Endogenously Expressed Apolipoprotein E Has Different Effects on Cell Lipid Metabolism As Compared to Exogenous Apolipoprotein E Carried on Triglyceride-Rich Particles†

Yuan-Yuan Ho,[‡,§] Maysoon Al-Haideri,[‡] Theodore Mazzone,[||] Tikva Vogel,[⊥] John F. Presley,[#] Stephen L. Sturley,[‡,§] and Richard J. Deckelbaum*[,‡,§]

Departments of Pediatrics, Pathology, and Institute of Human Nutrition, College of Physicians and Surgeons of Columbia University, New York, New York 10032, Department of Medicine and Biochemistry, Rush Medical College, Chicago, Illinois 60612, and Biotechnology General Ltd, Rehovot, Israel 76326

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ABSTRACT: Apolipoprotein E (apoE) on model triglyceride-rich particles (TGRP) increases triglyceride (TG) utilization and cholesteryl ester (CE) hydrolysis, independent of its effect on enhancing particle uptake. We questioned whether, under physiological concentrations, endogenously expressed apoE has similar effects on cellular lipid metabolism as compared to exogenous apoE. J774 macrophages, which do not express apoE, were engineered to express endogenous apoE by transfection of human apoE3 cDNA expression constructs (E^+) or control vectors (E^-) into the cells. To compare the effects of exogenous apoE and endogenous apoE on TGRP uptake, cells were incubated with or without apoE associated with ^{3}H-cholesteryl ether-labeled TGRP. Exogenous apoE enhanced TGRP uptake in both E^- and E^+ cells. E^- cells displayed significantly higher TGRP uptake than E^+ cells. Sodium chlorate, which inhibits cell proteoglycan synthesis, markedly diminished differences in TGRP uptake between E^- and E^+ cells, suggesting that endogenous apoE−proteoglycan interaction contributes to differences in uptake between the two cell types. Particle uptake by the LDL receptor, by the LDL receptor related protein, or by scavenger receptors were similar between E^- and E^+ cells indicating that endogenous apoE expression does not have a general effect on endocytic pathways. Exogenous apoE carried on TGRP stimulated TG utilization and CE hydrolysis in both cell types. However, TG utilization and CE hydrolysis were not affected by endogenous apoE expression. In conclusion, macrophage expression of apoE has very different effects on TGRP metabolism than exogenously supplied apoE. The fluorescence microscopy results in this study showing that exogenous apoE and endogenous apoE were confined in separate cellular compartments support the hypothesis that these differences resulted from distinct intracellular trafficking pathways followed by exogenous apoE bound to TGRP as compared to endogenous cell-expressed apoE.

Apolipoprotein E (apoE), a 34 kDa glycoprotein, is secreted by hepatic and many nonhepatic tissues (*1*, *2*) and primarily functions to facilitate the transport of lipid between these locations. It is a surface component of several classes of plasma lipoproteins, including HDL,[1] VLDL, and chylomicrons and a ligand for apoE receptors such as the LDL receptor (LDLR) and LDL receptor related protein (LRP) (*3*, *4*). ApoE further serves to enhance lipoprotein uptake by binding to cell surface heparan sulfate proteoglycans (*5*, *6*). The lipid transport properties of apoE play crucial roles in modulating the process of atherosclerosis.

ApoE is also important in the nervous system. In brain, apoE is produced by astrocytes, and its secretion increases locally during nerve injury (*7*, *8*). Epidemiological and laboratory studies have demonstrated a positive association between the apoE4 allele and the risk of developing familial Alzheimer's disease (*9*). Still, mechanisms of how apoE may affect the metabolic behavior of normal or degenerating neurons remain mostly undefined.

Physiologically, many cell types are simultaneously interacting with both lipoprotein-associated apoE in plasma

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* Corresponding author address: Department of Pediatrics and Institute of Human Nutrition, Columbia University, PH 15-1512, 630 W. 168th Street, New York, NY 10032. Tel: 212-305-4808. Fax: 212-305-3079. E-mail: rjd20@columbia.edu.

‡ Department of Pediatrics, College of Physicians and Surgeons of Columbia University.

§ Institute of Human Nutrition, College of Physicians and Surgeons of Columbia University.

|| Rush Medical College.

⊥ Biotechnology General Ltd.

Department of Pathology, College of Physicians and Surgeons of Columbia University.

[1] Abbreviations: apo, apolipoprotein; TGRP, triglyceride-rich particle; IDL, intermediate density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; LDLR: low-density lipoprotein receptor; LRP: low-density lipoprotein receptor related protein; HSPG: heparan sulfate proteoglycan; TG, triglyceride; FC, free cholesterol; CE, cholesteryl ester; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; α_2m, α_2-macroglobulin; Fα_2m, fluorescein-labeled α_2-macroglobulin; NL, neutral lipid.

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(exogenous apoE) and intrinsically expressed cellular apoE (endogenous apoE). In the case of Alzheimer's disease, apoE is found to be associated with both intracellular and extracellular pathological elements (*9, 10*). Previous studies have demonstrated that apoE of both exogenous and endogenous origins may modulate cell functions. For example, we have reported that exogenous apoE carried on triglyceride-rich particles (TGRP) modulate intracellular lipid metabolism by promoting particle triglyceride (TG) utilization and cholesteryl ester (CE) hydrolysis independent of its effect on enhancing model TGRP uptake (*11*). Exogenously provided apoE inhibits the proliferation of normal endothelial cells and tumor cells (*12, 13*)—an effect proposed to be related to the ability of apoE to compete with heparin binding growth factors for cell surface binding sites (*12, 13*). Endogenous cell-expressed apoE, much more than exogenous apoE, promotes cholesterol efflux from cells to HDL (*14*−*16*). Also, endogenous apoE expression inhibits steroidogenesis in mouse adrenal cells by suppressing protein kinase A and enhancing protein kinase C-mediated signaling pathways (*17, 18*).

Although both endogenous and exogenous apoE have biological functions, direct comparisons of the effects of exogenous versus endogenous apoE on cell lipid metabolism have not been reported. We questioned, therefore, whether in cells derived from the same origin would endogenously expressed cellular apoE affect TGRP metabolism in a manner similar to exogenous apoE carried on TGRP. Physiologically, macrophages secrete apoE in a quantity that is significantly lower than the concentration of apoE circulating in the plasma (*16, 19*−*21*). We were able to take advantage of this difference to sharply discriminate between the biological effects of endogenous apoE versus exogenous apoE carried on TGRP. To mimic physiological conditions, in this study, we used apoE-expressing macrophages (E^+ cells) producing amounts of apoE comparable to those produced by human monocyte-derived macrophages (*16, 19, 21*) and concentrations of exogenous apoE close to plasma apoE concentrations. By comparing the metabolism of TGRP in apoE-expressing cells (E^+ cells) to non-apoE-expressing cells (E^- cells) in the presence or absence of exogenous apoE, we demonstrate that endogenously expressed apoE has very different effects on cell lipid metabolism from exogenous apoE carried on TGRP. Exogenous apoE increases TGRP uptake, cell TG utilization, and CE hydrolysis, whereas endogenous apoE decreases TGRP uptake and does not affect TG and CE metabolism.

EXPERIMENTAL PROCEDURES

Materials. Triolein and cholesteryl oleate were obtained from NuChek Prep Inc. (Elysian, MN). Egg yolk phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Isotopically labeled compounds were purchased from DuPont-New England Nuclear (Boston, MA). Bovine serum albumin (BSA, fraction V containing less than 0.005% w/w fatty acid), Dulbecco's modified Eagle's medium (DMEM), heparin, and Hepes were obtained from Sigma (St. Louis, MO). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI), fluorescein-labeled reagents (including α_2-macroglobulin [α_2m] and wheat germ agglutinin), and rhodamine-labeled secondary antibody were obtained from Molecular Probes Inc. (Eugene, OR). L-Glutamine, penicillin−streptomycin, antibiotics used for selecting transfected cells, geneticin (G418), and serum free medium were purchased from Gibco (Grand Island, NY). The enzymatic colorimetric assay kits used to determine TG (triglyceride GPO−PAP test) and CE (cholesterol/HP) mass were obtained from Boehringer Mannheim (Indianapolis, IN). Silica Gel 60 for TLC was purchased from Merck (Darmstadt, Germany).

Cells. Cells stably expressing human apoE3 (E^+ cells) were established by cotransfection of human apoE3 cDNA expression vectors and control vectors (pSV2-neo) into J774A.1 mouse macrophages that do not express apoE, as previously detailed (*19*). Cells carrying pSV_2-neo alone were generated as vector control (E^- cells) cell lines (*19*). E^+ cells were clonally derived apoE-secreting cells. ApoE produced by E^+ cells displayed lipid and heparin binding properties similar to plasma apoE (*22, 23*). In addition, exogenous and endogenous apoE analyzed by SDS−polyacrylamide gel electrophoresis followed by immunoblotting assays showed identical mobility. These western immunoassays were performed using monoclonal anti-human apoE antibody E10. The E10 antibody recognizes the LDLR binding domain of apoE and was a generous gift from Dr. E. Krul and Dr. G. Schonfield at the University of Washington, St. Louis. E^- cells were derived from either a mixed culture after G418 selection or clonally derived. Three clones of E^+ cells and three clones of E^- cells were used for experiments. Monolayer cultures of E^- and E^+ cells were grown and maintained in growth media: DMEM containing 10% (v/v) fetal bovine serum, 200 μg/mL G418, 100 μg/mL streptomycin, 100 U/mL penicillin, and 292 μg/mL glutamine. G418 was removed from the growth medium 2 weeks before initiation of experiments. Cells were plated in growth medium without G418 at 1×10^5 cells/cm^2 48 h before each experiment and then switched to medium containing 10% (v/v) lipoprotein-deficient serum (LPDS), 100 μg/mL streptomycin, 100 U/mL penicillin, and 292 μg/mL glutamine 24 h prior to experiments. For fluorescence microscopy experiments, cells were plated on cover-slip bottom dishes (MatTek Co., Ashland, MA). Cells were maintained at 37 °C in an atmosphere containing 5% CO_2 in air.

Exogenous ApoE. Human apoE was produced in *Escherichia coli* by transforming cells with an expression vector containing the human apoE3 cDNA sequence encoding the entire mature apoE3 (*22*). This recombinant apoE has very similar biological activities to native human plasma apoE3 (*22*).

Preparation of TGRP, TGRP−ApoE, and Lipoprotein Particles. Triolein, cholesteryl oleate, and egg yolk phosphatidylcholine (EYPC) were combined in 1:1:2 weight ratio. Summation of TG and cholesteryl oleate concentrations gives the neutral lipid (NL) concentration. Depending on the purpose of the experiments, 0.25 mol % DiI/EYPC, ^{3}H-cholesteryl hexadecyl ether [cholesteryl-1,2-^{3}H] (a nondegradable marker; 2 μCi/mg of NL), or ^{3}H-cholesteryl oleate [cholesteryl-1,2,6,7-^{3}H] (2 μCi/mg of NL), was added as a marker for particle uptake. Most organic solvent was removed by evaporation under nitrogen. Remaining traces were removed by vacuum desiccation for 16 h. Sonication and ultracentrifugation were performed as previously detailed to generate IDL-size particles (25−30 nm diameter) with ratios of NL to phospholipid of about 2.0 (*11*). The final

TGRP triglyceride concentrations were determined by the TG GPO−PAP test. The cholesteryl oleate concentration was determined by the cholesterol/HP test. TGRP phospholipid concentration was measured by the Barlett (*24*) method. The weight ratio of TG:CE was 1.06 ± 0.09:1 (mean ± SD) for the TGRP used here ($n = 10$). The specific activities of the markers for the neutral lipids were determined by liquid scintillation counting. TGRP−apoE complexes were formed by incubating apoE with TGRP (with gentle inversion every 5 min) at room temperature for 20 min, sufficient time to ensure equilibrium binding (*11*). Fasting plasma from normolipidemic subjects was obtained, and LDL was isolated as previously described (*25*). All procedures for LDL preparation and storage (under argon gas) were at 4 °C. Modified lipoproteins including oxidized LDL (oxLDL) and acetylated LDL (acLDL) were prepared as previously described (*26*). LDL, acLDL, and oxLDL were iodinated with ^{125}I using the monochloride method as previously described (*25*, *27*, *28*). α_2m was iodinated with Na^{125}I using the chloramine T method as previously described (*29*).

Incubations. DMEM incubation medium contained 100 μg/mL streptomycin, 100 U/mL penicillin, 292 μg/mL glutamine, and 1% BSA. Prior to incubation, cells were washed with PBS at 37 °C. Then PBS was exchanged with experimental media. For experiments assessing TGRP uptake, cells were incubated with ^{3}H-cholesteryl ether-labeled TGRP, with or without exogenous apoE. For lipoprotein uptake experiments, cells were incubated with LDL, oxLDL, or acLDL for 4 h at 37 °C.

In experiments studying the effects of proteoglycan-mediated TGRP uptake, cells were preincubated with 0−60 mM Na_3ClO_4, an inhibitor of proteoglycan synthesis, for 72 h followed by TGRP incubation in the presence of 30 mM Na_3ClO_4. Trypan blue exclusion assays (*30*) confirmed that Na_3ClO_4 incubation did not cause cytotoxicity under the above experimental conditions. To determine if sodium chlorate actually decreased proteoglycan synthesis in our experimental system, proteoglycans in E$^-$ cells were labeled by incubating the cells for 72 h with 25 μCi/mL [^{35}S]sulfate. ^{35}S incorporation into cell proteoglycans was measured after precipitation with 3 vol of absolute ethanol containing sodium acetate (0.8 g/L) for 18 h at −20 °C as described (*31*).

For experiments studying CE hydrolysis, cells were incubated with ^{3}H-cholesteryl oleate-labeled TGRP. To estimate TG utilization, cells were incubated with ^{3}H-cholesteryl ether-labeled TGRP with the addition of ^{3}H-glycerol (5 μCi/mL) to measure TG synthesis. Incubations with TGRP or lipoprotein particles were performed at 37 °C on a rocker (Lab-Line Instruments, Inc., Melrose Park, IL), usually for 4 h unless otherwise specified. At the end of the incubation, the media were removed, and cells were chilled on ice and washed twice with ice-cold PBS containing 0.2% BSA (5 min washes) and twice with ice-cold PBS alone. Cells were then incubated with heparin (1400 U/mL of PBS) at 4 °C on a rocker for 1 h followed by the removal of heparin and two further PBS washes. At the end of each experiment, the monolayers were harvested by dissolution in 0.1 N NaOH, and protein concentration was determined by the Bio Rad method (*32*) using BSA as a standard.

Assays. For cell protein assays, cells were dissolved in 0.1 N NaOH. Protein concentration was measured by the Bio Rad method (*32*). To determine cell TG mass, the cell lipids were extracted, the hexane/2-propanol extract was dried, and the TG mass was assayed by GPO−PAP test. TGRP uptake was measured by cell-associated radioactivity of ^{3}H-cholesteryl ether, a nondegradable marker in the cell lysates, by scintillation counting (Tri-Carb liquid scintillation spectrometer 3255, Packard Instrs., Meriden, CT) with Ultima Gold scintillation fluid (Parkard, Meriden, CT). In previous work, we demonstrated that uptake assayed by these methods measured cell internalization and not surface adherence (*11*). TG synthesis was measured by addition of ^{3}H-glycerol (5 μCi/mL) to the incubation medium. Cell lipids including newly formed TG (containing ^{3}H-glycerol) were extracted by hexane/2-propanol and separated from diglycerides, monoglycerides, and phospholipids by TLC with the solvent system hexane/diethyl ether/acetic acid (70/30/1 v/v/v). TG utilization was calculated as follows:

$$\text{TG utilization} = \text{TG uptake} + \text{TG synthesis} - \text{net TG mass}$$

During these incubations, TG synthesis, measured by ^{3}H-glycerol incorporation into TG, was less than 1% of TG uptake and thus could be omitted from the above formula without affecting the results. Net TG mass was calculated by TG mass measured from cells incubated with TGRP and subtracting TG mass measured from the cells incubated with 1% BSA/DMEM only. The calculated TG utilization values were divided by TG uptake values to normalize for particle uptake differences (*11*).

To measure CE hydrolysis, after incubating cells with ^{3}H-cholesteryl oleate labeled TGRP, cell lipids were extracted with hexane/2-propanol (3/2 v/v) and separated from TG and other lipids by TLC with the solvent system hexane/diethyl ether/acetic acid (70/30/1 v/v/v). Cell extracts were assayed for ^{3}H-free cholesterol and ^{3}H-cholesteryl ester by TLC. Radioactivity was determined by liquid scintillation counting and normalized by cell protein or calculated as mass from the appropriate specific activity of the marker.

Fluorescence and Immunofluorescence Microscopy. To follow intracellular trafficking of TGRP and apoE, cells were incubated with apoE-associated, DiI labeled TGRP. All experimental procedures were performed at room temperature unless otherwise specified. For cellular localization of exogenous apoE carried on TGRP, cells were co-incubated with the TGRP−apoE complexes and fluorescein-labeled α_2m (Fα_2m) at 37 °C in DMEM, 1% BSA, and 20 mM Hepes, pH 7.4 (*33*, *34*). Cells were then washed four times with cold PBS followed by fixing for 30 min with 2% formaldehyde in PBS and four PBS washes. To determine the cellular localization of endogenous apoE, cells in coverslip bottom dishes were fixed for 30 min with 2% formaldehyde and then washed with PBS. For apoE immunodetection, cells were permeablized with saponin (1% BSA, 500 μg/mL saponin, and 20 mM glycine) for 20 min and incubated with monoclonal anti-apoE primary antibody E10 (1:200 dilution) for 1 h. For experiments aimed to identify Golgi regions, cells were further incubated with fluorescein-conjugated wheat germ agglutinin (100 μg/mL, 30 min) (*35*) and then washed three times (5 min/wash) with 1% BSA in PBS + 500 μg/mL saponin. Depending on the purpose of the experiment, either rhodamine or fluorescein-conjugated secondary goat anti-mouse IgG antibody (1:500 dilution in

1% BSA in PBS + 500 μg/mL saponin) was added to the cells and incubated for 1 h. After incubation, cells were washed twice with 1% BSA + 500 μg/mL saponin in PBS (5 min/wash) and once with PBS (10 min). Fluorescence images were obtained using a Zeiss LSM410 confocal laser scanning system attached to a Zeiss Axiovert 100TV inverted microscope (Jena, Germany). For observation of DiI and rhodamine fluorescence, a 568-nm excitation argon−krypton laser and a 590-nm long pass emission filter were used. For fluorescein fluorescence, a 488-nm excitation argon−krypton laser and a 515−540-nm band-pass emission filter were used. Images were recorded with a Pentium PC computer with Zeiss LSM software for image enhancement and analysis.

Statistical Analysis. Student's *t* tests were performed to test differences between the groups (with exogenous apoE vs without exogenous or E^- cells vs E^+ cells). A significant difference was determined at the $p < 0.05$ level.

RESULTS

Effects of Exogenous ApoE Carried on TGRP and Endogenously Expressed ApoE on TGRP Uptake. The association of exogenous apoE to TGRP enhances particle uptake in cultured cells and promotes particle lipid utilization after internalization (*11*). We first questioned whether endogenous apoE has similar effects on cell TGRP uptake. Shown in Figure 1A, endogenous apoE expression, unlike exogenous apoE, did not stimulate TGRP uptake. In fact, E^+ cells showed 30−50% lower TGRP uptake as compared to the control E^- cells. Figure 1B confirms previous data that addition of exogenous apoE to TGRP triggered a classic cellular response featured by the enhancement of particle uptake in both E^- and E^+ cells. These data also demonstrate that the substantial differences in TGRP uptake between E^+ cells and E^- cells persisted regardless of the presence or absence of exogenous apoE. The higher TGRP uptake in E^- cells as compared to E^+ cells was consistently observed in multiple clones of both cell types (Figure 1A and 1B inset). In results not shown, we confirmed that the differences in TGRP uptake between E^- and E^+ cells were maintained at various incubation times (5 min−4 h) and at different concentrations of TGRP (25−1000 μg of NL/mL). These results illustrate that endogenous apoE modulates TGRP uptake very differently than does exogenous apoE carried on TGRP.

We next explored the mechanisms that account for the different effects of exogenous apoE and endogenous apoE on TGRP uptake. In trying to define mechanisms underlying the effects of endogenous apoE on decreasing TGRP uptake, we compared the effects of the conditioned media from E^+ cells and E^- cells on TGRP uptake in both E^+ and E^- cells. Conditioned media from either E^+ or E^- cells had no effect on TGRP uptake in both cell lines, indicating that factors in the media or apoE secreted by E^+ cells did not contribute to differences in TGRP uptake (data not shown). Other experiments assessed possible differences at the cellular level between E^- and E^+ cells and included studies on levels of TGRP re-secretion into the media, lipoprotein lipase activities, and activities of endocytic pathways mediated by the LDLR and other receptors (including LRP and scavenger receptors). In these experiments, we found no differences that could explain the marked differences in TGRP uptake between the two cell types. For example, Figure 2 shows that E^- and E^+ cells have very similar levels of uptake of LDL, acetylated LDL, and α_2m, indicating that endogenous

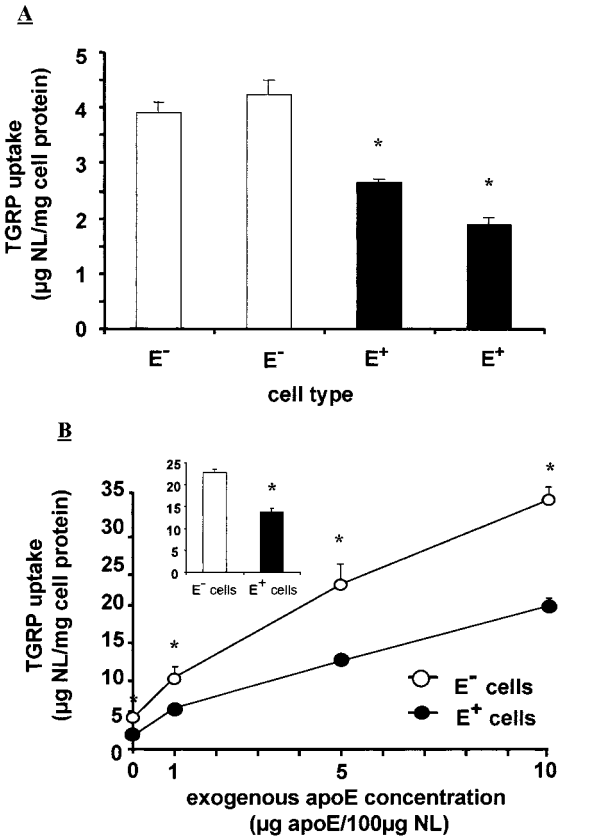

FIGURE 1: TGRP uptake in E^+ and E^- cells. Cells were incubated with ^{3}H-cholesteryl ether-labeled TGRP at the concentration of 250 μg of NL/mL. Particle uptake was measured after 4 h incubation at 37 °C. (A) TGRP uptake of independent clones of E^- and E^+ cells in the absence of exogenous apoE. (B) TGRP uptake in the presence of indicated amount of exogenous apoE. Inset, TGRP uptake of additional clones of E^- and E^+ cells in the presence of 5 μg of apoE/100 μg of NL. Results are means ± SD of three parallel experiments. An asterisk (*) indicates a significant difference between E^- and E^+ cells ($p < 0.05$).

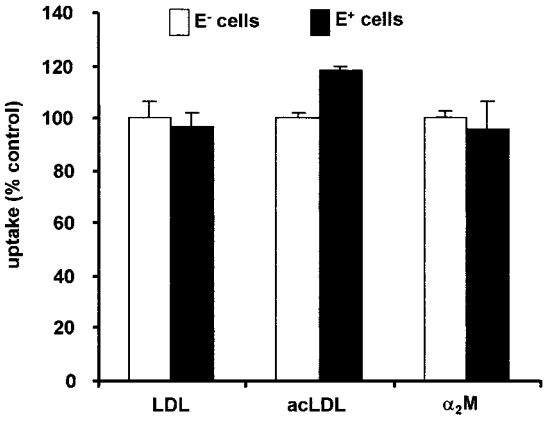

FIGURE 2: Endogenous apoE expression does not affect LDL and α_2m uptake. Cells were incubated with ^{125}I-labeled LDL (5 μg/mL, 4 h, 37 °C), acetylated LDL (acLDL) (5 μg/mL, 4 h, 37 °C), or α_2m (10 μg/mL, 15 min, 37 °C) followed by the measurement of ligand uptake by scintillation counting. Results for E^- cells are shown as controls at 100%. Cell association values for LDL, acLDL, and α_2m in E^- cells were 71.4 ± 4.53, 335.5 ± 7.11, and 64 ± 1.87 ng/mg of cell protein, respectively. Results are means ± SD of three parallel experiments.

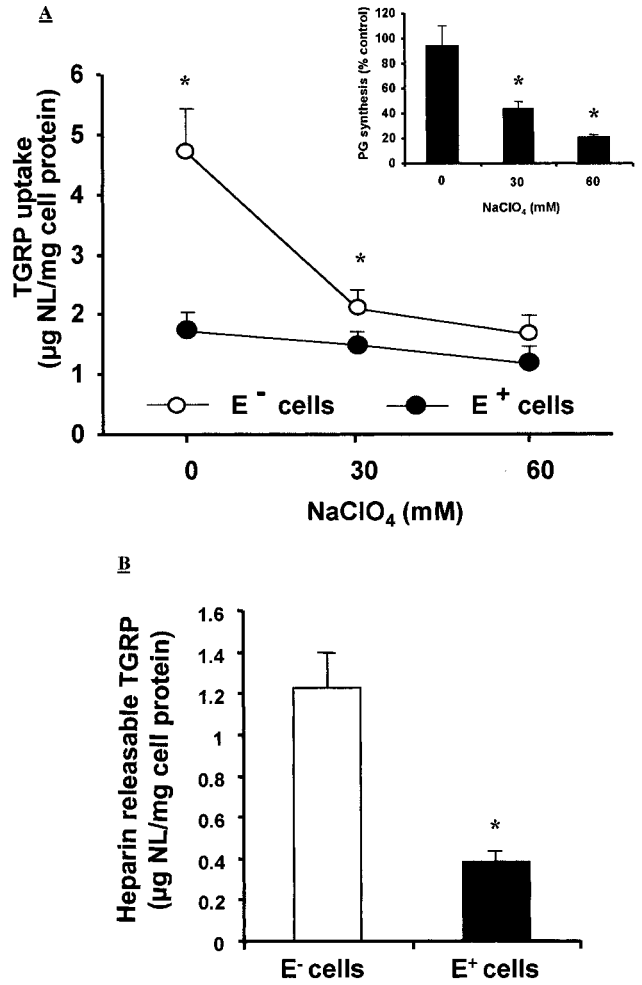

FIGURE 3: Proteoglycan-mediated TGRP uptake and cell surface TGRP binding. (A) Cells were preincubated for 72 h with 0, 30, and 60 mM sodium chlorate followed by incubation with apoE-associated TGRP (100 μg of NL/mL with addition of 1 μg of exogenous apoE/100 μg of NL) for 4 h at 37 °C in the presence of 30 mM sodium chlorate (for sodium chlorate pretreated cells only). Inset, cells were incubated with 25 μCi/mL [^{35}S]sulfate in the presence of indicated amount of sodium chlorate for 72 h. Production level of intracellular + pericellular proteoglycan (^{35}S incorporation per milligram of cell protein) of E^- cells without sodium chlorate treatment was set at 100%. Proteoglycan production was significantly inhibited by 30 and 60 mM sodium chlorate incubation. (B) TGRP cell surface binding was measured after heparin wash as described in Experimental Procedures. Heparin-releasable TGRP binding was determined by scintillation counting of radioactivity in the heparin wash media. Results are means ± SD of three parallel experiments. An asterisk (*) indicates a significant difference between E^- and E^+ cells ($p < 0.05$).

apoE expression does not affect the activity of LDLR, scavenger receptor, or LRP pathways, respectively.

Proteoglycan-Mediated TGRP Uptake. Cell surface proteoglycans serve as an important cell surface lipoprotein anchor and therefore may modulate lipoprotein internalization (*5*). In E^+ cells, sodium chlorate inhibits proteoglycan synthesis and releases cell surface proteoglycan-associated apoE into the media (*23*). We next questioned whether proteoglycan-mediated cell TGRP uptake is different between E^- and E^+ cells. Control experiments were performed to confirm the inhibitory effect of sodium chlorate on proteoglycan synthesis in the control E^- cells (Figure 3A, inset). Inhibition of proteoglycan synthesis by sodium chlorate not only decreased TGRP uptake in both E^- and E^+ cells but also substantially diminished the differences in TGRP uptake between the two cell types (Figure 3A). The decrease in TGRP uptake in response to sodium chlorate treatment was only statistically significant in E^- cells, not in E^+ cells. This inhibitory effect of sodium chlorate on TGRP uptake was confirmed at TGRP concentrations ranging from 50 to 250 μg of NL/mL. These results indicate that E^- cells contain sodium chlorate-sensitive TGRP uptake compartments that are not shared by E^+ cells. E^- cells also showed higher heparin-releasable TGRP binding than did E^+ cells in the presence of exogenous apoE (Figure 3B). This observation further supports the theory that E^- cells have a larger proteoglycan-mediated lipoprotein binding pool than do E^+ cells. Thus, differences in cell surface proteoglycan-mediated particle binding likely contribute to differences in TGRP uptake between E^- and E^+ cells.

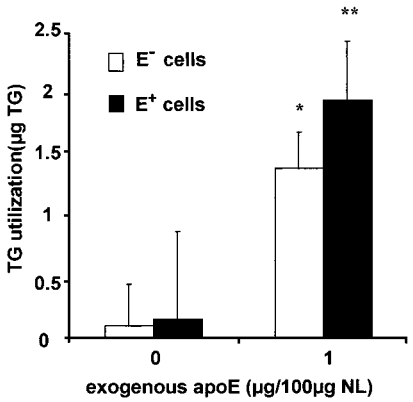

FIGURE 4: TG utilization in E^- and E^+ cells. TG utilization in cells incubated with 50 μg of NL/mL TGRP plus 5 μCi of ^{3}H-glycerol/mL in the presence or absence of exogenous apoE (1 μg of apoE/100 μg of NL) for 4 h at 37 °C. TG uptake, mass, and synthesis were measured, and TG utilization is calculated as described in Experimental Procedures. Results are means ± SD of four parallel experiments. Differences between E^- (*) or E^+ (**) cells incubated with TGRP in the absence and presence of apoE are significant at $p < 0.05$.

Effects of Exogenous ApoE versus Endogenous ApoE on Particle TG *Metabolism.* We have reported that exogenous apoE carried on TGRP enhances TGRP triglyceride metabolism independent of its effect on particle uptake (*11*). We next questioned whether endogenous apoE has similar effects on TG metabolism. Cellular TG synthesis and mass were measured, and TG utilization was calculated in the presence and absence of exogenous apoE as described in Experimental Procedures. In this series of experiments, we chose to use 50 μg of NL/mL TGRP concentration for assays (in both E^+ and E^- cells, TGRP concentrations ≤ 50 μg of NL/mL are associated with uptake levels that do not saturate cell TG hydrolysis systems). Consistent with our previous report (*11*), addition of exogenous apoE to TGRP stimulated TG utilization (Figure 4). In contrast, endogenous apoE expression did not significantly alter TG utilization.

Effects of Exogenous ApoE and Endogenous ApoE on Particle CE Hydrolysis. Our previous studies have also shown that exogenous apoE carried on TGRP enhances particle CE hydrolysis independent of its effects on TGRP uptake (*11*). To determine if endogenous apoE expression affects particle CE hydrolysis, cells were incubated with ^{3}H-cholesteryl oleate-labeled TGRP in the presence or absence of exogenous apoE at 37 °C for 4 h followed by the

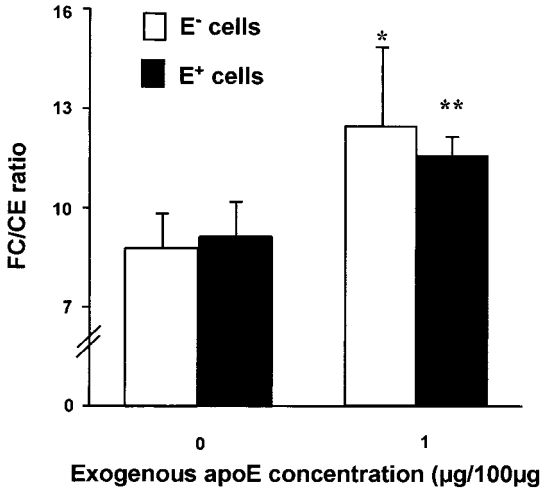

FIGURE 5: TGRP cholesteryl ester hydrolysis in E^- and E^+ cells. E^- and E^+ cells were incubated with 250 μg of NL/mL ^{3}H-cholesteryl ester-labeled TGRP in the presence or absence of exogenous apoE (1 μg of apoE/100 μg of NL) at 37 °C for 4 h. Cell ^{3}H-free cholesterol (FC) and ^{3}H-cholesterol ester (CE) were measured as described in Experimental Procedures, and CE hydrolysis is presented as cell FC/CE partitioning. Results are normalized for TG uptake and are means ± SD of six experiments. Differences between E^- (*) or E^+ (**) cells incubated with TGRP in the absence and presence of apoE are significant ($p < 0.05$).

assessment of cell-associated ^{3}H-free cholesterol (FC) and ^{3}H-CE content. Consistent with our previous results, addition of exogenous apoE to TGRP significantly increased FC/CE partitioning in both E^- and E^+ cells (Figure 5). However, endogenous apoE expression alone, unlike exogenous apoE, did not affect CE hydrolysis (Figure 5).

Cellular Localization of Exogenous TGRP ApoE and Endogenous ApoE. Exogenous apoE carried on TGRP has been shown to follow primarily endocytic trafficking pathways (*36*). In separate studies and in independent cell lines, endogenous apoE has been shown to mainly follow secretory pathways (*37, 38*). Whether apoE of different origins concentrates in the same cellular compartment in one cell type is unknown. Since different cellular compartmentalization could contribute to different bioactivities of apoE from different sources, we examined whether major separation of intracellular trafficking occurs for apoE from different origins in one cell type. Cellular localizations of exogenous apoE carried on TGRP and endogenous apoE were examined using immunostaining and fluorescence microscopy techniques (Figure 6). In Figure 6A, Fα_2m identified lysosomal compartments in E^- cells (*39*). This area colocalized with exogenous apoE carried on TGRP (Figure 6B). In contrast, endogenous apoE concentrated in a perinuclear cellular compartment (Figure 6C), and this area colocalized with regions marked by fluorescein-labeled wheat germ agglutinin, a Golgi marker (Figure 6D). The Golgi localization of endogenous apoE has also been reported in different cell types (*37, 38*). [Although previous studies have reported the cellular re-entry (*40*) of secreted apoE, this was not observed in our fluorescence microscopy experiments, also in keeping with low levels of apoE secretion into the media by the E^+ cells used here.] These results indicate separate and distinct intracellular trafficking routes of exogenous apoE carried on TGRP versus endogenous apoE. Our data thus demonstrate a major separation of cellular localization of exogenous versus endogenous apoE in one cell type. Figure 6E shows

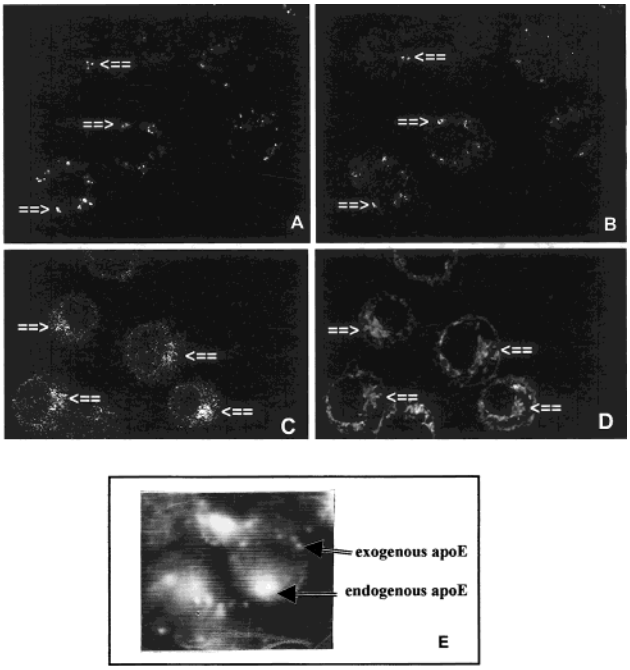

FIGURE 6: Cellular localization of exogenous apoE and endogenous apoE. Cells were incubated with (300 μg of NL/mL) TGRP + (1 μg/100 μg of NL) apoE (panels A, B, and E) for 15 min at 37 °C in the presence (for E^- cells in panels A and B) or absence (for E^+ cells in panels C−E) of 40 μg/mL Fα_2m. Cells were then washed, fixed, and permeabilized followed by apoE immunodetection using monoclonal anti-apoE antibody (E10) and rhodamine-labeled goat anti-mouse IgG secondary antibody. E^+ cells in panels C and D were further incubated with 100 μg/mL fluorescein-labeled wheat germ agglutinin for 30 min at room temperature. (A) Fα_2m marks lysosomal compartments in E^- cells; (B) rhodamine fluorescence of exogenous apoE in E^- cells. (C) Rhodamine fluorescence of endogenous apoE in E^+ cells. (D) Fluorescein-conjugated wheat germ agglutinin labels Golgi compartments in E^+ cells. Arrows in panels A and B show colocalization of Fα_2m and exogenous apoE in E^- cells; arrows in panels C and D indicate colocalization of endogenous apoE with wheat germ agglutinin. (E) Rhodamine fluorescence of exogenous apoE and endogenous apoE in E^+ cells.

that addition of TGRP-associated exogenous apoE (1 μg of apoE/100 μg of TGRP) to E^+ cells led to the concomitant identification of apoE in both lysosome (exogenous apoE) and Golgi (endogenous apoE) areas. Note the strong fluorescence intensity shown by endogenous apoE in the Golgi area relative to the lysosomes. This indicates that within E^+ cells there is likely higher abundance of endogenous apoE as compared to exogenous apoE internalized via TGRP.

We also questioned whether exogenous apoE carried on TGRP travels to the lysosomal compartments along with the lipid components of TGRP. Figure 7 shows the colocalization of DiI-labeled TGRP (Figure 7A) with exogenous apoE carried on TGRP (Figure 7B). Together with Figure 6A, which showed the lysosomal localization of exogenous apoE carried on TGRP, these results demonstrate that after internalization, exogenous apoE is co-transported with TGRP lipids to the lysosomal compartments.

DISCUSSION

Although a number of lines of evidence suggest different metabolic roles of exogenous versus endogenous apoE, little attention has been focused on direct discrimination between functions of exogenous apoE and endogenous apoE in the

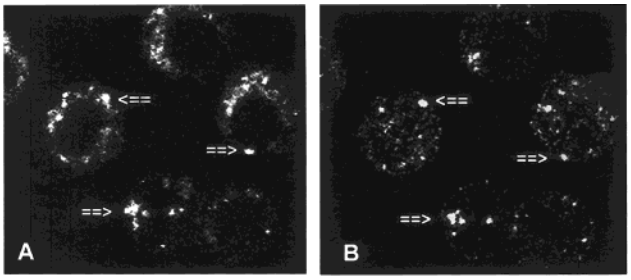

FIGURE 7: Cellular colocalization of exogenous apoE and TGRP. Cells were incubated with 300 μg of NL/mL DiI-labeled TGRP + 1 μg of exogenous apoE/100 μg of NL at 37 °C for 15 min and then processed as indicated under Experimental Procedures. (A) DiI fluorescence; (B) immunofluorescence of exogenous apoE stained by fluorescein-conjugated secondary antibody after anti-apoE primary antibody (E10) incubation. Arrows indicate colocalization of apoE with TGRP.

same cell type. Our results demonstrate that under a range of physiological concentrations, exogenous apoE associated with TGRP and endogenously expressed apoE have very different biological effects on TGRP uptake and metabolism in J774 cells, a macrophage cell line that normally does not express apoE.

After internalization, both exogenous apoE and TGRP target rapidly to the lysosomal compartment (Figure 7) where efficient TG and CE metabolism takes place (*41*), while endogenous apoE colocalizes with the Golgi compartment (Figure 6). It is likely that only apoE that is cotransported with lipids in TGRP to the lysosomes will exert effects on intracellular particle lipid metabolism. Supporting this hypothesis, we have shown that after internalization, exogenous apoE is degraded slowly, as compared to another apoprotein, apoB (*42*). This would allow exogenous apoE to be active intracellularly after internalization, perhaps by acting as a molecular chaperone. While our data and the data of others do not clarify how exogenous apoE exerts its effects on intracellular lipid metabolism, it is clear from these studies that endogenous apoE does not mimic these effects, perhaps because it is restricted to a separate cellular compartment (the Golgi areas). Thus, this study not only demonstrated different biological effects of exogenous versus endogenous apoE on lipid uptake and metabolism but also showed major separation of intracellular compartmentalization of apoE of different origins in the same cell type.

In contrast to the observation that exogenous apoE promotes TGRP uptake, TGRP uptake is significantly less in apoE-expressing macrophages (E^+ cells) as compared to non-apoE-expressing macrophages (E^- cells). Similarly, endogenous apoE expression has no effect on CE hydrolysis or TG utilization; however, exogenous apoE enhances particle TG and CE metabolism. Whether higher levels of endogenous apoE expression or secretion might modulate cell lipid metabolism by providing sufficient amount of apoE into the extracellular media to mimic the action of exogenous apoE, a mechanism suggested for the secretion−recapture pathway (*40*), requires further investigation. Nevertheless, our semiquantitative fluorescence microscopy data (Figure 6E) suggest that internalized exogenous apoE stimulated TG and CE metabolism at an intracellular concentration lower than the level of endogenous apoE expression (Figure 6E, compare fluorescence intensities of exogenous vs endogenous apoE). This indicates that the different biological effects of exogenous apoE versus endogenous apoE result from different cellular localizations rather than from differences in cellular concentrations.

Using a non-apoE-synthesizing cell line that was transfected to express but not secrete substantial amount of apoE, we avoided the confounding effect of E^+ cells secreting large quantities of apoE into the media to act like exogenous apoE (*22*). [Our conditioned media experiments showed that the secreted apoE did not stimulate TGRP uptake. As well, the fluorescence microscopy experiments (Figure 6C) showed no identifiable re-entry of secreted apoE.] Even though E^+ cells used here only secrete small quantities of apoE, endogenous apoE expression still modulated extracellular events such as TGRP uptake. However, this level of expression did not affect intracellular metabolic events such as TG and CE hydrolysis, indicating that the cellular localization of apoE dominates the bioactivity of the protein.

Exogenous apoE increases TGRP cell uptake by promoting the high affinity interaction of apoE-containing particles with cell apoE receptors including the LDLR and LRP (*3, 4*). At higher physiological particle concentrations, exogenous apoE can also mediate TGRP uptake by LDLR and LRP-independent pathways after cell surface proteoglycan binding (*5*). Previous studies have demonstrated that in the same E^+ cell lines and in other cell types, apoE "coats" the cell surface by binding to cell surface proteoglycans (*23, 43, 44*). Also, our recent studies showed that endogenous cell expression of apoE markedly decreases proteoglycan degradation (*45*), indicating a tight interaction between endogenous apoE and cell proteoglycans. One possible explanation for decreased TGRP uptake in apoE-expressing cells is that endogenously expressed apoE binds tightly with proteoglycans at both intracellular and cell surface sites making them unavailable for initial cell surface anchoring of TGRP. Supporting this hypothesis, inhibition of proteoglycan synthesis substantially diminished the differences in TGRP uptake between E^- and E^+ cells (Figure 3).

In other reports, endogenous apoE has been demonstrated to produce distinct structural/metabolic effects when compared to exogenous apoE. In defining the roles of apoE3 versus apoE4 isoforms in Alzheimer's disease models, cultured neuronal cells transfected with apoE3 versus apoE4 showed no differences in dendrite growth and other neuronal parameters unless the apoE re-entered cells with lipid particles (*46*). In another example, cell endogenous expression of apoE promotes cholesterol efflux from cultured cells (*14, 16*). In contrast, exogenous apoE carried on TGRP or on HDL-like particles is not as efficient in promoting cell cholesterol efflux (*15, 16*). One possible mechanism underlying this effect of endogenous expression of apoE enhancing cholesterol efflux is that upon secretion, it carries with it cell phospholipid with associated solubilized FC (*47*), a process in keeping with the strong lipid-binding properties of apoE.

ApoE secretion by macrophages at atherosclerotic lesion sites indicates a "local" role of apoE in modulating the processes of lesion development (*48*). Anti-atherogenic effects of apoE are well-demonstrated in induced mutant animal models. ApoE knockout mice are hypercholesterolemic and develop premature atherosclerosis (*49*). Expression of a human apoE3 transgene in apoE-deficient mice corrected the hypercholesterolemia and impeded the progres-

sion of atherosclerosis (*50*). Of interest, wild-type mice with bone marrow reconstitution of apoE-null macrophages develop markedly more advanced atherosclerosis than do those reconstituted with macrophages carrying the wild-type apoE gene, in the absence of significant differences in serum cholesterol levels or lipoprotein profiles (*51*). Also, macrophage-specific human apoE3 transgene expression in apoE-deficient mice is anti-atherogenic even in the face of persistent hyperlipidemia (*52*). These observations indicate that some anti-atherogenic effects of endogenous apoE may prove to be independent of its effects on plasma lipoprotein clearance, for example, by promoting cholesterol efflux from cells and/or by decreasing uptake of cholesterol-containing remnant TGRP.

Exogenous apoE carried on TGRP is believed to be anti-atherogenic, based on its ability to promote particle clearance from plasma. As increased particle uptake may result in pro-atherogenic lipid accumulation, exogenous apoE can be biologically protective only if the cells are capable of better metabolizing the internalized lipids. Our results support this hypothesis in that the ability of exogenous apoE to decrease cell lipid accumulation by promoting TG and CE metabolism accompanies its ability to enhance particle uptake (*11*). As well, since apoE expression does not enhance particle TG and CE metabolism, the suppression of TGRP uptake by endogenous apoE expression may help prevent excessive lipid accumulation in macrophages (and perhaps other apoE-expressing cells). Together with the observation that endogenous apoE increases cholesterol efflux from macrophages (*14*, *16*), we hypothesize that one biological significance of apoE expression by macrophages in the atherosclerotic lesion sites is to prevent/reduce cell lipid accumulation and subsequent foam cell formation, independent of its effect on plasma lipoprotein clearance.

Our study demonstrates that exogenous apoE and endogenous apoE not only follow distinct cellular trafficking pathways but also have very different effects on cell lipid metabolism. Different cellular localizations are likely to be important for the different effects of exogenous apoE versus endogenous apoE on modulating various cell functions. The elucidation of the biological effects of exogenous apoE and endogenous apoE on macrophage lipid metabolism may help to define better the roles of apoE in the process of atherosclerosis at the cellular level. Exploring the relationship between apoE and human pathophysiology and distinguishing the source and cellular localization of apoE will also facilitate the understanding of the multiple roles of apoE in different metabolic pathways.

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