

# Apolipoprotein E-deficient lipoproteins induce foam cell formation by downregulation of lysosomal hydrolases in macrophages

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**Abstract** Apolipoprotein E (apoE) deficiency has been suggested to induce foam cell formation. Using lipoproteins obtained from wild-type mice and apoE-deficient mice expressing apoB-48 but not apoB-100, we studied apoE-deficient lipoprotein-induced changes in lipoprotein catabolism and protein expression in mouse peritoneal macrophages (MPMs). Our data demonstrate that incubation of MPMs with apoE-deficient lipoproteins induced intracellular lipoprotein, cholesteryl ester, and triglyceride accumulation, which was associated with a time-related decline in apoE-deficient lipoprotein degradation in MPMs. Confocal microscopy analysis indicated that the accumulated lipids were localized in lysosomes. ApoE-deficient lipoproteins reduced the protein levels of lysosomal acid lipase, cathepsin B, and cation-dependent mannose 6 phosphate receptor (MPR46). Exogenous apoE reduced apoE-deficient lipoprotein-induced lipid accumulation and attenuated the suppressive effect of apoE-deficient lipoproteins on lysosomal hydrolase and MPR46 expression. Although oxidized lipoproteins also increased lipid contents in MPMs, exogenous apoE could not attenuate oxidized lipoprotein-induced lipid accumulation. Our *in vivo* studies also showed that feeding apoE-deficient mice a high-fat diet resulted in cholesteryl ester and triglyceride accumulation and reduced lysosomal hydrolase expression in MPMs. These data suggest that apoE-deficient lipoproteins increase cellular lipid contents through pathways different from those activated by oxidized lipoproteins and that reducing lysosomal hydrolases in macrophages might be a mechanism by which apoE-deficient lipoproteins result in intralysosomal lipoprotein accumulation, thereby inducing foam cell formation.—Wu, D., C. Sharan, H. Yang, J. S. Goodwin, L. Zhou, G. A. Grabowski, H. Du, and Z. Guo. **Apolipoprotein E-deficient lipoproteins induce foam cell formation by downregulation of lysosomal hydrolases in macrophages.** *J. Lipid Res.* 2007. 48: 2571–2578.

**Supplementary key words** oxidized lipoproteins • cholesteryl ester • mannose 6 phosphate receptor

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Increased plasma apolipoprotein B (apoB)-containing lipoproteins is an atherosclerosis risk factor (for review, see Refs. 1, 2). One mechanism by which apoB-containing lipoproteins induce atherosclerosis is by transforming macrophages into foam cells. The hallmark feature of foam cells, cholesteryl ester accumulation, can result from three mechanisms: 1) increased cholesteryl-rich lipoprotein uptake; 2) increased reesterification of extralysosomal free cholesterol (FC) by ACAT; and 3) reduced lysosomal cholesteryl ester hydrolysis. It has been suggested that apoB-containing lipoproteins must be modified (oxidized and acetylated) to induce foam cell formation. Modified apoB-containing lipoproteins have a reduced affinity for low density lipoprotein receptor (LDLR) and LDLR-related proteins but an increased affinity for scavenger receptors (SRs) and therefore can be internalized via SR-mediated pathways. Interestingly, lipoproteins with different modifications produce different cholesterol accumulation patterns. For example, acetylated LDL induces the accumulation of ACAT-derived cholesteryl esters in cytosolic lipid droplets (3), whereas oxidized LDL induces cholesterol accumulation in lysosomes (4). However, the underlying mechanisms by which lipoproteins with different modifications produce different lipid accumulation patterns remain largely unknown.

Recent studies indicate that without modification, the apoB-containing lipoproteins obtained from apoE-deficient (*ApoE*<sup>-/-</sup>) mice, which contain mainly apoB-48, can induce foam cell formation (5, 6). It has been suggested that

Abbreviations: apoB, apolipoprotein B; apoB-48R, apolipoprotein B-48 receptor; E<sup>-</sup>/B48, apolipoprotein E-deficient, apolipoprotein B-48-containing lipoprotein; EC, esterified cholesterol; FC, free cholesterol; LDLR, low density lipoprotein receptor; MPM, mouse peritoneal macrophage; MPR46, cation-dependent mannose 6 phosphate receptor; SR, scavenger receptor; SR-A, scavenger receptor class A; SR-BI, scavenger receptor class B type I; TBARS, thiobarbituric acid-reacting substances.

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an uncontrolled uptake of the apolipoprotein E-deficient, apolipoprotein B-48-containing lipoprotein (E<sup>-</sup>/B48) is a mechanism of foam cell formation (7) and that SRs, such as scavenger receptor class A (SR-A), scavenger receptor class B type I (SR-BI), CD36, VLDL receptors, and apolipoprotein B-48 receptors (apoB-48Rs), are responsible for the uncontrolled uptake of E<sup>-</sup>/B48 lipoproteins (8, 9).

In this report, we studied the mechanism underlying E<sup>-</sup>/B48 lipoprotein-induced foam cell formation using lipoproteins obtained from apoE-deficient mice expressing apoB-48 but not apoB-100 (*ApoB<sup>48/48</sup>/ApoE<sup>-/-</sup>*). Our data indicate that E<sup>-</sup>/B48 lipoprotein-induced macrophage foam cells are characterized by the accumulation of lipids in the lysosomes, which is associated with a time-related decline in E<sup>-</sup>/B48 lipoprotein degradation. Our data also demonstrate that the effect of apoE-deficient lipoproteins on macrophage SRs varied with the SR isoforms: the protein level of CD36 was increased, SR-BI was reduced, and SR-A and apoB-48R were not altered significantly. However, incubation of mouse peritoneal macrophages (MPMs) with E<sup>-</sup>/B48 lipoproteins reduced the protein level of lysosomal hydrolases. Our *in vivo* studies also demonstrate that high-fat diet-induced macrophage cholesteryl ester accumulation was associated with reduced protein levels of lysosomal hydrolases in apoE-deficient mice. These findings provide the first evidence that reduction in macrophage lysosomal hydrolases is a mechanism by which E<sup>-</sup>/B48 lipoproteins induce foam cell formation.

## MATERIALS AND METHODS

### Animals

Wild-type C57BL/6, *ApoE<sup>-/-</sup>*, and *ApoB<sup>48/48</sup>/ApoE<sup>-/-</sup>* mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *ApoE<sup>-/-</sup>* mice were generated by Piedrahita et al. (10) and were backcrossed to C57BL/6 for >10 generations. *ApoB<sup>48/48</sup>/ApoE<sup>-/-</sup>* mice were obtained by cross-breeding *ApoE<sup>-/-</sup>* mice with *ApoB<sup>48/48</sup>* mice. The *ApoB<sup>48/48</sup>* mice, which expressed only apoB-48 and not apoB-100, were generated by Farese et al. (11). All mice were studied at 3–4 months of age and were fed a chow diet containing ~5% fat and 19% protein by weight (Harlan Teklad, Madison, WI) unless indicated otherwise. All procedures for handling the animals were approved by the Institutional Animal Care and Use Committee of Meharry Medical College.

### Isolation and culture of MPMs

MPMs were prepared from *ApoE<sup>-/-</sup>* mice that had received an intraperitoneal injection of 2 ml of 3% thioglycollate broth 4 days earlier, as described by Li et al. (12). The cells were maintained in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under 5% CO<sub>2</sub> in 75 mm culture flasks (Corning, Inc., Corning, NY).

### Mouse lipoproteins

Wild-type and E<sup>-</sup>/B48 lipoproteins were prepared from wild-type and *ApoB<sup>48/48</sup>/ApoE<sup>-/-</sup>* mice, respectively. Mouse plasma mixed with 50 µM butylated hydroxytoluene was overlaid with a potassium bromide gradient solution (d = 1.063) and centrifuged at 120,000 rpm for 2 h with a Sorvall Discovery MI50 ultracentrifuge (Kendro Laboratory Products, Asheville, NC).

The mixture of VLDL and LDL was collected, dialyzed in PBS (pH 7.4) containing 10 mM EDTA for 48 h at 4°C, and filtered through a 0.45 µm filter (13).

The oxidized level of lipoproteins was determined using a thiobarbituric acid-reacting substances (TBARS) assay kit (ZeptoMetrix Co., Buffalo, NY). The oxidized lipoproteins used in this study were obtained by incubation of wild-type mouse lipoproteins with 5 µM CuSO<sub>4</sub> for 4 or 12 h at 37°C (14), which increased the TBARS level of the lipoproteins from 0.75 to ~3.4 and 8.7 nM/mg protein, respectively.

### Macrophage binding, association, degradation, and uptake of <sup>125</sup>I-labeled lipoproteins

Wild-type and E<sup>-</sup>/B48 lipoproteins were iodinated with <sup>125</sup>I as described previously (13). In the binding experiments, MPMs were prechilled on ice for 30 min and then incubated with the indicated concentrations of <sup>125</sup>I-labeled lipoproteins on ice for 4 h. After washing twice with ice-cold PBS, the cells were lysed with 0.5 M NaOH. Ten microliters of lysate aliquot was used for protein measurement, and the rest of the lysate was used to determine the radioactivity bound to the cells using a universal γ-counter (1282 Compugamma; Perkin-Elmer Life and Analytic Science, Shelton, CT).

In the association and degradation experiments, MPMs were incubated with 20 µg/ml <sup>125</sup>I-labeled lipoproteins at 37°C. At the indicated incubation times, 0.75 ml of culture medium was collected and mixed with 0.25 ml of 40% ice-cold TCA and 0.2 ml of 0.7 M AgNO<sub>3</sub>, incubated at room temperature for 30 min, and centrifuged at 2,500 rpm for 10 min (5). The radioactivity in the supernatants was determined with the 1282 Compugamma γ-counter. The amount of <sup>125</sup>I-labeled lipoprotein particles degraded by macrophages was estimated by the non-TCA-precipitable radioactivity in the culture medium, normalized by incubation time. After removing the culture medium, MPMs were washed twice with PBS and then dissolved in 0.5 N NaOH. A 10 µl lysate aliquot was used for protein measurement, and the rest of the lysate was used to determine cell-associated radioactivity using the 1282 Compugamma γ-counter. The amount of lipoproteins that were taken up by the MPMs was the sum of the lipoproteins that were associated with the cells and those that were degraded by the cells in an incubation time period. Thus, we calculated lipoprotein uptake using the following equation: uptake = association + degradation.

### Oil Red O lipid staining

MPMs grown in 75 mm culture flasks were incubated with 20 µg/ml lipoproteins for 48 h followed by a 12 h equilibrium in lipoprotein-free medium. After removing culture medium, MPMs were fixed with 4% paraformaldehyde for 20 min, rinsed with water, dipped in 60% isopropanol, and stained in 0.3% Oil Red O for 15 min to identify cholesteryl ester-containing lipid droplets. Cell nuclei were momentarily stained in hematoxylin. Lipid accumulation in the cells was evaluated using a Nikon Eclipse E600 microscope equipped with a high-resolution camera (CoolSnaps; Nikon Instruments, Inc.). The cells in which the Oil Red O-stained lipids occupied more than one-third of the cytoplasm were considered foam cells (15).

### Confocal microscopic localization of endocytic lipids

Wild-type and E<sup>-</sup>/B48 lipoproteins were fluorescently labeled by incubating 10 µl of 3 mg/ml DiI<sub>18</sub> (dissolved in dimethyl sulfoxide) per 1 ml of 0.5 mg/ml (protein) lipoproteins (16). The mixture was incubated under sterile conditions at 37°C for 12 h in the presence of 50 µM butylated hydroxytoluene and 10 µM EDTA, and labeled lipoproteins were reisolated by ultra-

centrifugation. MPMs grown on chamber slides were treated with a mixture of 0.2 mg/ml DiI-labeled lipoproteins and 2 mg/ml unlabeled lipoproteins for 48 h followed by a 12 h equilibrium in a lipoprotein-free medium. Thereafter, the cells were incubated for 1 h with 75  $\mu$ M LysoTracker Green (Invitrogen, Carlsbad, CA), which permeates the cellular membrane and is commonly used as a probe to localize lysosomes. After incubation, cells were rinsed three times with PBS and then fixed for 10 min with 2% paraformaldehyde at 4°C. After three rinses, the slide was mounted with a glass coverslip with ProLong mounting medium (Invitrogen). To assess the localization of DiI-lipoproteins and LysoTracker, images were obtained with a Nikon TE2000 C1 confocal system equipped with UV-Vis lasers. Images were acquired sequentially using a 488 nm laser line and emission between 505 and 550 nm for LysoTracker and a 543 nm laser line and emission between 560 and 615 nm for DiI. High-resolution (100 nm/pixel) Z-series images were obtained from macrophages with a  $\times 60$  (1.4 numerical aperture) Plan Apochromat oil-immersion objective and analyzed for colocalization. Overlay images were assembled, zoomed, and cropped using Nikon Elements 2.3 software.

### Western blot analysis of macrophage protein expression

MPMs grown in 75 mm culture flasks were incubated with 20  $\mu$ g/ml lipoproteins for 48 h. In the apoE treatment experiments, the indicated concentrations of mouse apoE (Chemicals International, Copenhagen, Denmark) were gently mixed with E $\gamma$ /B48 lipoproteins or oxidized lipoproteins at room temperature for 30 min before adding to the culture. After a 12 h equilibrium in a lipoprotein-free medium, cells were washed twice with ice-cold PBS and then lysed in extraction buffer (62.5 mM Tris, 100 mM DTT, 2% SDS, 10% glycerol, and 0.01% bromophenol blue, pH 6.8) on ice. The lysate was centrifuged at 14,000 *g* at 4°C for 5 min, and the resulting supernatant containing 10–40  $\mu$ g of proteins was separated on a 10 or 12% SDS-polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% BSA, the membrane was immunoblotted with antibodies against cathepsin B, cation-dependent mannose 6 phosphate receptor (MPR46), SR-A, CD36 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), SR-BI (Cell Signaling Technology, Danvers, MA), apoB-48R (Abnova Co., Taipei City, Taiwan), and lysosomal acid lipase (obtained from G.A.G.'s laboratory, Children's Hospital Research Foundation). After incubation with horseradish peroxidase-conjugated secondary antibodies and an ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ), protein fluorescence on the membrane was detected using a laser scanner (Typhoon 9410; Amersham Biosciences) and quantified using an image analysis system (ImageQuant; Amersham Biosciences).

### Cellular cholesterol measurement

MPMs grown in 75 mm culture flasks were incubated with 20  $\mu$ g/ml lipoproteins in the presence or absence of apoE for 48 h followed by a 12 h equilibrium period in a lipoprotein-free medium. After removing the culture medium, MPMs were scraped into 1 ml of ice-cold distilled water. Lipids were extracted, and cellular total cholesterol, FC, and triglycerides were determined using an enzymatic kit (Wako Chemicals, Richmond, VA) as described previously (13). The level of esterified cholesterol (EC) was calculated from the difference between total cholesterol and FC.

### In vivo model of foam cell formation

Feeding mice with a high-fat diet followed by intraperitoneal thioglycollate injection has been used to induce macrophage foam cell formation in vivo (17). Three month old *ApoE*<sup>-/-</sup> mice

were fed a high-fat diet containing 15% fat and 1.25% cholesterol by weight (Harlan Teklad) for 5 days, which increased the average plasma cholesterol of the *ApoE*<sup>-/-</sup> mice from 517 to 1,129 mg/dl. These mice were then intraperitoneally injected with 2 ml of 3% thioglycollate broth and fed a high-fat diet for another 4 days. MPMs were collected, and lipid and protein extracts were prepared for cellular lipid measurement and Western blot analysis as described above.

### Statistical analysis

Data are reported as means  $\pm$  SEM, and data distribution was examined by the Shapiro-Wilk normality test. Comparisons between treatment and control groups were performed with a Student's *t*-test, and differences were considered significant at *P* < 0.05. All statistical analyses were performed using Stastix software (Analytical Software, Tallahassee, FL). For experiments using 96-well plates, the value reported for each experiment is the average of four duplicate wells on the same plate. The number of experiments performed is indicated in the figure legends.

## RESULTS

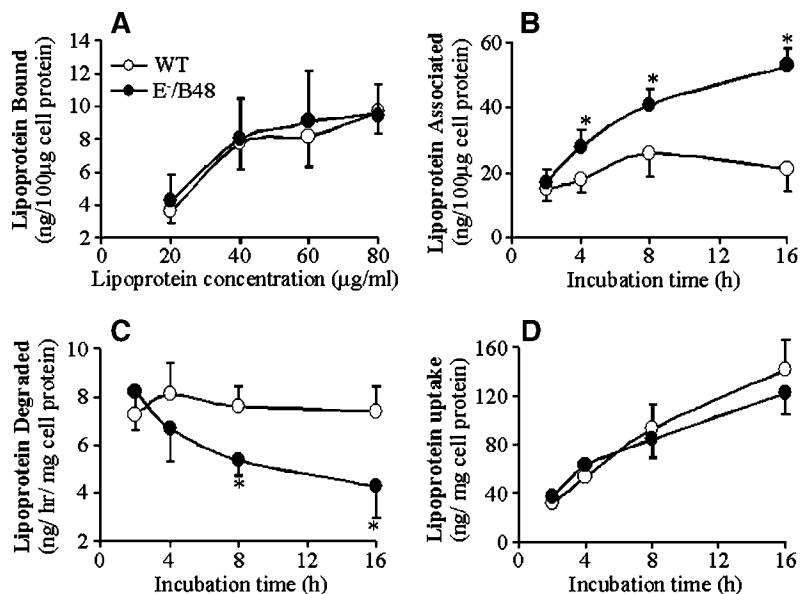
### Increased association and decreased degradation of E $\gamma$ /B48 lipoproteins in MPMs

The data in Fig. 1A illustrate that, at ice-cold temperature, the cell-bound lipoproteins increased with increasing concentrations of lipoproteins in the culture medium, plateauing at a concentration of 40  $\mu$ g/ml and remaining constant at concentrations up to 80  $\mu$ g/ml. The amount of wild-type and E $\gamma$ /B48 lipoproteins bound to the MPMs was comparable (Fig. 1A). The amount of lipoproteins associated with the cells at 37°C is a measure of the total lipoproteins bound to the cell surface and internalized into the endosomes and lysosomes. As Fig. 1B shows, the amount of wild-type lipoproteins associated with MPMs increased slightly with incubation time, peaked within 8 h, and remained unchanged thereafter. In contrast, the amount of E $\gamma$ /B48 lipoproteins associated with MPMs increased continuously over time, suggesting a time-related intracellular accumulation of E $\gamma$ /B48 lipoproteins.

To investigate the mechanism responsible for the increased E $\gamma$ /B48 lipoprotein association, we determined the uptake and degradation of wild-type and E $\gamma$ /B48 lipoproteins in MPMs. As Fig. 1C shows, the amount of wild-type and E $\gamma$ /B48 lipoproteins taken up by MPMs during the incubation time periods studied was comparable; however, MPMs showed significant differences in the degradation of these lipoproteins. The degradation rate of wild-type lipoproteins remained unchanged within the incubation time, whereas the E $\gamma$ /B48 lipoprotein degradation rate in MPMs declined after 8 h of incubation (Fig. 1D). These data suggest that reduced degradation could be a causal mechanism for E $\gamma$ /B48 lipoprotein accumulation in MPMs.

### E $\gamma$ /B48 lipoproteins result in lipid accumulation in MPM lysosomes

The Oil Red O staining technique has been used by many laboratories for the detection of lipid-laden foam cells (15). As Fig. 2 illustrates, cells without lipoprotein

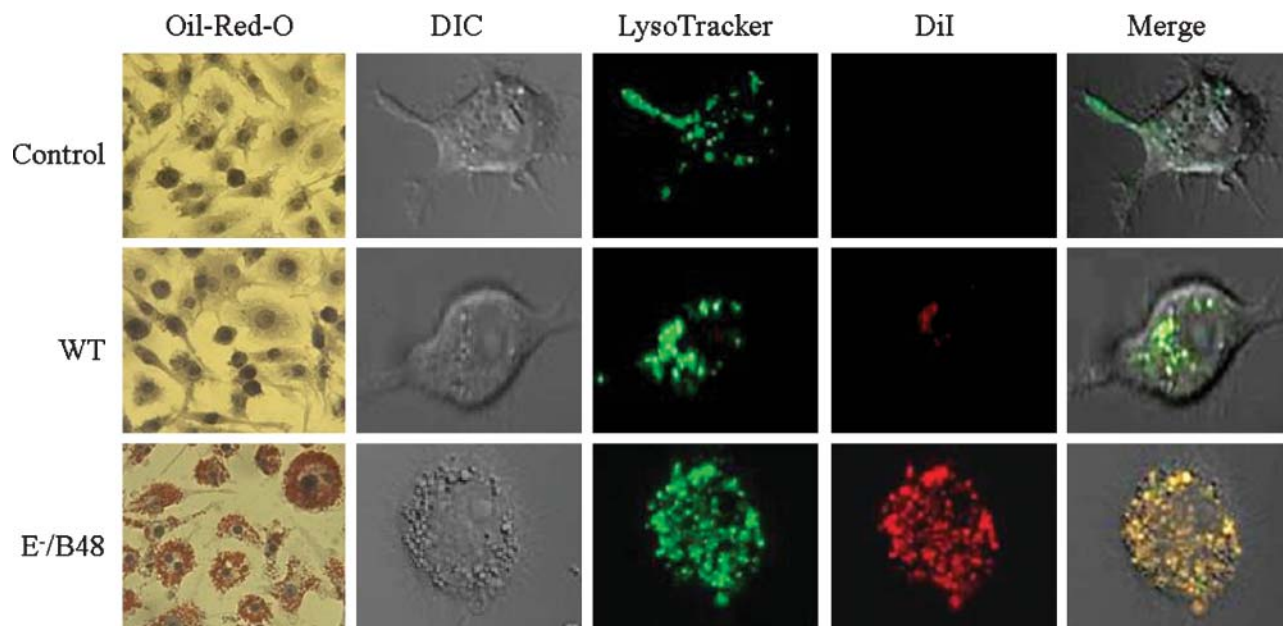


**Fig. 1.** Lipoprotein catabolism in mouse peritoneal macrophage (MPMs). A: MPMs were incubated with the indicated concentrations of <sup>125</sup>I-labeled wild-type (WT) or apolipoprotein E-deficient, apolipoprotein B-48-containing (E<sup>-</sup>/B48) lipoproteins at ice-cold temperature for 4 h, and the radioactivity bound to the cells was determined as described in Materials and Methods. B, C: MPMs were incubated with 20 μg/ml <sup>125</sup>I-labeled wild-type or E<sup>-</sup>/B48 lipoproteins at 37°C. At the indicated incubation times, culture medium was collected to determine the amount of degraded <sup>125</sup>I-labeled lipoprotein particles, and cells were lysed to estimate the amount of cell-associated <sup>125</sup>I-labeled lipoproteins. D: The uptake of lipoproteins was calculated as the sum of the associated and degraded lipoproteins at an incubation time period. Values represent means ± SEM of five experiments for each time point. \* *P* < 0.05 compared with the wild-type lipoprotein treatment.

treatment were negative for Oil Red O staining, and treatment of these cells with 20 μg/ml wild-type lipoproteins did not induce detectable Oil Red O staining. However, the same concentration of E<sup>-</sup>/B48 lipoproteins induced intracellular Oil Red O-stained lipid droplets, beginning at 24 h after treatment and becoming more prominent as the exposure duration increased. Within 48 and 72 h, ~60% and 90% of the MPMs, respectively, showed Oil Red O-stained lipid droplets occupying more than one-third of

the cytoplasm, whereas the cytoplasm of some cells was 100% lipids, giving these cells a foam cell-type appearance (Fig. 2).

In this study, confocal microscopy was used to localize the cellular compartment where the lipid droplets were accumulated. The representative images in Fig. 2 illustrate that the MPMs treated with or without lipoproteins showed cytoplasmic lysosomal staining with LysoTracker. However, the extent of staining was markedly greater in the cells



**Fig. 2.** E<sup>-</sup>/B48 lipoprotein accumulation in MPMs. MPMs were incubated with 20 μg/ml wild-type (WT) or E<sup>-</sup>/B48 lipoproteins or culture medium alone (control) at 37°C for 48 h, followed by a 12 h equilibrium phase in a lipoprotein-free medium. The cytoplasmic lipid droplets were visualized with a microscope after staining with Oil Red O. For intracellular localization of the accumulated lipoproteins, MPMs were incubated with 20 μg/ml DiI-labeled wild-type or E<sup>-</sup>/B48 lipoproteins or culture medium alone (control) at 37°C for 48 h. After a 12 h equilibration in a lipoprotein-free medium, MPMs were stained with LysoTracker Green. The bright-field differential interference contrast (DIC) images and the fluorescence images of intracellular DiI and LysoTracker were captured as described in Materials and Methods. The merged images are the overlay of the differential interference contrast, LysoTracker, and DiI images. Colocalization of DiI and LysoTracker staining indicates lipoprotein accumulation in the lysosomes.

treated with E<sup>-</sup>/B48 lipoproteins than in cells without lipoprotein treatment or cells treated with wild-type lipoproteins. Cells without lipoprotein treatment did not show DiI staining. The wild-type lipoprotein-treated cells were stained slightly by DiI in the cytoplasm (Fig. 2). In contrast, the E<sup>-</sup>/B48 lipoprotein-treated MPMs were stained significantly by DiI, and most of the DiI-stained lipid droplets overlapped the LysoTracker-labeled spots (Fig. 2), suggesting that the lipids accumulated in the cytoplasm were located in the lysosomes.

### E<sup>-</sup>/B48 lipoproteins downregulate lysosomal hydrolases in MPMs

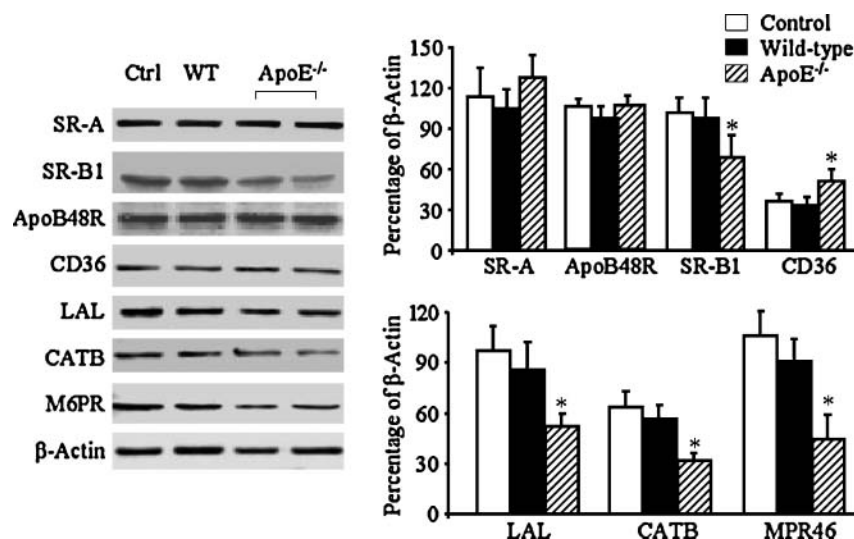
Having demonstrated a reduced degradation of E<sup>-</sup>/B48 lipoproteins in MPMs, we investigated whether E<sup>-</sup>/B48 lipoproteins alter the protein level of lysosomal hydrolases, which degrade the lipid and protein components of lipoproteins. We found that incubation of MPMs with 20 μg/ml wild-type lipoproteins did not significantly alter the protein level of cathepsin B and lysosomal acid lipase; however, the same concentration of E<sup>-</sup>/B48 lipoproteins significantly reduced the protein levels of these enzymes (Fig. 3). These data suggest that reduction in lysosomal hydrolases might be at least partially responsible for the reduced degradation of E<sup>-</sup>/B48 lipoproteins in MPMs. The data in Fig. 3 also indicate that E<sup>-</sup>/B48 lipoproteins significantly reduced the protein level of MPR46, a carrier protein that delivers lysosomal hydrolases from the Golgi network to endosomes and lysosomes. Reduction in MPR46 might therefore reduce the protein level of lysosomal hydrolases in endosomes and lysosomes.

A number of SRs have been suggested to mediate apoE-deficient lipoprotein endocytosis and to play a role in foam

cell formation (8). The present study examined the effect of lipoproteins on SR-A, apoB-48R, SR-BI, and CD36 expression. E<sup>-</sup>/B48 lipoproteins regulated the protein levels of various SRs selectively. For example, incubation of MPMs with E<sup>-</sup>/B48 lipoproteins did not alter the protein levels of SR-A and apoB-48R but significantly increased CD36 and reduced SR-BI protein levels (Fig. 3). In contrast, wild-type lipoproteins did not significantly alter the protein levels of these SRs in MPMs (Fig. 3).

### ApoE attenuates E<sup>-</sup>/B48 lipoprotein-, but not oxidized lipoprotein-, induced cellular events

In this report, we observed that the lipoproteins obtained from *ApoB<sup>48/48</sup>/ApoE<sup>-/-</sup>* mice contained more oxidized lipids than those obtained from wild-type mice ( $1.81 \pm 0.49$  vs.  $0.75 \pm 0.24$  nM TBARS/mg protein;  $P < 0.05$ ). To study whether lipoproteins alter lysosomal hydrolase expression and cellular lipid contents via increasing oxidative modifications, we treated MPMs with CuSO<sub>4</sub>-oxidized wild-type lipoproteins. At 20 μg/ml, the mildly oxidized lipoproteins (containing 3.4 nM TBARS/mg protein) barely altered the levels of macrophage lysosomal acid lipase, cathepsin B, MPR46, cholesteryl esters, and triglycerides within 48 h (Table 1). More severely oxidized lipoproteins (containing 8.7 nM TBARS/mg protein) increased cellular lipid contents but did not significantly alter the protein levels of lysosomal hydrolases and MPR46 in macrophages. In addition, the lipid accumulation pattern induced by oxidized lipoproteins appears to be different from that induced by E<sup>-</sup>/B48 lipoproteins. For example, oxidized lipoproteins increased not only cholesteryl esters and triglycerides but also FC in macrophages. The data in Table 1 also indicate that exogenous apoE did



**Fig. 3.** E<sup>-</sup>/B48 lipoprotein-induced changes in macrophage protein expression. MPMs were incubated with 20 μg/ml wild-type (WT) or E<sup>-</sup>/B48 lipoproteins or culture medium alone [control (Ctrl)] for 12 h. Left panel: Representative Western blots. Right panels: Levels of the indicated proteins expressed as the percentage of their immunoblot intensity relative to β-actin. Values represent means ± SEM of six separate experiments. \*  $P < 0.05$  compared with the control. ApoB-48R, apolipoprotein B-48 receptor; CATB, cathepsin B; LAL, lysosomal acid lipase; MPR46, cation-dependent mannose 6 phosphate receptor; SR-A, scavenger receptor class A; SR-BI, scavenger receptor class B type I.

TABLE 1. Effects of E<sup>-</sup>/B48 lipoproteins and oxidized lipoproteins on macrophage lipid contents and protein levels

Sample	Esterified Cholesterol	Free Cholesterol	Triglycerides	Lysosomal Acid Lipase	Cathepsin B	Cation-Dependent Mannose 6 Phosphate Receptor
	<i>μg/mg protein</i>	<i>% β-actin</i>				
Control	82 ± 18	149 ± 14	224 ± 26	103 ± 17	72 ± 9	96 ± 11
WT	95 ± 21	168 ± 19	267 ± 35	91 ± 21	69 ± 11	84 ± 16
OxWTm	103 ± 27	156 ± 15	269 ± 41	97 ± 22	76 ± 14	91 ± 13
OxWT	142 ± 27 <sup>a</sup>	242 ± 37 <sup>a</sup>	312 ± 46 <sup>a</sup>	123 ± 31	75 ± 8	79 ± 15
OxWT + apoE	204 ± 25 <sup>a,b</sup>	273 ± 32 <sup>a</sup>	378 ± 53 <sup>a</sup>	108 ± 29	67 ± 9	90 ± 13
E <sup>-</sup> /B48	257 ± 42 <sup>a</sup>	165 ± 24	486 ± 13 <sup>a</sup>	58 ± 16 <sup>a</sup>	31 ± 8 <sup>a</sup>	43 ± 7 <sup>a</sup>
E <sup>-</sup> /B48 + apoE	93 ± 19 <sup>b</sup>	172 ± 18	294 ± 87 <sup>b</sup>	97 ± 8 <sup>b</sup>	64 ± 8 <sup>b</sup>	104 ± 18 <sup>b</sup>

Mouse peritoneal macrophages were incubated with culture medium alone (control) or with 20 μg/ml native wild-type (WT), mildly oxidized wild-type (OxWTm), severely oxidized wild-type (OxWT), or native apolipoprotein E-deficient, apolipoprotein B-48-containing (E<sup>-</sup>/B48) lipoproteins in the presence or absence of mouse apolipoprotein E (apoE; 1.6 μg/ml) at 37°C for 48 h. After a 12 h equilibration in a lipoprotein-free medium, cells were lysed for the determination of cellular esterified cholesterol, free cholesterol, triglycerides, lysosomal acid lipase, cathepsin B, and cation-dependent mannose 6 phosphate receptor. Values represent means ± SEM of five separate experiments.

<sup>a</sup> *P* < 0.05 compared with the control.

<sup>b</sup> *P* < 0.05 compared with E<sup>-</sup>/B48 or oxidized lipoprotein treatment alone.

not reduce but aggravated oxidized lipoprotein-induced lipid accumulation (e.g., significantly increased cholesteryl ester and slightly increased FC and triglyceride levels in macrophages). Exogenous apoE, however, inhibited E<sup>-</sup>/B48 lipoprotein-induced changes in a concentration-dependent manner. We observed that apoE at a concentration of 0.8 μg/ml reduced ~60% of E<sup>-</sup>/B48 lipoprotein-induced cholesteryl ester accumulation in macrophages (data not show). When the concentration of apoE reached 1.6 μg/ml, E<sup>-</sup>/B48 lipoprotein-induced cholesterol and triglyceride accumulation vanished completely, and the lysosomal hydrolase and MPR46 levels remained unchanged at the control level (Table 1). These data suggest that E<sup>-</sup>/B48 and oxidized lipoproteins alter macrophage lysosomal hydrolase expression and lipid contents via different mechanisms.

### High-fat diet induces foam cell formation

In *ApoE*<sup>-/-</sup> mice fed a normal chow diet, the total cholesterol content in the peritoneal macrophages was ~183 μg/mg protein, and the EC/FC ratio was ~0.54 (Fig. 4). Feeding *ApoE*<sup>-/-</sup> mice a high-fat diet increased the macrophage total cholesterol content to ~393 μg/mg protein, which results entirely from an increased EC level. The FC level in the macrophages obtained from the high-

fat diet-fed mice was slightly lower than that obtained from the normal chow diet-fed mice (Fig. 4). An increase in EC and no change in FC raised the EC/FC ratio to ~1.8. The data in Fig. 4 also indicate that *ApoE*<sup>-/-</sup> mice on a high-fat diet significantly increased macrophage triglyceride content. These data suggest a reduced hydrolysis of cholesteryl esters and triglycerides in the macrophages obtained from the high-fat diet-fed mice.

To establish whether foam cell formation in vivo alters protein expression as occurred in vitro, we examined the effect of a high-fat diet on lysosomal hydrolase expression in MPMs. As the data in Fig. 4 show, the peritoneal macrophages obtained from *ApoE*<sup>-/-</sup> mice fed a high-fat diet had significantly reduced protein levels of lysosomal acid lipase and cathepsin B compared with cells obtained from *ApoE*<sup>-/-</sup> mice fed normal chow. These observations imply that E<sup>-</sup>/B48 lipoproteins are capable of suppressing lysosomal enzyme expression in macrophages in vivo.

### DISCUSSION

Under physiological conditions, LDL contains one molecule of apoB-100, whereas chylomicron remnants contain multiple molecules of apoE and a single apoB-48. The

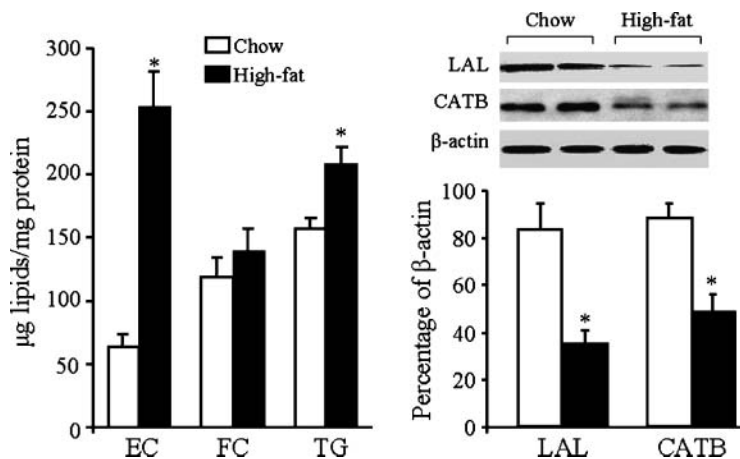


Fig. 4. High-fat diet-induced changes in cholesterol contents and lysosomal hydrolase levels in MPMs. Peritoneal macrophages were obtained from *ApoE*<sup>-/-</sup> mice fed high-fat or normal chow diets as described in Materials and Methods. Left panel: Lipids were extracted from the cells for the determination of total cholesterol, free cholesterol (FC), and triglycerides (TG). The amount of esterified cholesterol (EC) was calculated as the difference between the total cholesterol and FC. Right panels: Protein extracts were prepared from the peritoneal macrophages, and Western blot analysis was performed as described in Materials and Methods. The levels of the indicated proteins were expressed as the percentage of their immunoblot intensity relative to β-actin. Values represent means ± SEM of six separate experiments. \* *P* < 0.05 compared with mice fed normal chow. CATB, cathepsin B; LAL, lysosomal acid lipase.

apoB-100 and apoE molecules, by binding to cell surface LDLR and LDLR-related proteins, initiate receptor-mediated endocytosis (for review, see Ref. 18). Endocytosis of native apoB-containing lipoproteins barely transforms macrophages to foam cells; however, once modified by oxidation or acetylation, apoB-carrying lipoproteins gain the ability to trigger foam cell formation. It has been suggested that oxidized or acetylated apoB-carrying lipoproteins reduce their affinity for LDLR and LDLR-related proteins but have increased affinity for SRs. An uncontrolled uptake through SR-mediated pathways is a possible mechanism by which modified apoB-containing lipoproteins induce foam cell formation. Evidence from the present study and previous reports (5, 6) indicates that without oxidation or acetylation, the apoE-deficient, apoB-48-containing lipoproteins can transform macrophages to foam cells. A number of SRs, such as SR-A, SR-BI, CD36, Lox-1, VLDL receptors, and apoB-48R, have been suggested to mediate the endocytosis of apoE-deficient lipoproteins (8, 9). Data from the current study demonstrate that the effect of E<sup>-</sup>/B48 lipoproteins varied with SR isoforms. Incubation of MPMs with E<sup>-</sup>/B48 lipoproteins did not alter the protein level of SR-A and apoB-48R. However, E<sup>-</sup>/B48 lipoproteins significantly increased the protein level of CD36 and reduced SR-BI in MPMs. In addition, we observed that the ability of MPMs to take up wild-type and E<sup>-</sup>/B48 lipoproteins was comparable. These observations suggest that apoE deficiency enables apoB-48-containing lipoproteins to trigger foam cell formation through mechanisms other than increasing lipoprotein uptake.


The present report for the first time demonstrates that the lipid droplets in E<sup>-</sup>/B48 lipoprotein-induced foam cells were located mainly in the lysosomes. We also provide evidence that the degradation rate of E<sup>-</sup>/B48 lipoproteins in macrophages declines over time and that incubation of MPMs with E<sup>-</sup>/B48 lipoproteins reduces the protein levels of lysosomal acid lipase and cathepsin B. Lysosomal acid lipase is the sole hydrolase for the cleavage of cholesteryl esters delivered to the lysosomes, whereas cathepsin B is one of the lysosomal proteases for the degradation of endocytic proteins, including the protein components of lipoproteins. It is highly likely that interaction of macrophages with E<sup>-</sup>/B48 lipoproteins reduces the protein level of lysosomal hydrolases, which in turn reduces the degradation of the lipid and protein components of E<sup>-</sup>/B48 lipoproteins, leading to lipoprotein accumulation in the lysosomes and inducing foam cell formation. This presumption was supported by published evidence that superimposition of a lysosomal acid lipase mutation onto an *ApoE*-deficient background accelerated the development of atherosclerosis in mice (19), whereas intravenous administration of recombinant human lysosomal acid lipase into LDLR-deficient mice delayed the development of atherosclerotic lesions (20). Paradoxically, macrophages in the atherosclerotic lesions have been shown to increase the secretion of cathepsins in *ApoE*-deficient mice (21). The increased extracellular cathepsins are believed to play a role in the degradation of extracellular matrix during atherogenesis (22), although the mechanism un-

derlying the increased cathepsin secretion in atherosclerotic lesions has not been defined. Data from the present report indicate that E<sup>-</sup>/B48 lipoproteins reduce the protein level of MPR46, which delivers hydrolases to endosomes and lysosomes. Reduction in MPR46 may alter the distribution of cathepsin B and other lysosomal hydrolases: reducing their levels in the endosomes and lysosomes and increasing their levels in the extracellular space.

Previous studies have suggested that induction of lipid accumulation in the lysosomes also is a mechanism by which oxidized LDL transforms macrophages to foam cells. For example, Hoppe, O'Neil, and Hoff (4) reported that oxidized LDL initially induced lysosomal FC accumulation. As the accumulation of oxidized LDL progressed, cholesterol distribution shifted and cholesteryl esters accumulated in the lysosome. Data from the present study and a previous report (6) indicate that lipoproteins obtained from apoE-deficient mice contained more oxidized lipids than those obtained from wild-type controls. The present report, however, demonstrates that E<sup>-</sup>/B48 lipoprotein-induced cellular events cannot be explained by the increased oxidative modification. Our data show that a CuSO<sub>4</sub>-oxidized lipoprotein, which contained 2-fold more oxidized lipids than E<sup>-</sup>/B48 lipoproteins, neither reduced lysosomal hydrolases nor increased cellular lipid contents in macrophages. Although more severely oxidized lipoproteins increased cellular lipid contents, exogenous apoE did not attenuate but aggravated oxidized lipoprotein-induced lipid accumulation in MPMs. In contrast, exogenous apoE inhibited E<sup>-</sup>/B48 lipoprotein-induced lipid accumulation and attenuated the suppressive effect of E<sup>-</sup>/B48 lipoproteins on lysosomal hydrolases and MPR46 in macrophages. These data suggest that apoE-deficient lipoproteins induce macrophage lipid accumulation via pathways different from those activated by oxidized lipoproteins.

Another important finding of this report is that the E<sup>-</sup>/B48 lipoprotein-induced cellular events *in vitro* also occurred during *in vivo* foam cell formation. Our data demonstrate that feeding *ApoE*<sup>-/-</sup> mice a high-fat diet increased cholesteryl ester but did not alter the FC level in peritoneal macrophages and that a high-fat diet reduced the protein level of macrophage lysosomal acid lipase and cathepsin B. These observations confirmed the *in vitro* findings that suppression of lysosomal hydrolases was associated with the intracellular accumulation of lipids/lipoproteins in macrophage foam cells.

In summary, the present report for the first time characterizes macrophage foam cell transformation induced by E<sup>-</sup>/B48 lipoproteins, in which lipid droplets accumulate mainly in the lysosomes. Our data also demonstrate that the degradation rate of E<sup>-</sup>/B48 lipoproteins is reduced in macrophages over time and is associated with a reduced protein level of lysosomal acid lipase and cathepsin B. In addition, E<sup>-</sup>/B48 lipoproteins reduced the protein level of MPR46 in MPMs. Moreover, exogenous apoE prevented the E<sup>-</sup>/B48 lipoprotein-induced suppressive effect on lysosomal hydrolases and MPR46. In our *in vitro* studies, we observed that the protein level of macrophage lysosomal

hydrolases was reduced in the high-fat diet-fed *ApoE*<sup>-/-</sup> mice. These novel findings suggest that reducing the synthesis of lysosomal hydrolases and/or reducing the delivery of these enzymes to lysosomes might be mechanisms by which E7/B48 lipoproteins transform macrophages to foam cells. 

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