Characterization of Apolipoprotein-Mediated HDL Generation Induced by cAMP in a Murine Macrophage Cell Line†

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ABSTRACT: Murine macrophage RAW264 were investigated for their response to lipid-free apolipoproteins. Preincubation of the cells with 300 μM dibutyryl cyclic (dBc) AMP for 16 h induced specific binding of apolipoprotein (apo) A-I to the cells and apoA-I-mediated HDL formation with cellular lipids, neither of which was detected in the absence of dBcAMP. Dose-dependent changes of the apoA-I specific binding and the apoA-I-mediated cholesterol release were largely superimposable. ApoA-II also mediated lipid release after the treatment of the cells with dBcAMP and effectively displaced the apoA-I binding to the cells. In contrast, cellular cholesterol efflux to lipid microemulsion and to 2-(hydroxypropyl)-β-cyclodextrin was uninfluenced by the dBcAMP treatment. To induce the cellular reactivity with apoA-I, the incubation with dBcAMP required at least 6 h. Actinomycin D, cycloheximide, puromycin, and brefeldin A suppressed both the induction of apoA-I-mediated lipid release and the apoA-I specific binding to the cells. Analysis of the expression level of *ABC1* mRNA by using reverse transcription−polymerase chain reaction and oligonucleotide arrays revealed that *ABC1* mRNA was already expressed in the dBcAMP-untreated cells, and the dBcAMP treatment for 16 h enhanced its expression 9−13-fold. We conclude that dBcAMP selectively induces apolipoprotein-mediated cellular lipid release and accordingly high-density lipoprotein generation by inducing specific binding of apolipoprotein, but does not influence diffusion-mediated lipid efflux. The cell−apolipoprotein interaction seems to depend on cellular protein biosynthesis and transport. A substantial increase in the level of *ABC1* mRNA caused by the dBcAMP treatment indicates that ATP-binding cassette transporter 1, the protein product of *ABC1*, may directly be responsible for the interaction, but the question about the absence of the interaction with its baseline expression level remains.

All mammalian cells require cholesterol for biosynthesis of membranes. Cellular cholesterol can be derived by de novo synthesis or externally via the uptake of cholesterol-containing lipoprotein particles. On the other hand, extrahepatic peripheral cells lack metabolic pathways for cholesterol catabolism except for the cells in some specific organ with the enzymes for steroidogenesis or sterol 27-hydroxylase (*1*, *2*). Therefore, cholesterol molecules in most of the somatic cells need to be removed and transported to the liver where they are metabolized to bile acid and excreted. This is one of the key pathways for cholesterol homeostasis for both the whole body and peripheral cells. High-density lipoprotein (HDL)[1] is believed to play a central role in this reaction.

Two independent mechanisms have been proposed for cellular cholesterol removal by HDL (*3*, *4*). One is diffusion-mediated cholesterol exchange between plasma membrane and the HDL surface. The other is assembly of cellular lipids in the formation of new HDL particles by apolipoproteins having amphiphilic α-helical segments as they dissociate from HDL. The former is a physicochemical reaction that can be mimicked by cellular cholesterol diffusion to non-specific cholesterol acceptors such as lipid microemulsions (*5*) and cyclodextrin (*6*). The reaction is bidirectional, but the gradient of cholesterol content between the cell membrane and HDL surface maintained by lecithin:cholesterol acyltransferase can be a driving force for the net efflux (*7*). On the other hand, the latter reaction can be probed by cellular lipid release stimulated by lipid-free helical apolipoproteins (*8*, *9*).

The apolipoprotein-mediated cellular cholesterol release is characterized as a biological process that requires a specific interaction site(s) with apolipoproteins on the cell surface and a specific intracellular cholesterol trafficking system. The site for the interaction with apolipoprotein is cell type and

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[1] Abbreviations: HDL, high-density lipoprotein; apo, apolipoprotein; ABC1, ATP-binding cassette transporter 1, the protein product of *ABC1*; dBcAMP, dibutyryl cyclic AMP; DF, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; PBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin; CD, 2-(hydroxypropyl)-β-cyclodextrin; RT−PCR, reverse transcription−polymerase chain reaction; PKA, protein kinase A.

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stage specific (*10*, *11*), and protease sensitive (*12*), and the intracellular cholesterol trafficking is regulated by several signaling pathways (*11*, *13*).

A defect in the apolipoprotein (apo) A-I-mediated cellular lipid release has been demonstrated in fibroblasts from patients with Tangier disease, an autosomal recessive disorder characterized by the absence of plasma HDL cholesterol and deposition of esterified cholesterol in the reticuloendothelial system (*14*, *15*), while nonspecific cholesterol exchange between cells and HDL is retained. The results strongly indicated that the apolipoprotein-mediated lipid release is a major source of plasma HDL. Recent reports demonstrated that mutations in *ABC1*, a member of ATP binding-cassette transporter superfamily, are the cause of Tangier disease and other genetic HDL deficiencies (*16*−*20*). The proposed function of the protein product of this gene (ABC1) is to mediate transmembrane cholesterol transport (*21*). However, the exact role of ABC1 in apolipoprotein-mediated HDL generation has not yet been clarified.

Induction of apolipoprotein binding to the cell surface by cyclic AMP analogues has been reported in a mouse monocytic leukemia cell line, RAW264 (*22*, *23*). This cell line is therefore considered to be a good model for studying the nature of the apolipoprotein interaction site on the cell surface. We have characterized the apolipoprotein−cell interaction in detail in this cell line mainly by using apoA-I, a major apolipoprotein of HDL. We show that apolipoprotein-mediated cellular lipid release can be selectively induced by dibutyryl cyclic (dBc) AMP but not a diffusion-mediated pathway. The induced reaction is dependent on the newly synthesized protein(s) that are essential for both specific binding of apoA-I to the cells and cellular lipid release.

EXPERIMENTAL PROCEDURES

Cell Culture. Mouse monocytic leukemia cell line RAW264 was purchased from Riken Gene Bank (Tsukuba, Japan). Cells were maintained in a 1/1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) supplemented with 2% (v/v) TCM serum replacements (ICN 2010022) under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. We chose a chemically defined medium to avoid the possible influence of serum components that might affect the intracellular cAMP concentration and to maintain cellular cholesterol at low levels.

Apolipoproteins. ApoA-I and apoA-II were isolated from the human plasma HDL fraction (density, 1.09−1.21) and stored at −80 °C until they were used as described previously (*24*, *25*). The proteins were dissolved in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) by stirring at 25 °C for 2 h and then incubated at 37 °C for 30 min (*24*), and were sterilized by passing them through a 0.22 μm membrane filter (Costar 8110). The protein concentrations of the resulting solutions were adjusted to 1 mg/mL with PBS, and they were stored at 4 °C as stock solutions.

Cellular Lipid Release Assay. Cells were subcultured in six-well trays at a density of 1.2×10^6 cells/well with 2% TCM−DF medium. After incubation for 48 h, the cells were washed with PBS and were cultured for an additional 16 h in 0.1% bovine serum albumin (BSA)−DF medium with or without 300 μM dBcAMP and the indicated reagents. The cells were washed with PBS, and incubated in DF medium containing lipid acceptors [apoA-I, apoA-II, lipid microemulsion of egg phosphatidylcholine and triolein having a low-density lipoprotein size (*25*), and 2-(hydroxypropyl)-β-cyclodextrin (CD) (*6*)]. The lipid content in the medium and cells was determined after the indicated incubation time.

For lipid mass analysis, lipids in the medium and cells were extracted with chloroform/methanol (2/1) and *n*-hexane/2-propanol (1/1) mixtures, respectively. The chloroform/methanol extract was back-extracted with water to remove contaminating proteins and phenol red which would interfere with the following assays. The amounts of total cholesterol, free cholesterol, and choline phospholipids were measured by colorimetric enzyme assays using reagents for clinical tests for serum lipids (3/1 mixture of Kyowa Medics 31228-4 and 30753-2 for total cholesterol, 3/1 mixture of Kyowa Medics 30752-5 and 30753-2 for free cholesterol, and Wako 275-54001 for choline phospholipids) with some modification. In brief, samples or standards dissolved in 20 μL of 2-propanol were mixed with 150 μL of the reagents in microtiter wells and were incubated for 1 h at 37 °C. The absorbance was measured for the cholesterol and choline phospholipid assay at 555 nm with a reference at 700 nm and at 505 nm with a reference at 600 nm, respectively. The analyses were quantitative over a range of 0.08−20 μg for cholesterol and 0.2−30 μg for choline phospholipids.

To examine the cellular phospholipid compartment used for HDL assembly, choline-containing phospholipid was labeled with [*methyl*-^{3}H]choline. The cells in a six-well plate at a density of 2.0×10^6 cells/well were cultured with 2% TCM−DF medium for 24 h and then cultured in 0.1% BSA−DF medium with or without 300 μM dBcAMP for 24 h. For protocol A, 5 μCi/mL of [*methyl*-^{3}H]choline chloride was present in the medium throughout the incubation with dBcAMP. The cells were washed with PBS, and 0 or 15 μg/mL apoA-I in 0.1% BSA−DF medium was added. For protocol B, cells were incubated in Dulbecco's modified Eagle's medium containing 20 μCi/mL of [*methyl*-^{3}H]choline chloride for 30 min prior to the incubation with apoA-I. Choline phospholipids and metabolites in the cells and medium were analyzed after incubation for 2, 4, and 6 h with apoA-I as described previously (*26*, *27*).

ApoA-I Binding Assay. Alexa 488-labeled apoA-I was prepared according to the manufacturer's instructions (Molecular Probes P6466). Cells prepared in 12-well trays were put on ice and treated with ice-cold solutions thereafter, to avoid the potential influence of any cellular events such as the endocytosis and exocytosis of apolipoprotein proposed to be required for the reaction (*23*) and to minimize the lipid−protein interaction process to take place (such as assembly of the HDL particles, etc.). The cells were washed with 0.1% BSA−PBS medium and incubated with 10 μg/mL Alexa 488-labeled apoA-I in 0.1% BSA−PBS medium for 2 h. Then the cells were washed and incubated in 0.1% BSA−PBS medium with or without nonlabeled apolipoproteins for 4 h with one change of the solution. The cells were washed again, and the amount of Alexa 488-labeled apoA-I was measured with a FL600 fluorescent plate reader (BIO-TEK Inc.). Subsequently, cells were lysed in 0.1% SDS, and an aliquot of the lysate was processed to measure cellular DNA content with Hoechst 33258 (*28*). The extent of

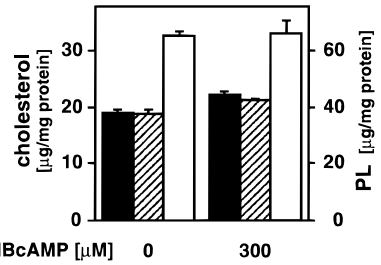

FIGURE 1: Cellular lipid content of RAW264 cells. Cells were subcultured in six-well trays at a density of 1.2×10^6 cells/well, and incubated for 48 h. The culture medium was replaced with DF medium containing 1 mg/mL BSA (0.1% BSA−DF) with or without 300 μM dBcAMP and incubated for an additional 16 h. Cellular lipid was extracted and its level measured as described in the text: (black columns) total cholesterol, (hatched columns) free cholesterol, and (white columns) choline phospholipid. Results represent the means ± the SD for three samples.

reversible binding of apoA-I was estimated by subtracting the nondisplaceable binding from the total binding as described previously (*29*).

Reverse Transcription−Polymerase Chain Reaction (RT−PCR). Total RNA fractions were isolated by acid guanidinium thiocyanate−phenol−chloroform extraction (*30*). For each reaction, 5 μg of total RNA was reverse-transcribed to cDNA using a Super Script Preamplification System (Gibco BRL). The resulting cDNA was then subjected to PCR. Two pairs of oligonucleotides with sequences, corresponding to nucleotides 341−361 (5′-TCCCGGCGAGGCTCCCGGTGT-3′) and 879−899 (5′-CAGCTCTTGGGCCAGGCCCCC-3′) of mouse *ABC1* cDNA (*31*), and nucleotides 566−585 (5′-ACCACAGTCCATGCCATCAC-3′) and 998−1017 (5′-TCCACCACCCTGTTGCTGTA-3′) of mouse glyceraldehyde-3-phosphate dehydrogenase cDNA (*32*) were used as PCR primers. Since the primer set for *ABC1* we used spans introns (*19*), we first examined its reliability to amplify exclusively *ABC1* mRNA. Reverse transcription followed by 30 repeated PCR cycles produced a band with predicted length (559 bp) with the mRNA samples from either untreated or dBcAMP-treated cells. No band was detected when the total RNA samples were processed without reverse transcriptase, and when PCR was performed with genomic DNA. Thus, it was confirmed that our primer pairs would not amplify any genomic DNA contaminated in the RNA samples. We also confirmed that the amplified fragment was indeed derived from *ABC1* mRNA by *Pst*I digestion since murine *ABC1* cDNA has a *Pst*I site at position 600 (*31*). The result showed complete digestion, yielding two fragments matching the expected products. The PCR products were also quantitated with Hoechst 33258.

Oligonucleotide Array Analysis. mRNA expression levels were analyzed by using GeneChip Expression Analysis Arrays (Affymetrix 900161) as described previously (*33*, *34*), except that 10 μg of total RNA prepared as described above was used to generate first-strand cDNA.

RESULTS

Lipid contents were analyzed for the cells that had and had not been subjected to the dBcAMP pretreatment. No significant difference was found with respect to the cellular total and free cholesterol and choline phospholipids between untreated and dBcAMP-treated cells (Figure 1).

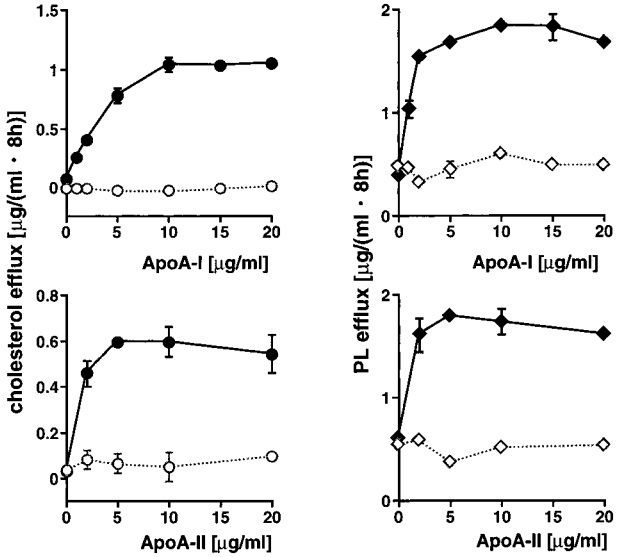

FIGURE 2: Release of cholesterol (left two panels) and choline phospholipids (right two panels) from RAW264 cells mediated by apoA-I (top two panels) and apoA-II (bottom two panels). Cells were cultured in the same manner as described in Figure 1. After incubation with (black symbols) and without (white symbols) 300 μM dBcAMP for 16 h, the cells were washed with PBS and incubated in 1 mL of 0.1% BSA−DF medium containing apolipoproteins at the indicated concentration per well. Medium was collected after 8 h for lipid analysis. Results represent means ± the SD for three samples. Error bars are not shown when they are found to lie within the symbols.

Typical profiles of lipid released by apoA-I and apoA-II from RAW264 cells that had and had not been subjected to the dBcAMP pretreatment are shown in Figure 2. Preincubation of the cells with 300 μM dBcAMP for 16 h induced apoA-I-mediated release of cholesterol and choline phospholipids, while no apoA-I-mediated lipid release was detected in the absence of dBcAMP (upper two panels). The release of the choline phospholipids appears to be more sensitive than that of cholesterol with respect to the apoA-I concentration dependency. The release by apoA-I (15 μg/mL) of phospholipid labeled with [*methyl*-^{3}H]choline was the same with the two protocols described in Experimental Procedures in which there was universal labeling of the cell phospholipid and pulse labeling of the newly synthesized phospholipid (2.58 ± 0.41 vs 2.17 ± 0.09% for phosphatidylcholine and 2.59 ± 0.57 vs 2.26 ± 0.24% for sphingomyelin, respectively). Therefore, no specific pool of choline phospholipid is used for the apoA-I-mediated release. Density gradient analysis of the medium revealed that the lipids were recovered in a fraction with a density of around 1.1 g/mL. ApoA-II also mediated the lipid release only when the cells were pretreated with dBcAMP (Figure 2, lower two panels). The dose-dependent curve of the reaction with apoA-II was similar to that of apoA-I with respect to the molar concentration of the proteins for both cholesterol and phospholipid. The data were analyzed with double-reciprocal plots after subtraction of the background, yielding apparent K_m values for cholesterol and choline phospholipids of approximately 1.1×10^{-7} and 5.2×10^{-8} M for apoA-I and 7.6×10^{-8} and 4.3×10^{-8} M for apoA-II, respectively (average of two experiments).

In contrast, cellar cholesterol efflux to lipid microemulsion and to CD was identical in the cells that had and

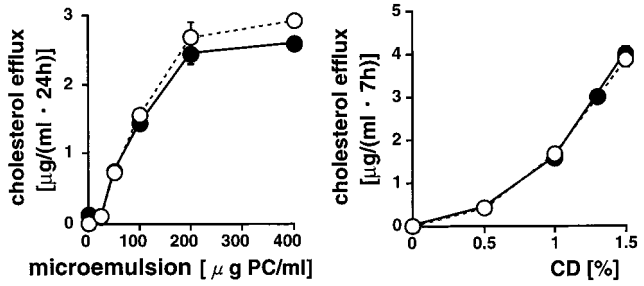

FIGURE 3: Cholesterol efflux from RAW264 cells to lipid microemulsions (left panel) and 2-(hydroxypropyl)-β-cyclodextrin (right panel). Cells were cultured and stimulated with (black symbols) or without (white symbols) dBcAMP. After being washed with PBS, the cells were incubated in 1 mL of 0.1% BSA−DF medium containing lipid microemulsions or 2-(hydroxypropyl)-β-cyclodextrin at the indicated concentration per well. The cholesterol level in the medium was determined after incubation for 24 and 7 h, respectively. Results are means ± the difference for two samples. Error bars are not shown when they are found to lie within the symbols.

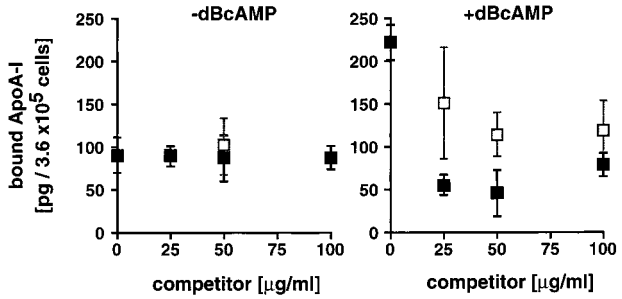

FIGURE 4: Displacement of the binding of the fluorescence-labeled apoA-I by unlabeled apoA-I and apoA-II to the RAW264 cellular surface. Cells were subcultured in 12-well trays at a density of 8 × 10^5 cells/well and incubated with or without dBcAMP as described in the legend of Figure 1. Binding of the labeled apoA-I was assessed as described in Experimental Procedures after displacement by the competitors, unlabeled apoA-I (□) or apoA-II (■) as described in the text. Results represent means ± the SD for three samples.

had not been subjected to the dBcAMP pretreatment (Figure 3).

Figure 4 demonstrates displacement of the binding of the fluorescence-labeled apoA-I by nonlabeled apoA-I and apoA-II. No displacement was demonstrated by either apolipoprotein when cells were not treated with dBcAMP (left panel). When the cells were pretreated with dBcAMP, the level of total binding of the labeled apoA-I substantially increased, and this increment was displaced by both apoA-I and apoA-II (right panel). Displacement was achieved at lower concentrations and more reproducible with apoA-II than with apoA-I, implying a higher specific binding affinity of apoA-II than apoA-I. The extent of specific apoA-I binding was thus calculated by subtracting the level of nonspecific fluorescence binding with unlabeled apoA-I or apoA-II from the level of total binding without unlabeled apolipoproteins. Figure 5 demonstrates the results with displacement by unlabeled apoA-II, showing that the dBcAMP treatment induced specific reversible binding of apoA-I, while there was no reversible apoA-I binding to the cells not treated with dBcAMP. The experiment with unlabeled apoA-I yielded essentially the same results, though the displacement was less complete than that by apoA-II (data not shown).

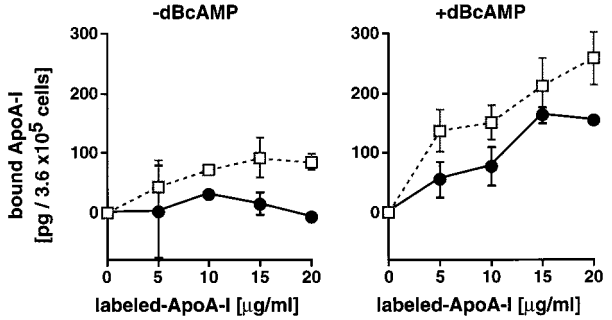

FIGURE 5: Reversible binding of apoA-I to RAW264 cells. Cells were prepared as described in the legend of Figure 4, and incubated with the labeled apoA-I at the indicated concentration with the dBcAMP-pretreated cells (right panel) and untreated cells (left panel), followed by displacement by 50 mg/mL unlabeled apoA-II. The extent of reversible binding (●) was calculated as displaceable binding by subtracting the level of binding after the displacement from the level of total binding without displacement (□). Results are means ± the SD for three samples.

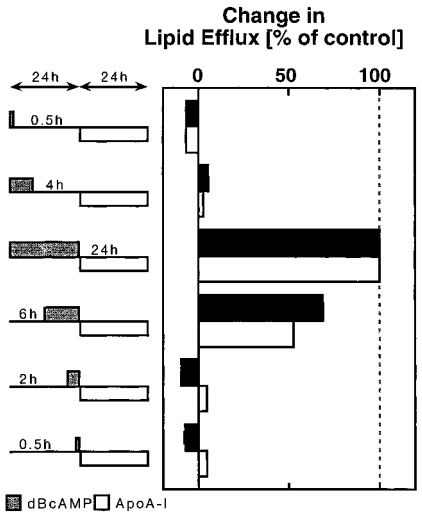

FIGURE 6: Effect of the timing of the pretreatment with dBcAMP on the apoA-I-mediated lipid release. Cells were subcultured in six-well trays at a density of 1.2 × 10^6 cells/well for 48 h. The cells were then incubated with 300 μM dBcAMP for the indicated periods of time. Cellular lipid release by 15 μg/mL apoA-I during the next 24 h was assessed. Data are shown as values relative to the positive control (samples from cells with the 24 h pretreatment) after subtracting the background (efflux to 0 μg/mL apoA-I). Results are means ± the difference for two samples of cholesterol (black columns) and a single-sample assay for phospholipid (white columns).

To investigate the induction mechanism for the generation of HDL, apoA-I-mediated lipid release was assessed after various incubation protocols of the cells with dBcAMP. The results are shown in Figure 6, demonstrating that at least 6 h was required for the induction to occur. The effect of various compounds on cellular metabolism and trans Golgi protein transport was studied on the induction of the apoA-I-mediated lipid release by dBcAMP (Figure 7). Actinomycin D, cycloheximide, puromycin, or brefeldin A was added to the medium during the dBcAMP treatment, and all repressed the induction of apoA-I-mediated lipid efflux in a dose-dependent manners. These compounds also suppressed the specific binding of apoA-I to the dBcAMP-treated cells (Figure 8).

Expression of the *ABC1* gene was examined in the RAW264 cells that had and had not been subjected to the

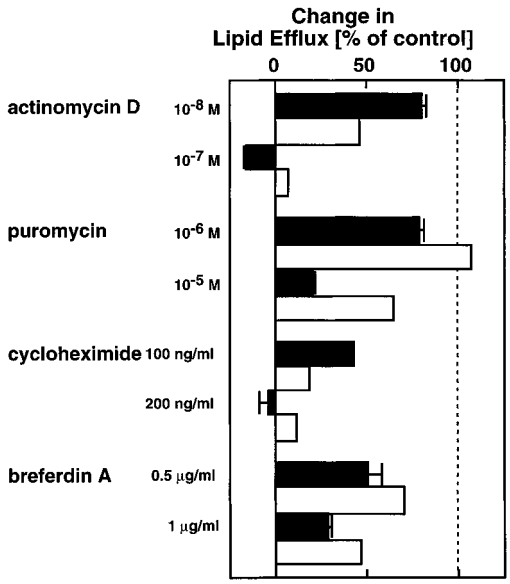

FIGURE 7: Effects of various metabolic inhibitory compounds on induction of apoA-I-mediated lipid release by dBcAMP. Cells were subcultured in six-well trays at a density of 1.2×10^6 cells/well and incubated for 48 h. The culture medium was replaced with 0.1% BSA−DF medium containing 300 μM dBcAMP and the indicated compounds, and the cells were cultured for an additional 16 h. Release of cholesterol (black columns) and phospholipid (white columns) by 15 μg/mL apoA-I was assessed after 24 h. The results are shown as values relative to positive control (samples from the cells cultured without inhibitors). The value of 100% was the same as that in Figure 6. Results represent means ± the SD for three samples except for a single-point assay for phospholipid with actinomycin D, puromycin, and cycloheximide.

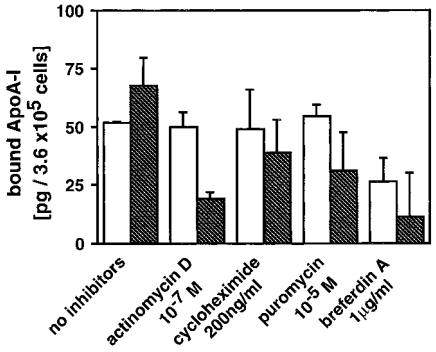

FIGURE 8: Effects of various metabolic inhibitory compounds on specific apoA-I binding induced by dBcAMP. Cells were subcultured in 12-well trays at a density of 8×10^5 cells/well and processed as described in the legend of Figure 7. Nonspecific (white columns) and specific (hatched columns) binding of apoA-I was then assessed. Results represent means ± the SD for four samples.

pretreatment with dBcAMP, since it is a causative gene of familial HDL deficiency, including Tangier disease (*16*−*18*, *20*). Figure 9 shows the results of RT−PCR analysis. *ABC1* mRNA was substantially expressed already in the cells without dBcAMP pretreatment. The level of *ABC1* mRNA relative to that of glyceraldehyde-3-phosphate dehydrogenase mRNA was increased 9.3-fold in dBcAMP-treated cells, indicating that dBcAMP upregulates *ABC1* mRNA transcription. Changes in mRNA expression were also analyzed quantitatively by using oligonucleotide arrays. The arrays contained probe sets for 6500 murine genes (approximately 50% are known genes and 50% are expressed sequence tags). Among the genes that have been examined, 1536 genes

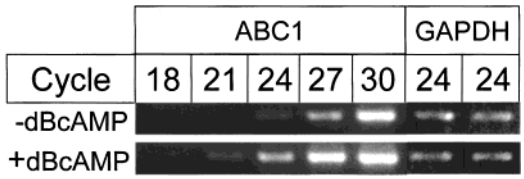

FIGURE 9: Analysis of *ABC1* mRNA in the dBcAMP-treated and untreated RAW264 cells. Total RNA fractions were prepared from RAW264 cells after treatment with 0 or 300 μM dBcAMP for 16 h. RT−PCR was performed by using primer pair oligonucleotides for *ABC1* cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. PCR was repeated for the indicated cycles, and the products were analyzed and visualized by 1.7% agarose/ethidium bromide gel electrophoresis with ultraviolet illumination.

demonstrated an average difference of >1000 arbitrary units in the dBcAMP-treated cells, indicating these genes are substantially expressed. Twenty-three of them exhibited an increase in the average difference of >10-fold in comparison with that of dBcAMP-untreated cells (Table 1). *ABC1* mRNA was expressed but not at a very high level in the dBcAMP-untreated cells, and the expression level increased 13.3-fold after dBcAMP treatment, in 13th place with respect to the increase among the 1536 genes mentioned above. There are two other probes for ATP-binding cassette transporter family genes in the arrays, *ABC2* and *ABC transporter-7*, but neither of them was expressed at a high level in either the untreated or dBcAMP-treated cells (Table 2).

DISCUSSION

dBcAMP induced apolipoprotein-mediated lipid release and, accordingly, the generation of HDL with cellular lipids from mouse macrophage cells RAW264, consistent with previous reports, such as the data presented in Figures 2, 3, and 5−7 (*22*, *23*). In contrast to this reaction, diffusion-mediated nonspecific cholesterol efflux to lipid microemulsions and CD was uninfluenced by the dBcAMP treatment (Figure 3). In addition, there was no change induced by dBcAMP in the cellular levels of cholesterol, free cholesterol, and phospholipid (Figure 1). Therefore, it is not highly likely that the effect of dBcAMP is secondary to redistribution of cellular cholesterol among the intracellular compartments. The data also indicated that the apolipoprotein-mediated lipid release was regulated by dBcAMP, while diffusion-mediated cholesterol efflux is independent of this stimulation. Thus, the data provided further supportive evidence for the hypothesis that these two pathways operate independent of each other (*3*, *4*).

Apolipoprotein-mediated cholesterol release is minimal (less than 1% of the total cholesterol mass) from the cells cultured in chemically defined medium without lipoprotein loading and without dBcAMP. This is much lower than the level of release reported in previous studies (*22*, *35*, *36*) where cells were cultured in serum-containing medium and loaded with modified LDL. These procedures may already have induced certain metabolic changes that may secondarily alter expression of certain genes. Indeed, when RAW264 cells were cultured in serum-containing medium in our laboratory, apolipoprotein-mediated cholesterol release from the dBcAMP-untreated cells was somewhat increased (data not shown).

Cellular response to lipid-free amphiphilic apolipoproteins varies with various cell types and stages. From this point of

Table 1: Gene Expression Upregulated by dBcAMP According to Oligonucleotide Array Analysis[a]

accession no.	description	AD-1	AD-2	FC
M15131	mouse interleukin 1-β (IL-1-β) mRNA, complete cds	11	35434	~1354.4
D12712	*Mus musculus* mRNA for type IV collagenase (gelatinase B)	−247	3170	~154.4
M60285	mouse cAMP-responsive element modulator (CREM) mRNA, complete cds	−25	3610	~151.6
X16490	mouse RNA for plasminogen activator inhibitor 2	−47	2804	~112.5
U44088	*M. musculus* TDAG51 (TDAG51) mRNA, complete cds	58	1633	26.8
M55154	mouse transglutaminase (TGase) mRNA, complete cds	410	11108	25.2
X57437	*M. musculus* mRNA for L-histidine decarboxylase	121	2501	23.2
M88242	mouse glucocorticoid-regulated inflammatory prostaglandin G/H synthase (griPGHS) mRNA, complete cds	162	3725	21.9
J03733	mouse kidney ornithine decarboxylase mRNA, clone pODC16, 3′ end	1938	43468	21.5
U73004	*M. musculus* secretory leukocyte protease inhibitor mRNA, complete cds	742	12696	16.8
X14432	mouse mRNA for thrombomodulin	293	4655	15.1
X53798	mouse mRNA for macrophage inflammatory protein 2 (MIP2)	1018	15306	14.2
X75926	*M. musculus* abc1 mRNA	203	2983	13.6
X62940	*M. musculus* TSC-22 mRNA	567	7720	13.6
U51805	*M. musculus* liver arginase mRNA, complete cds	87	1327	13.4
X15591	mouse ctla-2-α mRNA, homologue to cysteine protease proregion	116	1565	13.3
M74495	mouse adenylosuccinate synthetase mRNA, complete cds	318	4117	13.0
X59769	mouse Il-1r2 mRNA for the type II interleukin 1 receptor (cell line 70Z/3)	148	2027	12.2
V00727	provirus of a replication defective murine sarcoma virus (FBJ-MuSV) with c-fos(p55) and p15 E reading frames	1670	24102	12.0
M95200	mouse vascular endothelial growth factor mRNA, complete cds	1068	10846	11.4
M87966	*M. musculus* intracellular calcium-binding protein (MRP8) mRNA, complete cds	199	2426	11.2
M73748	mouse glycoprotein 38 mRNA, complete cds	893	10059	11.2
U28404	*M. musculus* macrophage inflammatory protein 1 α receptor gene, complete cds	292	3412	10.7

[a] The analysis was performed with the total RNA samples prepared in the same manner as described in the legend of Figure 9, as described in Experimental Procedures. The results are presented with normalization of the average for 1000. AD-1 and AD-2 are the average differences for the untreated and dBcAMP-treated cells (arbitrary units), respectively, and FC is a ratio of AD-2 to AD-1. A negative average difference value indicates higher scanned data of mismatch probes than that of complete match probes.

Table 2: Expression Levels of the ABC Transporter Gene Family Measured by Oligonucleotide Array Analysis[a]

accession no.	description	AD	AD-2	FC
X75926	*M. musculus* abc1 mRNA	203	2983	13.6
X75927	*M. musculus* abc2 mRNA	489	326	−1.2
U43892	*M. musculus* ABC transporter-7 mRNA, partial cds	275	227	−1.5

[a] AD-1 and AD-2 are average differences for the untreated and dBcAMP-treated cells (arbitrary units), respectively, and FC is a ratio of AD-2 to AD-1.

view, cells can be classified into the three categories. In group A, cells release both cholesterol and phospholipids to generate cholesterol-rich HDL. In group B, cells release phospholipid but not cholesterol to generate cholesterol-poor HDL. In group C, cells release neither cholesterol nor phospholipids and generate no HDL. Cells such as peritoneal macrophages (*5*, *8*, *9*, *37*), human fibroblasts (*37*), and THP-1 differentiated after PMA treatment (*38*, *39*) belong to the group A. Rat vascular smooth muscle cells grown under regular conditions (*10*, *11*, *37*) and undifferentiated THP-1 cells (*39*) belong to group B. Erythrocytes (*12*), cells loaded with probucol (*29*, *40*), and perhaps rat hepatoma cell line cells Fu5AH (*40*, *41*) belong to group C. RAW264 cells are classified as group C cells before the dBcAMP treatment and are converted to group A after the stimulation (*22*, *23*).

The K_m values for apoA-I and apoA-II were apparently in the same order with respect to their molarity (Figure 2), but apoA-II displaced labeled apoA-I more effectively than the unlabeled apoA-I (Figure 4). A higher tendency of apoA-I to self-associate in solution (*42*) may explain the reason for this, or the affinity for the cells of apoA-II may in fact be higher than that of apoA-I which can only be detected by direct competition. It should be noted that apoA-II binds more tightly to the HDL surface (*43*), and the apparent affinity of apoA-II was higher than that of apoA-I for the surface of the large lipid microemulsions (*25*). Thus, the difference in the interactions of apoA-I and apoA-II may have come from the difference in the affinity of these proteins for cellular lipid to assemble HDL particles rather than the direct affinity for cellular interaction site(s). However, the results of apoA-I binding displacement rather imply a higher affinity of apoA-II for the binding site.

Specific binding of ApoA-I was also induced by dBcAMP. This is also consistent with the previous report that described the binding by using only a very low apoA-I concentration of 0−500 ng/mL, which is not comparable to that used for lipid release (*22*). Specific binding of apoA-I defined as reversible binding to the cell surface (Figure 5) seems saturable, as well as the apoA-I-mediated release of phospholipid and cholesterol (Figure 2). However, phospholipid release reaches a plateau at the lowest concentration of apoA-I; the cholesterol release is saturated next, and the binding seems to be saturated at the highest concentration. This apparent difference in saturation seems to be consistent with the previous report (*40*). Such data may not withstand strict quantitative kinetic analysis due to the nature of the experiments, so one must be careful not to overinterpret such data. Nevertheless, the hypothetical view from these results is that the release of phospholipid and cholesterol are secondary reactions of the bound apoA-I, and the apparent K_m for the phospholipid release is substantially lower than that for the cholesterol release with respect to the bound apoA-I. If this view were true, apoA-I binding is necessary for the generation of HDL but a direct rate-limiting step is the recruitment of cellular phospholipid to apolipoprotein.

Differential regulation of HDL assembly by helical apolipoproteins with cellular phospholipid and mobilization of

cellular cholesterol for its incorporation into HDL were demonstrated by stimulation and inhibition of protein kinase C that indicates the involvement of intracellular signaling processes in this specific cholesterol mobilization (*11*, *13*). Such differential regulation was also implicated by changing the cellular cholesterol level (*38*). If the difference between the apparent K_m values of phospholipid release and cholesterol release by the bound apoA-I is real, it would provide additional support for the independent regulation of these two reactions (*11*, *13*). Differences were also observed in profiles of the inhibition of these parameters by various compounds (Figures 7 and 8).

dBcAMP is a membrane-permeable cAMP analogue and activates protein kinase A (PKA). PKA phosphorylates many substrate proteins, leading to rapid cellular effects (*44*, *45*). However, PKA can also affect specific gene expression through phosphorylating nuclear proteins (*46*). As shown in Figure 6, induction of the cellular reactivity to apoA-I by dBcAMP required incubation for more than 6 h. Therefore, the induction seems to involve expression of new protein(s) rather than a mechanism mediated by rapid protein phosphorylation. Enhancement of the apolipoprotein-mediated cellular cholesterol release by cAMP analogues was also demonstrated with several other types of cells (*35*, *40*, *47*), showing that the effect seems to involve a ubiquitous protein(s).

Regulatory mechanisms for the expression of *ABC1* have not been completely clarified. Change in the human *ABC1* mRNA level by differentiation of monocytes to macrophages in vitro as well as loading and removing cellular cholesterol was reported, suggesting a sterol-dependent regulatory mechanism (*35*, *48*). We found an increase in the level of *ABC1* mRNA in RAW264 cells by dBcAMP (Figure 9 and Table 1), consistent with results previously reported for human fibroblasts (*35*). Therefore, it is possible that the *ABC1* gene has a cAMP-responsible element(s) in its promoter as well as elements for sterol dependency (*48*). Overexpression of human *ABC1* mRNA in RAW264 cells resulted in an increase in the extent of apoA-I-mediated lipid release (*35*, *36*) which suggests an essential role for ABC1 in this reaction. It remains to be clarified whether specific apoA-I binding is also dependent on the expression of *ABC1*.

The ABC1 protein is also a substrate of PKA. ABC1 expressed in *Xenopus* oocytes was phosphorylated by PKA in vitro (*49*). Its activity as an anion transporter was enhanced after short-time treatment with PKA activators. However, phosphorylation does not appear to be essential for the activity of ABC1 since the basal anion transport activity was still higher in ABC1-expressing cells than in control cells even after the treatment with a PKA inhibitor (*49*). Thus, it is unlikely that enhancement of the apoA-I-mediated reactions can be attributed to the phosphorylation of the ABC1 already expressed by dBcAMP in RAW264 cells.

There have been no reports for an apolipoprotein binding protein(s) to mediate cellular lipid release and HDL assembly. Our data suggest that ABC1 might be essential. However, we cannot exclude the possibility that an additional protein(s) may be involved in the apolipoprotein−cell interaction to mediate the generation of HDL since the cells not treated with dBcAMP lack reactivity even with a certain level of *ABC1* expression.

A recent report indicated that the apolipoprotein undergoes binding to coated pits, cellular uptake, and then resecretion as HDL particles (*23*). On the other hand, the abnormal appearance of Golgi apparatus and impaired intracellular lipid transport were implicated in the macrophage of the patients with Tangier disease (*50*, *51*) and in the intestinal cells of the *abc1*-deficient mice (*36*). Thus, the assembly of HDL particles may take place intracellularly even with extracellular apolipoprotein. ABC1 may therefore not necessarily function in the plasma membrane.

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