ABCA1 Is the cAMP-inducible Apolipoprotein Receptor That Mediates Cholesterol Secretion from Macrophages*

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Lipoprotein (apo) A-I, the major protein component of plasma high density lipoprotein (HDL), removes both cholesterol and phospholipids from cells by an active secretory pathway controlled by an ABC transporter called ABCA1. This pathway is induced by cAMP and ABCA analogs in a cell-specific manner. Here we provide evidence that increased plasma membrane ABCA1 accounts for the enhanced apolipoprotein-mediated lipid secretion from macrophages induced by cAMP analogs. Treatment of RAW264 macrophages with 8-bromo-cAMP caused parallel increases in apoA-I-mediated cholesterol efflux, ABCA1 mRNA and protein levels, incorporation of ABCA1 into the plasma membrane, and binding of apoA-I to cell-surface ABCA1. All of these parameters declined to near base-line values within 6 h after removal of 8-bromo-cAMP, indicating that ABCA1 is highly unstable and is degraded rapidly in the absence of inducer. Thus, ABCA1 is likely to be the cAMP-inducible apolipoprotein receptor that promotes removal of cholesterol and phospholipids from macrophages.

Apolipoprotein (apo)A-I, the major protein component of plasma high density lipoprotein (HDL), removes both cholesterol and phospholipids from cells by an active transport pathway (1), which may account for the atheroprotective effect of HDL. An ATP-binding cassette transporter called ABCA1 (formerly ABC1) is a rate-controlling protein in this pathway (2). Mutations in ABCA1 impair apoA-I-mediated removal of cellular lipids and cause Tangier disease (2–9), a severe HDL deficiency syndrome characterized by sterol deposition in tissue macrophages (10) and prevalent atherosclerosis (11). Thus, this lipid secretory pathway plays an important role in macrophage lipid trafficking, lipoprotein metabolism, and protection against atherogenesis.

Cholesterol and cAMP analogs appear to induce the apolipoprotein-mediated lipid removal pathway by different mechanisms (2, 12–15). Sterol loading of macrophages and fibroblasts markedly elevates ABCA1 mRNA and protein levels (2, 15, 16), implying that excess sterols promote their own secretion by inducing ABCA1. Treatment of immortalized fibroblasts with a cAMP analog also induces apoA-I-mediated lipid efflux and ABCA1 expression, but these effects are usually modest and require some cholesterol loading of cells (2, 14). In contrast, apoA-I-mediated lipid efflux from several murine macrophage cell lines is highly inducible by cAMP analogs, even when the cells are in a cholesterol-depleted state (12, 13). This implies that these analogs can activate this pathway by sterol-independent mechanisms. This activation is associated with an increase in apolipoprotein-binding sites on the cell surface and expression of a 200-kDa plasma membrane protein that binds apoA-I (12, 13). These findings raise the possibility that the interaction of apoA-I with a cell-surface receptor is involved in lipid removal. Because its mass is similar to the plasma membrane apoA-I-binding protein, it is possible that ABCA1 is this putative receptor.

Here we investigate whether ABCA1 expression is also responsible for the apoA-I-mediated cholesterol efflux induced by cAMP in macrophages and whether this involves direct binding of apoA-I to plasma membrane ABCA1. We show that 8-Br-cAMP activates ABCA1 transcription in RAW264 macrophages, leading to increased incorporation of ABCA1 into the plasma membrane where it binds apoA-I. Thus, it is likely that ABCA1 is the cAMP-inducible apolipoprotein receptor that promotes secretion of lipids from macrophages.

EXPERIMENTAL PROCEDURES

Cell Culture and Cholesterol Efflux—Murine RAW264.7 macrophages were maintained in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum. To radio-label cellular cholesterol, [3H]cholesterol was added to the growth medium during the 72 h immediately preceding treatments. Cells were washed twice with PBS containing 1 mg/ml bovine serum albumin (BSA), incubated for 18–24 h with DMEM/BSA minus or plus 0.3 mM 8-Br-cAMP, and then chased with the same medium containing either no cholesterol acceptors, 5 µg/ml purified apoA-I, or 50 µg/ml HDL protein (14). Where indicated, HDL was pretreated with trypsin to digest active apolipoproteins and added to the medium at a concentration equivalent to untreated HDL based on phospholipid content (~40 µg/ml protein) as described (3, 14). After 2–6 h at 37 °C, cells were chilled on ice; medium was collected, and the [3H]cholesterol content of the medium and cells was measured. Results are presented as fraction of total [3H]cholesterol released into the medium after subtraction of values obtained in the absence of apoA-I or other acceptors.

mRNA Levels—Cellular mRNA levels were measured by quantitative PCR using the Gene Amp 5700 Sequence Detection System and a two-step RT-PCR, SYB green protocol (PerkinElmer Life Sciences) (2). Values for ABCA1 mRNA were normalized to cyclophilin mRNA as described (2). For mRNA stability studies, cells were incubated for 0–8 h with 5 µg/ml actinomycin D minus or plus 8-Br-cAMP. Half-life was determined by plotting the “ln” of ABCA1 mRNA levels and calculating time from the slope of the best fit line.

Cell-surface and Metabolic Labeling of ABCA1—For selective labeling of plasma membrane ABCA1, macrophages were incubated for 30 min at 0 °C with PBS containing 1 mg/ml sulfo-NHS-biotin to biotinylate cell-surface proteins (2). For metabolic labeling, cells were incu-
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bated with DMEM/BSA containing 10 μg/ml [125I]methionine. To isolate ABCA1, cell proteins were solubilized in PBS containing 1% Triton X-100 and protease inhibitors, and the extract was incubated overnight at 4 °C with antisera (1:200 dilution) against a synthetic peptide corresponding to the deduced 22-amino acid C terminus of ABCA1 (2). The antibody-antigen complex was isolated by protein A-coated magnetic beads (Dynal) and electrophoresed in SDS using a 6% polyacrylamide gel. [125I]Methionine-labeled ABCA1 was detected on dried gels by phosphorimaging (Cyclone, Packard Instrument Co.). To measure biotinylated ABCA1, proteins were transferred to nitrocellulose and identified with a streptavidin-horseradish peroxidase ECL assay (Bio-Rad). Immunoblots of whole-cell ABCA1 were performed by probing nitrocellulose with ABCA1 antisera (1:1,000 dilution).

Cell-surface and ABCA1 Binding of ApoAl—For the whole-cell binding assay, cells were incubated for 2 h at 37 °C with DMEM/BSA containing 1 μg/ml [125I]apoA-I and then washed at 0 °C twice with PBS/BSA and twice with PBS. Cell-associated radioactivity and cell protein were measured after digestion in 0.2 N NaOH. Results are expressed as nanograms of [125I]apoA-I/mg of cell protein. For the cross-linking studies, cells were chilled to 0 °C and incubated for 2 h on ice with HEPES-buffered DMEM/BSA plus 5 μg/ml [125I]apoA-I. Cells were then washed twice with ice-cold PBS, incubated for 30 min at 0 °C with PBS containing 1 mg/ml DSS, washed twice with cold PBS containing 20 mM glycine, and extracted with detergent for ABCA1 immunoprecipitation and SDS-PAGE. Radiolabeled ABCA1 in the dried gels was identified by phosphorimaging.

RESULTS AND DISCUSSION

Cholesterol efflux from cells occurs by both ABCA1-dependent and -independent mechanisms (1). Apolipoproteins are acceptors of cholesterol and phospholipids secreted by the ABCA1 pathway, whereas phospholipid particles are acceptors of cholesterol released from cells by other mechanisms. To examine the effects of cAMP on these different mechanisms, we measured the effects of 8-Br-cAMP on cholesterol efflux from RAW264 macrophages in the presence of different cholesterol acceptors (Fig. 1a). With untreated cells, lipid-free apoA-I had little ability to remove cholesterol from cells. Treatment of cells with 8-Br-cAMP, however, dramatically induced apoA-I-mediated cholesterol efflux associated with increased cell-surface apoA-I binding, similar to what was reported by Smith et al. (12). This cAMP analog also stimulated apoA-I-mediated phospholipid efflux (not shown). HDL particles, which contain both apolipoproteins and phospholipids, promoted cholesterol efflux from untreated cells, and this was further enhanced by 8-Br-cAMP. When we incubated cells with trypsinized HDL, which lacks active apolipoproteins (3, 14), cholesterol efflux from untreated cells was comparable to that seen with native HDL, but 8-Br-cAMP had no effect. These results show that this cAMP analog induces not only the apolipoprotein-mediated, ABCA1-dependent cholesterol efflux pathway in RAW264 macrophages.

We examined the stability of the cAMP-induced cholesterol removal pathway by measuring cholesterol efflux to apoA-I after removing 8-Br-cAMP from the medium. Results showed that apoA-I-mediated cholesterol efflux decreased slightly during a second 24-h period of incubation with 8-Br-cAMP (Fig. 1b). Without 8-Br-cAMP, however, apoA-I-mediated cholesterol efflux returned to near baseline values within 6 h. These results indicate that this cholesterol efflux pathway is unstable and rapidly loses activity after the cAMP analog is removed.

We tested whether the reversible increase in apoA-I-mediated cholesterol efflux induced by 8-Br-cAMP was associated with changes in cellular ABCA1 mRNA and protein levels. Treatment of cells with 8-Br-cAMP caused a time-dependent increase in ABCA1 mRNA that reached 20-fold over untreated cells by 24 h (Fig. 2a). This time course is similar to the increase in apoA-I-mediated cholesterol efflux induced by cAMP analogs (12, 13). ABCA1 mRNA returned to baseline levels within only 6 h after removal of 8-Br-cAMP from the medium. Immunoblots revealed that ABCA1 protein was expressed at near undetectable levels in untreated cells (Fig. 2b). As with mRNA, ABCA1 protein progressively increased during 24-h incubations with 8-Br-cAMP and almost completely disappeared within 4 h after the analog was removed. Thus, the reversible induction of cholesterol efflux caused by addition and removal of 8-Br-cAMP paralleled changes in ABCA1 mRNA and protein levels, consistent with this transporter controlling the activity of the apolipoprotein-mediated lipid removal pathway. These data also show that both mRNA and protein for ABCA1 are highly unstable and are rapidly degraded in the absence of inducer.

To determine if 8-Br-cAMP influences mRNA stability, we measured the effects of 8-Br-cAMP on mRNA turnover. The mRNA induced by 8-Br-cAMP was degraded with half-lives of 2.4 and 2.8 h during subsequent incubations without and with 8-Br-cAMP, respectively (Fig. 2c). Thus, 8-Br-cAMP induces ABCA1 mRNA by activating transcription rather than stabilizing mRNA.

We also tested the possibility that inhibition of protein degradation could contribute to the cAMP-stimulated expression of ABCA1. We labeled cellular proteins for 24 h with [35S]methionine in the presence of 8-Br-cAMP, incubated cells with or without 8-Br-cAMP in the absence of radiolabel, and measured [35S]-labeled ABCA1 after isolation by immunoprecipitation and SDS-PAGE. The rates of turnover of prelabeled ABCA1 were
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Fig. 2. Reversible induction of ABCA1 in RAW264 macrophages by 8-Br-cAMP. a, macrophages were incubated without or with 8-Br-cAMP, and mRNA levels were measured in triplicate (± S.D.) by quantitative PCR at the times indicated. At the arrow, cells were washed once and incubated with 8-Br-cAMP-free medium. b, macrophages were incubated for the indicated hours with 8-Br-cAMP. For the right lane (24+4), cells treated with 8-Br-cAMP for 24 h were chased for 4 h without analog. ABCA1 protein levels were measured by immuno blot analysis of the same amount of total cell protein per lane (200 µg) from the same experiment. c, cells were incubated for 24 h with 8-Br-cAMP and chased with medium containing actinomycin D minus or plus 8-Br-cAMP, and mRNA levels were measured in triplicate (± S.D.).

By using a cell-surface biotinylation protocol, we determined if 8-Br-cAMP caused a reversible increase in the plasma membrane content of ABCA1, where it would have direct access to apoA-I. We detected very little ABCA1 on the surface of un treated RAW264 cells. Treatment with 8-Br-cAMP for 24 h markedly increased cell-surface ABCA1 (Fig. 3a). When we incubated cAMP-induced cells with fresh medium lacking 8-Br-cAMP, the amount of cell-surface ABCA1 decreased to basal levels between 2 and 6 h. The parallel changes in total cell protein (Fig. 2b) indicate that this decrease reflects ABCA1 turnover rather than redistribution between cellular compartments. Thus, plasma membrane as well as whole-cell ABCA1 is rapidly degraded when transcription is arrested. These changes in plasma membrane ABCA1 could account for the reversible stimulatory effects of 8-Br-cAMP on cholesterol removal by apoA-I.

Even when induced to high levels, ABCA1 does not promote secretion of lipids from cells unless apolipoproteins are present (3, 12, 14), indicating that apolipoproteins are required partners for this pathway. Conditions that induce ABCA1 and apolipoprotein-mediated lipid efflux also increase binding of apoA-I to cells (Fig. 1a) and to a 200 kDa plasma membrane protein in another macrophage cell line (13). These findings raise the possibility that binding of apolipoproteins to ABCA1 on macrophages is necessary for removing lipids. To test this possibility, we incubated cells with 125I-labeled apoA-I at 0 °C, treated cells with the cross-linking agent DSS, and isolated ABCA1 from detergent extracts by immunoprecipitation and SDS-PAGE. Results with cAMP-treated cells show a single 125I-labeled protein band with an apparent mass of 250–300 kDa (Fig. 3b), consistent with one or two apoA-I molecules (28 kDa) cross-linked to one ABCA1 molecule (248 kDa). This band was below the level of detection when cells were not treated with 8-Br-cAMP and almost completely disappeared when the cAMP analog was removed for 4 h. These results strongly suggest that apoA-I binds directly to cAMP-induced ABCA1 on the surface of RAW264 macrophages. Because it occurs at 0 °C, this binding is likely to represent an early step in the process of removing lipids from cells. The low efficiency of the cross-linking method does not allow us to estimate the fraction of apoA-I binding to cells attributable to ABCA1 interactions. It is reasonable to assume, however, that this is a significant fraction, as 8-Br-cAMP treatment almost tripled cellular binding of apoA-I (Fig. 1a). These studies implicate ABCA1 as the cholesterol efflux-promoting HDL receptor initially described nearly 20 years ago (17).

Studies of human disease (10, 11) and mouse models (8, 9) revealed that ABCA1 plays a critical role in clearing excess cholesterol from macrophages and has a major impact on lipoprotein metabolism and atherogenesis. It is now apparent that multiple processes modulate the activity of ABCA1. Induction of ABCA1 by cholesterol and cAMP appears to occur by separate and synergistic mechanisms (2, 12–15), presumably at the level of transcription. In addition, the discovery that ABCA1 is an apolipoprotein receptor suggests that receptor-
ligand interactions are involved in regulating cholesterol trafficking. Because of its rapid turnover, stabilization of ABCA1 protein may also contribute to its increased activity. Genetic and environmental factors that impair any of these regulatory mechanisms may contribute to the low plasma HDL phenotype associated with cardiovascular disease. These findings also illustrate the feasibility of developing agonists for ABCA1 that enhance cholesterol secretion from macrophages above that induced by sterols alone.

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