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Cellular Sphingolipids Regulate Macrophage Apolipoprotein E Secretion[†]

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ABSTRACT: Macrophage-derived apolipoprotein E (apoE) in the vessel wall has important effects on the vessel-wall response to atherogenic injury. The current studies characterize a novel post-transcriptional pathway for the regulation of apoE secretion from macrophages. Treatment of J774 macrophages transfected to constitutively express a human apoE3 cDNA (to constitutively secrete a physiologic level of apoE) with sphingomyelinase led to a reduction of apoE secretion by nearly 50%. Increasing cellular ceramide by inhibiting ceramide degradation or by the direct treatment of cells with exogenous ceramide also reduced apoE secretion without a concomitant increase in cellular retention of newly synthesized apoE. Reducing cellular sphingomyelin (SM) by inhibiting its synthesis also reduced apoE secretion, but in this case, reduced apoE secretion was accompanied by increased cellular retention of apoE. The effect of sphingomyelin depletion to decrease apoE secretion and increase its cellular retention was dependent upon the presence of intact C-terminal amphipathic lipid-binding domains in apoE. ApoE expression also increased sphingomyelin secretion from macrophages, and this sphingomyelin was co-localized with apoE in secreted lipoprotein particles. The importance of sphingomyelin for apoE secretion and cell retention was confirmed using a Chinese hamster ovary model, in which cellular sphingolipids (both ceramide and sphingomyelin) are reduced secondary to absent serine palmitoyltransferase activity. Our results show that cellular sphingolipids, ceramide and sphingomyelin, have important effects on the post-transcriptional handling of nascent apoE by macrophages. Increased cellular ceramide reduces apoE secretion without increased cell retention, consistent with enhanced degradation of newly synthesized apoE. Reduction of cell SM also reduces apoE secretion, but this is associated with increased cellular retention of newly synthesized apoE. The dependence for this effect on the C-terminal domain of apoE suggests a model in which the SM content of intracellular membranes modulates the secretion of nascent apoE via the interaction with amphipathic lipid-binding domains.

Macrophages are a prominent feature of clinical and experimental atherosclerosis (*1*, *2*). Monocyte-derived macrophages are among the first cell type to accumulate in nascent atheromas, and the lipid-engorged mature macrophage gives rise to the prototypical foam cell of advanced atheroma. A major mechanism by which macrophages can influence vessel-wall health relates to the biologic function of macrophage products secreted in the vessel wall. Apolipoprotein E (apoE) is the only apolipoprotein produced by macrophages and is a major secretory product of macrophages. Conversely, macrophages are the major source of apoE in the vessel wall (*3*−*6*). In a majority of *in vivo* models of experimental atherosclerosis, macrophage-derived apoE has been demonstrated to suppress the development of the atherosclerotic lesion in the vessel wall, and a significant portion of this atheroprotection can be ascribed to local effects of apoE in the vessel wall (*7*). Multiple pathways could account for the atheroprotection related to macrophage-derived apoE in the vessel wall. Macrophage-derived apoE enhances sterol efflux from macrophages by ABCA1-dependent and -independent mechanisms (*8*−*10*), enhances sterol efflux to mature high density lipoprotein (HDL) particles (*8*, *11*), influences arterial smooth muscle cell migration and proliferation, modulates endothelial cell inflammatory responses, and may promote an antioxidant milieu in the vessel wall (*12*, *15*).

The impact of macrophage-derived apoE on atherosclerosis makes it important to understand the regulation of macrophage apoE expression and secretion. Previous work has demonstrated that transcription of the apoE gene is regulated by cellular sterols via a liver X receptor (LXR)-dependent pathway (*5*, *16*). The macrophage differentiation state and inflammatory cytokines also impact apoE gene transcription (*17*, *18*). A large fraction of newly synthesized apoE in macrophages is degraded prior to secretion, and there are important post-transcriptional and post-translational loci for control of macrophage apoE secretion (*19*−*23*). We have previously shown that the segregation of newly synthesized apoE to a pool destined for secretion versus degradation can be influenced by the sterol content of intracellular macrophage membranes (*21*, *24*). Enrichment of macrophages with sterols, derived from either modified low density lipoprotein

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(LDL) or oxysterols, increases the sterol/phospholipid ratio of an intracellular membrane compartment responsible for the degradation of nascent apoE and increases the secretion of nascent apoE (*21*, *24*).

A substantial body of literature exists suggesting that sphingomyelinase (SMase)[1] expression in the vessel wall with local degradation of sphingomyelin (SM) has an important effect on atherosclerosis (*25*, *26*) A relationship between macrophage apoE and SM metabolism has been suggested by the observation that the expression of human apoE3 in macrophages significantly increases the efflux of choline-containing phospholipids from these cells (*8*). In view of the above considerations, we initiated a series of studies to investigate a potential role for macrophage SM in modulating macrophage expression of apoE, using multiple approaches for perturbing cellular SM. Because of our previous observations that cholesterol loading of macrophages with subsequent elevation of the cholesterol/phospholipid ratio of intracellular membranes leads to increased apoE secretion by working at a post-translational locus, we focused on a post-translational locus of control. In view of the important relationship between cellular SM and ceramide metabolism, a potential role for the latter sphingolipid was also tested.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS), recombinant protein G-agarose, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen. Bovine serum albumin (BSA), Triton X-100, D-609, D-erythro-2(*N*-myristoylamino)-1-phenyl-1-propanol (D-MAPP), deoxycholate, sphingomyelinase-C, and C6-ceramide were obtained from Sigma. Goat apoE antiserum was from International Immunology Corp. [^{35}S]Methionine and heparin-sepharose CL-6B were purchased from GE Healthcare. All other materials were from previously published sources (*8*, *10*, *17*−*24*).

Cell Culture. J774 macrophages, which do not express their endogenous apoE gene, were transfected to stably express human apoE3 at a physiologic level. The production and characterization of this cell line has previously been described in detail (*19*). J774-Δ202 cells are J774 cells that express a truncated apoE3, in which amino acids between 203 and 299 have been deleted. This deletion eliminates the C-terminal amphipathic lipid-binding domains. The production and characterization of this cell line has been described in detail (*24*). J774-C cells were transfected to express a neomycin-resistant construct only. J774 cell lines were maintained in DMEM with 10% FBS until used for experiments. For experiments, the cells were plated into 6-well dishes at a density of 1×10^6 cells per well. D-609 was used at a final concentration of 100 μM for 5 h in 0.2% BSA/DMEM. D-MAPP was used at a final concentration of 5 μM for 24 h in 0.2% BSA/DMEM. Exogenous C6 ceramide was used at a final concentration of 5 μM in 0.2% BSA/DMEM for 4 h. Sphingomyelinase-C treatment of cells was performed for 1 h at 37 °C at a final concentration of 50 mU/mL in 0.2% BSA. After these treatments, cells were washed with prewarmed methionine-free DMEM and incubated for 30 min with methionine-free DMEM containing 150 μCi/mL [^{35}S]methionine. At that time, some cells were harvested for the measurement of labeled apoE content within cells, and the balance were chased in 0.2% BSA/DMEM containing 500 μM unlabeled methionine for the times indicated in the figures.

LY-B and LY-B/cLCB1 (LY-B/c) cells were obtained from Dr. K. Hanada (National Institutes of Infectious Diseases, Tokyo, Japan). LY-B cells lack serine palmitoyltransferase (SPT) activity and thus are unable to synthesize sphingolipids (*27*). LY-B/c cells are LY-B cells genetically complemented to restore endogenous sphingolipid synthesis (*27*) and were used as control cells for LY-B. Both LY-B and LY-B/c cells were maintained in 10% FBS in F12 medium. LY-B and LY-B/c cells were transfected to stably express a human apoE3 cDNA under the control of the cytomegalovirus (CMV) promoter in a constitutive manner. To examine the effect of sphingolipid deficiency, we used a protocol previously described to reduce SM and ceramide levels in LY-B cells (*27*); incubating these cells in a sphingolipid-deficient medium containing 1% Nutridoma-SP, 0.1% FBS, and 10 μM sodium oleate.

Immunoprecipitation and Measurement of Cellular and Secreted ApoE. Quantitative immunoprecipitation of apoE was performed as previously described in detail (*19*). Briefly, labeled samples were precleared with 25 μL of nonimmune goat serum for 2 h at 4 °C, followed by incubation with 60 μL of protein G-agarose beads for 2 h at 4 °C. The samples were centrifuged, and the supernatant was incubated with 18 μL of apoE antiserum for 2 h at 4 °C. Protein G-agarose beads were added for an additional 2 h at 4 °C. After the protein G-agarose beads were washed, the samples were heated for 3 min at 90 °C to release the apoE−IgG complex from the beads and applied to 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The apoE signal was detected with a Molecular Dynamics Phosphorimager and quantitated using the ImageQuant software program. For a direct comparison of the level of apoE synthesis or secretion, immunoprecipitations were started with equal numbers of trichloroacetic acid (TCA)-precipitable counts for the specific pulse or chase time; therefore, changes in apoE are already corrected for any changes in total protein synthesis or secretion and comparisons can only be made for equivalent times of pulse or chase. For a calculation of the percent of apoE secretion and cell retention, the radioactivity in apoE present in the medium or cells (respectively) at indicated chase times is divided by total labeled cellular apoE measured immediately after the 30 min pulse-labeling incubation.

Measurement of Phospholipids. To assess the effect of D-609 and D-MAPP treatment on cellular ceramide and SM, J774-E cells were labeled for 24 h with 2 μCi/mL ^{14}[C]serine and 2.5% FBS/DMEM containing 10 μM unlabeled serine. After 24 h, either D-609 or D-MAPP were added at the concentrations and times described in each figure. Cellular lipids were extracted using hexane/isopropyl alcohol (3:2, v/v) and separated by thin-layer chromatography (TLC) as previously described (*8*). SM and ceramide spots were scraped, and their radioactivity was quantitated in a liquid scintillation counter.

[1] Abbreviations: SM, sphingomyelin; SMase, sphingomyelinase; D-MAPP, D-erythro-2(*N*-myristoylamino)-1-phenyl-1-propanol; SPT, serine palmitoyltransferase; CHO, Chinese hamster ovary; PC, phosphatidylcholine; LY-B/c, LY-B/cLCB1.

For measurement of SM and phosphatidylcholine (PC) radioactivity secreted from macrophages, J774-C and J774-E cells were cultured in T75 flasks to 90% confluency (3 flasks for each cell type). Cells were labeled with 0.5 μCi/mL ^{14}-[C]choline in DMEM and 10% FBS for 3 days. After this incubation, cells were washed 3 times with DMEM and 0.1% BSA and equilibrated in the same medium for 24 h. Conditioned medium was collected by incubating the cells in DMEM and 0.1% BSA for another 2 h. The supernatants were spun down at 1800 rpm for 5 min to remove floating cells and concentrated with MY-10 centriprep to reduce the volume to 1−2 mL (3 runs, 40 min each at 3000 rpm and 4 °C). The concentrated samples were centrifuged at 100000*g* for 30 min at 4 °C to remove membrane debris. Lipids in the samples were extracted, and phospholipid subclasses were analyzed by TLC. SM and PC spots were harvested, and radioactivity was quantitated.

To measure SM and PC in apoE particles, J774-E cells were cultured in T75 flasks until 90% confluent. Cells were washed 3 times with DMEM and incubated with DMEM and 0.1% BSA for 2 h to remove any residual serum-derived apoE. After washing, conditioned medium was generated from six T75 flasks of J774-E cells by incubating the cells in DMEM and 0.1% BSA for 24 h. The floating cells were removed by centrifugation at 10 000 rpm for 5 min, and protease inhibitors were added. ApoE-conditioned medium was loaded onto a heparin sepharose column to separate the conditioned medium into a fraction containing apoE (bound) and a fraction depleted of apoE (unbound). The recovery and purity of apoE in the bound fraction was confirmed using immunoblot and silver staining of SDS−PAGE gels. The bound and unbound fractions were concentrated and subject to liquid chromatography/mass spectrometry (LC/MS) analysis. LC/MS analyses of the molecular species of PC and SM were kindly preformed by Dr. Su Chen (Chainon Bio, Albany, NY) on an LCQ ion-trap LC/MS system (ThermoFisher, CA) equipped with an electrospray ion source. The high-performance liquid chromatography (HPLC) separations were carried out using a silica column (3-mm Spherisorb silica, 2.0 × 150 mm; Waters, MA), which was eluted with a linear gradient of 100% solvent A (80:19.5:0.5 chloroform/methanol/30% ammonium hydroxide; v/v/v) to 100% solvent B (60:34.5:5:0.5 chloroform/methanol/water/30% ammonium hydroxide; v/v/v/v) for 15 min and then in 100% solvent B for another 15 min. A total of 20 μL of the sample was injected into the LC column, and the flow rate was at 0.35 mL/min. Positive-ion mass spectra were obtained in the mass range of 300−1000 Da, with the source temperature set at 350 °C. Semiquantitative analyses were performed with the help of di-17:0-PC (100 μg/mL) and 17:0-lyso PC (100 μg/mL) internal standards. The (protonated) molecular species of various phospholipids were identified and quantified from their peak areas in comparison to the peak areas of internal standards. The SM/PC ratios were calculated from the total areas of all PC and SM species.

For measurement of PC and SM mass in LY-B or LY-B/c cells, total lipids were extracted and the lipids were separated on silica gel TLC plates with the solvent system of chloroform/methanol/water (65:25:4, v/v/v). The spots were visualized by exposure to iodine vapor. Spots corresponding to standards for PC and SM were scraped from the plate, and the lipid phosphorus was determined by the modified Bartlett procedure (*8*) using KH_2PO_4 as a standard.

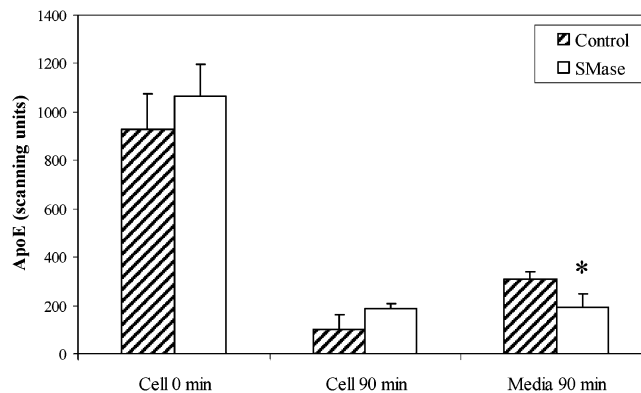

FIGURE 1: Exogenous sphingomyelinase-C treatment reduces apoE secretion from macrophages. J774-E cells were treated with 50 mU/mL of sphingomyelinase-C for 60 min in DMEM. The cells were then labeled with 150 μCi/mL [^{35}S]methionine at 37 °C for 30 min. Cells were harvested immediately after 30 min or chased for an additional 90 min. ApoE was isolated from cells and medium and quantitated (after correcting for incorporation of the radioisotope into total protein) as described in the Experimental Procedures. Values shown are mean ± standard deviation (SD) of triplicate samples. (*) $p < 0.05$. Results shown are representative of two similar experiments, each done in triplicate.

Statistics. Statistical comparisons were performed using analysis of variation (ANOVA; SPSS, Chicago, IL); $p < 0.05$ was considered statistically significant. Each experiment presents results representative of two to three experiments, each done in triplicate.

RESULTS

SMase activity has been detected in human plaques and has been postulated to promote atherosclerotic lesion development (*28*, *29*). The first experiment evaluated the effect of SMase treatment on the post-transcriptional handling of apoE in macrophages. The results in Figure 1 demonstrate that treatment of J774-E cells with SMase reduced apoE secretion by nearly 50%. There was no significant effect of SMase treatment on apoE present in the cell immediately after the 30 min pulse (cell 0 min) or the amount of apoE retained in the cell after a 90 min chase (cell 90 min).

SMase treatment of cells reduces cellular SM and increases cellular ceramide content. We first evaluated any role of increased cellular ceramide for the effect of SMase by treating J774-E cells with D-MAPP, an inhibitor of ceramidase-mediated ceramide breakdown (*30*). As shown in Table 1, the treatment of J774-E cells with D-MAPP for 24 h significantly increased the amount of serine radioactivity that could be found in ceramide without significantly affecting that found in SM. D-MAPP treatment of J774-E cells had no effect on cellular apoE at 0 or 90 min but led to a 46% reduction of apoE secretion at 90 min (Figure 2A). The results in Figure 2B show that the effect of D-MAPP on apoE secretion can be reproduced by direct treatment of cells with exogenous ceramide. Incubation of J774-E cells with ceramide for 4 h had no effect on cellular apoE either at 0 or 60 min but significantly reduced secretion of apoE at 60 min. The lack of an increase in cellular apoE after the chase incubations, despite an approximate 50% reduction in apoE secretion and initially equal levels of cellular apoE at 0 min,

Table 1: Effect of D-MAPP Inhibition of Ceramidase on Sphingomyelin and Ceramide in J774-E Cells[a]

	sphingomyelin (dpm/mg of cell protein)	ceramide (dpm/mg of cell protein)
control	$4.8 \pm 0.4 \times 10^6$	$5.7 \pm 0.6 \times 10^5$
D-MAPP	$4.3 \pm 0.3 \times 10^6$	$9.1 \pm 0.7 \times 10^5$ [b]

[a] J774-E cells were incubated in serine-free DMEM with 0.2% BSA containing 2 μCi/mL ^{14}[C]serine and 10 μM unlabeled L-serine at 37 °C for 24 h. The cells were then treated with 5 μM D-MAPP for 24 h in 0.2% BSA/DMEM. The level of radioactivity in sphingomyelin and ceramide was determined by thin-layer chromatography as described in the Experimental Procedures. Mean ± SD of triplicate samples. [b] $p < 0.05$ for the D-MAPP treatment compared to the control.

Table 2: Effect of D-609 Inhibition of Sphingomyelin Synthesis on Sphingomyelin and Ceramide in J774-E Cells[a]

	sphingomyelin (dpm/mg of cell protein)	ceramide (dpm/mg of cell protein)
control	$3.2 \pm 0.3 \times 10^6$	$1.5 \pm 0.2 \times 10^5$
D-609	$1.5 \pm 0.2 \times 10^6$ [b]	$1.3 \pm 0.2 \times 10^5$

[a] J774-E cells were incubated in serine-free DMEM with 0.2% BSA containing 2 μCi/mL ^{14}[C]serine and 10 μM unlabeled L-serine at 37 °C for 24 h. The cells were then treated with 100 μM D-609 for 5 h in 0.2% BSA/DMEM. The level of radioactivity in sphingomyelin and ceramide was determined by thin-layer chromatography as described in the Experimental Procedures. Mean ± SD of triplicate samples. [b] $p < 0.05$ for the D-609 treatment compared to the control.

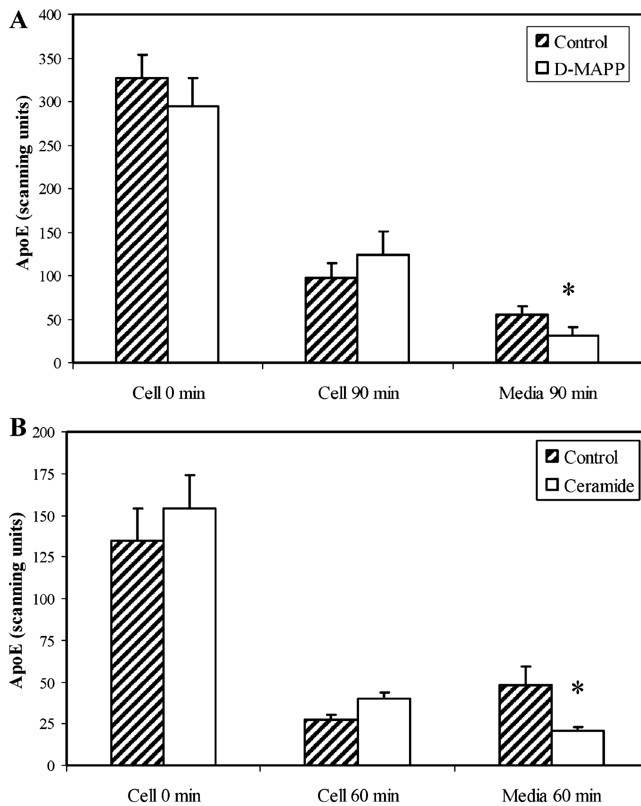

FIGURE 2: Ceramide decreases apoE secretion from macrophages. (A) J774-E cells were treated with 5 μM D-MAPP for 24 h in 0.2% BSA/DMEM to inhibit the degradation of cellular ceramide. The cells were then incubated with methionine-free DMEM containing 150 μCi/mL [^{35}S]methionine at 37 °C for 30 min. Some cells were harvested for the measurement of cellular apoE content, and the rest were chased for 90 min and then harvested for the measurement of cellular and medium apoE as described in the Experimental Procedures. Values shown are the mean ± SD of triplicate samples. (*) $p < 0.05$. (B) Ceramide at a concentration of 5 μM was incubated with J774-E cells for 4 h in 0.2% DMEM. The cells were labeled with 150 μCi/mL [^{35}S]methionine at 37 °C for 30 min. At that time, some cells were harvested for the measurement of cellular apoE, and the balance were chased for 60 min and then harvested for the measurement of cellular and medium apoE. Values shown are mean ± SD of triplicate samples. (*) $p < 0.05$. ApoE was isolated and quantitated (after correcting for radioisotope incorporation into total protein) as described in the Experimental Procedures. Results shown are representative of two similar experiments, each done in triplicate.

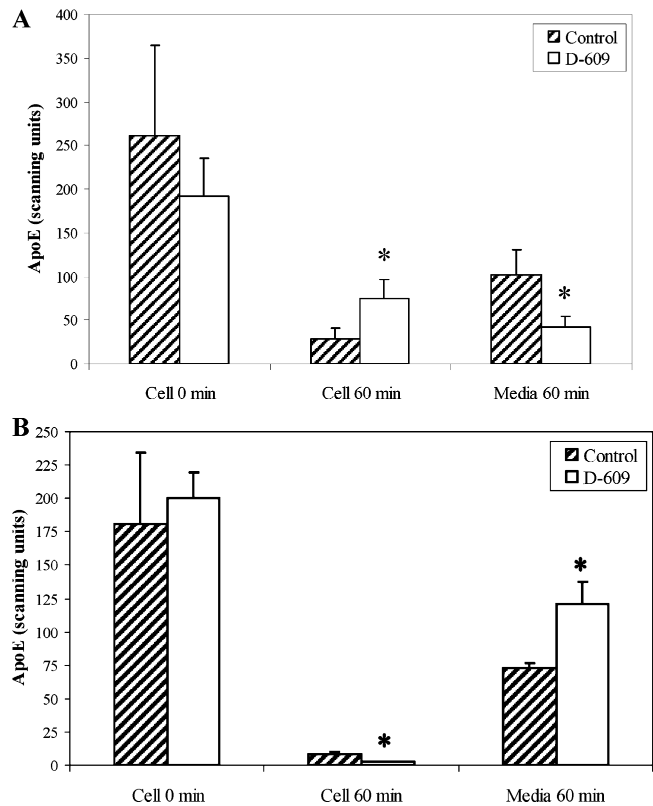

FIGURE 3: Inhibition of cellular sphingomyelin generation inhibits apoE secretion from macrophages. (A) J774-E cells were treated with 100 μM D-609 for 5 h in 0.2% BSA/DMEM to inhibit the generation of sphingomyelin. Cells were then incubated with methionine-free DMEM containing 150 μCi/mL [^{35}S]methionine for 30 min at 37 °C. Some cells were harvested for the measurement of cellular apoE content, and the rest were chased for 60 min as described in the Experimental Procedures. Values shown are the mean ± SD of triplicate samples. (*) $p < 0.05$. (B) Experiment described above was repeated in J774-Δ202 cells. (*) $p < 0.05$. ApoE in cells and media were isolated and quantitated (after correcting for radioisotope incorporation into total protein) as described in the Experimental Procedures. The results shown are representative of two similar experiments, each done in triplicate.

is consistent with rapid degradation of newly synthesized apoE in cells treated with exogenous ceramide or D-MAPP.

We next turned to an evaluation of the role of cellular SM on the post-transcriptional handling of apoE by macrophages. J774-E cells were incubated with D-609, which has been previously shown to inhibit cellular generation of SM (*31*). The addition of D-609 to J774-E cells for 5 h led to a significant reduction in radioactive serine found in SM, with no effect on levels found in cellular ceramide (Table 2). Treatment of J774 cells with D-609 for 5 h produced effects on both cellular and secreted apoE (Figure 3A). Cellular apoE at 0 min tended to be lower in D-609-treated cells; however, this difference did not reach statistical significance. Treated cells displayed reduced apoE secretion and also increased the cellular retention of apoE at 60 min.

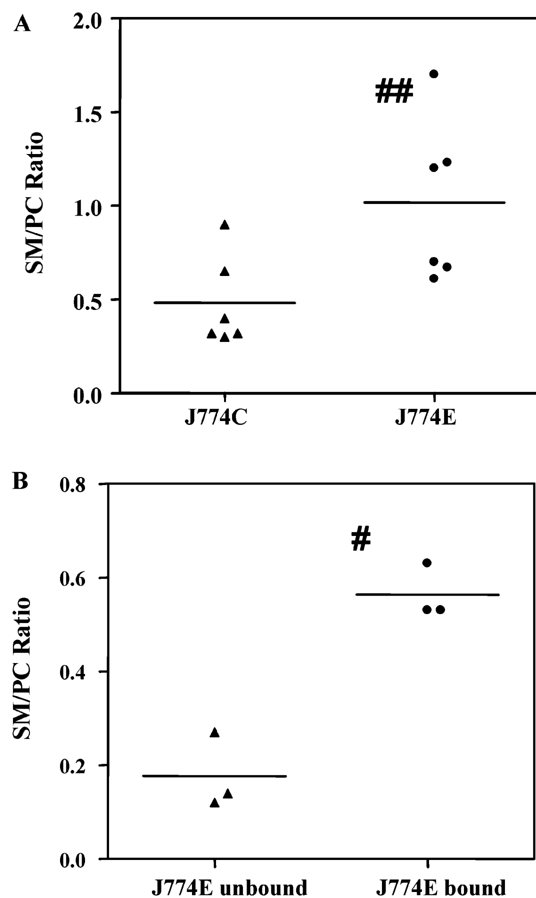

FIGURE 4: ApoE expression increases the SM/PC ratio of lipids secreted from macrophages. (A) J774-C and J774-E cells in T75 flasks were labeled with 0.5 μCi/mL [14][C]choline in DMEM and 10% FBS for 3 days. After incubation, cells were washed and equilibrated in DMEM and 0.1% BSA for 24 h. Conditioned medium was collected by incubating the cells in DMEM and 0.1% BSA for 24 h. The supernatants were pooled from three T75 flasks and centrifuged to remove floating cells and membrane debris. Lipids in the samples were extracted, and radioactivity in SM and PC were measured as described in the Experimental Procedures. Each point shown represents measurements made from three flasks of pooled medium. The horizontal line shows the means, which are significantly different at (##) $p < 0.05$. (B) J774-E-conditioned medium was collected from six T75 flasks after incubating cells in DMEM and 0.1% BSA for 24 h. The medium was divided into apoE-enriched (bound) and apoE-poor (unbound) fractions as described in the Experimental Procedures. Lipids in the bound and unbound fractions were extracted and subject to LC/MS analysis. Each point represents individual measurements on medium pooled from six T75 flasks. The horizontal line shows the means, which are significantly different at (#) $p < 0.01$.

ApoE is an amphipathic protein with a strong affinity for binding phospholipid, a property largely mediated by C-terminal α-helical domains (*32*, *33*). We next evaluated if the apoE C-terminal amphipathic α-helical domains were necessary for the effect of SM deficiency on apoE secretion and cellular retention. Figure 3B shows the effect of treating J774-Δ202 cells, which express a C-terminal truncated apoE mutant, with D-609. Immediately after pulse-labeling, there was no difference in cellular apoE. Completely opposite to what was observed for native apoE, at 60 min, cellular apoE is significantly reduced and apoE secreted into the medium is significantly increased in J774-Δ202 cells treated with D-609. These results indicate that the apoE C-terminal lipid-binding domains are required for the reduction in macrophage apoE secretion and its increased cellular retention, produced by cellular SM deficiency.

Table 3: Cellular SM/PC Molar Ratio in LY-B and LY-B/c Cells Incubated in FBS or Nutridoma[a]

	cell SM/PC molar ratio	
	Nutridoma	FBS
LY-B/c	0.34 ± 0.10	0.28 ± 0.14
LY-B	0.18 ± 0.05[b]	0.31 ± 0.10[b]

[a] LY-B or LY-B/c cells were incubated in 10% FBS or sphingolipid-deficient Nutridoma for 48 h before being harvested for measurement of SM and PC mass as described in the Experimental Procedures. The values shown are the mean ± SD of six replicate samples. [b] $p < 0.05$ for LY-B in FBS versus LY-B in Nutridoma.

We further evaluated an important link between macrophage apoE and SM metabolism by evaluating if secreted apoE and SM were co-localized within secreted lipoprotein particles. The results in Figure 4A show that SM secretion relative to PC secretion was significantly increased (2-fold) in the cells secreting apoE. It has previously been shown that the presence of amphipathic apolipoproteins can increase the release of lipid microparticles from cells but that these microparticle lipids are not always contained within the amphipathic apoprotein-containing particle (*32*). To address the important question of whether the increment in SM secreted from apoE-expressing cells was a direct reflection of the phospholipids contained within the apoE particle, we directly measured PC and SM mass in isolated apoE particles. ApoE particles in macrophage-conditioned medium were immobilized on heparin sepharose (bound, Figure 4B), and SM and PC were measured and compared to that in the medium without apoE particles (unbound). The SM/PC ratio was 3-fold higher in phospholipids bound to apoE compared to the non-apoE-containing medium fraction. These results indicate that the endogenous expression of apoE leads to the formation of a secreted lipoprotein particle containing both apoE and cellular SM.

The mechanism of action of D-609 can produce increased cell ceramide along with decreased cell SM (*31*), and the results in Table 2 (from whole cell extracts) do not rule out such an increase in a subcellular compartment critical for apoE processing. To clearly separate the effect of decreased cell SM from an increase in ceramide on apoE secretion, we took advantage of a cell system in which a decrease in cell SM can be produced with no potential for an increase in ceramide (*27*). LY-B cells lack SPT activity, leading to a reduction in total endogenous sphingolipid synthesis. Incubation of LY-B cells in sphingolipid-deficient medium has been shown to reduce both SM and ceramide (*27*). As a control for these cells, we used LY-B/cL1B cells, which are LY-B cells genetically complemented to replace defective SPT activity (*27*). In Table 3, we show the effect of incubating LY-B and LY-B/c cells in FBS or in sphingolipid-deficient Nutridoma for 48 h. The cellular SM/PC ratio was similar in LY-B and LY-B/c cells grown in FBS and in LY-B/c cells grown in either Nutridoma or FBS. Incubation of LY-B cells in Nutridoma, however, led to a significant reduction in the cellular SM/PC molar ratio compared to LY-B cells grown in FBS.

We next evaluated the effect of the incubation in sphingolipid-deficient Nutridoma on the post-translational handling

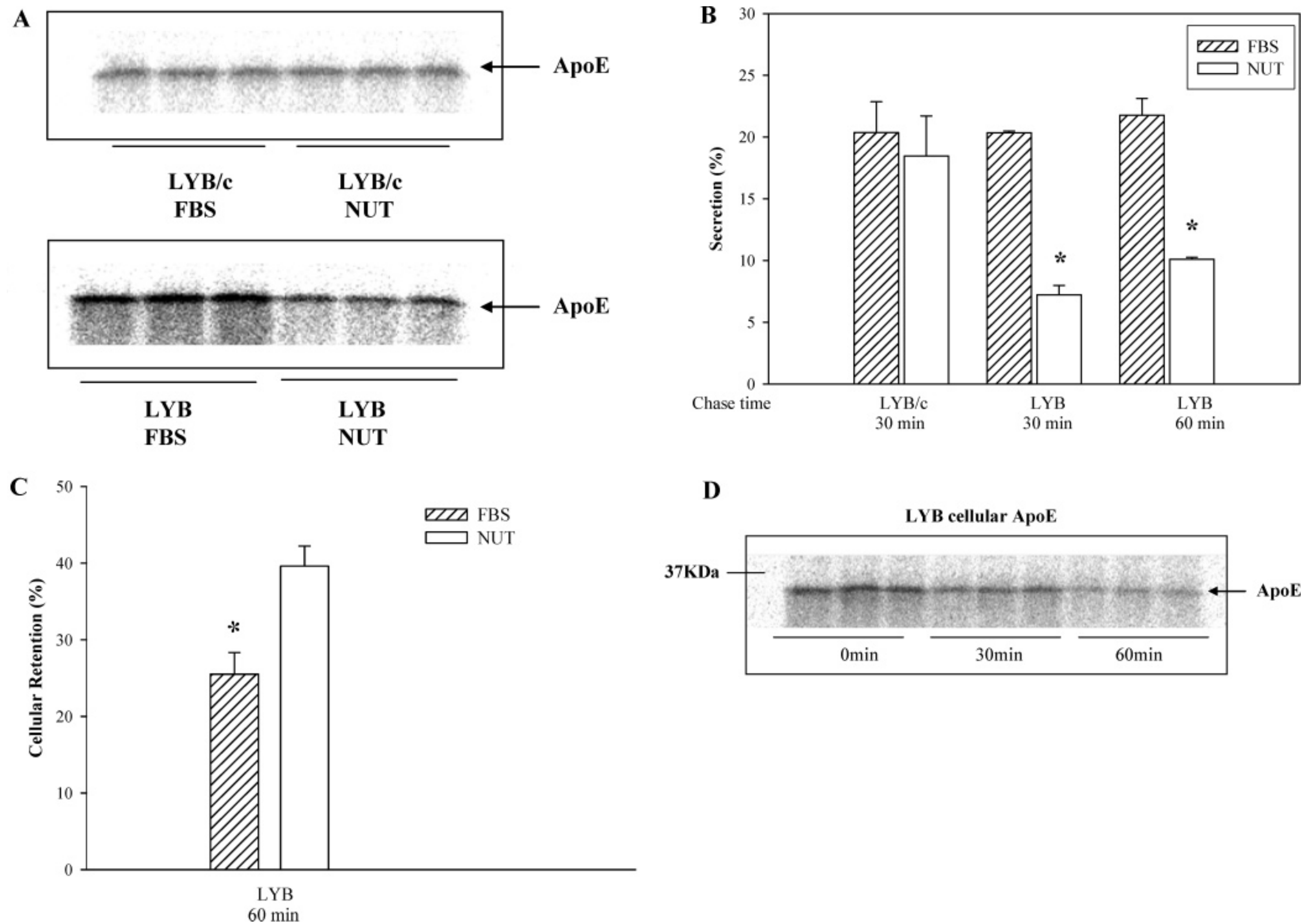

FIGURE 5: Cellular retention and secretion of apoE in sphingolipid-deficient LY-B cells. (A) SDS−PAGE of apoE recovered from LY-B and LY-B/c cells maintained in FBS or sphingolipid-deficient Nutridoma (Nut) immediately after a 30 min pulse labeling. (B) Percent apoE secretion in LY-B and LY-B/c cells in FBS or sphingolipid-deficient Nutridoma at the chase times shown. (*) $p < 0.01$ for Nutridoma versus FBS. (C) Cellular retention of apoE in LY-B cells in FBS or sphingolipid-deficient Nutridoma at 60 min. (*) $p < 0.01$ for Nutridoma versus FBS. (D) SDS−PAGE of cellular apoE from LY-B cells in Nutridoma at the times shown.

of apoE. Each cell line was stably transfected to constitutively express a human apoE3 cDNA. First, LY-B and LY-B/c cells grown in Nutridoma or FBS were pulse-labeled for 30 min and harvested for analysis of cellular apoE. SDS−PAGE of cellular apoE isolated immediately after the 30 min pulse is shown in Figure 5A. Incubation of LY-B/c cells in Nutridoma had no effect on cellular apoE present after the 30 min pulse-labeling period compared to FBS (top panel). However, incubation of LY-B cells in Nutridoma led to a substantial decrease in cellular apoE in cells after the 30 min pulse-labeling (bottom panel). Because LY-B cells express a human apoE cDNA (under the control of the CMV promoter) in a constitutive manner, the decreased apoE in LY-B cells incubated with Nutridoma is consistent with co-translational suppression of apoE synthesis or co-translational destabilization of apoE.

Figure 5B shows that, when pulse-labeled LY-B or LY-B/c cells are chased for 30 min, the incubation in Nutridoma significantly suppresses the secretion of apoE only from LY-B cells. LY-B cells in FBS and LY-B/c cells in Nutridoma or FBS secrete approximately 20% of their apoE by 30 min. LY-B cells incubated in Nutridoma, however, secrete approximately 7% of apoE. If the chase time in LY-B cells is extended to 60 min, the suppression of apoE secretion is maintained. There was also a significant increase in cellular retention of apoE in sphingolipid-deficient cells (Figure 5C). The electrophoretic migration pattern of apoE present in SM-deficient LY-B cells is presented in Figure 5D. We have previously shown that nascent apoE appears as a single sialic-acid-poor isoform after a short pulse-labeling (*23*, *34*). This single band is observed in LYB cells at 0 min (Figure 5D). We have also shown that nascent apoE acquires sialic acid residues in the Golgi, leading to the transient appearance of cellular isoforms with higher apparent molecular weight (MW) on SDS−PAGE, and that these higher MW isoforms are rapidly secreted (*23*, *34*). We therefore next determined if the incremental apoE retained (i.e., not secreted) in SM-deficient LYB cells migrated as a sialic-acid-rich or -poor isoform on SDS−PAGE. Post-Golgi, sialic-acid-rich apoE isoforms migrate above the sialic-acid-poor isoforms up to approximately 37 kDa. As shown in Figure 5D, at 30 or 60 min of chase, there is no evidence of heterogeneity in apoE molecular weight compared to 0 min, suggesting that the incremental apoE retained in SM-deficient cells remains in a nascent sialic-acid-poor form.

DISCUSSION

Our results identify a new post-translational pathway for the regulation of apoE secretion in macrophages. SMase treatment of cells, which decreases SM and increases

ceramide, reduces apoE secretion by working at a post-transcriptional locus. Increasing macrophage ceramide by inhibiting the degradation of endogenous cellular ceramide or by treating macrophages with exogenous ceramide also decreases apoE secretion. Increasing cellular ceramide levels has been shown to produce apoptosis in other model systems (*30*). However, the relationship between cellular ceramide metabolism and induction of apoptosis is complex (*35*). We did not evaluate apoptosis in these experiments; however, the effect of ceramide on apoE secretion was specific and did not reflect a generalized effect on total protein synthesis and/or secretion. We, therefore, feel it unlikely that this specific effect on apoE secretion could be related to cellular apoptosis Reduction of macrophage-derived apoE in response to apoptosis, however, would also be expected to have a proatherogenic influence in the artery wall. In previous studies, we have shown that ceramide has little effect on apoE gene transcription in mouse peritoneal macrophages (*18*). The current studies, therefore, identify a new locus of control for cellular ceramide on macrophage apoE expression.

Our results also establish that reduction of cellular SM has an important effect on apoE secretion. Inhibition of cellular SM synthesis reduces apoE secretion by over 50% and, different from the effect of ceramide, results in a significant retention of cellular apoE. We have previously shown that increasing the cholesterol/phospholipid molar ratio of internal macrophage membranes leads to increased secretion of apoE, an effect that can be transduced at a post-translational regulatory locus. We began the current studies with the hypothesis that altering the cholesterol/phospholipid molar ratio of cell membranes by reducing SM content would also increase apoE secretion. Surprisingly, our results demonstrate that the reduction of cellular SM, by either promoting its hydrolysis or inhibiting its synthesis, significantly reduces apoE secretion.

ApoE amphipathic α-helical segments, primarily within the C-terminal third of the protein, have a high affinity for binding phospholipid (*32*, *33*). Further, apoE conformation is influenced by the presence and the composition of lipid with which it is associated. The influence of associated lipids on apoE conformation has important functional consequences, because different conformations present different epitopes when mapped with monoclonal antibodies, and manifest differential receptor-binding affinities (*32*, *33*, *36*). The requirement for C-terminal lipid-binding domains implicates the need for apoE binding to intracellular lipids or membranes for cellular SM deficiency to modulate nascent apoE retention and secretion. SM deficiency likely alters the composition of cellular membranes and could thereby influence the conformation of nascent apoE associated with these membranes. The increased secretion of SM with apoE expression and the co-localization of secreted SM with apoE in secreted lipoprotein particles suggest the importance of a SM-containing membrane. The SM content of the Golgi is in rapid equilibrium with SM in the plasma membrane, and SM enriched microdomains have been described in Golgi membranes (*37*, *38*). Sialic acid residues are added to apoE in the distal Golgi, and SM deficiency leads to cellular retention of newly synthesized apoE in a sialic-acid-poor isoform. It is therefore possible to speculate that SM deficiency may produce a conformational change in apoE proximal to or at the distal Golgi and thereby modulate the progression of apoE along the secretory pathway. This potential model will require further experimental evaluation.

The relationship between the circulating lipoprotein SM level and atherosclerosis remains controversial, with some studies showing protection and others showing increased susceptibility to atherosclerosis with increased circulating SM levels (*39*, *40*). SMase activity in the vessel wall has been proposed to be proatherogenic. ApoE in the vessel wall has a well-established role for modulating atherogenesis. The observations in this paper detail an important relationship between macrophage SM levels and apoE production; a relationship that could have important implications for the vessel wall homeostatic response to atherogenic insult. Reduction of macrophage SM results in a decreased secretion of apoE, and this could be expected to promote atherosclerosis based on the overall atheroprotective role of apoE in the vessel wall. The potential *in vivo* relevance of the impact of the macrophage ceramide/SM level on apoE secretion relates to work showing that abundant SMase can be detected in atherosclerotic lesions (*28*). This secreted SMase is the product of the acid SMase gene; however, hydrolytic activity has been demonstrated at neutral pH against modified lipoproteins or lipoproteins obtained from vessel wall atherosclerotic lesions (*41*). Several types of arterial wall cells secrete SMase, and among these, endothelial cells are most active (*42*). Also interesting, in view of recent information suggesting inflammation plays an important role in atherogenesis, inflammatory cytokines markedly upregulate the secretion of SMase from endothelial cells (*42*). Most cellular SM is located on the outer leaflet of the plasma membrane, but plasma membrane SM is in rapid equilibrium with SM content of intracellular membranes. Secretion of SMase by endothelial cells in the vessel wall could impact macrophage SM and ceramide content, especially in microenvironments of increased acidity that may occur in inflammatory and atherosclerotic lesions (*43*−*45*).

In summary, our results establish a new and important role for cellular sphingolipids in the post-transcriptional handling of macrophage apoE. ApoE is the only apolipoprotein secreted by macrophages, and macrophages are the major source of apoE in the vessel wall. *In vivo* mouse models have established a key role played by macrophage-derived apoE for defending vessel wall homeostasis against atherogenic injury. The presence of SMase in the vessel wall and its increased production by endothelial cells at the site of inflammation could promote the development of atherosclerotic lesions by reducing the release of macrophage-derived apoE in the vessel wall.

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