# Intracellular cholesterol mobilization involved in the ABCA1/apolipoprotein-mediated assembly of high density lipoprotein in fibroblasts

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**Abstract Differential regulation has been suggested for cellular cholesterol and phospholipid release mediated by apolipoprotein A-I (apoA-I)/ABCA1. We investigated various factors involved in cholesterol mobilization related to this pathway. ApoA-I induced a rapid decrease of the cellular cholesterol compartment that is in equilibrium with the ACAT-accessible pool in cells that generate cholesterol-rich HDL. Pharmacological and genetic inactivation of ACAT enhanced the apoA-I-mediated cholesterol release through upregulation of ABCA1 and through cholesterol enrichment in the HDL generated. Pharmacological activation of protein kinase C (PKC) also decreased the ACAT-accessible cholesterol pool, not only in the cells that produce cholesterol-rich HDL by apoA-I (i.e., human fibroblast WI-38 cells) but also in the cells that generate cholesterol-poor HDL (mouse fibroblast L929 cells). In L929 cells, the PKC activation caused an increase in apoA-I-mediated cholesterol release without detectable change in phospholipid release and in ABCA1 expression. These results indicate that apoA-I mobilizes intracellular cholesterol for the ABCA1-mediated release from the compartment that is under the control of ACAT. The cholesterol mobilization process is presumably related to PKC activation by apoA-I.**—Yamauchi, Y., C. C. Y. Chang, M. Hayashi, S. Abe-Dohmae, P. C. Reid, T-Y. Chang, and S. Yokoyama. **Intracellular cholesterol mobilization involved in the ABCA1/apolipoprotein-mediated assembly of high density lipoprotein in fibroblasts. *J. Lipid Res.* 2004.** 45: **1943–1951.**

**Supplementary key words** ATP binding cassette transporter A1 • acyl-coenzyme A:cholesterol acyltransferase • apolipoprotein A-I • protein kinase C

Cholesterol has various important biological functions, such as regulation of the structure and function of cellular membranes, covalent modification of protein, and biosynthesis of steroid hormones and bile acids as their precursors. Cellular cholesterol content and its distribution are therefore tightly regulated by various factors, and intracellular cholesterol trafficking is closely related to its cellular homeostasis. One of the sensing sites of cellular cholesterol level is the endoplasmic reticulum, where various important molecules for cholesterol homeostasis are located. Sterol regulatory element binding proteins (SREBPs) and their related elements are identified as a system to regulate various genes for cholesterol biosynthesis and its uptake. ACAT is also in the endoplasmic reticulum and functions to reduce excess free cholesterol by its esterification. On the other hand, cellular cholesterol is released to the extracellular environment primarily for its catabolism, because cholesterol is hardly metabolized in most somatic cells (1). This is also recognized as one of the crucial factors in cholesterol homeostasis in peripheral cells. ACAT reaction and cholesterol release are both active systems to protect cells from the membrane-toxic excess accumulation of free cholesterol.

Cellular cholesterol is removed in two distinct pathways by HDL to be transported to the liver for degradation to bile acids. Cellular cholesterol is actively released by lipid-free apolipoproteins that dissociate from HDL (2) to form new HDL particles with cellular phospholipid, whereas cholesterol molecules leave the cell surface to HDL by passive diffusion, which is enhanced by extracellular cholesterol esterification in HDL (1). It has been demonstrated that cells from patients with Tangier disease, a familial HDL deficiency, lack apolipoprotein-mediated lipid

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Abbreviations: apoA-I, apolipoprotein A-I; DOG, *sn*-1,2-dioctanoylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; SREBP, sterol regulatory element binding protein.

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release (3), and mutations have been identified in the gene *abca1* of these patients (4–6). Numerous studies were carried out to characterize this gene and its product, ABCA1, including its overexpression by cDNA transfection (7, 8), cyclic AMP analog treatment (9, 10), and stimulation by liver X receptor and/or retinoid X receptor ligands (11, 12), and demonstrated that ABCA1 mediates the HDL assembly by apolipoprotein with cellular lipids. In addition, findings with knockout mice (13, 14) and transgenic mice (15) of *abca1* confirmed an essential role of this molecule in the generation of plasma HDL. It has thus been established that ABCA1 is a rate-limiting factor of apolipoprotein-mediated lipid release and subsequent HDL assembly. However, it remains to be addressed how this protein mediates the reaction.

We recently reported that apolipoprotein-mediated releases of cholesterol and phospholipid are differentially regulated. Fibroblast cell lines can apparently be categorized into three groups: cells that generate *1*) cholesterol-rich HDL, *2*) cholesterol-poor HDL, and *3*) no HDL, after apolipoprotein A-I (apoA-I) exposure (16). This report demonstrated that ABCA1 expression is required for apoA-I-mediated phospholipid release and for the subsequent generation of HDL particles, rather than a direct requirement for cholesterol release and increase of cholesterol content in the HDL. Caveolin-1 was previously shown to be involved in the enrichment of cholesterol in the HDL generated by the apolipoprotein-mediated reaction in certain types of cells (17–19). However, L929 cells, for example, abundantly express both ABCA1 and caveolin-1 and yet generate only cholesterol-poor HDL (16). Thus, regulation of cholesterol enrichment of the HDL generated by an ABCA1/apolipoprotein system seems multifactorial. An additional factor(s) may be required to induce cellular cholesterol release for the apolipoprotein/ABCA1 pathway. In this article, mobilization of intracellular cholesterol for its release by this pathway was investigated. We show that protein kinase C (PKC) and ACAT-1 activities are involved in regulating the rate of intracellular cholesterol mobilization for ABCA1-mediated cholesterol release by apoA-I.

## MATERIALS AND METHODS

### Materials

ApoA-I was prepared from fresh human plasma HDL as described (20). Phorbol 12-myristate-13-acetate (PMA) and 4α-PMA were purchased from Wako (Osaka, Japan), and *sn*-1,2-dioctanoylglycerol (DOG) was from Seikagaku Corporation (Tokyo, Japan). An ACAT inhibitor, F12511 (21), was a gift of Pierre Fabre Research (Castres Cedex, France) to T.Y.C.

### Cell lines and cell culture

The fibroblast cell lines WI-38 (a human fibroblast cell line), L929 (a mouse fibroblast cell line), and COS-7 (a monkey fibroblast cell line) were incubated as described (16). Human embryonic kidney-derived cell line HEK293 and a clone of its stable human ABCA1-green fluorescent protein transfectant (293/2c) were maintained in DMEM with 10% FBS as reported (22, 23), and this clone has been extensively studied (22–24). ACAT-1-deficient CHO cells, AC29 (25), its parental 25RA cells (26), and AC29 stably expressing human ACAT-1 (AC29/hACAT1) were grown in a 1:1 mixture of DMEM and Ham's F12 supplemented with 10% FBS plus 10 μg/ml gentamycin. 25RA cells have a gain-of-function mutation in SREBP cleavage-activating protein, resulting in constitutive activation of the proteolytic cleavage of SREBPs (27). The AC29/hACAT1 cell line was generated by transfection of pcDNA3 (Invitrogen) harboring human ACAT-1 cDNA (1397–4011 bp region, including the full-length open reading frame) (28). The pCMV4 plasmid containing human ACAT cDNA K1 (28) was digested by *Sal*I and *Sma*I. The resulting human ACAT-1 cDNA fragment was subcloned into *Eco*RV sites of pcDNA3, and AC29 cells were transfected with the plasmid by using Lipofectamine reagent (Invitrogen). A stable clone was isolated by the selection of G-418 resistance and further verified by the presence of cytoplasmic cholesteryl ester lipid droplets as visualized with a phase-contrast microscope. The clone was designated AC29/hACAT1, and it showed expression of the 50 kDa human ACAT-1 as confirmed by Western blotting (data not shown). Its enzyme activity is described in **Table 1**.

### Cellular lipid release

Cells grown at a confluent stage in six-well trays were incubated with or without apoA-I for the indicated periods of time in the presence of 0.1% fatty acid-free BSA, except that 0.02% BSA was used for HEK293 cells. After the incubation, lipid in medium and cells was extracted, and free cholesterol, total cholesterol, and choline-containing phospholipid were then determined enzymatically by the method described (10, 16). Alternatively, cellular lipids were radiolabeled with [$^3$H]cholesterol (NEN Life Science Products, Inc., Boston, MA) or with [$^3$H]choline chloride (NEN Life Science Products, Inc.) for 20–24 h, and the cells were incubated under the indicated conditions after washing with PBS. Cellular and medium lipids extracted were separated by TLC, and radioactivity of the desired lipid was determined by scintillation counting.

### Measurement of the free cholesterol pool available for ACAT

The ACAT-accessible cholesterol pool in the cells was estimated by measuring the incorporation of [$^{14}$C]oleic acid into

TABLE 1. Cellular cholesterol and ACAT activity in CHO mutants examined

| Variable | 25RA | AC29 | AC29/hACAT1 |
|---|---|---|---|
| Lipid droplets | + | − | + |
| Total cholesterol | 68.8 ± 2.9 | 35.9 ± 1.7 | 87.7 ± 4.3 |
| Free cholesterol | 43.8 ± 2.8 | 33.7 ± 1.2 | 62.7 ± 3.4 |
| Cholesteryl ester | 25.0 ± 1.9 | 2.2 ± 0.5 | 25.0 ± 1.4 |
| Phospholipid | 100.7 ± 5.7 | 116.2 ± 4.4 | 129.1 ± 6.2 |
| Intact cell assay | 10,388 ± 49 | 78 ± 4 | 11,488 ± 899 |
| In vitro assay | 40 ± 5 | 0 ± 0 | 89 ± 5 |

25RA, AC29, and AC29/hACAT1 cells were grown in medium containing 10% FBS. Cellular lipid contents were measured by enzymatic colorimetric assays as described in Materials and Methods after incubation of cells in medium with 0.1% BSA for 24 h. Cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. The data represent means ± SD of triplicate assays and are expressed as micrograms of lipid per milligram of cell protein. ACAT activity in these cells was determined by the intact cell ACAT assay and by the in vitro ACAT assay as described in Materials and Methods. These data represent the average ± variation between duplicate assays expressed as disintegrations per minute per milligram of cell protein for the intact cell assay or as picomoles per minute per milligram of cell protein for the in vitro assay.

cholesteryl ester in 1 h. After incubation of the cells in six-well trays at 37°C with or without stimulants (apoA-I, PMA, or DOG) for various periods of time in 0.1% or 0.02% (only for HEK293 cells) BSA-containing medium, the cells were further incubated in the presence of 1.5 or 1.0 μCi/ml [1-$^{14}$C]oleic acid (NEN Life Science Products, Inc.) for 1 h at 37°C in the same condition. After the cells were washed three times with ice-cold PBS, cellular lipids were extracted and separated by TLC to measure radioactivity in cholesteryl ester.

### ACAT assays

ACAT activity was determined by two different methods: intact cell ACAT assay and in vitro ACAT assay. In the intact cell assay, cells grown in medium containing 10% FBS were incubated with [$^{3}$H]oleate in BSA for 20 min and the incorporation of [$^{3}$H]oleate into cholesteryl ester was measured as described (29). The in vitro ACAT assay was performed as described previously (30). Briefly, whole cell extract prepared by hypotonic shock was solubilized, and ACAT was then placed in mixed micelles. ACAT activity was probed by measuring the incorporation of [$^{3}$H]oleoyl-CoA into cholesteryl ester.

### PKC assay

PKC activation was measured as described (24). Briefly, cells in a confluent stage in 100 mm dishes were incubated in the medium with 0.1% BSA for 20–24 h before stimulation by apoA-I (10 μg/ml) for various periods of time or with 160 nM PMA for 20 min as a positive control. The membrane fraction was prepared, and PKC activity in the membrane fraction (5 μg of protein) was determined by using a MESACUP Protein Kinase Assay Kit (Medical and Biological Laboratories) according to the manufacturer's instruction.

### Immunoblotting of ABCA1

Total membrane fraction or total cell lysate was prepared, and ABCA1 was analyzed by immunoblotting with the rabbit antiserum against the C-terminal peptide of human ABCA1 as described (16, 31, 32). Consistency of protein loading was confirmed by Coomassie Brilliant Blue staining of the electrophoretic gels or by immunoblotting of β-actin using anti-β-actin monoclonal antibody (clone AC-74 from Sigma). The signal intensity of ABCA1 was measured with NIH Image 1.61 software, and fold change in ABCA1 level was analyzed.

## RESULTS

### Change of the cellular cholesterol pool available to ACAT as induced by apoA-I

To elucidate the mechanisms for the cholesterol enrichment of HDL generated by the apoA-I/ABCA1 pathway, we used WI-38 human fibroblasts, L929 mouse fibroblasts, and COS-7 monkey fibroblasts to represent the cells that generate cholesterol-rich HDL, cholesterol-poor HDL, and no HDL by apoA-I treatment, respectively. As we reported previously (16), WI-38 cells released both cholesterol and phospholipid, L929 cells predominantly released phospholipid, and COS-7 cells released neither cholesterol nor phospholipid upon incubation with apoA-I (**Fig. 1A, B**). The ratio of cholesterol to phospholipid in the conditioned medium was therefore higher in WI-38 cells than L929 cells (**Table 2**), reflecting the lipid profiles of the HDL fraction generated by these cells (16). The release of cholesterol and phospholipid by apoA-I from WI-38 was linear up to 24 h (Fig. 1C).

Change in the cellular ACAT-accessible cholesterol pool by apoA-I was estimated in these three fibroblast cell lines. The ACAT-accessible cholesterol pool was probed by measuring the incorporation of [$^{14}$C]oleic acid into cholesteryl ester. In contrast to the linear time course of apoA-I-mediated cholesterol release (Fig. 1C), the cholesterol pool rapidly decreased within the initial few hours after exposing the cells to apoA-I in WI-38 (**Fig. 2**), consistent with our previous reports (33, 34). Decrease of the ACAT-accessible cholesterol pool by apoA-I was also shown in BALB/3T3 (a mouse fibroblast cell line) and MRC-5 (a human fibroblast cell line) (by 17% and 24%, respectively, from the control at the 3 h incubation time with apoA-I),

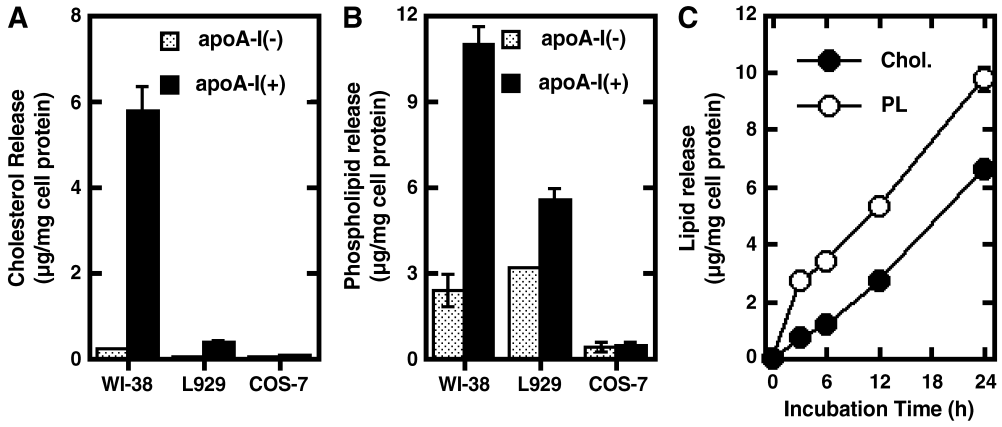


**Fig. 1.** Release of cellular lipid by apolipoprotein A-I (apoA-I) in fibroblast cell lines. WI-38, L929, and COS-7 cells were incubated with (+) and without (−) 10 μg/ml apoA-I for 24 h, and cholesterol (A) and choline-phospholipid in the medium (B) were measured. The time course of apoA-I-mediated cholesterol and phospholipid release in WI-38 cells is shown in C. Cholesterol (Chol.) and choline-phospholipid (PL) were measured enzymatically as described in Materials and Methods. The data represent mean ± SD of triplicate assays.

TABLE 2. Characterization of apoA-I-mediated reactions in the cells examined

| Variable | WI-38 | L929 | COS-7 |
|---|---|---|---|
| Cholesterol release by apoA-I | + | − | − |
| PL release by apoA-I | + | + | − |
| HDL generation[a] | + | + | − |
| FC/PL in the HDL[b] | 0.65 | 0.14 | NA[c] |
| ABCA1 expression[a] | + | + | − |
| Caveolin-1 expression[a] | + | + | + |
| Cholesterol translocation by apoA-I | + | − | − |
| ABCA1 increase by apoA-I | + | ± | NA |
| PKC activation by apoA-I | + | − | − |
| Cholesterol translocation by PMA | + | + | − |
| ABCA1 increase by PMA | + | − | NA |
| PKC activation by PMA | + | + | + |

apoA-I, apolipoprotein A-I; FC, free cholesterol; PKC, protein kinase C; PL, phospholipid; PMA, phorbol 12-myristate-13-acetate.

[a] From ref. (16)

[b] Determined from the results shown in Fig. 1.

[c] NA, not applicable.

both of which generate cholesterol-rich HDL in the presence of apoA-I. In contrast, no change of the ACAT-accessible cholesterol pool size was observed in L929 cells or in COS-7 cells that generate cholesterol-poor HDL or no HDL by apoA-I, respectively (Fig. 2).

To confirm a relationship between the ABCA1-mediated cholesterol release by apoA-I and the decrease of the ACAT-accessible cholesterol pool, HEK293 cells stably expressing human ABCA1 (293/2c) were compared with nontransfected HEK293 cells. Wild-type HEK293 did not express ABCA1 at a detectable level by Western blotting (23) and released neither phospholipid nor cholesterol by apoA-I (**Fig. 3A, B**). ApoA-I also failed to reduce the ACAT-accessible cholesterol pool in the cells (Fig. 3C). In contrast, 293/2c cells demonstrated an apoA-I-mediated release of cholesterol and phospholipid and a decrease of ACAT-accessible cholesterol by apoA-I (Fig. 3). It is thus clear that apoA-I reduces the ACAT-accessible pool as it mediates cholesterol removal in the presence of ABCA1 activity.

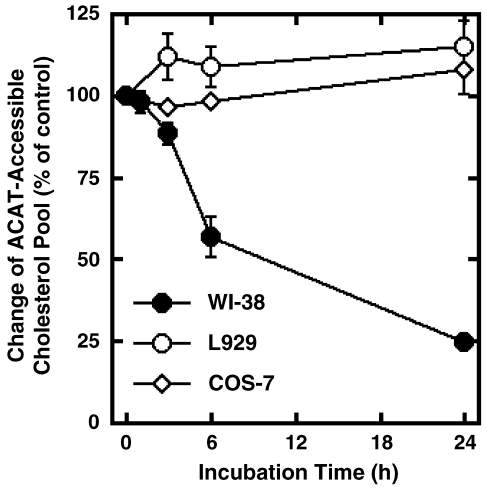


**Fig. 2.** Change of the ACAT-accessible cholesterol pool by apoA-I. Cells were incubated with or without 10 μg/ml apoA-I for the indicated times, and 1.5 μCi/ml [$^{14}$C]oleic acid was included during the final 1 h for measurement of its incorporation into cholesteryl ester. Data represent means ± SD for percentage of control (incubation without apoA-I) based on percentage of cholesteryl [$^{14}$C]oleate to the total cellular incorporation of [$^{14}$C]oleic acid. The control values were all of approximately the same order of magnitude, such as $4 \times 10^4$ dpm/mg cell protein for WI-38, $3 \times 10^4$ dpm/mg for COS-7, and $4 \times 10^4$ dpm/mg for L929.

### Effect of ACAT enzyme activity on apoA-I-mediated cholesterol release

To examine the role of the ACAT enzyme in the regulation of the cholesterol pool available for ABCA1-mediated cholesterol release by apoA-I, we treated cells with a potent and specific ACAT inhibitor. Treatment of the CHO cell-derived clone, 25RA, with an ACAT inhibitor, F12511, resulted in a substantial increase in apoA-I-mediated cholesterol release (**Fig. 4A**). It also caused an increase in phospholipid release, although smaller than the cholesterol increase (Fig. 4A). Treating cells with the ACAT inhibitor F12511 caused ABCA1 upregulation (Fig. 4C), consistent with our previous finding in mouse peritoneal macrophages using a different ACAT inhibitor (35). To avoid the use of ACAT inhibitors that may cause nonspecific side effect(s), we examined ACAT-1-deficient cells, AC29 (25), and AC29 stably expressing human ACAT-1 (AC29/hACAT1) were also examined to assess the role of ACAT. Table 1 shows cellular cholesterol and ACAT activity in these CHO mutants. ApoA-I-mediated cholesterol release was 9-fold higher in the AC29 cell than its parental cell, 25RA (Fig. 4B). Expression of human ACAT-1 in AC29 cells partially reversed the apoA-I-mediated cholesterol release, although it was still higher than that in 25RA cells, presumably because of the higher free cholesterol level in AC29/hACAT1 cells than in 25RA cells (Table 1). Phospholipid release by apoA-I was also enhanced in AC29 cells, although not as much as the cholesterol release; phospholipid release was slightly higher in AC29/hACAT1 than in 25RA cells (Fig. 4B). We next examined the cellular ABCA1 levels in these cells treated with or without apoA-I. ABCA1 levels in these mutants were counterregulated by the expression of ACAT, as shown in Fig. 4C. ApoA-I further increased ABCA1 even in the ACAT-deficient cells (Fig. 4D). These results suggested that the mechanisms that cause the increase in ABCA1 protein content by inactivation of ACAT and by exposure to apoA-I are different. Inactivation of ACAT may cause an increase of ABCA1 expression as a result of transcription activation (35), whereas lipid-free apolipoprotein stabilizes the cellular ABCA1 protein against degradation (32). Irrespective of the mechanisms involved, change in ABCA1 expression seems correlate with the increase of apoA-I-mediated phospholipid release rather than cholesterol release (Fig. 4).

### PKC activation induces the translocation of intracellular cholesterol from the ACAT-accessible pool for apoA-I-mediated release

It has been reported that PKC is involved in the change of the ACAT-accessible cholesterol pool in rat vascular

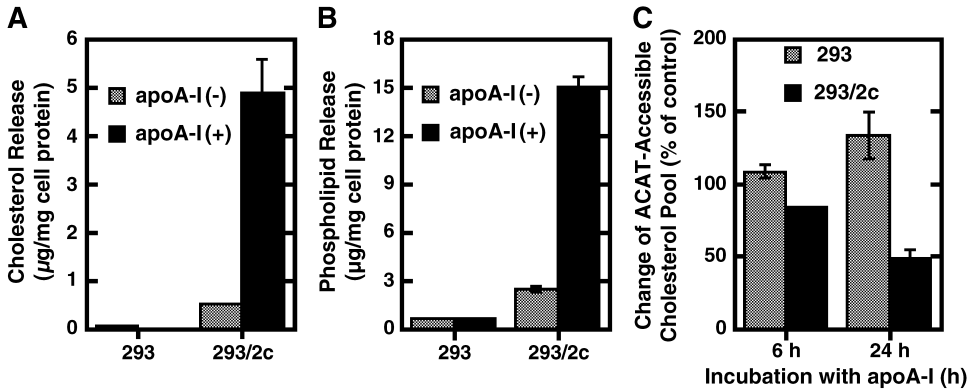


**Fig. 3.** ABCA1 requirement for apoA-I-induced intracellular cholesterol translocation. apoA-I-mediated cholesterol (A) and phospholipid (B) release and change of the ACAT-accessible cholesterol pool by apoA-I (C) in ABCA1-transfected (293/2c) and wild-type HEK293 (293) cells were examined. A, B: apoA-I-mediated cholesterol and phospholipid release were determined enzymatically after incubation of cells with and without apoA-I (10 μg/ml) for 24 h. C: Change of the ACAT-accessible cholesterol pool was assayed by incorporation of 1.0 μCi/ml [$^{14}$C]oleic acid into cholesteryl ester as described for Fig. 2. The data represent means ± SD of triplicate determinations as expressed as percentage of control (without apoA-I).

smooth muscle cells and mouse peritoneal macrophages (33, 34). To extend these early studies, we treated various cell types with PKC activators to monitor the effect of the change in the ACAT-accessible cholesterol pool. The change of the cholesterol pool was demonstrated with the short-term treatment of cells with 160 nM PMA, which leads to PKC activation (**Fig. 5A**). WI-38 and L929 showed decreases of this cholesterol pool by PKC activation in the absence of cholesterol acceptor, whereas a reduction was not observed in COS-7, in which ABCA1 expression is not detected. Treatment with DOG, another PKC activator, also induced the reduction of the ACAT-accessible cholesterol pool in WI-38 (data not shown). In contrast, 4α-PMA, a control compound of PMA that possesses no stimulating effect on PKC, had no effect on the reduction of the ACAT-accessible cholesterol pool in WI-38 (data not shown). Thus, PKC seems to trigger cholesterol translocation from the ACAT-accessible pool.

PKC activation by apoA-I was previously demonstrated in WI-38 human fibroblasts (24). We examined whether apoA-I can also activate PKC in L929 and COS-7 cells. Both cells were treated with apoA-I for 5–120 min, and the membrane-associated PKC activities were then measured. We found no PKC activation by apoA-I at any point during this time course in these cell lines. The control experiment showed that a 20 min PMA treatment increased membrane-associated PKC activity in both cell lines: 1.6-fold in COS-7 cells and 2.1-fold in L929 cells. Thus, apoA-I failed in the activation of PKC, reduction of the ACAT-accessible cholesterol pool, and induction of cholesterol release in L929, whereas pharmacological activation of PKC induced the reduction of this cholesterol compartment. Therefore, we examined the effect of PMA on the apoA-I-mediated cholesterol release in this cell line (Fig. 5B). L929 cells were pretreated with 160 nM PMA for 30 or 60 min before incubation with apoA-I. A significant increase by PMA treatment was observed in the apoA-I-mediated release of cholesterol ($P < 0.05$, Student's *t*-test) when measured as the short-term release of [$^{3}$H]cholesterol (Fig. 5B), although it was still poor and no measurable mass was detected in the medium because it was still under the detection limit of the assay method. The apoA-I-dependent cholesterol release was increased by ~2.7 times. The release of phosphatidylcholine and sphingomyelin by apoA-I was not influenced by PMA, resulting in cholesterol "enrichment" in the conditioned medium. Thus, PKC activation induced intracellular cholesterol translocation from the ACAT-accessible pool, presumably to the site for the apoA-I/ABCA1-mediated release in L929 cells.

### Change in ABCA1 protein level by apoA-I and PMA

We examined the effect of apoA-I and PMA on change in ABCA1 expression level. As we reported (24), apoA-I treatment resulted in an increase of ABCA1 in WI-38 as a result of the retardation of proteolytic degradation (**Fig. 6A**). On the other hand, apoA-I failed to increase ABCA1 protein in L929 within 4 h (Fig. 6A), although a longer incubation (24 h) increased it to some extent (data not shown). The PMA treatment that leads to PKC activation resulted in an increase of ABCA1 by 1.4-fold in WI-38 cells in 1 h, consistent with our previous report (24), whereas the same treatment did not affect ABCA1 expression in L929 (Fig. 6B). Therefore, the stimulations that lead to ABCA1 stabilization in WI-38 human fibroblasts were inefficient in L929 cells.

## DISCUSSION

We have suggested differential regulation of cholesterol release and phospholipid release in the apolipoprotein/ABCA1 pathway to generate HDL, based on the following observations. Cholesterol contents in the HDL generated by the apolipoprotein-cell interaction is cell specific (16,

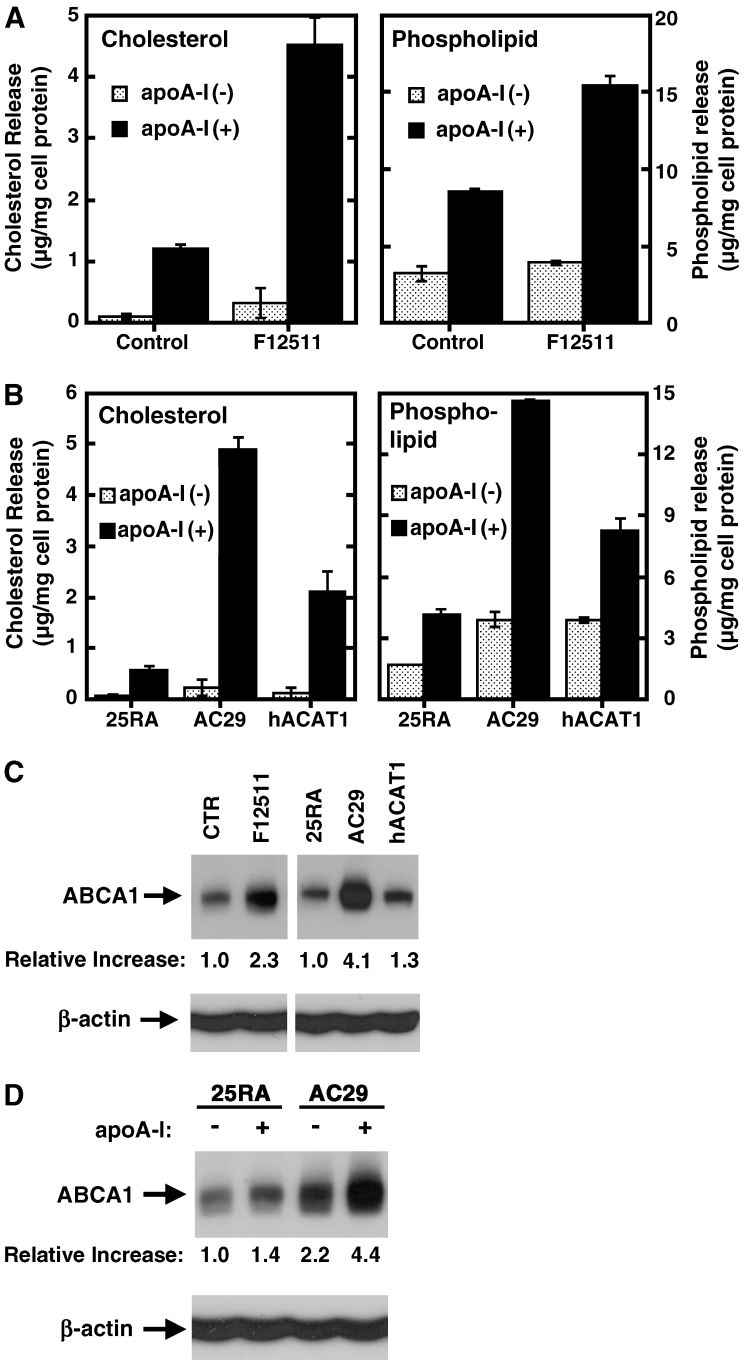


**Fig. 4.** Effect of ACAT activity on apoA-I-mediated cholesterol release and cellular ABCA1 levels. A: 25RA cells were seeded into six-well trays at a density of $1.5 \times 10^5$ cells/well and were grown for 3 days. The cells were incubated with (+) and without (−) 5 μg/ml apoA-I in the presence or absence of the ACAT inhibitor F12511 (400 nM) for 24 h, and release of cholesterol and choline-phospholipid were measured. The data represent means ± SD in triplicate assays. B: apoA-I-mediated release of cholesterol and phospholipid from ACAT-deficient cells. The CHO mutant, 25RA, its ACAT-deficient mutant, AC29, and human ACAT-1-expressing AC29 cells, AC29/hACAT1, were grown as described above. The cells were then incubated with (+) and without (−) 5 μg/ml apoA-I for 24 h, and releases of cholesterol and choline-phospholipid were measured. The data represent means ± SD of the apoA-I-dependent lipid release in triplicate assays. C, D: Cellular ABCA1 protein level was examined in CHO mutant cells. CHO cells (25RA) were incubated with and without an ACAT inhibitor, F12511 (400 nM) [F12511 and CTR (control), respectively] for 24 h in the medium containing 10% FBS (C, left panel). The right panel of C shows the results with 25RA, its ACAT-deficient mutant AC29, and AC29 transfected with ACAT-1 (AC29/hACAT1) under the same incubation conditions without an ACAT inhibitor. The effect of apoA-I on ABCA1 levels was examined for 25RA cells and AC29 cells as incubated with (+) and without (−) 5 μg/ml apoA-I for 24 h in 0.1% BSA. Equal amounts of whole cell lysate protein (80 μg protein/lane) from the cells indicated were subjected to immunoblot analysis using anti-ABCA1 antibody or anti-β-actin antibody as a loading control. The signal intensity of ABCA1 was measured as described in Materials and Methods, and relative increases of ABCA1 are indicated. The data represent mean values of two or three separate scanning results, and similar results were obtained in two separate experiments. Expression of β-actin did not change between the cells compared.

36). Apolipoprotein-mediated cellular cholesterol release was accompanied by a rapid reduction of the intracellular pool of cholesterol available to ACAT within the initial few hours in mouse peritoneal macrophages, whereas cholesterol release was linear for at least 24 h (33). PKC inhibitors and activators modulated both cholesterol content in the HDL generated by the apolipoprotein-cell interaction and change in the ACAT-accessible cholesterol pool in certain cells under certain conditions (33, 34). More recent studies have shown that caveolin-1 is involved in cholesterol enrichment of the HDL generated by apoA-I-mediated lipid release in THP-1 cells (10) and that plasma membrane lipid composition modulates apoA-I/ABCA1-mediated cholesterol release but not phospholipid release (37). On the other hand, pharmacological inhibition of ACAT increased ABCA1 through the enhancement of its transcription (35). Thus, in the current work, we attempted to establish a role of ACAT in the apoA-I/ABCA1-mediated HDL assembly and investigated potential factors involved in the mobilization of intracellular cholesterol for HDL assembly.

Table 2 summarizes the apoA-I-mediated reactions in the fibroblasts examined. ABCA1 was expressed in WI-38 and L929 (24). Consequently, HDL was generated by apoA-I with WI-38 and L929, but no HDL was produced with COS-7. However, HDL produced with L929 contained almost no cholesterol (16). ApoA-I induced the reduction of the ACAT-accessible cholesterol pool in WI-38 cells but not in COS-7 or L929, neither of which exhibits cholesterol re-

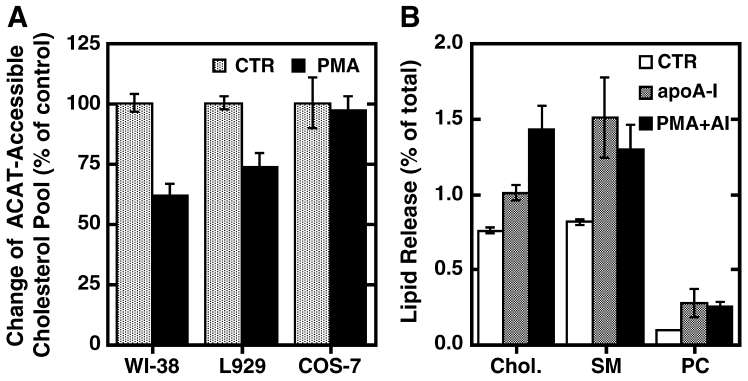


**Fig. 5.** Effect of phorbol 12-myristate-13-acetate (PMA) on the ACAT-accessible cholesterol pool and apoA-I-mediated cholesterol release in L929. A: Cells grown in six-well trays were stimulated with 160 nM PMA in the presence of 1.5 μCi/ml [$^{14}$C]oleic acid for 1 h, and incorporation of the radioactivity into cholesterol ester was measured as in Materials and Methods. The data points represent means ± SD of triplicate assays. CTR, control. B: L929 cells were incubated with 5 μCi/ml [$^{3}$H]cholesterol or 5 μCi/ml [$^{3}$H]choline chloride for 24 h as described in Materials and Methods. The cells were treated with or without 160 nM PMA for 30 min. Cells were washed three times with PBS followed by incubation of the cells with or without 10 μg/ml apoA-I for 3 h. Radiolabeled cholesterol (Chol.), phosphatidylcholine (PC), and sphingomyelin (SM) in the medium and cells were determined by TLC. The data represent means ± SD of triplicate assays expressed as percentage of release of the respective lipid.

lease by apoA-I. The reduction of the ACAT-accessible cholesterol pool by apoA-I was also observed in HEK293 stably expressing ABCA1 but not in wild-type HEK293 cells. Thus, the reduction of this compartment is related to the release of cholesterol by the apoA-I/ABCA1 reaction but not directly to ABCA1 expression and the generation of HDL with cellular phospholipid. These results indicate that cholesterol is mobilized from the ACAT-accessible pool for cholesterol enrichment of the HDL to be generated by the apolipoprotein/ABCA1-mediated reaction. Inactivation of ACAT-1 resulted in increases in both ABCA1 expression and lipid release, but the increase in ABCA1 expression related more directly to the apoA-I-mediated phospholipid release than did the cholesterol release. The change in cholesterol release by apoA-I was almost twice as great as the changes in phospholipid release and ABCA1 expression. These results are consistent with the finding of an increase of HDL-cholesterol in ACAT-1-deficient mice (38). We thus propose that ACAT-1 enzyme activity directly modulates the ABCA1/apolipoprotein-mediated HDL assembly by regulating both ABCA1 expression and the mobilization of cellular cholesterol.

As mentioned above, PKC activity seems to modulate the ACAT-accessible cholesterol pool (33, 34). For further characterization of this phenomenon, various fibroblast cells were treated with a PKC activator. Direct activation of PKC by PMA induced a reduction of the ACAT-accessible cholesterol pool in most of the cell types that produce cholesterol-rich HDL. Interestingly, PMA decreased the ACAT-accessible cholesterol in L929 cells. In these cells, apoA-I produced cholesterol-poor HDL but failed to reduce the ACAT-accessible cholesterol compartment. Accordingly, HDL produced from the PMA-treated L929 was relatively "enriched" with cholesterol. Therefore, PKC activation seems to trigger cellular cholesterol mobilization.

It remains to be investigated how apoA-I and/or PKC stimulates intracellular cholesterol transport. Relevant to this question is the finding that phosphorylation of caveolin-1 at serine 80 may modulate its cholesterol binding and apoA-I-mediated cholesterol release (39). Vesicular transport is also a focus of the study of cholesterol trafficking. ABCA1 is localized in intracellular compartments such as endosomes and the Golgi (13, 40). ApoA-I stimulates vesicular transport from the Golgi to the plasma membrane (41), and transport of lipids from the Golgi to

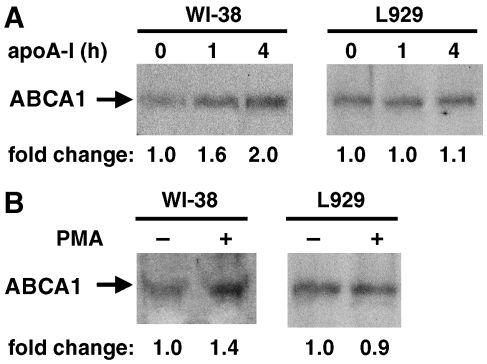


**Fig. 6.** Change of ABCA1 protein by apoA-I or PMA in WI-38 and L929 fibroblast cells. Cells were incubated with apoA-I (10 μg/mL) for the indicated times (A) or with 320 nM PMA for 1 h (B), and the membrane fraction was then prepared. Equal amounts of membrane protein (100 μg/lane for WI-38 and 200 μg/lane for L929) were subjected to immunoblotting using anti-ABCA1 antibody. The signal intensity for ABCA1 was measured as described in Materials and Methods, and relative changes of ABCA1 are shown. The data represent means of two or three scanning results, and similar results were obtained in two separate experiments. Consistency of the protein loading was verified by Coomassie Brilliant Blue staining of the gels (data not shown).

the plasma membrane is defective in the ABCA1-deficient cells (13). In addition, ABCA1 is reportedly involved in late-endosome vesicular trafficking (42). However, none of these reports directly indicate the involvement of PKC in the modulation of vesicular transport or ABCA1 localization.

ABCA1 is protected from calpain-mediated proteolytic degradation in the presence of lipid-free apolipoprotein (32, 43). We have recently demonstrated that apoA-I activates PKCα to phosphorylate and stabilize ABCA1 (24). In that study, we found a greater effect of PKC inhibitors on apoA-I-mediated cholesterol release when the inhibitors prevented both cholesterol and phospholipid release (24). In the current paper, we demonstrate that PKC also plays a role in the intracellular translocation of cholesterol for ABCA1-mediated HDL assembly by apoA-I. Thus, these dual effects of PKC activation may account for the difference of the inhibitory effect of PKC inhibitors on apoA-I-mediated cholesterol and phospholipid release.

However, apoA-I and PMA both failed to increase ABCA1 in L929 mouse fibroblast cells, inconsistent with our previous reports showing that release of phospholipid, presumably sphingomyelin, induces PKC activation by phosphatidylcholine-specific phospholipase C-mediated diacylglycerol production, leading to phosphorylation and stabilization of ABCA1 in WI-38 human fibroblasts (24). Another mouse fibroblast cell line, BALB/3T3, and mouse peritoneal macrophages both showed very poor increases of ABCA1 by apoA-I (16, 35). In addition, PKC inhibitors prevented only apoA-I-mediated cholesterol release in mouse macrophages (34). These results may indicate insufficiency of the PKC signaling pathway to regulate ABCA1 stabilization in murine cells.

In summary, the results in this report fit the conclusion that PKC plays a role in the apolipoprotein/ABCA1-mediated cholesterol release by inducing not only ABCA1 phosphorylation and stabilization but also intracellular cholesterol mobilization for its release, at least in human cells. ApoA-I mobilizes intracellular cholesterol from the ACAT-accessible compartment for ABCA1-mediated release via a process involving PKC signaling. In addition, ACAT-1 directly controls cholesterol availability for ABCA1-mediated release.

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