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.**

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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),

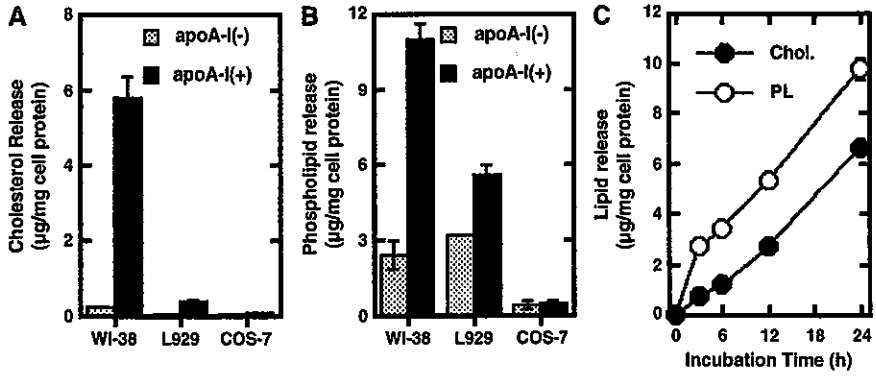

Fig. 1. Release of cellular lipid by apolipoprotein A-I (apoA-I) in fibroblast cell lines. WI-38, L929, and COS-7 cells were incubated with (+) and without (−) 10 μg/ml apoA-I for 24 h, and cholesterol (A) and choline-phospholipid in the medium (B) were measured. The time course of apoA-I-mediated cholesterol 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.

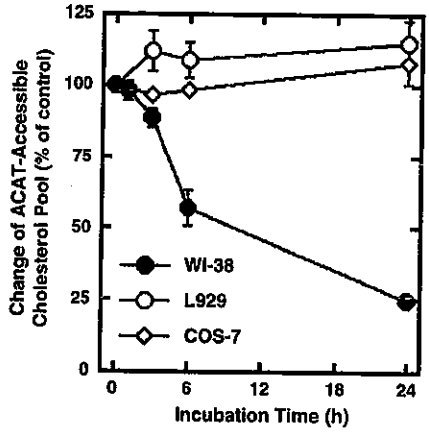

Fig. 2. Change of the ACAT-accessible cholesterol pool by apoA-I. Cells were incubated with or without 10 μg/ml apoA-I for the indicated times, and 1.5 μCi/ml [^{14}C]oleic acid was included during the final 1 h for measurement of its incorporation into cholesteryl ester. Data represent means ± SD for 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 4×10^4 dpm/mg cell protein for WI-38, 3×10^4 dpm/mg for COS-7, and 4×10^4 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

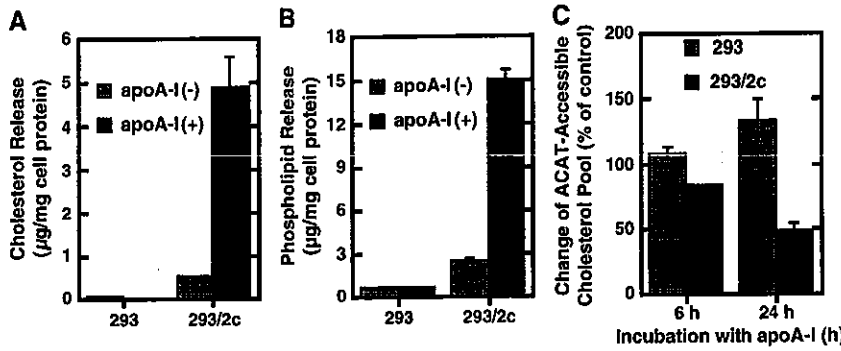

Fig. 3. ABCA1 requirement for apoA-I-induced intracellular cholesterol translocation. apoA-I-mediated cholesterol (A) and phospholipid (B) release and change of the ACAT-accessible cholesterol pool by apoA-I (C) in ABCA1-transfected (293/2c) and wild-type HEK293 (293) cells were examined. A, B: apoA-I-mediated 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,

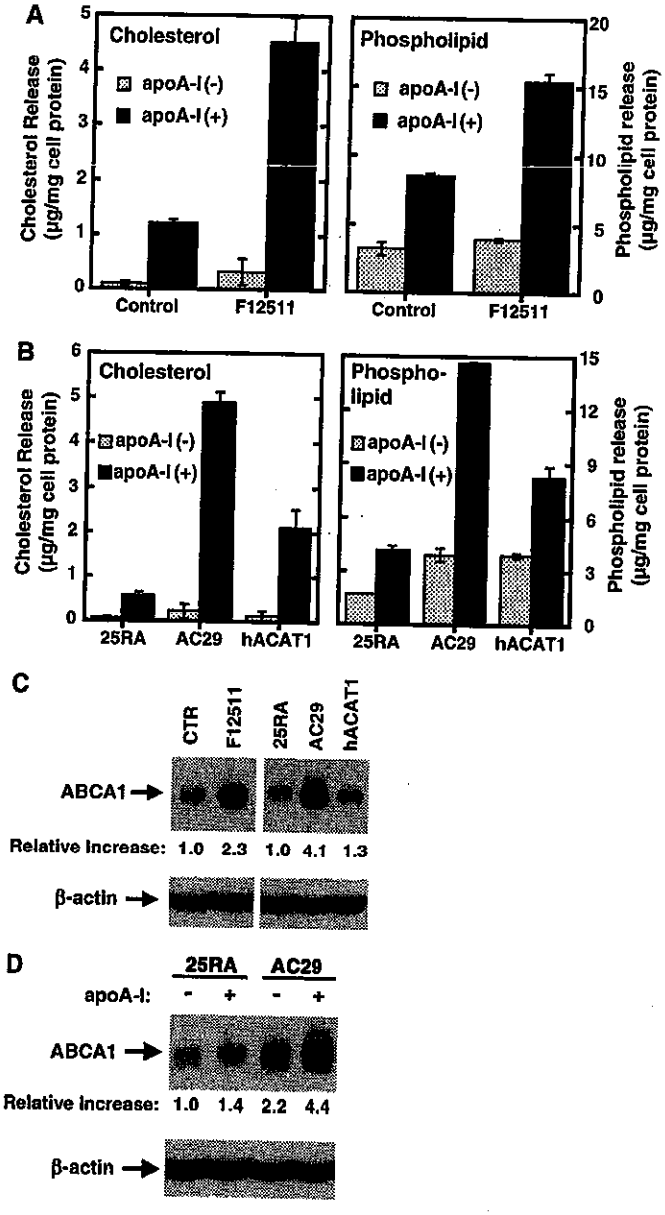

Fig. 4. Effect of ACAT activity on apoA-I-mediated cholesterol release and cellular ABCA1 levels. A: 25RA cells were seeded into six-well trays at a density of 1.5×10^5 cells/well and were grown for 3 days. The cells were incubated with (+) and without (−) 5 μg/ml apoA-I in the presence or absence of 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-

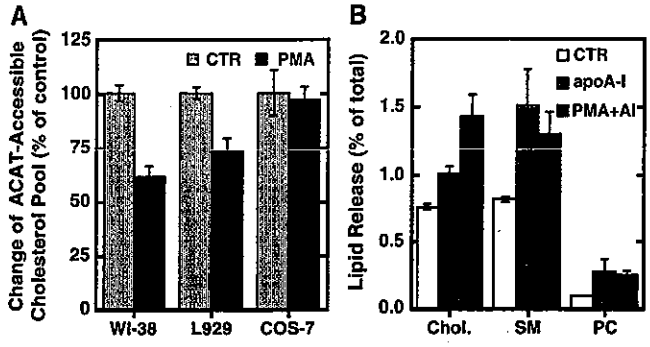

Fig. 5. Effect of phorbol 12-myristate-13-acetate (PMA) on the ACAT-accessible cholesterol pool and apoA-I-mediated cholesterol release in L929. A: Cells grown in six-well trays were stimulated with 160 nM PMA in the presence of 1.5 μCi/ml [^{14}C]oleic acid for 1 h, and incorporation of the radioactivity 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

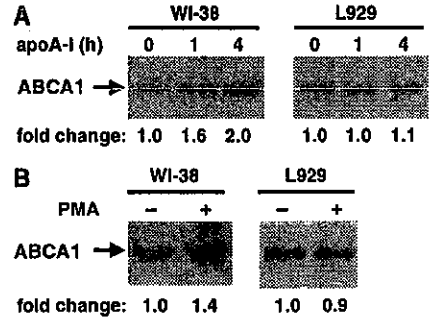

Fig. 6. Change of ABCA1 protein by apoA-I or PMA in WI-38 and L929 fibroblast cells. Cells were incubated with apoA-I (10 μg/mL) for the indicated times (A) or with 320 nM PMA for 1 h (B), and the membrane fraction was then prepared. Equal amounts of membrane protein (100 μg/lane for WI-38 and 200 μg/lane 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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Apolipoprotein A-I induces translocation of protein kinase Cα to a cytosolic lipid-protein particle in astrocytes

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Abstract **Apolipoprotein A-I (apoA-I) induces the translocation of newly synthesized cholesterol as well as caveolin-1 to the cytosolic lipid-protein particle (CLPP) fraction in astrocytes before its appearance in high density lipoprotein generated in the medium (Ito, J., Y. Nagayasu, K. Kato, R. Sato, and S. Yokoyama. 2002. Apolipoprotein A-I induces translocation of cholesterol, phospholipid, and caveolin-1 to cytosol in rat astrocytes. *J. Biol. Chem.* 277: 7929–7935). We here report the association of signal-related molecules with CLPP. ApoA-I induces rapid translocation of protein kinase Cα to the CLPP fraction and its phosphorylation in astrocytes. ApoA-I also induces the translocation of phospholipase Cγ to CLPP. Diacylglyceride (DG) production is increased by apoA-I in the cells, with a maximum at 5 min after the stimulation, and the increase takes place also in the CLPP fraction. An inhibitor of receptor-coupled phospholipase C, U73122, inhibited all the apoA-I-induced events, such as DG production, cholesterol translocation to the cytosol, release of cholesterol, and translocation of protein kinase Cα into the CLPP fraction. CLPP may thus be involved in the apoA-I-initiated signal transduction in astrocytes that is related to intracellular cholesterol trafficking for the generation of high density lipoprotein in the brain.**—Ito, J-i., H. Li, Y. Nagayasu, A. Kheirollah, and S. Yokoyama. **Apolipoprotein A-I induces translocation of protein kinase Cα to a cytosolic lipid-protein particle in astrocytes.** ***J. Lipid Res.*** **2004. 45: 2269–2276.**

Supplementary key words caveolin-1 • phospholipase C • phosphatidylinositol turnover • cholesterol

The main apolipoproteins in mammalian cerebrospinal fluid (CSF) are apolipoprotein A-I (apoA-I) and apoE (1–3), which are present as HDL and play major roles in intercellular cholesterol transport in the brain (4), being segregated by the blood-brain barrier from the lipoprotein system in the systemic circulation. Astrocytes and partly microglia cells generate cholesterol-rich HDL by endogenous apoE along with cellular cholesterol and phospholipid (5–9). These HDLs may transport cholesterol to the neural cells where it is required via the cellular receptors that recognize lipid-bound apoE (10). ApoE-HDL was indeed shown to play a critical role in wound healing of the brain (11). ApoA-I is also found in human CSF as the second major apolipoprotein, with a concentration almost equivalent to that of apoE (12–14), but the source of this protein is unclear. No neural cell is believed to produce apoA-I, whereas the brain capillary endothelial cells produce apoA-I, although it is uncertain whether it is secreted into the CSF (15, 16). Some authors propose that the apoA-I in the systemic circulation is transported across the blood-brain barrier (3, 4).

In addition to the production of apoE-HDL, astrocytes interact with exogenous apoA-I to generate phospholipid-rich and cholesterol-poor HDL (5, 17, 18). The physiological relevance of this observation in human brain has been supported by the facts that the apoA-I concentration in CSF is high enough to carry this reaction (13, 14) and that apoA-I dissociates from HDL to interact with the cells (19). The cholesterol-rich apoE-HDL and cholesterol-poor apoA-I-HDL may play differential roles in intercellular cholesterol transport in the brain.

In a previous paper, we demonstrated transient translocation of newly synthesized cholesterol and phospholipid to the cytosol from the endoplasmic reticulum and Golgi apparatus when exogenous apoA-I interacted with rat astrocytes and generated HDL (17, 20, 21). Transient translocation of caveolin-1 to the cytosol was also induced in a similar time-dependent manner to the lipid translocation (20). The lipids and caveolin-1 in the cytosol were recovered along with cyclophilin A in the cytosolic fraction, having the same density as plasma HDL [cytosolic lipid-protein particle (CLPP)]. The CLPP is a particle composed of proteins and lipids such as cholesterol, sphingomyelin,

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Abbreviations: apoA-I, apolipoprotein A-I; apoE-KO mouse, apoE knockout C57BL/6 mouse; CLPP, cytosolic lipid-protein particle; CSF, cerebrospinal fluid; DG, diacylglyceride; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; PI, phosphatidylinositol.

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and phosphatidylcholine with a diameter of 17–18 nm and a density of 1.08–1.12 g/ml (20). Cyclosporin A, a cyclophilin A inhibitor, inhibited this apoA-I-induced translocation and also apoA-I-mediated cholesterol release. Caveolin-1 is believed to play an important role in intracellular cholesterol trafficking, so that it is rational to hypothesize that CLPP is involved in the intracellular cholesterol transport stimulated by extracellular apoA-I for the generation of HDL. We attempted to investigate potential signaling pathways in astrocytes for apoA-I to stimulate lipid trafficking in relation to the function of CLPP. Protein kinase Cα and its related signaling molecules were found associated with this particle when cells were stimulated by apoA-I.

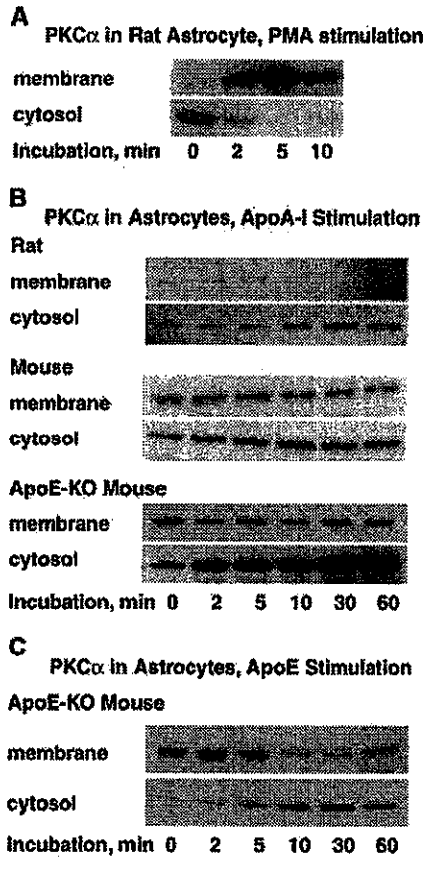

Fig. 1. Redistribution by apolipoprotein A-I (apoA-I) of protein kinase Cα (PKCα) in astrocytes. A: Rat astrocytes were treated with 200 nM phorbol 12-myristate 13-acetate (PMA). The membrane fraction protein (15 μg/lane) and the cytosol protein (50 μg/lane) were analyzed for protein kinase Cα by immunoblotting. Translocation of protein kinase Cα was demonstrated from the cytosol to the membrane. B: Astrocytes of rat, mouse, and apoE knockout C57BL/6 mouse (apoE-KO mouse) were incubated with 5 μg/ml apoA-I for the indicated period of time in 0.02% BSA/F-10, 0.02% BSA/DMEM, and 0.02% BSA/DMEM, respectively. The cytosol protein (30 μg/lane) and the membrane protein (15 μg/lane) were analyzed for protein kinase Cα. C: Astrocytes of an apoE-KO mouse were incubated with 5 μg/ml apoE. The same analysis was performed for protein kinase Cα.

MATERIALS AND METHODS

Materials

ApoA-I was prepared from freshly isolated human HDL by delipidation and anion-exchange chromatography according to the method described elsewhere (22). ApoE was prepared from hyperlipidemic human plasma as previously described (23). Inhibitors of receptor-coupled phospholipase C and its inactive analog, U73122 and U73343 (24), were purchased from WAKO Pure Chemical.

Cell culture

Astrocytes were prepared according to the method previously described from the cerebrums of 17 day old fetal Wistar rat (25), C57BL/6 mouse, and apoE knockout C57BL/6 mouse (apoE-KO mouse) purchased from Taconic/IBL (Germantown, NY/Fujioka,

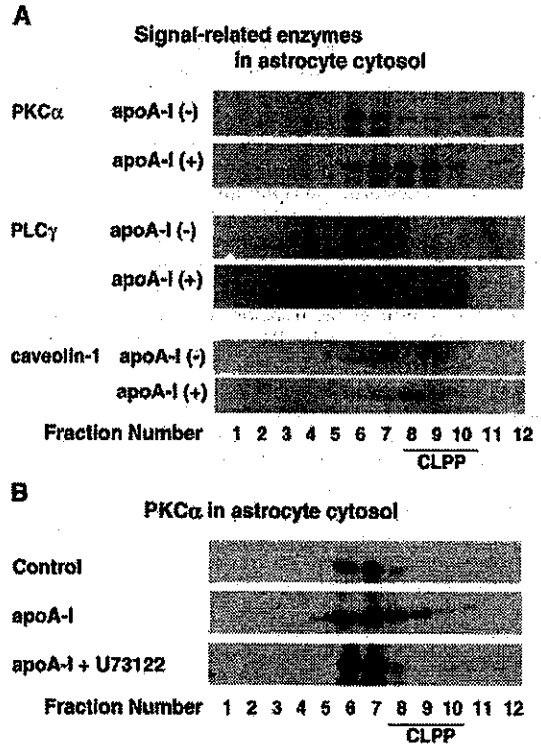

Fig. 2. Redistribution by apoA-I of protein kinase Cα (PKCα) and phospholipase Cγ (PLCγ) in cytosol of mouse astrocytes. A: After washing and medium replacement with 0.02% BSA/DMEM, apoE-KO mouse astrocytes were incubated with or without apoA-I (5 μg/ml) for 5 min. The cytosol (350 μg protein/7 ml) was prepared from the cells and centrifuged on the sucrose solution (18 ml) with a density of 1.17 g/ml at 49,000 rpm for 48 h and separated into 12 fractions from the bottom. Protein was precipitated with 10% TCA and analyzed by SDS-PAGE and Western blotting using rabbit anti-protein kinase Cα, mouse anti-phospholipase Cγ, and rabbit anti-caveolin-1 antibodies. CLPP, cytosolic lipid-protein particle. B: The cytosol (380 μg/7 ml) was prepared from apoE-KO mouse astrocytes treated with apoA-I (0 or 5 μg/ml) for 5 min with or without a 5 min pretreatment with 10 μM U73122. The cytosol was centrifuged as described in A and separated into 12 fractions from the bottom. The 10% TCA-precipitated protein of each fraction was analyzed by SDS-PAGE and Western blotting using rabbit anti-protein kinase Cα.

Japan). After removal of the meninges, the cerebral hemisphere was cut into small pieces and treated with 0.1% trypsin solution in Dulbecco's phosphate-buffered saline (DPBS) containing 0.15% glucose (0.1% trypsin/DPBS/G) for 3 min at room temperature. The cell pellets obtained by centrifugation at 1,000 rpm for 3 min were cultured in F-10 medium containing 10% fetal calf serum (10% FCS/F-10) for rat astrocytes or 15% FCS/DMEM for mouse astrocytes at 37°C for 1 week. The cells were treated with 0.1% trypsin/DPBS/G containing 1 mM EDTA again and then cultured in 10% FCS/F-10 or 15% FCS/DMEM using a six-well multiple tray for 1 week. Human fibroblast cell line WI-38 cells (RIKEN Cell Bank) were grown in 10% FCS/DMEM.

Cytosol preparation and density gradient ultracentrifugation analysis

Cytosol of astrocytes was prepared according to the method of Thom et al. (26). Cell pellet was obtained by centrifugation at 1,000 rpm for 10 min after washing the cells with DPBS four times and harvesting them with a rubber policeman. The pellet was treated with cold 0.02 M Tris-HCl buffer, pH 7.5 containing a protease inhibitor cocktail (Sigma) for 15 min, with 10 s of strong agitation (25 times) every 5 min. The cell suspension was centrifuged at 2,000 *g* for 20 min for preparation of the denuclear-supernatant fraction, and the supernatant was centrifuged at 367,000 *g* for 30 min at 4°C to obtain a cytosol fraction. The cytosol (7 ml) was overlaid on top of the sucrose solution at the density of 1.17 g/ml (18 ml) and centrifuged at 49,000 rpm for 48 h at 4°C using a Hitachi RP50T rotor. The solution in the centrifuge tube was collected from the bottom into 12 fractions.

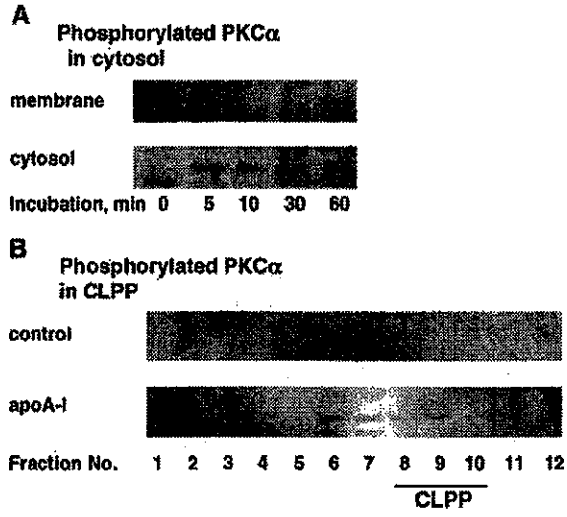

Fig. 3. Phosphorylation of protein kinase Cα (PKCα) in apoA-I-stimulated mouse astrocytes. A: The cytosol and membrane fractions were prepared from mouse astrocytes pretreated with 5 μg/ml apoA-I for the indicated periods of time in fresh 0.02% BSA/DMEM. Each sample was analyzed by SDS-PAGE (40 μg/lane for the cytosol fraction and 25 μg/lane for the membrane fraction) and Western blotting using goat anti-phospho-protein kinase Cα at residue serine-657 (Santa Cruz Biotechnology). B: The cytosol fraction (267 μg/7 ml) from the cells pretreated with or without apoA-I (5 μg/ml) for 5 min was centrifuged at 49,000 rpm for 48 h on 1.174 g/ml sucrose solution (18 ml) and separated into 12 fractions. Each fraction was analyzed by SDS-PAGE and Western blotting using goat anti-phospho-protein kinase Cα at residue serine-657 after precipitation with 10% TCA.

Caveolae/rafts preparation from the membrane fraction

The membrane fraction was prepared by centrifugation at 17,000 *g* for 60 min or 367,000 *g* for 30 min from the denuclear-supernatant fraction. The membrane pellet in 0.75 ml of 0.02 M Tris-HCl buffer containing a protease inhibitor cocktail was sonicated six times every 10 s at level 6 with a Taitec UP-55 homogenizer. After adjustment of the membrane solution to 30% sucrose by adding 0.75 ml of 60% sucrose solution and mixing, 1.5 ml of 10% sucrose solution was overlaid, followed by centrifugation at 367,000 *g* for 60 min. The sample was collected from the bottom of the centrifugation tube into five fractions and analyzed by SDS-PAGE (0.5% SDS/12.5% polyacrylamide gel). The caveolae/rafts fraction was recovered as fraction 3.

Western blotting

The membrane fraction was prepared and sonicated in 0.02 M Tris-HCl buffer, pH 7.5, containing protease inhibitor cocktail (Sigma). Protein was precipitated by centrifugation at 15,000 rpm for 20 min in the presence of 10% TCA from cytosol or the sonicated membrane fraction. The resolubilized protein pellet was applied to SDS-PAGE and transferred to a Sequi-Blot™ polyvinylidene fluoride membrane (Bio-Rad). The membrane was immunostained with rabbit anti-protein kinase Cα (Sigma), mouse anti-phospholipase Cγ (BD Transduction Laboratories), rabbit anti-caveolin-1 (Santa Cruz Biochemistry), and goat anti-phospho-PK-Cα (Ser-657) (Santa Cruz Biochemistry) antibodies.

De novo syntheses and release of lipid

Astrocytes at a confluent cell density were washed with DPBS four times and incubated in 0.1% BSA/F-10 for rat astrocytes or 0.1% BSA/DMEM for mouse astrocytes and WI-38 cells for 24 h. To measure de novo syntheses and release of cholesterol and phospholipid, the cells were incubated with [^{3}H]acetate (20 μCi/ml; New England Nuclear) in fresh 0.02% BSA/F-10 or 0.02% BSA/DMEM for various periods of time. After the cells were washed three times with cold DPBS, lipid was extracted from the cells or from the conditioned medium with hexane-isopropanol (3:2, v/v) solvent mixture or chloroform-methanol (2:1, v/v) mixture, respectively, and analyzed by TLC on Silica Gel-60 plates (E. Merck, Darmstadt, Germany) according to the method previously described (27). The cells were incubated with [^{3}H]acetate (20 μCi/ml) or [^{14}C]glycerol (0.2 μCi/ml; Amersham Biosciences) for various periods of time. The diacylglyceride (DG) was extracted from the cells, followed by TLC with diethylether-benzene-ethanol-acetic acid (200:250:10:1, v/v) solvent (16).

TABLE 1. Increase of DG production by apoA-I in mouse astrocytes

Apolipoprotein	Membrane	Cytosol	Total
ApoA-I (−)	23,451 ± 607	4,237 ± 154	27,688 ± 761
ApoA-I (+)	20,692 ± 1,159	15,708 ± 369	36,400 ± 790

apoA-I, apolipoprotein A-I; DG, diacylglyceride. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM. After washing and medium replacement with fresh 0.02% BSA/DMEM, the cells were incubated with or without 5 μg/ml apoA-I for 5 min. The denuclear-supernatant fraction was prepared as described in Materials and Methods. The cytosol and total membrane fractions were prepared by centrifugation at 367,000 *g* for 30 min as the supernatant and the pellet, respectively. Lipid was extracted from the total membrane fraction (62 μg of protein) and the total cytosol (347 μg/7 ml), and radioactivity in DG was determined after separation by TLC according to the method described in Materials and Methods. Each value represents the average and SEM of triplicate samples in total dpm.

RESULTS

When rat astrocytes were stimulated with 200 nM phorbol 12-myristate 13-acetate, protein kinase Cα was translocated from the cytosol to the membrane fraction (**Fig. 1A**). To our surprise, however, apoA-I induced the translocation of protein kinase Cα in the reverse direction, from the membrane to the cytosol fraction, in the astrocytes prepared from rats, wild-type mice, and apoE-KO mice, at 2–10 min after stimulation (Fig. 1B). The effect of apoA-I was smaller in wild-type mice than in apoE-KO mice, perhaps because of baseline autocrine stimulation by apoE in the former cells. This was confirmed by the effect of apoE on the cells of an apoE-KO mouse to demonstrate the similar translocation of protein kinase Cα to that by apoA-I (Fig. 1C). This result also indicated that the reaction is not apoA-I-specific and seems helical apolipoprotein-specific. A small increase of the membrane-bound enzyme was observed by long-term incubation in the apoE-KO cells for an unknown reason.

The cytosol was analyzed by density gradient ultracentrifugation for change in the distribution of protein kinase Cα after the 5 min stimulation by apoA-I in apoE-KO mouse astrocytes, because the increase of protein kinase Cα by apoA-I was most prominent in this type of cell. **Figure 2A** demonstrates that protein kinase Cα increased in the CLPP fractions (fractions 8–10) by apoA-I stimulation for 5 min. Interestingly, phospholipase Cγ also increased in the same fraction at 5 min after apoA-I stimulation. Caveolin-1 was recovered in this fraction and apoA-I caused its further increase, consistent with our previous findings with rat astrocytes (20). The increase of protein kinase Cα in the CLPP fraction was reversed by a receptor-coupled phospholipase C inhibitor, U73122 (Fig. 2B). Faint bands of protein kinase Cα were also observed in the lower density fractions of the control cells and the U73122-treated cells. These fractions are to be investigated further.

It is an important question whether protein kinase Cα is activated when it is translocated to CLPP by apoA-I stimulation. The activity of protein kinase Cα is reportedly associated with its phosphorylation at the serine-657 residue (28). The phosphorylated enzyme was probed by a specific antibody, and it increased in the astrocyte cytosol of apoE-KO mouse after the 5 min stimulation by apoA-I (**Fig. 3A**). When the cytosol was analyzed by density gradient ultracentrifugation, the phosphorylated protein kinase Cα was increased in the CLPP fractions (fractions 8–10), although a major portion of the phosphorylated enzyme was in the heavier fraction (fractions 6 and 7) (Fig. 3B).

As apoA-I may initiate signal transduction, the production of DG was monitored in mouse astrocytes when apoA-I was added to the medium (5 μg/ml) (**Table 1**). DG pro-

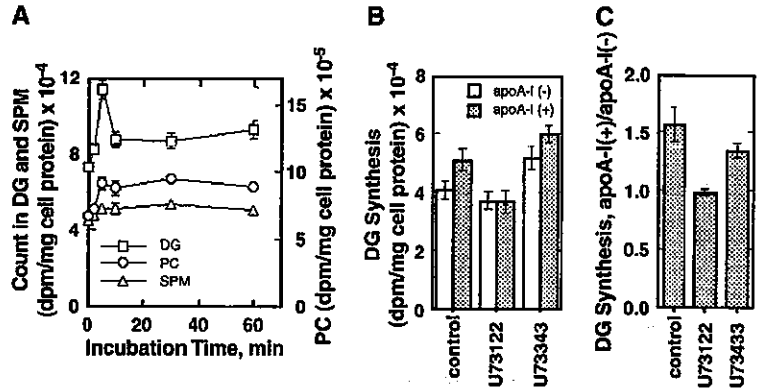

Fig. 4. Increase of diacylglyceride (DG) production by apoA-I and the effect of U73122 in mouse astrocytes. A: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of DMEM medium containing 0.02% BSA (0.02% BSA/DMEM). After three complete washes with Dulbecco's phosphate-buffered saline containing 0.15% glucose (DPBS/G), the cells were incubated for 60 min in fresh 0.02% BSA/DMEM. ApoA-I (5 μg/ml) was added to the medium at 0, 30, 50, 55, 58, and 60 min after the start of the incubation, to make the incubation periods with apoA-I 60, 30, 10, 5, 2, and 0 min. Lipid was then extracted with hexane-isopropanol (3:2) from the whole cells and separated by TLC. Radioactivity was determined for DG, sphingomyelin (SPM), and phosphatidylcholine (PC). Each data point represents the average and SEM of triplicate samples. B: Rat astrocytes were treated with (dotted columns) or without (open columns) apoA-I (5 μg/ml) in 0.02% BSA/F-10 in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 2 h. After three washes with DPBS, the cells were incubated for 1 h with 0.2 μCi/ml [^{14}C]glycerol in fresh 0.02% BSA/F-10. Lipid was extracted from the cells and separated by TLC. Radioactivity was determined for DG. Each data point represents the average and SEM of triplicate samples. C: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi/ml [^{3}H]acetate in 0.02% BSA/DMEM and washed three times with DPBS. The cells treated with U73122 (10 μM) or U73343 (10 μM) in 0.02% BSA/DMEM for 30 min were incubated with apoA-I (0.5 μg/ml) for 5 min. After washing, lipids were extracted from the cells and analyzed by TLC, and radioactivity was determined for DG. The data are expressed as the ratio of DG synthesis with apoA-I against that without apoA-I. Data represents mean ± SE for three measurements.

duction transiently increased at 5 min of incubation with apoA-I (**Fig. 4A**). This is distinct from the sphingomyelin replenishment reaction to generate DG with respect to the time course (21). This rapid and transient increase of DG implied the involvement of phosphatidylinositol (PI) turnover and the activation of phospholipase Cγ. This view was supported by the finding that U73122 suppressed the increase of DG production by apoA-I but U73343, an inactive analog of U73122, did not (Fig. 4B, C). These findings were also identical in human fibroblast WI-38 (**Fig. 5**). The site of this DG increase was analyzed in mouse astrocytes (**Fig. 6**). DG in the membrane fraction was mainly localized in the caveolin-1-rich caveolae/rafts fraction and did not show significant change by apoA-I stimulation (Fig. 6A). On the other hand, cholesterol and phosphatidylcholine in the cytosol were recovered in the fraction at a density of 1.07–1.12 g/ml (CLPP) (Fig. 6B). Unlike our previous finding in rat astrocytes under stimulation by apoA-I for 90 min (20), treatment of the cells with apoA-I for 5 min was not long enough to cause significant translocation of cholesterol and phospholipid to this fraction. However, apoA-I induced the increase of DG in this fraction by 5 min incubation (Fig. 6C). U73122 canceled the apoA-I-induced cholesterol translocation to the cytosol and its release by apoA-I (**Fig. 7**).

DISCUSSION

We recently reported that exogenous apoA-I induces the transient translocation of caveolin-1 and newly synthesized cholesterol to CLPP that also contains cyclophilin A in rat astrocytes (20). As many previous reports indicated that helical apolipoproteins, especially apoA-I, initiate intracellular signal transduction (29, 30), it is important to clarify whether this cholesterol translocation is induced by a specific signal(s) or by other mechanism such as a metabolic cascade triggered by the removal of lipid by apolipoprotein (31). We here investigated the association of signal-relating molecules with CLPP induced by apoA-I in astrocytes, indicating the potential involvement of this particle in signal transduction to mobilize cholesterol for the generation of HDL.

The results are summarized as follow. *1*) ApoA-I rapidly induced the translocation of phospholipase Cγ and protein kinase Cα to the CLPP fraction, and the latter was phosphorylated. The translocation of protein kinase Cα was inhibited by a receptor-coupled phospholipase C inhibitor, U73122. *2*) DG transiently increased by apoA-I at the 5 min incubation, and this increase was suppressed by U73122. The increase of DG was not observed in the membrane fraction but in the CLPP fraction. *3*) U73122 also suppressed both the apoA-I-mediated cholesterol release and related changes in cholesterol metabolism, such as cholesterol translocation to the cytosol.

These findings are consistent with the view that apoA-I initiates rapid signal transduction by receptor-coupled phospholipase C-mediated DG production, presumably through a PI turnover pathway. In most of the initiation of signal transduction, the activation of phospholipase Cγ occurs through the interaction of its SH-2 domain with a receptor that is tyrosine-autophosphorylated by binding a specific ligand, and DG is generated in the plasma membrane through the enhancement of PI turnover (32). Therefore, activation of the signaling pathway is associated with translocation of the signal-related enzymes from the cytosol to the membrane. To our surprise, apoA-I induced the translocation of phospholipase Cγ from the membrane to the cytosol in astrocytes. Further analysis of the cytosol revealed that the increase was in the CLPP fraction, and the increase of DG also takes place in this fraction rather than in the membrane. It is still unknown whether phospholipase Cγ is translocated to CLPP after its activation in the plasma membrane or is activated in the CLPP after the translocation. We were unable to detect the tyrosine-phosphorylated phospholipase Cγ in CLPP (data not shown). Nevertheless, it appears reasonable to assume that DG is generated in the CLPP fraction by the phospholipase Cγ translocated to this fraction. At present, we do not know the mechanisms by which phospholipase Cγ is translocated to CLPP and its activation. Phospholipase Cγ has a pleckstrin homology domain to bind PI 4,5-bisphosphate selectively (33). If PI turnover is triggered to produce this molecule in the CLPP by apoA-I stimulation, phospholipase Cγ may then be translocated to the CLPP. Also, we

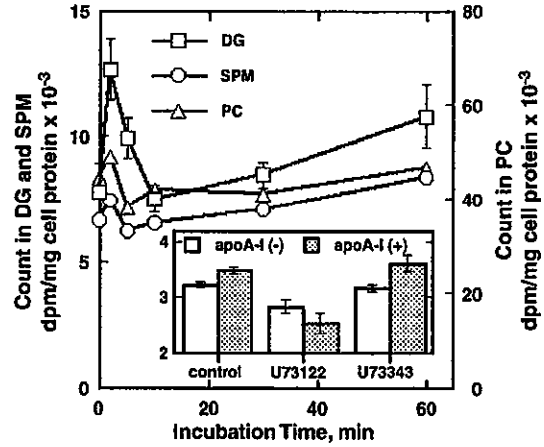

Fig. 5. Increase of DG production by apoA-I and the effect of U73122 on DG production in WI-38 cells. WI-38 cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM. The cells were incubated with apoA-I (5 μg/ml) for 0, 2, 5, 10, 30, and 60 min as described for Fig. 4A. Lipid was then extracted from the whole cells and separated by TLC for the determination of DG, sphingomyelin (SPM), and phosphatidylcholine (PC). Each data point represents the average and SEM of triplicate samples. In the inset, WI-38 cells were treated with (dotted columns) or without (open columns) apoA-I (5 μg/ml) in 0.02% BSA/DMEM in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 2 h. After washing three times with DPBS, the cells were incubated for 1 h with 20 μCi/ml [^{3}H]acetate in fresh 0.02% BSA/DMEM with or without U73122 or U73433. Lipid was extracted from the cells and separated by TLC for DG determination. Each data point represents the average and SEM of triplicate samples.

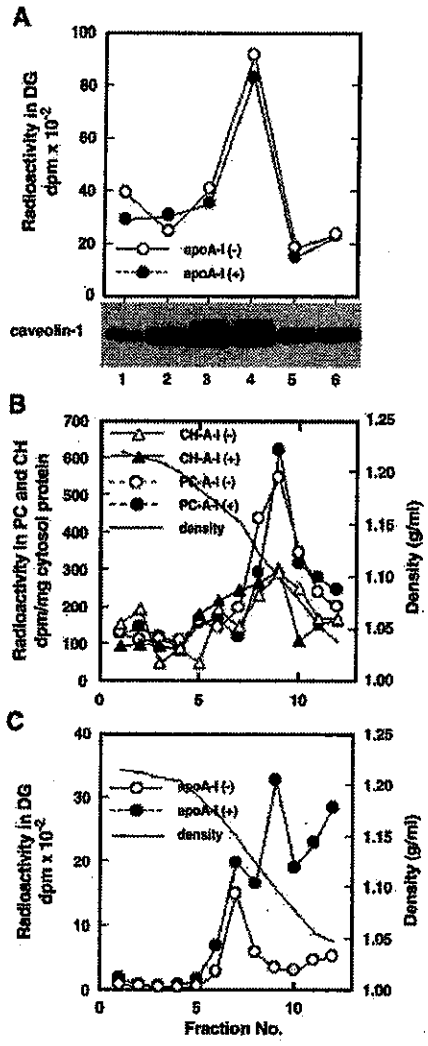

Fig. 6. Increase of DG by apoA-I in the cytosol of astrocytes. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM and then treated with (closed symbols) or without (open symbols) 5 μg/ml apoA-I for 5 min after washing and medium replacement with fresh 0.02% BSA/DMEM. The denuclear-supernatant fraction was prepared from the cells according to the method described in Materials and Methods. The cytosol and total membrane fractions were prepared by centrifugation at 367,000 *g* for 30 min as the supernatant and the pellet, respectively. A: The membrane fraction (60 μg of protein) was sonicated and analyzed by ultracentrifugation as described in Materials and Methods. The samples were separated into a pellet fraction (fraction 1) and five fractions (fractions 2–6 from the bottom to the top). Each fraction was subjected to SDS-PAGE and analyzed by Western blotting using a rabbit anti-caveolin-1 antibody (gel at bottom). Lipid was extracted from each membrane fraction and analyzed by TLC to determine radioactivity in DG. B and C: The cytosol fraction (350 μg protein/7 ml) was overlaid on top of the sucrose solutions at a density of 1.17 g/ml (18 ml) and centrifuged at 49,000 rpm for 48 h. The solution in the centrifuge tube was collected from the bottom into 12 fractions, and lipids were extracted. Radioactivities of phosphatidylcholine (PC; circles) and cholesterol (CH; triangles) (B) and of DG (C) were determined after the lipid was separated by TLC.

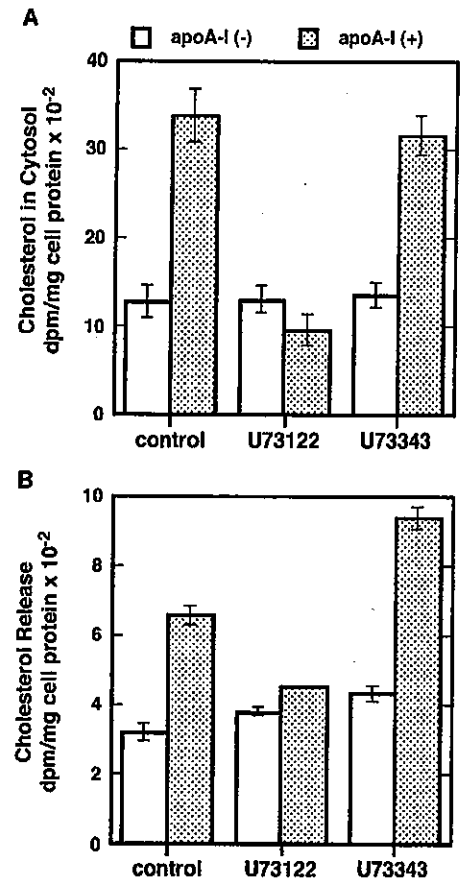

Fig. 7. Effects of U73122 on cholesterol trafficking in mouse astrocytes. A: The cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM followed by washing and medium replacement with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were treated with (dotted columns) or without (open columns) 5 μg/ml apoA-I in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 90 min. After washing, the cytosol was prepared and lipid was extracted. Radioactivity of cholesterol was determined after separation of lipid by TLC. B: The cells were labeled for 16 h with 20 μCi/ml [^{3}H]acetate in 0.02% BSA/DMEM, and the medium was replaced with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were incubated with (dotted columns) or without (open columns) 5 μg/ml apoA-I in the presence or absence of U73122 (10 μM) or U73343 (10 μM) for 4 h. Lipids were extracted from the conditioned medium, and radioactivity in cholesterol was determined.

cannot completely exclude the possibility of the participation of phospholipase Cβ in DG production.

The increase of DG production by apoA-I was accompanied by the translocation of protein kinase Cα to the cytosol in the astrocytes of rat, mouse, and apoE-KO mouse. Thus, the reactions seem to be independent of the influence of endogenously synthesized apoE in astrocytes. The increase of protein kinase Cα in the cytosol was again exclusively in the CLPP fraction. U73122 inhibited the translocation of protein kinase Cα to CLPP, so that it is reasonable to assume that this translocation occurs downstream

of DG production as a signal initiated by apoA-I. This view is consistent with our previous findings that the differentiated rat vascular smooth muscle cells that produce cholesterol-poor HDL by apolipoproteins generate cholesterol-rich HDL after stimulation of protein kinase C by phorbol ester and that protein kinase C inhibitors decreased the apoA-I-mediated cholesterol release in macrophages (34). Further investigation is required to clarify whether the translocation of these signal-related molecules takes place to the same lipid-protein particle or to different particles that happen to have the same density.

In agreement with our previous finding that apoA-I induces the translocation of caveolin-1 and newly synthesized cholesterol to the CLPP fraction, this fraction may play a role in intracellular cholesterol transport to the plasma membrane when HDL is generated by apoA-I and may also provide a site for the initiation of signal transduction to induce such cholesterol trafficking. Interestingly, protein kinase Cα phosphorylated at serine-657 was mainly recovered from the free protein fraction in cytosol, although it is increased in the CLPP fraction also by apoA-I stimulation (28). This finding indicates the possibility that the enzyme is translocated to the CLPP and dissociated from the particle by serine phosphorylation. There is no further information for the reactions after the activation of protein kinase Cα.

This rapid initiation of the signaling cascade by apoA-I is apparently different from the relatively slower generation of DG by phosphatidylcholine-specific phospholipase C in the replenishment reaction for sphingomyelin when it is removed by the HDL assembly reaction by apoA-I with cellular lipid (21). This slower reaction is associated with the stabilization of ABCA1 (31). The rapid reaction seems to involve phospholipase Cγ and PI turnover, so that it should be initiated by the interaction of apoA-I with a receptor-like signal-mediating membrane protein, whether directly or indirectly. Although many reports indicated the initiation of the signaling cascade by apoA-I or HDL, there is no clear indication of the signal-mediating membrane protein that may directly interact with apolipoprotein or HDL (35–40). ABCA1 has been identified as a key protein for the generation of HDL by apolipoprotein from cellular lipid, but it is still unclear whether this protein interacts directly with apolipoprotein to generate HDL or plays an indirect role for the HDL assembly reaction (41–45). ABCA1 is an essential molecule for the reaction to generate HDL by apoA-I. Our preliminary experiments indicated the presence of ABCA1 in astrocytes but less stabilization effect by apoA-I. A recent report indicated that ABCA1 is required for the generation of apoE-HDL in the brain (46). However, it is unclear whether ABCA1 is a signal-mediating receptor in the reactions presented in this article.

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On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway

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Abstract The mechanism for the assembly of HDL with cellular lipid by ABCA1 and helical apolipoprotein was investigated in hepatocytes. Both HepG2 cells and mouse primary culture hepatocytes produced HDL with apolipoprotein A-I (apoA-I) whether endogenously synthesized or exogenously provided. Probucol, an ABCA1 inactivator, inhibited these reactions, as well as the reversible binding of apoA-I to HepG2. Primary cultured hepatocytes of ABCA1-deficient mice also lacked HDL production regardless of the presence of exogenous apoA-I. HepG2 cells secreted apoA-I into the medium even when ABCA1 was inactivated by probucol, but it was all in a free form as HDL production was inhibited. When a lipid-free apoA-I-specific monoclonal antibody, 725-1E2, was present in the culture medium, production of HDL was suppressed, whether with endogenous or exogenously added apoA-I, and the antibody did not influence HDL already produced by HepG2 cells. We conclude that the main mechanism for HDL assembly by endogenous apoA-I in HepG2 cells is an autocrine-like reaction in which apoA-I is secreted and then interacts with cellular ABCA1 to generate HDL.—Tsujita, M., C-A. Wu, S. Abe-Dohmae, S. Usui, M. Okazaki, and S. Yokoyama. **On the hepatic mechanism of HDL assembly by the ABCA1/apoA-I pathway.** *J. Lipid Res.* **2005.** 46: 154–162.

Supplementary key words cholesterol • high density lipoprotein • hepatocytes • HepG2 • probucol • apolipoprotein A-I • ATP binding cassette transporter A1

High density lipoprotein is produced by the reaction of helical apolipoprotein and ABCA1 (1). This is considered a main source of plasma HDL, because familial HDL deficiency (Tangier disease) has been identified as the defect of HDL assembly by this reaction (2) caused by the mutation of ABCA1 (3–5). The ABCA1/apolipoprotein reaction is also an important pathway of cellular cholesterol release for its conversion to bile acids in the liver, along with an alternative nonspecific diffusion pathway accelerated by cholesterol esterification with LCAT on HDL particles (1). The main site for HDL production is generally thought to be the liver and intestine, where the cells synthesize helical apolipoprotein, mainly apolipoprotein A-I (apoA-I), and produce HDL, presumably upon the interaction of this apolipoprotein with its own ABCA1 by removing cellular lipid (6–8). However, it is unclear in which step of the apoA-I production and secretion this reaction takes place for the assembly of HDL. HDL particles have never been clearly identified in the secretory pathway of any HDL-producing cell, including hepatocytes.

Probucol is an inhibitor of apoA-I-mediated cellular cholesterol release and HDL assembly (9, 10) and has been identified as an inactivator of ABCA1 (11). We used this compound in LCAT-deficient mice in attempting to suppress the two major cholesterol-release pathways of somatic cells described above (12). To our surprise, no systemic cholesterol accumulation was observed, indicating that cholesterol may leave cells by a nonspecific pathway and that many extracellular acceptors can act as cholesterol transporters, such as albumin and blood cells. However, cholesterol content increased only in the liver when probucol inhibited the ABCA1 pathway in the cholesterol-fed LCAT-deficient mice. Thus, the liver seems to be a major organ from which cholesterol release requires the ABCA1 pathway, and these results indicate that the liver is a major source of plasma HDL (13), consistent with other reports using genetically engineered animals (6–8).

It is thus important to characterize how HDL is assembled in hepatocytes with helical apolipoproteins synthesized by hepatocytes interacting with their own ABCA1. We used the human hepatoma cell line HepG2 and mouse primary culture hepatocytes as model systems. To identify

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Abbreviations: apoA-I, apolipoprotein A-I; FCS, fetal calf serum; MEM-α, minimum essential medium Eagle α modification.

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a role of ABCA1 in HDL production by hepatocytes, we used probucol to inactivate ABCA1 as well as ABCA1-deficient mice. A monoclonal antibody specific against lipid-free apoA-I was used as a tool to trap lipid-free apoA-I to examine whether apoA-I is secreted from the cells as a free form before it interacts with ABCA1 of the cells to generate HDL.

MATERIALS AND METHODS

Apolipoprotein, lipoprotein, and an anti-apoA-I monoclonal antibody

ApoA-I and apoA-II were purified from human HDL fraction using delipidation and anion-exchange column chromatography in 6 M urea as previously described (13, 14). Apolipoproteins were dissolved in 50 mM sodium phosphate buffer, pH 7.4, containing 6 M guanidine-HCl and thoroughly dialyzed against 10 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl (PBS). For the specific binding study, apoA-I was labeled with ^{125}I as previously described (9) except for the use of Iodo-Beads to activate ^{125}I. The iodine-labeled apoA-I was concentrated by a Ultrafree-15 centrifugal filter device (Millipore Corp.). One milliliter of ^{125}I-apoA-I solution was dissolved in an equal volume of 6 M guanidine-HCl in 50 mM sodium phosphate buffer, pH 7.4, and dialyzed against PBS to remove unbound ^{125}I and guanidine-HCl. Probucol was kindly provided by Daiichi Pharmaceutical Co. LDL was isolated by sequential ultracentrifugation, and control and probucol-containing LDL were prepared by the method previously described (9). A monoclonal antibody (IgG) against lipid-free apoA-I, 725-1E2, was among the antibodies provided by Daiichi Pure Chemicals (Tokyo, Japan) and characterized in our laboratory as described previously (15). Mouse IgG was purchased from Chemicon International and used as a nonspecific control for 725-1E2.

HepG2 cells

HepG2 cells (American Type Culture Collection; ATCC HB8065) were maintained in minimum essential medium Eagle α modification (MEM-α) supplemented with 10% fetal calf serum (FCS) and antibiotics (5 U/ml penicillin and 5 μg/ml streptomycin).

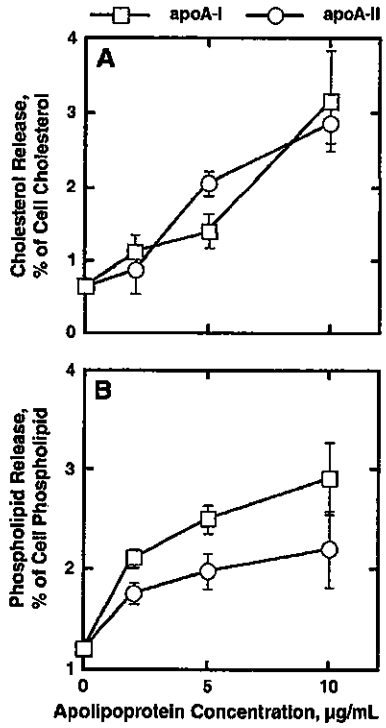

Fig. 1. Apolipoprotein-mediated lipid release from HepG2 cell. Cells were incubated for 16 h with minimum essential medium Eagle α modification (MEM-α) containing 0.02% BSA and the indicated concentration of apolipoprotein A-I (apoA-I) or apoA-II. Cellular lipid released into the conditioned medium was extracted by organic solvent and analyzed by colorimetric enzymatic assay for cholesterol and choline-phospholipid as described. The values represent means ± SEM for three determinations. Open circles, exogenous human apoA-I; open squares, exogenous human apoA-II.

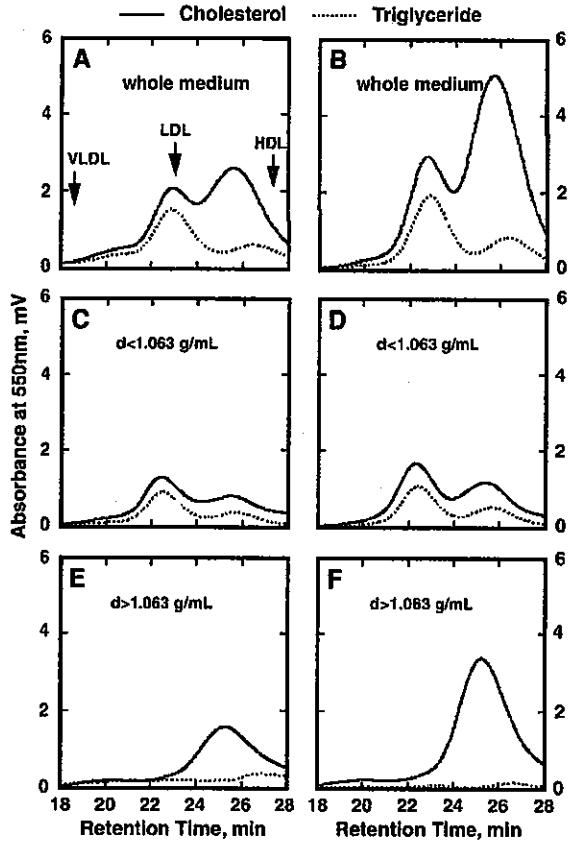

Fig. 2. Lipoprotein analysis of the culture medium of HepG2 cells by HPLC. Cells were incubated with MEM-α containing 0.02% BSA with (B, D, F) or without (A, C, E) 10 μg/ml human apoA-I for 16 h. The conditioned medium of HepG2 cells (100 μl) was analyzed by the HPLC lipoprotein analysis system using two tandem gel-permeation columns (Lipopropak XL; 7.8 mm × 300 mm; Tosoh). The elution profile was monitored by an online assay system for total cholesterol (solid lines) and triacylglycerol (dotted lines). A and B: Whole conditioned medium. C and D: d > 1.063 g/ml fraction. E and F: d < 1.063 g/ml fraction. The eluting positions of human plasma VLDL, LDL, and HDL are indicated by arrows in A.

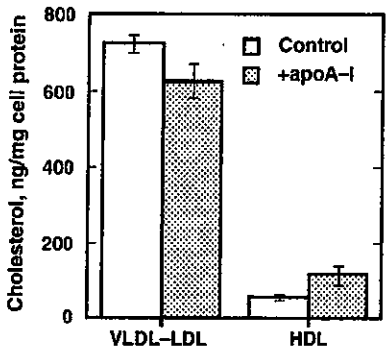

Fig. 3. Analysis of lipoprotein generated by mouse primary culture hepatocytes. Primary culture hepatocytes of C57Bl/6 mice were harvested using the Hanks'-EDTA and collagenase two-medium method described in Materials and Methods. The cells were incubated in DMEM (high glucose)/BSA medium with or without human apoA-I for 16 h. The conditioned medium was separated by ultracentrifugation at a density 1.063 g/ml. The data represent means ± SEM of three determinations. Difference in HDL between control and apoA-I (+) is significant by $P < 0.005$.

For the individual experiments, cells were subcultured onto 35 or 60 mm plates at the density of 0.7 to 1 × 10^6 cells/ml and maintained in MEM-α with 10% FCS and antibiotics (5 U/ml penicillin and 5 μg/ml streptomycin) by changing the medium every 2 days. On the fifth day, when the cells were 80–90% confluent, the cells were washed extensively with MEM-α and incubated with MEM-α containing 0.02% BSA and antibiotics with and without exogenous human apoA-I and human apoA-II for 16 h. The conditioned medium was collected for further lipid analysis. To load probucol, cells at 65–75% confluence were incubated with LDL or probucol-containing LDL (50 μg/ml as protein) in MEM-α for 24 h before the lipid-release assay.

Mouse primary hepatocytes

C57Bl/6 mice (9 weeks old) were purchased from the local experimental animal supplier. The mice were fed with normal mouse chow or chow containing 0.5% (w/w) probucol ad libitum. ABCA1-deficient mice were bred from ABCA1 heterozygote mice (DBA/1-Abca1^{tm1Jdm}/J) purchased from Jackson's Animal Laboratories (Stony Brook, NY). The genotypes of all offspring were determined by PCR analysis of tail DNA. The oligonucleotide primer sets 5′-TGGGAACTCCTGCTAAAAT-3′, 5′-CCATGTGGTGTGTAGACA-3′ and 5′-TTTCTCATAGGGTTGGTCA-3′, 5′-TGCAATCCATCTTGTTCAAT-3′ were used to determine the wild-type and mutant alleles, respectively. PCR was performed according to the genotyping method provided by the animal supplier except for an annealing temperature of 58°C. The mouse primary hepatocytes were harvested and cultured according to the method by Noga et al. (16). Mice were anesthetized and the liver was perfused with Hanks' salt solution without calcium containing 0.5 mM EGTA and Hanks' salt solution with calcium and magnesium containing 75 U/ml collagenase (type IV) at 37°C. The hepatocytes were isolated by low-gravity centrifugation and placed onto sterilized collagen-coated 60 mm culture dishes (1.8 × 10^6 cells/dish). After 2 h, when cells were attached as a monolayer, the unbound cells were washed with DMEM (high glucose) containing 0.02% BSA. The cells were incubated with and without apoA-I for 16 h, and the conditioned medium was collected after the incubation. All of the experiments were completed within 24 h after harvesting the cells. The experimental procedure had been approved by the Animal Welfare Committee of Nagoya City University Graduate School of Medical Sciences according to institutional guidelines.

Lipoprotein analysis in the conditioned medium

Lipoprotein in the conditioned medium of the hepatocytes was analyzed by HPLC using a gel-permeation column(s) (Lipopropak XL; 7.8 mm × 300 mm; Tosoh) with 0.05 M Tris-buffered acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min and an online enzymatic lipid-detection system (10, 17–19). The conditioned medium was centrifuged at 10,000 rpm for 5 min to remove cell debris, and a 200 μl aliquot was applied for HPLC analysis. The method was thoroughly validated against the reference methods of ultracentrifugation and of Superose gel-permeation chromatography, including the criteria of subfraction analysis of HDL (17, 19). In some experiments, the VLDL/LDL fraction ($d < 1.063$ g/ml) and the HDL fraction ($1.063 < d < 1.21$ g/ml) were isolated from the conditioned medium by sequential ultracentrifugation at 1.063 g/ml and 1.21 g/ml in a Himac CS120GX (Hitachi) at 99,000 rpm for 4 h. Lipid was extracted from the total conditioned medium and the VLDL/LDL and HDL fractions with organic solvent, and cholesterol and choline-phospholipid were determined with colorimetric enzyme assay kits (Kyowa Medics Co., Ltd., for cholesterol and Wako Pure Chemical Industries, Ltd., for phospholipid) (20). Electrophoretic analysis was also performed for the medium using a Beckman Paragon System on an agarose gel.

Apolipoprotein analysis

Apolipoproteins in the HepG2 conditioned medium were analyzed by immunoblotting using rabbit antiserum raised against human apoA-I and goat anti-human apoB IgG (affinity purified; Academy Bio-Medical Co., Inc.). The distribution of apoA-I and apoB in the HPLC-fractionated samples was analyzed. The eluent was fractionated every 30 s. After adding 4 μg of BSA to each sample, protein was precipitated with 15% (w/v) trichloroacetic acid for 30 min on ice and recovered by centrifugation at 15,000 rpm for 10 min. The precipitated protein was washed with 1 ml

TABLE 1. Chemical compositions of cellular lipids in HepG2 cells

Sample	Total Cholesterol	Triglyceride	Phospholipid	Probucol
		μg/mg protein		
Control LDL				
exo-apoA-I(−)	22.02 ± 0.93	10.09 ± 2.90	201 ± 5.85	0.00
exo-apoA-I(+)	21.25 ± 0.35	13.91 ± 5.62	213 ± 6.61	0.00
Probucol LDL				
exo-apoA-I(−)	19.84 ± 0.63	15.12 ± 1.72	169 ± 10.45	1.01 ± 0.21
exo-apoA-I(+)	21.17 ± 0.84	19.08 ± 2.48	185 ± 8.60	1.17 ± 0.27

exo-apoA-I, exogenous apolipoprotein A-I. Cellular lipid was analyzed by enzymatic methods, and probucol was measured using an HPLC method.