Sterol Efflux Mediated by Endogenous Macrophage ApoE Expression Is Independent of ABCA1

Zhi Hua Huang, Chen-Yi Lin, John F. Oram, Theodore Mazzone

Abstract—Sterol efflux importantly contributes to preservation of cellular cholesterol homeostasis, and multiple pathways may be involved for mediating such efflux. Recently, an important role has been ascribed to ABCA1 in facilitating lipid efflux from cells, including macrophages, to extracellular lipid-free apolipoproteins. Macrophages are relatively unique among cells because they express apoprotein E (apoE) as a major protein product, and this endogenous expression of apoE increases sterol and phospholipid efflux from macrophages. The studies in this article were designed to test whether the sterol efflux mediated by the endogenous expression of apoE in macrophages was dependent on ABCA1 expression. These studies were facilitated by comparing apoE-expressing J774 cells (J774E+) with nonexpressing parental cells (J774E−). Sterol efflux was higher from J774E+ cells compared with J774E− cells, but the increment in efflux between these cell types was not increased by induction of ABCA1 expression with cAMP. Induction of ABCA1 with cAMP, however, did increase sterol efflux to exogenously added apoA1 from both cell types. Inhibitors of ABCA1 activity significantly reduced (by 40% to 50%) sterol efflux from both J774E+ and J774E− cells treated with cAMP and apoA1. This inhibitor did not, however, reduce the increment in sterol efflux due to the expression of endogenous apoE. The results of these studies indicate that the increment in sterol efflux mediated by the endogenous expression of apoE in macrophages does not depend on ABCA1 expression or activity. (Arterioscler Thromb Vasc Biol. 2001;21:2019-2025.)

Key Words: atherosclerosis ■ macrophages ■ apolipoprotein E ■ sterol efflux ■ ABCA1

Accumulation of cholesterol in the vessel wall is the hallmark of human and experimental atherosclerosis. Most of this vessel wall cholesterol is derived from circulating apoB-containing lipoproteins, and a great deal of data in humans and animals has demonstrated the importance of increased delivery by these lipoproteins for the development of vessel wall lesions.1 More recently, however, the importance of cholesterol removal mechanisms for preserving vessel wall cholesterol homeostasis has been appreciated.2,3 In experimental animals, altering the efficiency of sterol removal from the vessel wall has a profound effect on the development of atherosclerotic lesions. Human diseases thought to be associated with decreased removal of cholesterol from vessel wall cells are also characterized by accelerated atherosclerosis.4

There are likely multiple mechanisms that contribute to sterol efflux from vessel wall cells, including aqueous diffusion and scavenger receptor class B type 1–mediated efflux.5–7 Most recently, the importance of the ATP binding cassette A1 (ABCA1) transporter protein has been emphasized for facilitating sterol efflux to lipid-free apoA1 from multiple cell types.8 A defect in expression of this protein underlies Tangier disease and accounts for the dramatic reduction in sterol efflux to lipid-free apoA1 observed in cells derived from Tangier disease patients.2,8 Extensive study of the ABCA1 transporter in cultured cells has indicated that it may bind to apoA1 to facilitate sterol efflux.9,10 In the absence of ABCA1, there is markedly reduced sterol efflux to lipid-free apoA1, although sterol efflux to protein-free phospholipid vesicles, cyclodextrins, albumin, or trypsinized HDL is maintained.

The macrophage is a cell type of major importance for understanding the pathophysiology of atherosclerosis. Macrophages are among the earliest cell type to accumulate after hyperlipemic insult to the vessel wall, are a prominent source of vessel wall foam cells, and produce a number of the cytokines and proteases found in atheroma. Necrosis or apoptosis of vessel wall macrophages secondary to the excessive intracellular accumulation of sterol11 may be important for the genesis of many of the features of complicated vessel wall lesions. Sterol efflux from this cell type, then, is likely to be important for maintaining normal vessel wall homeostasis. ABCA1 is expressed in macrophages and is important for cholesterol efflux to lipid-free A1, as has been found in other cells. However, macrophages are unique among vessel wall cells in that they express apoE as a major protein product. Endogenous expression of apoE in the macrophage facilitates sterol efflux from these cells.12–14 This

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endogenous apoE-dependent increment in efflux can be observed in the absence of specific extracellular sterol acceptors but is magnified when HDL₃, 2-hydroxypropyl \( \beta \)-cyclodextrin (\( \beta \)CD), or phosphatidylcholine (PC) vesicles are incubated with cells.\textsuperscript{12,14} In transgenic and knockout mouse models, it has been shown that macrophage-specific expression of apoE in the vessel wall can be atheroprotective, and its role in facilitating sterol efflux from macrophages likely contributes to this protection. Although it has been well established that ABCA1 is required for sterol efflux from macrophages to exogenous lipid-free apoA1, there is less information regarding whether ABCA1 expression is required for the sterol efflux that results from endogenous expression of apoE in the macrophage. The studies in this report were designed to test whether the increment in sterol efflux mediated by the endogenous expression of apoE in macrophages was dependent on ABCA1 expression.

**Methods**

**Materials**

Purified apoA1 isolated from human HDL and purified apoE isolated from human VLDL were purchased from Calbiochem. cAMP and 4,4'-disothiocyanoatostilbene-2,2'-disulfonic acid (DIDS) were purchased from Sigma. All other materials were from previously identified sources.\textsuperscript{12,14}

**Cells**

The generation and characterization of the apoE-expressing (J774E') and the nonexpressing (J774E) J774 macrophages have been previously described in detail.\textsuperscript{12,14,17} Both cell lines were transfected with a neomycin resistance vector, selected, and maintained in neomycin (400 \( \mu \)g/mL); J774E' cells also received a human apoE3 cDNA under the control of a constitutively expressed cytomegalovirus promoter. The apoE-expressing cell line chosen for these studies constitutively secretes 1.0 \( \mu \)g of apoE per milligram of cell protein over 24 hours, leading to concentrations in the medium of 0.8 to 1.4 \( \mu \)g/mL. This amount approximates physiological levels of apoE secreted by mouse peritoneal macrophages and human monocyte-derived macrophages. These cells were plated at 1 to 2\( \times \)10⁶ cells per well in 6-well plates and were used for experiments 2 to 3 days after plating. Resident mouse peritoneal macrophages were harvested from CD-1 mice (Jackson Laboratories, Bar Harbor, Me) by lavage with phosphate-buffered saline as previously described in detail.\textsuperscript{18} These cells were plated at 2 to 3\( \times \)10⁶ cells per well in 6-well plates and used for experiments with 1 week.

**Immunoblot Analysis**

Cellular ABCA1 levels were detected by Western blot by using an antibody raised against the 22–amino-acid C-terminus of ABCA1 as previously described.\textsuperscript{9} Cell monolayers were extracted in a buffer containing 50 mM Tris, 2% SDS, 0.1 mol/L \( \beta \)-mercaptoethanol, and 0.5 mM EDTA. Two hundred micrograms of cell protein per lane was loaded onto SDS–polyacrylamide gel electrophoresis gels for analysis. Detection was accomplished with an HP Scanjet 11cx (Hewlett-Packard).

**Cholesterol Efflux Assay**

Cholesterol efflux from cells was measured as previously described.\textsuperscript{14} Cells were plated and labeled with \([\text{H}]\)cholesterol (1 \( \mu \)Ci/mL) for 48 hours in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum. The \([\text{H}]\)cholesterol was added in an ethanol vehicle, and the final ethanol concentration in the medium was <0.4%. Before the start of the efflux incubation, the cells were rinsed 3 times with DMEM plus 0.1% bovine serum albumin (BSA), incubated in DMEM plus 0.5% BSA at 37°C for 2 hours, and then rinsed again in DMEM alone. The efflux time course was initiated by the addition of 0.1% BSA in DMEM plus any additions indicated in the figures or figure legends. Aliquots of the medium were sampled at the time points indicated and centrifuged at 10,000 rpm\( \times \)15 minutes to pellet the detached cells and cellular debris. The \([\text{H}]\)radioactivity in the supernatant was quantified by liquid scintillation counting. The efflux is expressed as a percentage, calculated as the \([\text{H}]\)cholesterol radioactivity released divided by that measured in cells at the beginning of the efflux incubation. At the completion of each experiment, cell monolayers were washed 3 times with PBS dissolved in 0.1N NaOH and assayed for protein and cholesterol mass. There were no differences in cellular sterol mass or cell protein between J774E' and J774E cells.

**ApoE Secretion**

Secretion rates of apoE were measured by biosynthetically labeling apoE by incubating the cells with \([\text{35S}]\)methionine (50 \( \mu \)Ci/mL) with 10 \( \mu \)mol/L unlabeled methionine. After 6 hours, culture supernatants were collected for the quantitative immunoprecipitation and quantification of secreted apoE, as previously described in detail.\textsuperscript{13} Levels of labeled apoE in the media were corrected for any differences in total protein secretion. Treatment with cAMP and DIDS was done exactly as described for the sterol efflux experiments.

**Other Analyses**

Cell protein was measured by using a DC protein assay kit (Bio-Rad). The cholesterol mass in extracts of cells was measured by gas-liquid chromatography with coprostanol as the internal standard as previously described.\textsuperscript{14} Statistical comparisons were made by ANOVA with SPSS software (SPSS Inc).

**Results**

**Induction of ABCA1 With cAMP Does Not Increase the Increment in Sterol Efflux Mediated by the Endogenous Expression of Macrophage ApoE**

We have previously shown that endogenous apoE expression leads to increased lipid efflux from macrophages.\textsuperscript{12,14} Specifically, expression of physiological levels of human apoE in the J774 macrophage line (which does not express its own apoE gene) significantly increases cholesterol and phospholipid efflux. This increase can be observed in the absence of extracellular sterol inhibitors and is magnified by addition of HDL₃, \( \beta \)CD, or PC vesicles. In the first series of experiments, we evaluated efflux from the apoE-expressing J774 cells (J774E') and control nonexpressing cells (J774E) in the presence or absence of cAMP. We performed this experiment because in multiple cell types, including J774 macrophages, it has been shown that cAMP treatment enhances ABCA1 expression and results in increased sterol efflux to exogenously added lipid-free apoproteins.\textsuperscript{9,19} Figure 1 shows the results of an experiment conducted in the absence of exogenously added sterol acceptors in J774E' and J774E cells. As we have previously shown,\textsuperscript{14} cholesterol efflux was significantly higher from J774E' cells compared with J774E' cells (compare left and right panels). Sterol efflux was higher in J774E' cells compared with J774E cells in both DMEM with 0.1% BSA (2.3-fold higher, \( P<0.01 \)) and DMEM alone (2.9-fold higher, \( P<0.01 \)). cAMP treatment did not increase sterol efflux from either J774E' or J774E cells. Therefore, the difference in efflux between cell types (ie, endogenous apoE-dependent efflux) is not enhanced as a result of cAMP induction of ABCA1 expression. To further confirm this result in another model, we evaluated the effect of cAMP treatment on sterol efflux from mouse peritoneal macrophages that express their endogenous apoE gene. These results are shown in Figure 2. Similar to the results in the
transfected J774 model, induction of ABCA1 with cAMP treatment did not increase sterol efflux from mouse peritoneal macrophages.

Sterol Efflux From J774E⁺ and J774E⁻ Cells to Exogenous ApoA1 Is Increased by cAMP

The above results indicated that the increment in sterol efflux due to endogenous apoE expression was not enhanced by cAMP treatment to induce ABCA1 expression. To confirm that the cAMP treatment was inducing ABCA1-dependent efflux, we measured sterol efflux to exogenous lipid-free apoA1 from each cell type in the presence and absence of cAMP. These results are shown in Figure 3. At 12 hours, sterol efflux to apoA1 was 8% and 16% from J774E⁻ cells without and with cAMP, respectively, a 2-fold increase (left panel). At the same time, sterol efflux from J774E⁺ cells was 3% and 10% without and with cAMP, respectively, a 3.3-fold increase (right panel). Therefore, cAMP treatment significantly increased ABCA1-dependent efflux from each cell type. cAMP induction of efflux in each cell type required 6 to 8 hours into the time course. This result is consistent with the reported time course for ABCA1 induction by cAMP in J774 cells.¹⁹ Although sterol efflux was significantly increased in the presence of cAMP plus apoA1 from J774E⁺ cells, the absolute level of efflux remained lower than that achieved from J774E⁻ cells under the same incubation conditions (compare left and right panels). This difference was unexpected and was observed in apoE-expressing clones derived from multiple transfections and from clones expressing both higher and lower levels of apoE than the clone used for these experiments (not shown). This apoE-dependent change in sterol efflux to apoA1 is different from what was observed after addition of βCD or PC vesicles to J774E⁺ and J774E⁻ cells. With the use of βCD or PC vesicles as acceptors, the absolute level of sterol efflux was substantially greater from J774E⁻ cells than from J774E⁺ cells.¹⁴

ABCA1 Protein Level Is Reduced in J774E⁻ Compared With J774E⁺ Cells but Is Induced by cAMP in Both Cell Types

There are multiple potential explanations for the lower absolute level of sterol efflux to apoA1 as a result of apoE expression in J774E⁻ cells. However, the fact that this was observed only to apoA1, an ABCA1-specific efflux agent, and not to βCD or phospholipid vesicles led us to first examine ABCA1 expression levels in these 2 cell lines. The results of representative experiments are shown in Figure 4. In the top panel of Figure 4, we show an immunoblot analysis...
Effect of cAMP on Sterol Efflux to Exogenous Lipid-Free ApoE

Other laboratories have reported that cAMP induction of ABCA1 expression can enhance sterol efflux to exogenously added lipid-free apoE. This situation is different from the above results with endogenously expressed apoE. We further confirmed this difference between endogenous and exogenous apoE-dependent pathways by evaluating the effect of cAMP on sterol efflux from J774E cells in the presence of exogenously added lipid-free apoE. The results of a representative experiment are shown in Figure 5. Addition of cAMP led to a significant increase in sterol efflux to exogenous lipid-free apoE added at 1 and 9 μg/mL. Therefore, different from what is observed with sterol efflux dependent on endogenous apoE expression (Figures 1 and 2), cAMP-induced expression of ABCA1 is associated with increased efflux to exogenously added lipid-free apoE. The accumulation of apoE in the medium that results from the endogenous expression of apoE (even after cAMP stimulation; see above) does not increase efflux. This finding is likely related to the decreased ABCA1 levels in apoE-expressing cells (even after cAMP stimulation; Figure 4) and to functional differences in the nature of the particle formed by endogenous expression versus the exogenous addition of apoE that we have previously observed.14

ABCA1 Inhibitors Do Not Reduce the Increment in Sterol Efflux Dependent on Endogenous ApoE Expression

On the basis of the above observations, we predicted that inhibitors of ABCA1 activity would reduce sterol efflux from both J774E and J774E cells incubated with exogenous apoA1 plus cAMP but not reduce the efflux due to the endogenous expression of apoE. We tested this prediction in the experiments shown in Figure 6. In the top panel of this figure, we show the effect of DIDS, an ABCA1 inhibitor, on sterol efflux from J774E and J774E cells that were treated with cAMP and incubated with apoA1. In the presence of...
apoE-expressing macrophages with parental macrophages that did not express apoE. Although our data do not exclude the participation of ABCA1 in facilitating endogenous apoE-dependent efflux under any condition (see below), they do establish that this efflux can be independent of ABCA1 expression. This conclusion is supported by several lines of evidence. First, sterol efflux is higher from J774E + cells compared with J774E − cells in 0.1% BSA, even though ABCA1 levels (by immunoblot) and activity (by efflux to lipid-free apoA1) were substantially lower in J774E − cells. Second, cAMP induction of ABCA1 levels significantly increased efflux from both J774E + cells and J774E − cells to exogenous lipid-free apolipoproteins but did not enhance the increment in efflux resulting from the endogenous expression of apoE in the absence of exogenously added apolipoproteins. Third, inhibition of ABCA1 activity significantly reduced efflux from cAMP-treated J774E + cells and J774E − cells to lipid-free apoA1 but did not reduce the increment in efflux resulting from the expression of endogenous apoE in macrophages. These results taken in aggregate establish that the increment in efflux, due to the endogenous expression of apoE in macrophages, does not depend on ABCA1 expression or activity.

Our finding that ABCA1 levels were markedly reduced as a result of apoE expression in macrophages was unexpected. A recent report21 also suggested an interaction between ABCA1 and apoE expression in the macrophage. In those studies, cAMP enhanced apoE secretion, and ABCA1 antisense oligonucleotides or inhibitors of ABCA1 reduced apoE secretion. On the basis of those observations, it was concluded that ABCA1 expression facilitated apoE secretion from macrophages. We observed similar effects of modulating ABCA1 activity on apoE secretion in our cell model. Our observation that increased apoE expression reduced ABCA1 expression, however, indicates that the relationship between ABCA1 and apoE expression in macrophages is complex. Both apoE and ABCA1 share common regulatory signals for gene transcription, being modulated by intracellular oxysterols via liver X receptor elements.22–24 Changes in the intracellular flux or metabolism of oxysterols due to apoE expression may, therefore, modulate expression of the ABCA1 gene. ABCA1 and apoE could also interact posttranslationally. These interactions could modulate the subcellular distribution of these proteins or their degradation rates and could be influenced by numerous factors, such as lipid flux in the cell. Alternatively, changes in ABCA1 level after changes in apoE expression may not reflect direct interaction but instead may reflect an indirect cellular adaptation. The potential complexity that underlies changes in ABCA1 expression, after changes in apoE expression, requires additional studies in isolated cell systems. After additional mechanistic information is available, it may be worthwhile to investigate cells isolated from various models of engineered mice. A model incorporating a bone marrow transplant approach would have the benefit of minimizing potential developmental differences (eg, hyperlipidemia) that could confound comparison of macrophages harvested from mouse models. Regardless of the nature of the relationship between apoE expression and ABCA1 level, the higher sterol efflux that we observed as a result of endogenous apoE expression, in the presence of reduced ABCA1 levels, underscores the

**Discussion**

The studies in this article provide new insight into the relationship between ABCA1 expression and the sterol efflux that results from the endogenous expression of apoE in macrophages. The experiments were facilitated by comparing
independence of this efflux pathway from ABCA1. The observation that endogenous expression of apoE can enhance sterol efflux independent of ABCA1 has important implications for understanding cholesterol homeostasis in the macrophage and for designing therapeutic interventions for atherosclerosis.

The data in Figure 5 indicate that efflux to exogenous lipid-free apoE at 1 μg/mL is enhanced by a small but significant amount by the addition of cAMP. However, the cAMP-related increase in sterol efflux is much larger in the presence of a higher concentration (9 μg/mL) of exogenous lipid-free apoE. This result is consistent with recently reported results showing that cAMP enhances sterol efflux from J774 macrophages to exogenously added lipid-free apoE at a concentration of 20 μg/mL. Therefore, at higher extracellular concentrations of lipid-free apoE, cAMP induction of ABCA1 expression can importantly contribute to sterol efflux. This mechanism could also relate to the previously reported results showing that cAMP increased sterol efflux from RAW 264.7 cells transfected to express apoE.

In those studies, because of the high level of transgene expression, extracellular apoE concentrations reached 14 μg/mL. The data above suggest that at this high concentration of extracellular apoE, ABCA1-dependent mechanisms are involved in sterol efflux. The results in this report indicate that when apoE is expressed at a lower and more physiological level, the sterol efflux due to the endogenous expression of apoE is not dependent on ABCA1. This supports a conclusion, based on previously reported observations, that endogenous expression of apoE and exogenous addition of apoE facilitate macrophage sterol efflux through distinct pathways. A functional difference (with respect to sterol efflux) in the nature of the extracellular particle formed by endogenous expression versus exogenous addition of apoE has been previously documented. This conclusion is further supported by results in this report: although cAMP and DIDS can modulate the extracellular accumulation of apoE, they do not modulate sterol efflux due to the endogenous expression of apoE. Therefore, mechanisms of efflux beyond the extracellular accumulation of apoE must be considered. For example, we have recently shown that retention of endogenous macrophage apoE in a pericellular proteoglycan matrix is important for stimulating sterol efflux from macrophages.

In summary, multiple pathways are likely involved in sterol removal from peripheral cells. From a physiological and human disease standpoint, these pathways are highly important in macrophages because of the likely importance of the macrophage in preserving vessel wall sterol homeostasis. Pathways for sterol efflux from macrophages include aqueous diffusion; ABCA1-dependent efflux; scavenger receptor class B type 1–dependent efflux; efflux resulting from the 27-hydroxylation of sterol, a pathway dependent on the endogenous expression of apoE; and perhaps pathways still to be identified.

We have previously shown that macrophage apoE gene transcription increases after small positive changes in macrophage sterol level, and this transcriptional response has recently been demonstrated to be dependent on liver X receptor signaling. We have also previously shown that the posttranslational degradation of apoE in macrophages is modulated by cellular sterol level. The observation that endogenous apoE expression facilitates macrophage sterol efflux, independent of ABCA1 expression, presents a question regarding the relative importance of these separate pathways for preserving macrophage sterol balance. Future studies in isolated cells can evaluate the contribution of redundant and independent pathways for defending macrophage cholesterol homeostasis.

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References


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