments, it was interesting that the amount of labeled apoE present within cells immediately after pulse-labeling was different between WT and Ldlr cells for all of the apoE isoforms. Although these differences could represent true changes in initial synthesis, we cannot rule out some contribution of differences in a very rapid initial degradation process. This problem can usually be addressed by shortening pulse-labeling times; however, this was impractical in our studies because of the very low secretion rates for some of the apoE isoforms, leading to difficulty in reliably measuring secreted apoE with shorter labeling times. The basis for the differences between WT and Ldlr cells immediately after pulse-labeling will require additional work. It is also important to recognize that the results of our experiments do not distinguish between the effects of an overall increase in LDL receptor expression and an increase in human LDL receptor expression.

The results in this report not only show that the LDL receptor is involved in regulating the secretion of apoE from macrophages but, importantly, also demonstrate that the LDL receptor expression level regulates apoE-dependent sterol efflux from macrophages. The changes in apoE-mediated sterol efflux produced by increased LDL receptor expression paralleled the LDL receptor-mediated changes in apoE secretion. In conclusion, the regulation of apoE secretion and apoE-mediated sterol efflux by the LDL receptor establishes a regulatory interaction between two branches of macrophage sterol homeostatic pathways. This interaction would allow macrophages to rapidly respond to changes in sterol flux that occur as part of their differentiated function.█

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