Cyclic AMP Induces Apolipoprotein E Binding Activity and Promotes Cholesterol Efflux from a Macrophage Cell Line to Apolipoprotein Acceptors

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RAW 264 mouse macrophage cells were stably transfected with human apolipoprotein E (apoE) expression vectors. Clonal derivatives were characterized for expression of the human apoE2, apoE3, and apoE4 isoforms. An apoE4-expressing clonal cell line and a non-expressing clonal control cell line were loaded overnight with either [3H]cholesterol or [3H]choline. The cells were washed and incubated for 24 h in serum-free medium with or without the addition of 8-bromo-cyclic AMP (8-Br-cAMP). Only the apoE-secreting cells and only in the presence of 8-Br-cAMP released large amounts of labeled cholesterol or phosphatidylcholine into the medium. Mass analyses of cellular free and esterified cholesterol confirmed the results of the labeling studies; a decrease in cellular cholesterol content was observed in the 8-Br-cAMP-treated apoE-secreting cells, concurrent with an increase in cholesterol found in the medium. FPLC analysis of the medium demonstrated that 8-Br-cAMP treatment of the apoE-secreting cells led to an increased size fraction and amount of a peak of secreted cholesterol which co- migrated with apoE. The 8-Br-cAMP-mediated increase in cholesterol efflux was also observed in non-apoE-secreting cells incubated with exogenous apoE or apoAI, and the effect of apoE was saturable. The apoE2, apoE3, and apoE4 isoforms were equally efficient in promoting 8-Br-cAMP-dependent cholesterol efflux. Reductive methylation of apoE abolished its ability to promote 8-Br-cAMP-dependent cholesterol efflux. Brefeldin A and monensin, inhibitors of protein processing through the Golgi, both blocked the 8-Br-cAMP stimulation of cholesterol efflux to exogenous apoE. 8-Br-cAMP induced specific apoE and apoAI binding, but not apoE degradation, by the RAW cells. We present a model wherein cAMP induces a membrane apolipoprotein receptor that does not lead to endocytosis and degradation, but instead promotes the transfer of lipids to apolipoproteins, which can then be released from the cell.

During atherogenesis, peripheral blood monocytes traverse the arterial endothelium and differentiate into mature tissue macrophages. During this differentiation, macrophages induce

the expression of both the scavenger receptor and apolipoprotein E (apoE)\(^1\) \(1, 2\). The scavenger receptor mediates the binding and uptake of modified lipoproteins leading to an accumulation of cellular cholesterol, which is stored primarily as cholesterol esters \(3\). When macrophages in the arterial intima become cholesterol laden, they are referred to as foam cells, due to their characteristic appearance in histologic specimens. The presence of clustered foam cells in a fatty streak is the earliest, and reversible, stage of atherosclerosis, which occurs even during adolescence \(4\). ApoE is a ligand necessary for uptake by the LDL receptor and the LDL receptor related protein and can mediate the macrophage binding and uptake of LDL, VLDL, and \(\beta\)-VLDL \(5, 6\), and could therefore mediate macrophage cholesterol accumulation. On the other hand, apoE secretion by macrophages could play a role in cholesterol removal from macrophages. This latter hypothesis is attractive due to the observed up-regulation of macrophage apoE mRNA, synthesis, and secretion by cholesterol loading \(7, 8\). It has been proposed that apoE may function directly in promoting the efflux of macrophage cholesterol, or it may function indirectly by binding to high density lipoprotein (HDL), which acts as an acceptor of macrophage cholesterol, and targets it for uptake by the liver \(9\). The role of macrophage apoE secretion is not well understood; however, recent experiments using transgenic mice that express apoE only in macrophages prove that macrophage apoE is antiatherogenic, although the mechanism of the protective effect of apoE was not elucidated \(10\).

Basu et al. \(9\) reported in 1983 that apoE and cholesterol are secreted by independent pathways in mouse peritoneal macrophages. This conclusion was based on two observations; 1) cholesterol efflux occurs only when an exogenous acceptor is present while apoE secretion is constitutive, and 2) cholesterol efflux occurs at a dose of the protein secretion inhibitor monensin that significantly reduces immunoprecipitable apoE secretion \(9\). However, lipid free apoE and apoAI have been shown to act as acceptors of cholesterol from cholesterol-loaded macrophages \(11\). In the present study a novel model system was created to reevaluate the role and mechanism of apoE in macrophage cholesterol efflux. The RAW 264 mouse macrophage cell line, which expresses the scavenger receptor \(12, 13\), but does not express endogenous apoE \(14\), \(^2\) was stably trans-
fectcd with apoE expression vectors. These apoE-secreting cells, when cholesterol-loaded, secreted increased cholesterol or phospholipid into medium devoid of any exogenous cholesterol acceptors, but only in the presence of a cAMP analogue. ApoE can exert its effect on cholesterol efflux when supplied exogenously to RAW cells in the presence of 8-Br-cAMP. Our data suggest a model that CAMP induces a cellular apoE receptor, which we propose to mediate the assembly of lipids with apoE and the subsequent release of the lipoprotein particle.

MATERIALS AND METHODS

Plasmid Construction—The human apoE expression vector was derived from plasmid pEA 10 from which the apoE λ clone (15). A HindIII linker was inserted into the first exon at the unique AatII site at position +24 relative to the start of transcription. DNA from this artificial HindIII site to the EcoRI site 626 base pairs 3′ of the apoE polyadenylation site was cloned into pUC18. A 343-base pair PvuII to HindIII fragment of SV40, containing the viral early promoter and enhancer, was cloned into the artificial HindIII site of the apoE plasmid using HindIII linkers. ApoE2 and apoE3 derivatives were generated by polymerase chain reaction (PCR) mutagenesis of the apoE4 expression plasmid and confirmed by DNA sequencing.

Stable Transfection of RAW 264 Cells—RAW 264.7 cells obtained from American Type Culture Collection were plated into 55-mm culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Cells were transfected with the apoE expression plasmid and 5 μg of pSV2neo (16) using the calcium phosphate method, as described previously (17). After 4 h of incubation with the DNA solution, the cells were shocked with 30% dimethyl sulfoxide in phosphate-buffered saline (PBS) for 4 min at room temperature. The cells were washed and fed with growth medium. On the following day, the cells were treated with 800 μg/ml Geneticin (Life Technologies, Inc.) to select for cells stably integrating the neo+ gene. Two days later, the cells were trypsinized and plated into 100-mm culture dishes and fed with the selection medium. Individual colonies were picked 7–10 days later and expanded for analysis of apoE gene incorporation and expression. Initial analysis of genomic DNA prepared fromneo+ clones was by PCR specific for the human apoE gene using primers E7 and E9 as described previously (18). Expression of human apoE mRNA was analyzed by an RNase protection assay (19). Confirmation of ubiquitous apoE expression by the clonal cell lines was obtained by immunohistochemistry as described below.

ApoE Immunoassays—Human apoE ELISA assays were performed with the use of Trasylol, Rockville, MD, using human apoE serum standards provided by Dr. Peter Alapoukie (Oklahoma Medical Research Foundation). Immunohistochemistry for human apoE was performed on adherent cells fixed in methanol for 2 min; all incubations were performed at room temperature. Cells were treated with casein blocking buffer (Pierce) for 10 min, followed by a 60-min incubation with the goat anti-human apoE antisera (INCstar, 1:100). After washing, the cells were incubated with horseradish peroxidase-conjugated mouse anti-goat IgG (1:250). Staining was done with the 1-Step chloronaphthol reagent (Pierce). The dot blot immunassay was performed by spotting 5μl of each sample and known dilutions of the human apoE standard onto nitrocellulose. The blots were blocked and then probed with goat anti-human apoE antisera (1:1000). After washing, the blots were incubated with horseradish peroxidase-conjugated mouse anti-goat IgG (1:5000). The washed blots were developed in enhanced chemiluminescent substrate (ECL, Amersham) and exposed to x-ray film. The films were scanned by laser densitometry and the concentrations of apoE were determined by comparison with the known standards.

Radioelaboring and Immunoprecipitation of ApoE—Cells were labeled with Tran35S-label (mixture of methionine and cysteine, ICN) in methionine-free DMEM. To 1 ml of the collected medium, 50 μl of a protease inhibitor mixture (1 mg/ml aprotinin, 6 mg/ml benzamidine, 100 mM EDTA, and 0.1 mg/ml leupeptin) and 5 μl of 200 mM phenylmethylsulfonyl fluoride were added. After centrifugation to remove cellular debris, radioactivity incorporated into protein was determined by trichloroacetic acid precipitation. Equal amounts of conditioned medium were added to 0.5 ml of buffer 3 (PBS containing 0.05% Tween 20) and 3 μl of goat anti-human apoE antisera. After rocking overnight at 4°C, 50 μl of a 10% protein A-Sepharose suspension was added for an additional hour. The Sepharose beads were pelleted and washed three times with buffer 3. The immunoprecipitated protein was eluted with SDS sample buffer and run on a discontinuous 10% SDS-polyacrylamide gel. Gels were stained, destained, and then processed for fluorographs by soaking for 30 min each in water and 1% sodium salicylate (pH 6.0) (20).

[3H]Cholesterol Pulse-Chase Studies—Cells in 24-well dishes were cholesterol-loaded and -labeled overnight in 0.5 ml of DMEM supplemented with 50 μM/ml 1 μl glucose, 10 μM/ml 200 mM glutamine, and 0.5% BSA (Sigma A6003) (referred to as DGG) and with [3H]cholesterol (Amersham), which had been preincubated for 30 min at 37°C with acetyltransferase low density lipoprotein (AcLDL), to a concentration of 0.33 μCi/ml [3H]cholesterol and 50 μg/ml AcLDL. AcLDL was prepared from human LDL as described previously (21). On the following day, the cells were washed three times in PBS, 0.2% BSA and chased with 0.5–1 ml of DGG; 8-Br-cAMP (Sigma) was included as indicated. After the 24-h chase, a 100-μl aliquot of medium was taken for chemical analysis of radioactivity and the cells were dissolved in 0.5 ml of 0.2 M sodium hydroxide, and the radioactivity in an aliquot was determined. The percentage of [3H]cholesterol secreted was calculated by dividing the medium-derived counts/min/dish by the sum of the medium-derived plus cell-derived counts/min. In some experiments, cells were chased with serum-free medium conditioned by incubating for 24 h with non-cholesterol-loaded apoE-secreting cells. In other experiments, cells were chased with DGG plus apoE-secreting RAW cells; apoE was purified from the conditioned medium by heparin-Sepharose Pharmacia chromatography, as described previously (22). Purified human apolipoprotein AI (apoAI) was obtained from PerImmune. Reductive methylation of apoE-conditioned medium was performed with sodium borohydride and formic acid. The lipid extract was then subjected to heptane/diethyl ether/acidic acid (70:15:1.5) (23). The cholesterol and cholesterol olate standards migrated with Ret values of 0.22 and 0.90, respectively. The plate was divided into five zones and scraped for determination of radioactivity.

[3H]Phospholipid Pulse-Chase Studies—Cells in 24-well dishes were cholesterol-loaded and cholesterol labeled overnight in 0.5 ml of DGGB supplemented with 1.0 μCi/ml [3H]cholesterol (Amersham) and 50 μg/ml AcLDL. On the following day, the cells were washed twice in PBS, 0.2% BSA and chased with 1 ml of DGGB with or without the addition of 0.3 mM 8-Br-cAMP. After 24 h, the chase medium was extracted by the addition of four volumes of chloroform:methanol (2:1). The organic layer was dried under nitrogen, and the radioactivity was determined by liquid scintillation spectrometry. Cell-associated radioactivity determined after the cells were dissolved in 0.5 ml of 0.2 M sodium hydroxide. The percentage of secreted [3H]phosphatidylcholine and sphingomyelin was calculated by dividing the medium-derived lipid extracted (counts/min/dish) by the sum of the medium-derived plus cell-derived counts/min.

Cholesterol Mass Analysis—Cholesterol and cholesterol ester mass analyses were performed on 55-mm cultures washed with PBS, 0.2% BSA and twice with PBS. Lipids were extracted in hexane/isopropanol (3:2), and 40 μl of 1 mg/ml corospondan in dimethyl sulfoxide was added as an internal standard for sample recovery. Coprosanol standard was added to tissue culture medium before extraction in medium:methanol: chloroform (3:4:8). The solvent from ether plate or medium extraction was removed and dried under nitrogen. The organic residue was dissolved in a small volume of carbon disulfide, and free cholesterol was determined by injection into a Perkin Elmer 8500 gas chromatograph with a 6-meter column packed with OV-17 and 3% WHP 160/120. Total cholesterol was determined in a similar manner after saponification of the lipid extract in tetramethylammonium hydroxide for 1 h at 80°C. Cholesterol esters were calculated as the difference between the total and free cholesterol. The protein content of the extracted plates was determined by a modified alkaline Lowry procedure (26).

FPLC Analysis of Conditioned Media—ApoE-secreting and control cells were cultured in p150 dishes and cholesterol-loaded and -labeled overnight by incubation with 50 μg/ml AcLDL and 0.33 μCi/ml [3H]cholesterol as described in DGG. Cells were then chased for 24 h in 20 ml of DGGB with or without the addition of 0.3 mM 8-Br-cAMP. The chase media from two dishes were pooled and concentrated to 1.5 ml with a Centriprep 10 (Millipore). 0.5-ml aliquots were separated by FPLC using two Superose 6 (Pharmacia) columns in series and eluted at 0.3 ml/min with 1 mM EDTA in 0.15 M sodium chloride. 1-ml fractions were collected, and [3H]cholesterol radioactivity was determined in 0.1-ml aliquots. The
apoE concentration in each fraction was determined by the quantitative immunodot blot assay, described above.

**ApOE and ApoAI Binding, Association, and Degradation Studies**—50 μg of apoE2 (PerImmune) in 0.1 M sodium borate was iodinated with 250 μCi of 125I-Bolton-Hunter reagent (DuPont NEN), according to the manufacturer’s specifications. The radiolabeled apoE was purified by Sephadex G-50 chromatography, in a column preabsorbed with 0.1% gelatin. The specific activity of the 125I-apoE2 was 3717 cpm/μg apoE, determined by γ counting and the apoE ELISA assay. Binding was performed in 24-well dishes, which were preincubated overnight with 50 μg/ml AcLDL in DGBB with or without the addition of 0.3 mM 8-Br-cAMP. On the following day the cells were put on ice, washed twice with PBS, 0.2% BSA, and incubated on ice for 1 h with 50–500 ng/ml 125I-apoE2 in the absence (total binding) or presence (nonspecific binding) of 50 μg/ml unlabeled apoE2 (purified from apoE2-secreting cells). The cells were washed once in cold PBS, 0.2% BSA and twice in cold PBS, and dissolved in 0.5 ml of 0.2 N sodium hydroxide. 125I radioactivity was determined from a 0.1-ml aliquot, and cell protein was determined on a 20-μl sample using the alkaline modification of the Lowry assay (26). Specific binding was calculated as the total binding minus the nonspecific binding, and was normalized per mg of cell protein. Cellular association and degradation of 125I-apoE2 was determined in cells preincubated with AcLDL and ± 8-Br-cAMP as above. Cells were incubated for 3 h at 37°C with 200 ng/ml 125I-apoE2 in the absence or presence of both 0.3 mM 8-Br-cAMP and 20 mg/ml unlabeled apoE2. Cellular association of 125I-apoE was determined after solubilizing the cells in 0.5 ml of 0.2 N sodium hydroxide, and was normalized per milligram of cell protein. 125I-ApoE degradation was determined from the 125I-tyrosine recovered in the media, by a trichloroacetic acid precipitation protocol in which free 125I was excluded (27). Specific uptake and degradation were calculated by subtracting the nonspecific uptake and degradation measured in the presence of 100-fold excess unlabeled apoE2. ApoAI was purified from human HDL and labeled with 125I by the iodine monochloride method, as described previously (28). The specific activity was determined by γ counting and by an apoAI turbidimetric assay (INCell). ApoAI binding was performed as above with 500 ng/ml 125I-apoAI in the absence or presence of 20 μg/ml amounts of various apolipoprotein competitors. Trypsin treatment (250 μg/ml) was performed immediately prior to binding for 15 min at 37°C, followed by extensive PBS washing.

**Statistical Analysis**—Tests of significance and linear regression analysis were performed using the InStat statistical software program from GraphPad Software (San Diego, CA).

## RESULTS

RAW 264 cells were co-transfected, as described above, with human apoE expression vectors driven by the SV40 promoter and a neomycin resistance plasmid. Stably transfected cells expressing the neomycin resistance gene were selected in medium containing 800 μg/ml Geneticin. In order to obtain a population of cells that uniformly made apoE, individual colonies were picked, expanded, and screened for the presence of the human apoE gene by PCR analysis of genomic DNA. Expression of human apoE mRNA was analyzed by an RNase protection assay, and secretion of apoE into the medium was analyzed by ELISA. Four clonal lines were selected and characterized: 1) a line serving as a negative control, which was transfected with the neomycin resistance gene alone and did not express apoE; 2) a line expressing apoE4; 3) a line expressing apoE3; and 4) a line expressing apoE2. ApoE immunohistochemistry revealed the absence of staining of the control clone and the uniformly positive apoE staining of the apoE-expressing cell lines (data not shown). Cells were loaded for 4 h with 150 μCi/ml [35S]methionine plus cysteine, and the total secreted proteins along with immunoprecipitated human apoE were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Only the apoE-secreting cells yielded labeled immunoprecipitated protein that migrated as a doublet of approximately 35.5 and 34 kDa, presumably due to glycosylation (data not shown).

For cholesterol pulse-chase studies, the control and apoE4-secreting cells were labeled by incubating overnight in serum-free medium with 50 μg/ml human AcLDL that had been preincubated with [3H]cholesterol. Upon a subsequent 24-h chase period in serum-free, BSA-containing medium without the addition of 8-Br-cAMP, there was no difference in cholesterol efflux between cell types (Fig. 1A). However, if the cells were treated with 0.3 mM 8-Br-cAMP, there was a dramatic increase in cholesterol efflux from the apoE4-secreting cells. The apoE- and 8-Br-cAMP-dependent effect on cholesterol efflux in pulse-chase studies was observed consistently in multiple independent experiments performed over a 3-year period. This effect was also observed in independently isolated clones of apoE3- and apoE2-expressing stably transfecteds. The absolute percent of cholesterol efflux from the apoE4-secreting cells in the basal and 8-Br-cAMP-treated states was variable, with 8-Br-cAMP effect ranging from 2-fold to over 8-fold. The identity of the labeled material released by the [3H]cholesterol-loaded apoE-secreting cells into the chase medium was investigated. Over 96% of the radioactivity was recovered in the organic fraction after extraction. This organic extracted material was subjected to silica gel thin layer chromatography, and greater than 96% of the radioactivity migrated in the free cholesterol zone.

Many control experiments were performed to probe the specificity of the apoE and 8-Br-cAMP response. We demonstrated that the active cAMP analogues dibutyryl-cAMP and 8-chlorophenylthio-cAMP were as effective as 8-Br-cAMP in promoting cholesterol efflux from the apoE4-secreting cells, while 8-Br-cGMP and two inhibitors of ACAT (58–035 and C1–976) had little effect (data not shown). The apoE and 8-Br-cAMP effect on cholesterol efflux was not dependent upon the addition of
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BSA to the chase medium, although the fold effect was lower in the absence of BSA (data not shown). BSA, presumably by blocking nonspecific protein binding sites on the culture dishes, led to increased accumulation of apoE in the medium, which varied with different lots of BSA. Treatment with 0.3 mM 8-Br-cAMP, in an experiment that led to a 4-fold increase in [3H]cholesterol efflux, had no effect on apoE levels in the chase medium (14 ± 2 µg/ml). In dose-response experiments, the maximal dose of 8-Br-cAMP in promoting cholesterol efflux from apoE-secreting cells was determined to be 0.1–0.3 mM, and the half-maximal effect was calculated to be at approximately 40 µM (data not shown). Progesterone, which is reported to block cholesterol translocation from lysosomes (29), decreased the basal level of cholesterol efflux by 30%, but did not inhibit the increased efflux mediated by 8-Br-cAMP (data not shown).

Phospholipid efflux was determined in experiments where cells were labeled with [3H]choline and chased in serum-free conditions in the presence or absence of 8-Br-cAMP. Organic solvent extraction of the chase media was used to determine radioactivity in phosphatidylcholine and sphingomyelin. Similar to the cholesterol efflux effect, there was a large 8-Br-cAMP-dependent increase in phospholipid efflux from the apoE-secreting cells, but not from the control cells. (Fig. 1B). Phospholipid efflux from apoE-secreting cells was stimulated by 8-Br-cAMP even if the cells were not cholesterol-loaded, although the stimulation fold was reduced by 19% compared with AcLDL-loaded cells (data not shown).

In addition to determining the efflux of labeled cholesterol, we also directly measured the mass of free and esterified cholesterol in apoE-secreting cells with or without 8-Br-cAMP treatment. ApoE-secreting cells grown in 10% FCS contained approximately 15 µg of free cholesterol/mg of cell protein, and less than 1 µg/mg of cholesterol esters. ApoE-secreting cells were cholesterol-loaded by overnight incubation with 50 µg/ml AcLDL, and then chased for an additional 24 h with or without the addition of 0.1 mM 8-Br-cAMP (Fig. 2). AcLDL incubation dramatically increased the levels of both free and esterified cholesterol. The cellular free, esterified, and total cholesterol were less in the 8-Br-cAMP chased cells compared with the control chased cells. These decreases occurred in the 8-Br-cAMP-treated cells without a substantial change in the ratio of free to esterified cholesterol (1.16 versus 1.19 for control and treated cells, respectively). The medium free cholesterol was also determined, revealing a substantial accumulation of cholesterol mass only in medium from the treated cells (18.4 ± 2 µg/mg cell protein), an amount roughly equal to the decrease in cellular total cholesterol comparing 8-Br-cAMP-treated cells with untreated cells (16.4 ± 2 µg/mg cell protein). The sum of the total cellular and medium cholesterol was similar for the control and treated cells. While the control cells secreted only approximately 1% of their total cholesterol during the 24-h chase period, the 8-Br-cAMP-treated cells secreted close to 20% of their total cholesterol. These cholesterol mass determinations confirmed both the results and the magnitude of the response of the [3H]cholesterol labeling studies.

The nature of the secreted apoE and cholesterol in the serum-free media from control cells (non-apoE-secreting) and apoE-secreting cells in the absence or presence of 8-Br-cAMP was probed by subjecting concentrated [3H]cholesterol chase media to FPLC size exclusion chromatography (Fig. 3). The basal cholesterol efflux in control cells and in apoE-secreting cells not treated with 8-Br-cAMP, was found in two small peaks, the first at the void volume of the column (elution ml 17), and the second in a fraction slightly smaller than human HDL (ml 36). ApoE from the cells not treated with 8-Br-cAMP was heterogeneous in size (ml 30 through 40). 8-Br-cAMP treatment of the apoE-secreting cells led to no change in the void volume cholesterol fraction, but did lead to a dramatic rise in the amount of cholesterol recovered in a fraction corresponding to the size of large HDL (ml 30). ApoE shifted to a slightly larger and less heterogeneous distribution (ml 27–35), and the peak of apoE corresponded to the new cholesterol peak (ml 30), suggesting that this cholesterol is associated with apoE. The
invariant cholesterol peak found in the void volume appears to be due to large membrane fragments, as observed by negative staining transmission electron microscopy (data not shown), as previously characterized from macrophage cultures in the laboratory of H. Kruth (30). The cholesterol peak which eluted at ml 36 from the apoE-secreting cells in the absence of 8-Br-cAMP is probably not apoE-associated, as the identical peak was observed in the control cells.

We considered two hypotheses for the role of apoE in the apoE- and cAMP-dependent cholesterol efflux. 1) ApoE acts intracellularly only within the cells that synthesize it, perhaps by affecting membrane trafficking and/or by complexing with cholesterol for co-secretion from the cell; and 2) apoE is secreted and acts extracellularly as a cholesterol acceptor. To test these hypotheses, we chased [3H]cholesterol-loaded control cells in serum-free medium conditioned by apoE4-secreting cells. The apoE-conditioned medium led to a dramatic increase in cholesterol efflux only in the presence of 8-Br-cAMP (Fig. 4A). We also partially purified apoE from the conditioned medium by heparin Sepharose chromatography. This material which was >50% apoE by SDS-PAGE, and whose major contaminant was BSA, was also effective in promoting cholesterol efflux in an 8-Br-cAMP-dependent fashion, and the apoE3 and apoE2 isoforms were as effective as apoE4 (Fig. 4A). The transcriptional inhibitor actinomycin D, added to the 24-h chase medium at 50 ng/ml, blocked the 4-fold 8-Br-cAMP-mediated increase in [3H]cholesterol efflux from control cholesterol-loaded cells to apoE-conditioned medium (Fig. 4B). The apoE dose response of this effect was examined using purified apoE4 (Fig. 4C). ApoE led to a dose-dependent increase in 8-Br-cAMP-mediated cholesterol efflux, which was saturable at about 20 μg/ml apoE. Therefore, we accepted the second hypothesis, that apoE promoted cholesterol efflux by its activity as an extracellular acceptor. However, we were still left with the question of what the 8-Br-cAMP was doing that allowed for the apoE to act as a lipid acceptor.

To further characterize the composition of the secreted apoE, apoE2 was partially purified from apoE2-secreting cells that had been cholesterol-loaded and chased in the absence or presence of 8-Br-cAMP. There was only a trace of cholesterol in the apoE purified from the control-treated cells, as the cholesterol to apoE mass ratio averaged 2.3% ± 1.2% (n = 3). However, the apoE purified from the 8-Br-cAMP-treated cells contained significantly higher levels of cholesterol, comprising 55% ± 27% of the apoE mass (n = 3, p < 0.03 by a two-tailed t test). Similarly, the phospholipid to apoE mass ratio was increased over 10-fold in the apoE purified from the 8-Br-cAMP-treated cells. The apoE purified from control and 8-Br-cAMP-treated cells contained 6.9% ± 2.6% and 83.9% ± 35.4% phospholipid to apoE mass ratio, respectively (n = 3, p < 0.02).

The time course of the 8-Br-cAMP effect was examined in [3H]cholesterol-loaded control cells chased in DGGB alone or in the presence of purified apoE4, apoAI, or HDL (Fig. 5). Large amounts of cholesterol efflux from the apoE- and apoAI-chased cells occurred only in the presence of 8-Br-cAMP. There was >6 h of time lag before the effect of 8-Br-cAMP was observed, with the effect just starting by 8 h, and fully expressed by 24 h. HDL supported two types of cholesterol efflux, 8-Br-cAMP-independent and -dependent cholesterol efflux. The 8-Br-cAMP-dependent portion of cholesterol efflux to HDL had a time course identical to that for efflux to purified apoE and apoAI. Cholesterol efflux from [3H]cholesterol-loaded control cells was also increased in a dose-dependent manner by incubation with small unilamellar vesicles of egg phosphatidylcholine; however, this efflux was not further increased by 8-Br-cAMP treatment (data not shown).

The saturability of exogenous apoE in promoting 8-Br-cAMP-dependent cholesterol efflux (Fig. 4B) led us to wonder whether apoE might be acting via interaction with a cellular receptor. ApoE4-conditioned medium was modified by reductive methylation of lysine residues using sodium borohydride and formaldehyde. This modification has been shown to inhibit apoE’s interaction with the LDL receptor (23). Before modification, this batch of apoE4-conditioned medium gave rise to a 7.4-fold 8-Br-cAMP-mediated increase in cholesterol efflux, while after reductive methylation, there was only a 1.3-fold effect of 8-Br-cAMP on cholesterol efflux. This result supports the notion that
apoE’s cholesterol accepting activity is mediated by its interaction with a cellular receptor, although other explanations of this result are possible, such as inhibition of apoE’s lipid binding activity.

Based on the notion that the cholesterol accepting activity of apoE is receptor-mediated, we developed the hypothesis that 8-Br-cAMP acts via the induction of this membrane-bound apoE receptor. We examined the effects of monensin and brefeldin A, two inhibitors of trans Golgi protein transport, which effectively block the delivery of newly synthesized proteins destined either for secretion or for residence in the plasma membrane. As expected, these inhibitors blocked 8-Br-cAMP-dependent cholesterol efflux from the apoE4-secreting cells (Fig. 6A) as well as apoE secretion (data not shown). These inhibitors also blocked 8-Br-cAMP-dependent cholesterol efflux from the control cells chased with apoE4-conditioned medium (Fig. 6B). This result supports the notion that the 8-Br-cAMP effect on cholesterol efflux to exogenous apoE is mediated by a newly synthesized secreted or plasma membrane protein.

The effect of 8-Br-cAMP on apoE binding, association, and degradation by control cells was determined directly by iodination of commercially obtained, baculovirus-derived, purified apoE2. We first determined that this material, similar to apoE-conditioned medium or our own partially purified apoE, was capable of promoting 8-Br-cAMP-dependent cholesterol efflux from control cells (data not shown). We performed 125I-apoE2 binding experiments at 0 °C to cells which had been cholesterol-loaded and treated in the absence or presence of 8-Br-cAMP for 24 h. The experiment shown in Fig. 7 is representative of three such experiments, all of which showed that 8-Br-cAMP led to the induction of a specific apoE2 binding activity. Nonlinear regression analysis of this experiment estimated the affinity of this receptor for apoE to be on the order of 50 nM, while the maximal binding was estimated to be 30 ng of apoE/mg of cell protein. Specific association and degradation of 125I-apoE2 by control cells pretreated with or without 8-Br-cAMP was determined by incubation at 37 °C for 3 h (Fig. 8). Specific cell association of 125I-apoE2 was increased by over 3-fold ($p < 0.002$) in the 8-Br-cAMP-treated cells, while specific degradation declined. Thus 8-Br-cAMP was shown to directly induce apoE binding and cell
association without increasing degradation. This apoE binding activity is not the LDL receptor, as apoE2 is defective in LDL receptor binding activity. In addition apoE2 worked as an extracellular cholesterol acceptor as efficiently as apoE4 (Fig. 4A). We also ruled out the LDL receptor related protein as this apoE binding activity, as lactoferrin, a competitive inhibitor of apoE binding to the LDL receptor related protein (31), did not inhibit the effect of apoE on 8-Br-cAMP-dependent cholesterol efflux (data not shown). 8-Br-cAMP also led to an almost 7-fold increase in apoAI binding, which was completely inhibited by 40-fold excess unlabeled apoAI, apoE2, apoE3, or apoE4 (Fig. 9). Thus the same cAMP inducible receptor appeared to bind both apoE and apoAI. Trypsin pretreatment led to a 70% reduction in apoAI binding, implying the protein nature of the 8-Br-cAMP induced apoAI binding activity.

\[^{3}H\]Cholesterol-loaded primary cultures of thioglycolate elicited mouse peritoneal macrophages derived from apoE-deficient mice (32) were chased with purified apoE4. ApoE4 led to a large increase in \[^{3}H\]cholesterol efflux, although this efflux was not dependent upon 8-Br-cAMP (data not shown), as it is in the RAW cells. These data confirm previous studies that purified apolipoproteins including apoE were capable of promoting cholesterol efflux from primary murine macrophages in the absence of cAMP analogues (11). Thus, the ability of apoE to act as an extracellular cholesterol acceptor is constitutive in cultured mouse peritoneal macrophages, while it is dependent upon 8-Br-cAMP in RAW cells.
its role in HDL retroendocytosis has also been proposed (41). Our analysis of this literature is that cholesterol efflux to HDL is composed of two separate components which can vary in their importance in various cell types, the passive diffusion component of cholesterol to a particle with a lower FC/PL ratio than the plasma membrane, and an apolipoprotein-mediated component. Apolipoprotein-mediated cholesterol efflux may be constitutively expressed in some cells and stimulated in other cells by activators of protein kinase A or C, and can be blocked by the trans Golgi inhibitors monensin and brefeldin A (38, 42, 43). In the present study we utilize a macrophage cell line as a model for arterial foam cells, and we have focused on the apolipoprotein-mediated component of cholesterol efflux, using apoE as it is the apolipoprotein synthesized by macrophages.

The effects of apoE on cholesterol efflux have been examined previously in various cell systems. Basu et al. reported that apoE secretion and cholesterol efflux from cultured mouse peritoneal macrophages are via independent pathways, partially on the finding that cholesterol is not apparently released into the medium in the absence of an external cholesterol acceptor such as fetal calf serum, while apoE secretion is constitutive, and that monensin could inhibit apoE secretion at doses which did not block cholesterol efflux to HDL (9). However, in these experiments the amount of cholesterol released into the medium was never directly determined after the 24-h chase, nor was the concentration of apoE in the chase medium, and the effect of apoE on cholesterol efflux was not directly examined as apoE is constitutively expressed by these cells (9). The finding that monensin blocks apoE secretion at doses which do not block apparent cholesterol efflux to the fetal calf serum acceptor does not disprove that apoE can play a role in cholesterol efflux. Direct evidence that apoE can promote cholesterol efflux from mouse peritoneal macrophages was demonstrated by Hara and Yokoyama (11). They demonstrated that exogenously supplied apoE along with apoAI, and apoAI, but not apoCIII, are capable of promoting cholesterol efflux from macrophages in a dose- and time-dependent, but saturable and constitutive fashion (11). In the present RAW cell experiments, we have found conditions in which efficient cholesterol efflux to serum-free chase medium is dependent upon endogenously produced apoE, or exogenously supplied apoE or apoAI, and the presence of a cAMP analogue. The reported dose response and saturability of exogenous apoE in promoting cholesterol efflux from peritoneal macrophages (11) is very similar to our results in 8-Br-cAMP-treated RAW cells (Fig. 4A).

Similar to our cell system, Mazzone and Reardon made stably transfected J774 cell lines which secrete apoE (44). In these cells there was no effect of apoE secretion on basal cholesterol efflux, but the addition of a cAMP analogue led to a small increase in labeled cholesterol efflux (Fig. 4). The apparent reason for the small effect observed in their study compared with the large effect observed in the current study is that Mazzone’s J774 cells, with apoE expression, driven by the metallothionein IIA promoter, accumulated less than 1 μg/ml apoE in the chase medium, while our RAW cells, with apoE expression driven by the SV40 promoter, could achieve levels of >10 μg/ml apoE in the BSA-containing 24-h chase medium. In a non-macrophage system, the effect of apoE expression on stably transfected C127 (murine mammary derived) cells has been assessed (45). After a 40-h incubation in serum-free medium, the apoE-expressing cells accumulated ~10 μg/ml apoE in the chase medium and secreted significantly more phospholipids and cholesterol than the control cells; furthermore, incubating C127 cells with 3–10 μg/ml exogenous apoE led to a dose-dependent increase in labeled lipid efflux from these cells. Therefore, C127 cells, like mouse peritoneal macrophages, can constitutively release cholesterol to apoE, unlike RAW cells, and perhaps J774 cells, where cholesterol efflux is dependent upon treatment with a cAMP analogue. In addition, the importance of apoE as a cholesterol acceptor was recently demonstrated in cholesterol-labeled fibroblasts chased for 1 min with apoE-deficient murine plasma, which had only 5% of the cholesterol release compared with normal murine plasma (46).

Our data support an extracellular role for apoE in cholesterol efflux, rather than intracellular assembly and secretion of an apoE and cholesterol containing particle. Next, examining the role of the cAMP analogue in cholesterol efflux in the current study, we ruled out the possibility that 8-Br-cAMP acts solely by altering the ratio of free to esterified cholesterol. We demonstrated that two ACAT inhibitors, which greatly increased the free to esterified cholesterol ratio, failed to promote cholesterol efflux as 8-Br-cAMP did. In addition, the ratio of free to esterified cholesterol was not substantially altered by 8-Br-cAMP treatment of the apoE-secreting cells (Fig. 2).

Since 8-Br-cAMP seems not to be working via increasing cholesterol ester hydrolysis there must be other effects, either on cellular cholesterol trafficking or on the assembly of lipids with apolipoprotein acceptors. There is previous evidence for effects of cAMP on cholesterol trafficking (47, 48). However, the results of our studies support the alternative model, that 8-Br-cAMP leads to increased cholesterol efflux to apolipoprotein acceptors via the induction of an apolipoprotein plasma membrane receptor (Figs. 7–9). In addition, the inability 8-Br-cAMP to increase cholesterol efflux to phosphatidylcholine liposomes supports our conclusion that 8-Br-cAMP leads to the induction of an apolipoprotein receptor. However, cAMP may lead to many additional cellular responses in RAW cells, including altering prostaglandin synthesis and cell morphology (49, 50). In the present system there was an 8-h time lag before 8-Br-cAMP led to increased cholesterol efflux to exogenous apoE, apoAI, or HDL (Fig. 5). This is sufficient time to allow for the initiation of transcription of a non-constitutively expressed gene. Actinomycin D completely inhibited the 8-Br-cAMP-mediated cholesterol efflux of control cells to apoE-containing chase medium, supporting the notion that the 8-Br-cAMP effect is mediated transcriptionally. In addition the ability of monensin and brefeldin A to block the 8-Br-cAMP-mediated cholesterol efflux to apoE (Fig. 6) is consistent with the activity of these inhibitors to block the insertion into the plasma membrane of a newly synthesized apolipoprotein receptor. Mendez has previously found that these inhibitors were able to inhibit cholesterol efflux from fibroblasts to HDL by 40% (42). Although Mendez’s result could be due to the inhibition of cholesterol transport to the plasma membrane, it is also possible in this case that 40% of the cholesterol efflux by fibroblasts to HDL is mediated by a plasma membrane apolipoprotein receptor, which would be sensitive to these inhibitors.

We propose that lipid efflux to apolipoprotein acceptors is mediated by this apolipoprotein receptor lipid transfer activity. Although 8-Br-cAMP is required for this activity in RAW cells, it is expressed constitutively in other cell types, such as C127 cells and primary cultures of mouse peritoneal macrophages (Refs. 11 and 45, and our results). Whether this activity is constitutive or inducible in arterial macrophages is not known. We do not know at this time whether this receptor activity is similar to the previously characterized putative HDL receptor (39) or the recently characterized scavenger receptor B1, which can mediate selective lipid transfer from HDL to cells (51).

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