Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7

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Abstract **The assembly of HDL by helical apolipoprotein and cellular lipid was studied using HEK293 cells to which ecdysone-inducible human ABCA1 or human ABCA7 was transfected. Expression of both ABCA1 and ABCA7 was induced linearly proportional to ponasterone A concentration in the medium. In the experimental conditions used, the ABC protein expression levels limited the rate of lipid release when the apolipoprotein concentration was high, and the apolipoprotein concentration was rate-limiting when the ABC protein expression levels were high. When ABCA1 expression increased in conditions in which it was rate-limiting, relative cholesterol content to phospholipid increased in the HDL produced. In contrast, it was constant when ABCA7 expression increased. To investigate the background mechanism, the HDL particles were analyzed by density gradient ultracentrifugation and high performance lipid chromatography. The ABCA1-mediated reaction produced two distinct HDLs, large cholesterol-rich and small cholesterol-poor particles, and the ABCA7-mediated reaction generated mostly small cholesterol-poor particles. The increase of HDL assembly with the increase of ABCA1 expression was predominant in large cholesterol-rich particles, whereas only small cholesterol-poor HDL increased as ABCA7 expression increased. We conclude that ABCA1 generates cholesterol-rich and cholesterol-poor HDL and that the former is more prominently dependent on the increase of ABCA1 expression. ABCA7 produces this HDL subfraction only as a very minor component.**—Hayashi, M., S. Abe-Dohmae, M. Okazaki, K. Ueda, and S. Yokoyama. **Heterogeneity of high density lipoprotein generated by ABCA1 and ABCA7.** *J. Lipid Res.* **2005. 46: 1703–1711.**

Supplementary key words ATP binding cassette transporter A1 • ATP binding cassette transporter A7 • cholesterol • ecdysone-inducible

ABCA1 mediates the assembly of HDL with extracellular helical apolipoprotein and cellular lipid (1). This reaction is the major source of plasma HDL (2–4) and one of the rate-liming reactions for the regulation of its level (5, 6). The reaction mediates the generation of HDL particles with apolipoprotein, primarily recruiting cellular phospholipid (7). Cholesterol content in these particles is independently regulated by various cellular factors, potentially including protein kinase C and related signaling machineries (8–10), caveolin-1 (11), acyl-CoA:cholesterol acyltransferase (10, 12), and also perhaps other factors relating to intracellular cholesterol trafficking pathways (13). When ABCA1 is transfected to HEK293 cells, which otherwise do not express ABCA1, phospholipid and cholesterol are both released and cholesterol-rich HDL is generated upon incubation of the cells with apolipoprotein A-I (apoA-I) (14–16). ABCA7 also mediates the generation of HDL with apolipoproteins when transfected to HEK293 cells, but the relative cholesterol content to phospholipid in the HDL was lower than that produced by the ABCA1-mediated reaction (16, 17). The relative increase of cholesterol release seemed greater than that of phospholipid when ABCA1 protein level was upregulated by dibutyryl cAMP and phorbol ester (16), so we wondered whether the expression level of ABCA proteins is also a factor that regulates cholesterol content in the HDL. There are many reports that ABCA1 expression can be induced by various factors such as cAMP analogs (18–20), phorbol ester (10, 11), and ligands of liver X receptor or retinoid X receptor, including alteration of cellular cholesterol level (21–24). However, these compounds influence other cellular conditions and sterol metabolism and may not be suitable for examining the isolated effect of the expression level of the ABCA proteins. To address this question,

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Abbreviations: apoA-I, apolipoprotein A-I; DF, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium; GFP, green fluorescent protein.

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we used the ecdysone-inducible mammalian expression system (Invitrogen), based on the ability of the insect molting hormone 20-H ecdysone to activate gene expression via the ecdysone receptor (25). Mammalian cells are not responsive to ecdysone or its analog ponasterone A, so the effect of these reagents is minimized other than the expression of a specific gene designed to react. With these cell systems, the expression of ABCA1 and ABCA7 was regulated quantitatively and the generation of HDL was examined accordingly.

Interestingly, the cholesterol content in HDL increased in a dose-dependent manner with ABCA1 but not with ABCA7. The ABCA1-mediated reaction produced large cholesterol-rich and small cholesterol-poor HDL particles, and the former predominantly increased as ABCA1 increased. On the other hand, ABCA7 produced predominantly small cholesterol-poor HDL even when its expression increased.

MATERIALS AND METHODS

Apolipoproteins

ApoA-I and apoA-II were isolated from fresh human HDL (26) and dissolved (19) in phosphate-buffered saline as stock solutions (1 mg/ml) to be stored at 4°C as described elsewhere.

DNA construction and transfection

Full-length cDNAs for human ABCA1 and human ABCA7 were cloned as described previously (15, 16) and subcloned into pIND vector (Invitrogen). ABCA1 cDNA within pEGFP-N1 was digested with *NotI* and ABCA7 cDNA within pEGFP-N1 was digested by *NotI* and *Hind*III. The purified inserts were ligated into pIND to obtain pIND-ABCA1-green fluorescent protein (GFP) and pIND-ABCA7-GFP. HEK293 expressing ecdysone receptor was purchased from Invitrogen and maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DF) supplemented with 10% (v/v) FBS (Hyclone) containing 400 μg/ml Zeocin (Invitrogen). The cloned cDNAs were transfected with Lipofectamine PLUS reagent (Invitrogen) according to the manufacturer's recommendation. Cells permanently introduced were selected with G418, and clones with higher level expression of the fusion proteins were further selected by FACStar (Becton Dickinson).

Evaluation of ABCA1 and ABCA7 expression levels

Total membrane fraction was prepared, and the expression of ABCA1 and ABCA7 was analyzed by immunoblotting using rabbit polyclonal anti-GFP antibody (Molecular Probes) (16).

Cellular lipid-release assay

Cells were seeded onto six-well trays at a density of 1.4×10^6 cells/well with 10% FBS-DF medium and incubated for 24 h. After treatment with ponasterone A of the ABCA1- and ABCA7-transfected cells for 17 and 24 h, respectively, cellular lipid release was induced in the presence of apoA-I or apoA-II (1–20 μg/ml) in DF containing 0.02% BSA and ponasterone A. Lipid was extracted from the medium, and free cholesterol and choline-phospholipid were measured in separate enzymatic assay systems (19).

Density gradient analysis

Cells were subcultured on 100 mm dishes at a density of 8.0×10^6 cells/dish, treated with ponasterone A, and stimulated by apoA-I (2 or 10 μg/ml) for 24 h in 5 ml/dish DF medium containing 0.02% BSA. The media from two dishes were combined and centrifuged to remove cell debris, and 8 ml of the supernatant was processed for sucrose density gradient ultracentrifugation (27). The solution was collected from the bottom of the tube

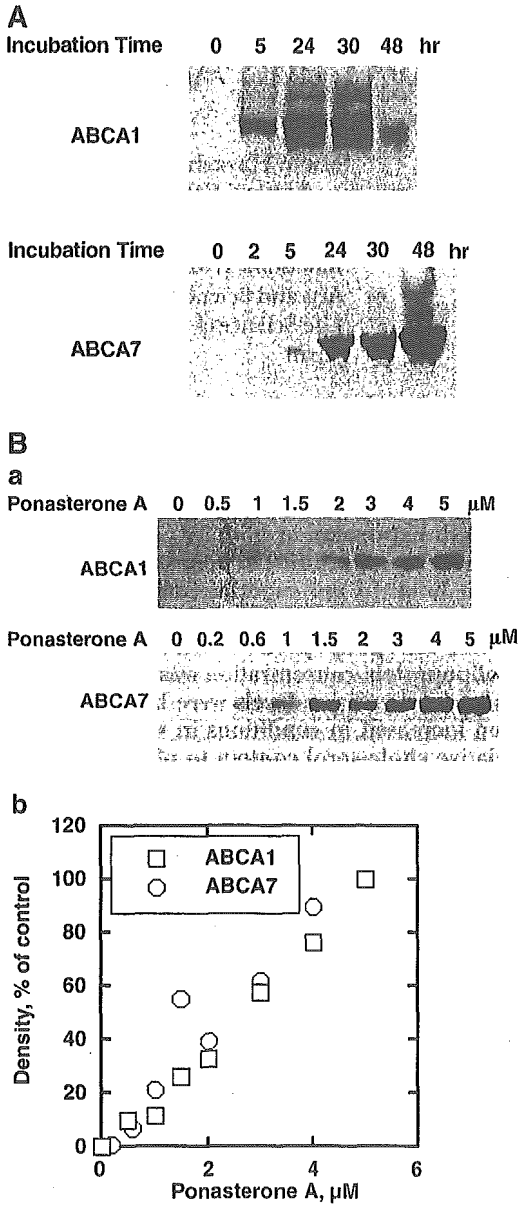

Fig. 1. Induction of green fluorescent protein (GFP)-tagged ABCA1 or ABCA7 expression by ponasterone A. A: The ecdysone-inducible cells were cultured for 24 h and incubated for the indicated times in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% FBS in the presence of 5 μM ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting against GFP. B: The ecdysone-inducible cells were cultured for 24 h and incubated for 17 h (ABCA1) or 24 h (ABCA7) in the presence of various concentrations of ponasterone A. The membrane fraction (100 μg of protein) was analyzed for ABCA1 or ABCA7 by Western blotting. The graph represents the density of the bands from the immunoblot quantitated by digital scanning in an Epson GT9500. The results are expressed as percentage of control (5 μM ponasterone A) for ABCA1 and ABCA7 (squares and circles, respectively).

into 13 fractions. The cholesterol and choline-phospholipid contents as well as the density were determined for each fraction (19).

Lipoprotein analysis by HPLC

Lipoproteins in the conditioned medium were analyzed with an HPLC system (28) at Skylight Biotech, Inc. (Akita, Japan). The conditioned medium was centrifuged at 10,000 rpm for 5 min to remove cell debris, and a 200 μl aliquot was applied for an HPLC system using two tandem gel permeation columns (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. 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 (29).

RESULTS

Induction of ABCA1 and ABCA7 by ponasterone A

GFP-tagged ABCA1 or ABCA7 cDNA with the ecdysone-inducible mammalian expression system was transfected to HEK293 cells that stably express the ecdysone receptor. It was previously confirmed that attachment of GFP to the C terminus of ABCA1 or ABCA7 did not influence their ability to produce HDL (16, 30). Expression of the ABCA proteins was examined by immunoblotting against GFP. With 5 μM ponasterone A treatment, expression of the GFP-tagged protein became apparent within 5 h, and it increased for 24 h in ABCA1 cells and for 48 h in ABCA7 cells (**Fig. 1A**). Therefore, 24 h induction was chosen as the optimum condition for ponasterone A to induce ABCA1 and ABCA7 for comparison. The dose-dependence of ABCA1 or ABCA7 expression was examined using various concentrations of ponasterone A. The immunoreacting bands of ABCA1-GFP and ABCA7-GFP both linearly increased ponasterone A in a dose-dependent manner (R^2 = 0.99 and 0.94, respectively) (Fig. 1B). At higher concentrations of ponasterone A (>10 μM), the expression levels seemed to reach a maximum (data not shown). The apoA-I-mediated lipid release increased by ponasterone A in a dose-dependent manner (**Fig. 2**). ABCA7 mediated cholesterol release less than ABCA1 with respect to its relative amount of phospholipid release.

Characterization of the lipid release mediated by ABCA proteins and apolipoproteins

Figure 3 demonstrates the release of cholesterol and phospholipid from HEK293 cells by apoA-I in the presence of ABCA1 and ABCA7. Cholesterol was almost all in a free form, and the amount of cholesteryl ester was negligible. When ABC protein expression is high (at high ponasterone A concentrations), apoA-I concentration limits the rate of lipid release. On the other hand, the expression levels of ABCA proteins limit the rate with the ponasterone A concentration used when apoA-I concentration is 10 and 20 μg/ml. **Figure 4** shows the results of similar experiments with apoA-II. The release of cholesterol and phospholipid by apoA-II appeared similar to the results

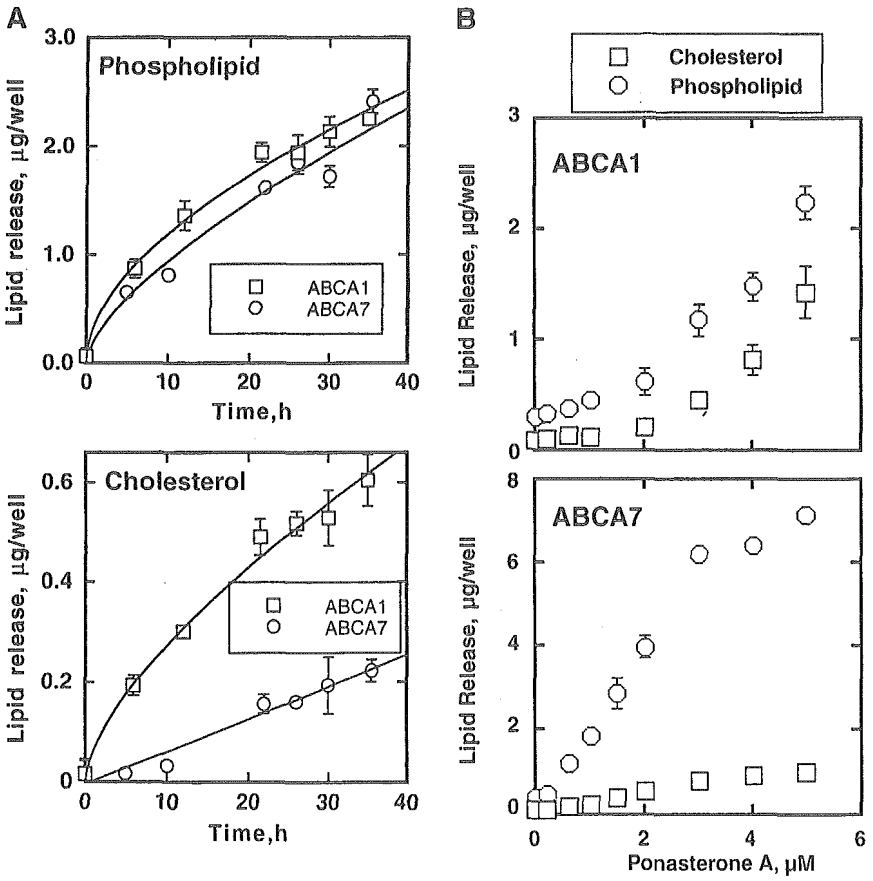

Fig. 2. Apolipoprotein A-I (apoA-I)-mediated release of cholesterol and choline-phospholipid. The ecdysone-inducible cells were incubated with the indicated concentrations of ponasterone A for 17 or 24 h. After washing with buffer H, the cells were incubated with apoA-I. A: Time-dependent profiles of the lipid release at 5 μM ponasterone A. B: Lipid release by apoA-I for 24 h at various concentrations of ponasterone A. Cholesterol (squares) and choline-phospholipid (circles) were determined in the medium. Data represent means ± SD for three samples.

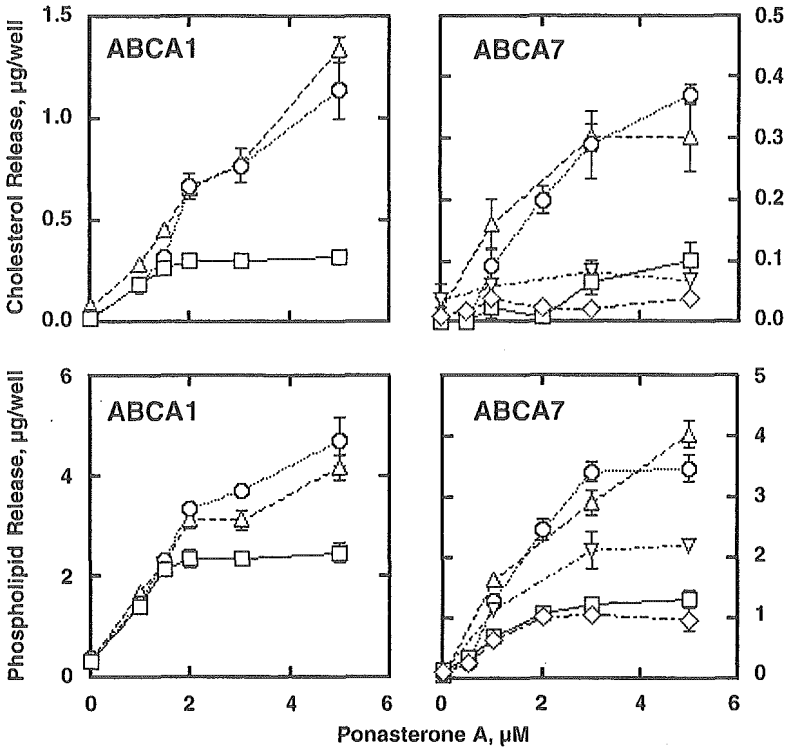

Fig. 3. Lipid release by apoA-I mediated with ABCA1 or ABCA7. The cells were induced for the expression of ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with various concentrations of apoA-I [1, 2, 5, 10, and 20 μg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were measured in the medium. Total cellular cholesterol and choline-phospholipids were 17.5 ± 0.7 and 91.2 ± 2.5 μg/well in the ABCA1-expressing cells and 24.1 ± 2.5 and 116.2 ± 5.8 mg/well in the ABCA7-expressing cells (average ± SD for six samples).

with apoA-I with respect to rate-limiting profiles. When apoA-II concentration is as low as 1 and 2 μg/ml, it limits the rate of lipid release. On the other hand, ABCA1 or ABCA7 limits the rate when apoA-II concentration is as high as 5 and 10 μg/ml.

In the conditions in which ABCA proteins are rate-limiting, the ratio of cholesterol to phospholipid was examined in the released lipid (**Fig. 5**). When the expression of ABCA1 increases by increasing ponasterone A from 1 to 5 μM, the relative content of cholesterol in the released lipid increased by both apoA-I and apoA-II, from 0.1 to 0.4 and from 0.04 to 0.17, respectively (Fig. 5). In contrast, it remained constant at a low level when the expression of ABCA7 increased (Fig. 5).

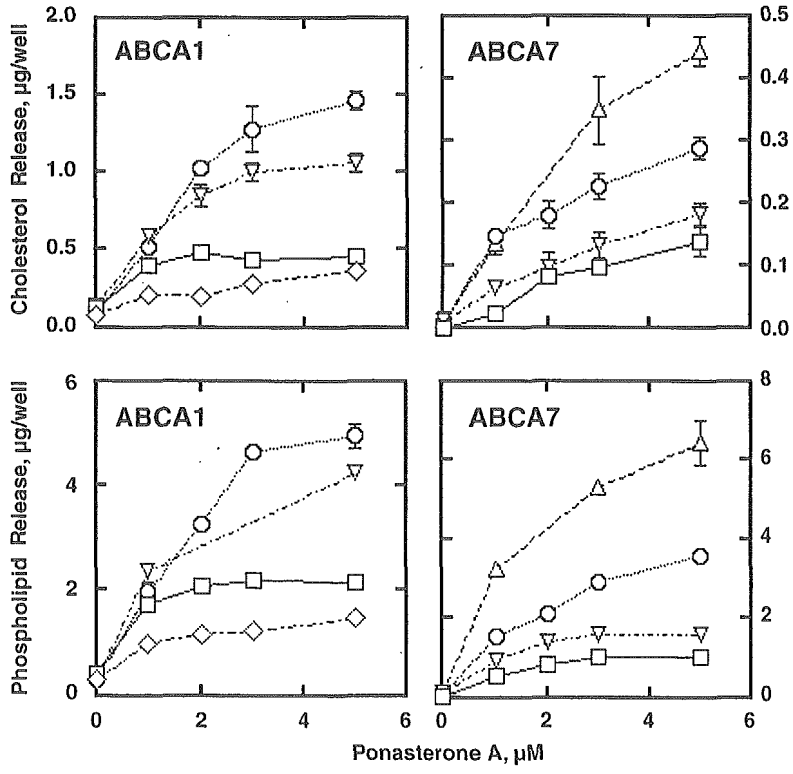

Fig. 4. Lipid release by apoA-II mediated with ABCA1 or ABCA7. The cells were induced for ABCA1 or ABCA7 in the presence of ponasterone A at 17 and 24 h, respectively. After washing, the cells were incubated with apoA-II [1, 2, 5, 10, and 20 μg/ml (diamonds, squares, inverted triangles, circles, and triangles, respectively)] for 24 h. Cholesterol and choline-phospholipid were determined in the medium.

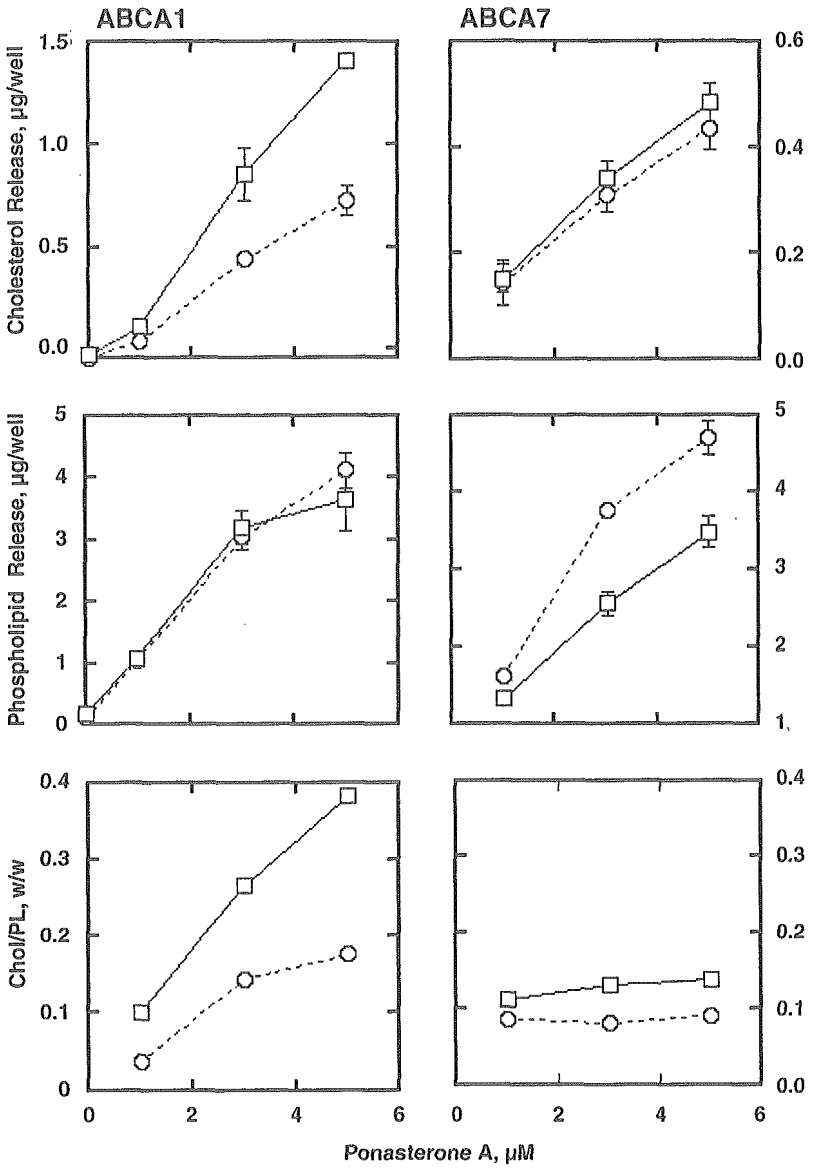

Fig. 5. Cholesterol-phospholipid ratio in the lipid released by apoA-I and apoA-II (squares and circles, respectively) mediated with ABCA1 or ABCA7. Expression of ABCA1 or ABCA7 was induced by ponasterone A, and cellular lipid release was induced by apolipoprotein (10 μg/ml). The ratio of cholesterol to phospholipid (Chol/PL) in mass is shown in the two bottom panels.

Density gradient ultracentrifugation analysis of the conditioned media

The conditioned media with apoA-I of HEK293 cells transfected with ABCA1 or ABCA7 were analyzed by density gradient ultracentrifugation. Both cholesterol and phospholipid were recovered in the fractions, with a density range of HDL to confirm that the lipid was released to form HDL particles. The density of the peak was 1.08 g/ml with the ABCA1-transfected cells and 1.10–1.11 g/ml with the ABCA7-transfected cells (**Fig. 6**), consistent with our previous observation (16). Density profiles of lipid distribution for the ABCA1-transfected cells appear with a shoulder at ~1.11 g/ml, whereas those for the ABCA7-transfected cells were rather symmetric, with a slight irregularity in the lower density side.

Analysis of the media by HPLC

To examine the heterogeneity of the HDL generated, the conditioned media were analyzed by HPLC with a gel permeation column. Elution profiles of cholesterol and phospholipid were monitored online. **Figures 7** and 8 show the results with the apoA-I-conditioned media of the ABCA1- and ABCA7-transfected cells. The medium of the ABCA1-transfeted cells contained two peaks: large cholesterol-rich particles with a diameter of 13 nm, and small cholesterol-poor particles with a diameter of 10 nm. In contrast, the ABCA7-transfected cells generated a predominant peak that is small and cholesterol-poor, accompanied by large particles as a very minor component. These profiles were essentially the same in the conditioned media of HEK293 cells to which ABCA1 or ABCA7 was transiently expressed without the GFP tag (data not shown). When the lipid release was increased either by increasing ABCA1 expression (Fig. 7) or by increasing apoA-I (Fig. 8), a more prominent increase was induced in the large cholesterol-rich particles, and accordingly, the relative amount of cholesterol to phospholipid in the media increased.

In contrast, small cholesterol-poor particles were still major components of the HDL generated by the apoA-I-ABCA7-mediated reaction. This profile did not change even when ABCA7 expression was increased, resulting in

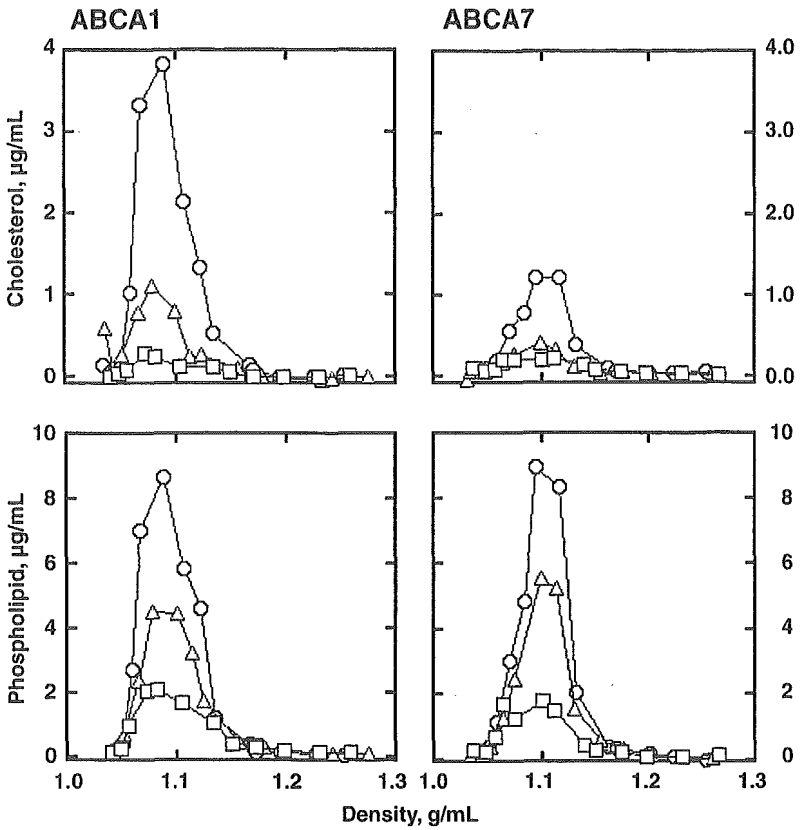

Fig. 6. Density gradient analysis of the lipids released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with 5 mM ponasterone A for 17 and 24 h, respectively, and cellular lipid was released by apoA-I [2, 4, and 10 μg/ml (squares, triangles, and circles, respectively)] for 24 h. The medium was analyzed by density gradient ultracentrifugation as described in Materials and Methods. Each fraction was determined for density and concentration of cholesterol and phospholipid.

the relative cholesterol content remaining constantly low (Fig. 7). The same tendency was observed when apoA-I was rate-limiting and increased the generation of HDL from the ABCA7-transfeted cells (Fig. 8).

Component analysis of the peaks (31) estimated that the large HDL particles generated by the ABCA1-apoA-I reaction contained cholesterol with 35–38% weight of phospholipid and that the small HDL particles contained

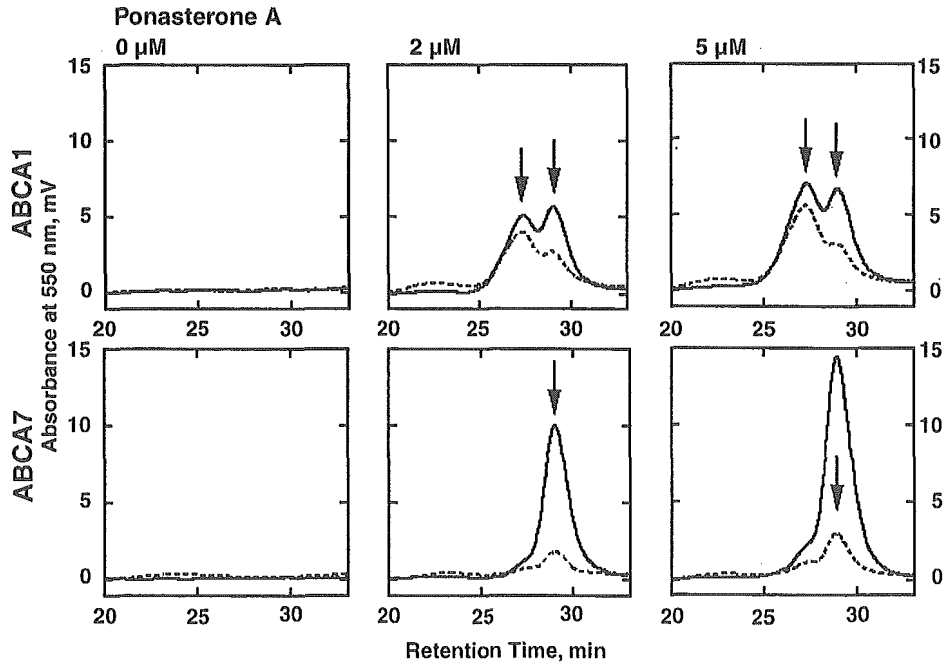

Fig. 7. HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. Lipoprotein analysis of the culture medium of ABCA1- or ABCA7-induced HEK293 cells by HPLC. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (2 and 5 μM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (10 μg/ml) for 24 h. The conditioned medium (200 μl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

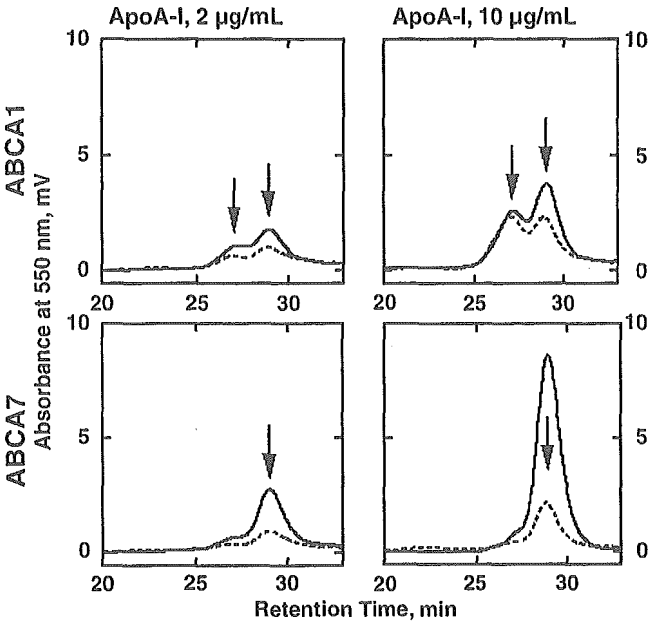

Fig. 8. HPLC analysis of the lipid released by ABCA1- or ABCA7-expressing cells. The cells were induced for the expression of ABCA1 or ABCA7 by incubating with ponasterone A (5 μM) for 17 and 24 h, respectively, and cellular lipid was released by apoA-I (2 and 10 μg/ml) for 24 h. The conditioned medium (100 μl) was analyzed by the HPLC lipoprotein analysis system by monitoring cholesterol (broken lines) and phospholipid (solid lines). The arrows indicate eluting positions of the particles with diameters of 13 and 10 nm.

10–13%. A major HDL component generated by the ABCA7-apoA-I reaction contained 8–11%.

DISCUSSION

ABCA1 is a key cellular factor for the generation of plasma HDL (2–6). ABCA7 was also demonstrated to mediate a similar reaction to ABCA1 to generate HDL in vitro (16, 17). There are several differences between the ABCA1- and ABCA7-mediated reactions with respect to cholesterol content in the HDL generated and posttranscriptional regulation. Recent studies indicated that ABCA7 does not play a major role in lipid release from macrophages (32, 33). However, it may contribute to a source of plasma HDL, at least in female mice (33), and it may be involved in lipid metabolism in kidneys (32) and adipose tissues (33).

The levels of expression of ABCA proteins may also affect these reactions, which could have significant influence on the nature of the products, such as their cholesterol content. However, the regulation of expression of ABCA proteins is multifactorial, including the liver X receptor/retinoid X receptor system (21–24), cAMP (18–20), the calcium-signaling pathway (34), and the peroxisome proliferator-activated receptor α-related system (35, 36). The content of cholesterol in the product HDL is regulated somewhat independently of the HDL assembly reaction itself, potentially with the involvement of such factors as caveolin-1 (11), protein kinase C-related signals (8–10), and intracellular cholesterol level and its esterification (10, 12). To characterize the HDL assembly reaction by ABCA proteins, it is thus important to control the expression level of these proteins without influencing other cellular factors.

We established the HEK293 cell systems with ecdysone-inducible ABCA1 and ABCA7. Both proteins are tagged with GFP, which was shown previously not to interfere with the function of either protein and enabled us to estimate the protein expression levels on a common immunoreactivity basis.

The results of the experiments are summarized below. Expression of ABCA1 and ABCA7 were both linearly proportional to the concentration of ponasterone A in the culture medium. The release of cellular cholesterol and phospholipid is limited by both ABCA protein expression levels and extracellular apolipoprotein levels. When ABCA proteins are rate-limiting, cholesterol release relative to phospholipid increased as ABCA1 expression increased, whereas it remained constant when ABCA7 expression increased. Increase of ABCA1 expression and apolipoprotein concentration both resulted in the increase of cholesterol content in HDL, and it was attributed to expansion of production of this component. On the other hand, ABCA7 mediated the generation of HDL particles that are predominantly small and cholesterol-poor. Thus, ABCA1 is more effective in releasing cellular cholesterol than is ABCA7 by producing large cholesterol-rich HDL. Both ABCA1 expression and apolipoprotein concentration increased relative to the release of cholesterol to phospholipid, and this was attributable to the expansion of production of this component.

Heterogeneity in nascent HDL particles produced by cell-apolipoprotein interaction has been reported in a previous study. When CHO-C19 cells were incubated with 8 μg/ml apoA-I for 24 h, 9.0 and 11.2 nm particles were produced (37). In another experiment with J774 macrophages, incubation with apoA-I led to the formation of more than one type of apoA-I-containing lipid particles, 9 and 12 nm in diameter, having cholesterol-phospholipid ratios of 1:1 to 1:3 (mol/mol), respectively (38). We also reported density profiles of HDL generated by peritoneal macrophages and apoA-I, which suggested similar heterogeneity of the HDL particles (8). The physicochemical and molecular bases for the size heterogeneity of discoidal HDL can be discussed (39), but it is not clear how such a mechanism can be applied when HDL is organized upon the apolipoprotein interaction with ABCA1 and membrane lipid. Lipid composition and particle size are related to the structure of specific domains of apoA-I on HDL (40). ABCA1 may induce specific modification of apoA-I conformation, either directly or indirectly, and such a change may cause parallel changes in its ability to bind to phospholipid bilayers and to the integration of cholesterol (41).

A higher expression level of ABCA1 causes the increase of large cholesterol-rich HDL particles. Although the exact mechanism by which ABCA1 functions in the membrane is not known, a few interesting findings have been

reported. ABCA1 is said to alter the microenvironment of the plasma membrane and influence lipid-protein complex formation in the membrane (42). It forms an oligomeric structure and is related to its function for the biogenesis of nascent apoA-I-containing HDL (43). If oligomeric ABCA1 produces cholesterol-rich HDL, it is consistent with the view that a higher expression of ABCA1 and therefore an increase of oligomeric ABCA1 in the membrane may cause more production of cholesterol-rich particles. The apparent biphasic increase of the lipid release by the increase of ABCA1 expression (Fig. 2B) may also be consistent with this view. ABCA7 may not act in such a manner, and production of cholesterol-poor HDL simply increased when its expression increased. Tall and colleagues (32) suggested that the small amount of cholesterol release by the ABCA7-mediated reaction is attributable to its nonspecific acquisition by the phospholipid-HDL. It is not clear whether the small HDLs demonstrated in the present study gain cholesterol only by such a mechanism.

We have demonstrated a fundamental difference between ABCA1-mediated and ABCA7-mediated HDL assembly. ABCA1 mediates two types of HDL particles, large cholesterol-rich and small cholesterol-poor particles, and the generation of large cholesterol-rich particles is perhaps responsible for the efficient release of cell cholesterol. ABCA7 mediates the generation of only the latter type of particle. At higher expression, ABCA1 produces predominantly more large cholesterol-rich particles, whereas ABCA7 increases the production of only small cholesterol-poor HDL. The results presented here provide a new biochemical basis for understanding an HDL assembly pathway that involves ABCA1 or ABCA7.

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Apolipoprotein A-I increases association of cytosolic cholesterol and caveolin-1 with microtubule-cytoskeletons in rat astrocytes

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Running Title: ApoA-I induces cholesterol traffic in astrocytes

Key words: HDL, apoA-I, apoE, ABCA1 cholesterol trafficking

Abbreviations used: apo, apolipoprotein; HDL, high density lipoprotein; CLPP, cytosolic lipid-protein particle; BIM, Bisindolylmaleimide I; DPBS, Dulbecco's phosphate buffered saline; BSA, bovine serum albumin; SDS-PAGE, 10 % polyacrylamide gel electrophoresis with 0.5 % sodium dodecylsulfate; rMT, reconstituted microtubule-like filament; TLC, thin layer chromatography; PIP2, phosphatidylinositol 4,5-bisphosphate.

ABSTRACT

Apolipoprotein (apo) A-I induces rapid translocation of protein kinase Cα and phospholipase Cγ, and slow translocation of caveolin-1 and newly synthesized cholesterol to the cytosolic lipid-protein particle (CLPP) fraction in rat astrocytes (J. Biol. Chem., 277, 7929-7935, 2002, J. Biol. Chem., 277, 44709-44714, 2002 and J. Lipid Res., 45, 2269-2276, 2004). In order to understand the function of CLPP, we investigated the interaction with cytoskeletons of CLPP-related proteins such as caveolin-1 and protein kinase Cα and of CLPP-related lipids in rat astrocytes. In the condition that microtubules were depolymerized, association of cytosolic caveolin-1 with protein kinase Cα and α-tubulin was enhanced when the cells were treated with apoA-I for 5 min. This association was suppressed by a scaffolding domain-peptide of caveolin-1. Association with the microtubule-like filaments of cytosolic lipids, caveolin-1 and protein kinase Cα was also increased by the apoA-I treatment and inhibited by the scaffolding domain peptide. Paclitaxel (taxol), a compound to stabilize microtubules, suppressed the apoA-I-mediated intracellular translocation and release from the cells of the *de novo* synthesized cholesterol and phospholipid. The findings suggested that the association of CLPP with microtubules is mediated by a scaffolding domain of caveolin-1, induced by apoA-I and involved in regulation of intracellular cholesterol trafficking for assembly of cellular lipids to apoA-I-HDL.

Key words: CLPP (cytosolic lipid-protein particle), astrocytes, apolipoprotein A-I, caveolin-1, microtubule, α-tubulin, cholesterol,

Running Title: Increase of cholesterol association with microtubules by apoA-I

Apolipoprotein (apo) E and apoA-I are main apolipoproteins in the central nervous system (Roheim et al. 1979; Chiba et al. 1991; Fujita et al. 1999; DeMattos et al. 2001; Koch et al. 2001; Thomas et al. 2001). They are present as high density lipoproteins (HDL) in the cerebrospinal fluid and thought to play an important role in intercellular cholesterol transport in the brain (Ito and Yokoyama 2004). While apoE is produced by astrocytes and microglias and secreted as HDL(Boyles et al. 1985; Ito et al. 1999; Xu et al. 2000), apoA-I is from an unknown source(s) except for a report that endothelial cells in the brain secrete it (Weiler-Guttler et al. 1990; Mockel et al. 1994; Panzenboeck et al. 2002). *In vitro*, astrocytes generate cholesterol-rich HDL with the endogenous apoE, and cholesterol-poor HDL through the interaction with the exogenous apoA-I (Ito et al. 1999).

We have reported that the transient translocation of newly synthesized cholesterol and phospholipid to the cytosol from the endoplasmic reticulum and Golgi apparatus occurs when exogenous apoA-I interacts with rat astrocytes and generates HDL for 60 - 90 min (Ito et al. 2002b; Ito et al. 2002a). The translocation of caveolin-1 to the cytosol was also induced by apoA-I in a similar time-dependent manner to the lipid translocation. We identified the cytosolic lipid-protein particle (CLPP) with a density of 1.09 – 1.16 g/mL and a diameter of 17 – 18 nm like plasma HDL, containing caveoin-1 and cyclophilin A, and the translocated lipids and caveolin-1 were recovered in this fraction. Cyclosporin A, a cyclophilin A inhibitor, inhibited the apoA-I-induced cholesterol translocation to the cytosol and also apoA-I-mediated cholesterol release. Caveolin-1 is believed to play an important role in intracellular cholesterol trafficking(Fielding et al. 1997; Uittenbogaard et al. 1998; Arakawa et al. 2000; Sviridov et al. 2001). Therefore, it is rational to hypothesize that the caveolin-1-associated CLPP is involved in intracellular cholesterol transport and generation of HDL when the astrocytes interact with apolipoprotein. We recently reported that apoA-I induces translocation of phospholipase Cγ and production of diacylglyceride to and in the CLPP

fraction, and translocation and activation of protein kinase Cα to and in the CLPP fraction within 5 minutes of the incubation with apoA-I, more rapidly than the translocation of lipids and caveolin-1, suggesting an initial signal transduction induced by apoA-I (Ito et al. 2004).

Based on these findings, we attempted to examine the hypothesis that CLPP participates in the intracellular cholesterol transport in astrocytes and, if so, CLPP may directly interact with cytoskeletons.

Methods

Materials

ApoA-I was highly isolated by delipidation of human HDL and anion-exchange chromatography (Yokoyama et al. 1982). Paclitaxel (taxol), that binds to the N-terminal of β-tubulin and stabilizes microtubules (Schiff and Horwitz 1980; Manfredi et al. 1982; Haber et al. 1995), and colchicines, that disrupt microtubules, were purchased from Sigma. Mouse anti-α-tubulin and mouse anti-β-actin antibodies were purchased from Sigma, rabbit anti-caveolin-1 antibody was from SANTA CRUZ, and mouse anti-protein kinase Cα antibody was from BD Transduction. A peptide representing a scaffolding domain of caveolin-1 (DGIWKASFTTFTVTKYWFYR) (Sargiacomo et al. 1995; Carman et al. 1999) was synthesized by Sigma. Bisindolylmaleimide 1 (BIM) was purchased from CALBIOCHEM.

Cell culture

Astrocytes were prepared from the cerebrum of 17-day fetal Wistar rat according to the method described previously (Lim et al. 1973; Ito et al. 1999). 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 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 at

37 °C for 1 week. The cells were treated with DPBS containing 0.1 % trypsin and 1 mM EDTA again and then cultured in the F-10 medium/10 % fetal calf serum using a 6-wells multiple tray (Costar 3516) or 10-cm-diameter culture dish (TPP93100) for 1 week. The cells were incubated with 5 μg/ml apoA-I for various periods of time in the F-10 medium containing 0.02 % bovine serum albumin (BSA)(BSA/F-10) (Ito et al. 2002b; Ito et al. 2004).

Cytosol preparation and density gradient ultracentrifugation analysis

The cytosol of astrocytes was prepared according to the method of Thom et al (Thom et al. 1977; Ito et al. 2002b; Ito et al. 2004). Briefly, the cell pellet was obtained by centrifugation at 1,000 rpm for 10 min after washing with DPBS four times and harvesting with a rubber policeman. The cell pellet was treated with cold hypotonic buffer, 0.02 M Tris-HCl, pH 7.5, containing a protease inhibitors cocktail (Sigma), for 15 min with 25 times of strong mixing per 10 sec every 5 min, in which condition the microtubules of astrocytes are depolymerized. The cell suspension was centrifuged at 5,000 rpm (1,000 x g) for 20 min for preparation of the denuclear-supernatant fraction, and it was further centrifuged at 90 krpm (367,000 x g) for 30 min at 4 °C in a Hitachi S100AT6 rotor to obtain the cytosol fraction that is to contain depolymerized microtubules components. For density gradient ultracentrifugation analysis, this cytosol fraction (7 mL) was overlayered on the top of the sucrose solution with a density of 1.17 g/mL (18 mL) and centrifuged at 49 k rpm for 48 h at 4°C by using a Hitachi RP50T rotor (Ito et al. 2002b). Centrifugation was performed at 15°C for the cytosol with reconstituted microtubule-like filament (rMT) described later. The solution in the centrifuged tube was collected from the bottom into 12 fractions. Cytosol proteins were analyzed by 10 % polyacrylamide gel electrophoresis with 0.5 % sodium dodecylsulfate (SDS-PAGE) and Western blotting. Density gradient fractions needed to be treated with 10 % trichloroacetic acid to precipitate protein before the analysis.

Immunoprecipitation

Immunoprecipitation of caveolin-1 was carried out by incubation of the cytosol fraction with a rabbit anti-caveolin-1 antibody and protein G-Sepharose (Amersham Bio) at 4°C for 2 h. The Sepharose fraction was washed 5 times with 0.02 M Tris-buffered saline containing protease inhibitors cocktail, and analyzed by SDS-PAGE and Western blotting.

Reconstitution of microtubule-like filaments

The microtubule protein was depolymerized by treating rat astrocytes with a cold hypotonic buffer. Both α–tubulin and β–actin were recovered by over 90 % in a cytosol fraction from the cells whether treated with or without apoA-I. The cytosol fraction containing depolymerized microtubule components was prepared from rat astrocytes as described above, and incubated with 100 μM GTP and 2 mM $MgCl_2$ at room temperature for 20 min (Lee and Timasheff 1975; Mithieux et al. 1986). After centrifugation at 80 k rpm (290,000 x g) at 20°C for 30 min, the rMT was obtained as a pellet and used for analysis by SDS-PAGE and Western blotting. The 52 kDa protein was recovered more in the pellet of the first centrifugation at 15 k rpm (10,000 x g) that represents longer filaments than in a pellet of the second centrifugation at 80 krpm (290,000 x g) (Figure 1A). This band was superimposed with the anti-α-tubulin antibody-positive band by Western blotting (Figure 1A). The 52 kDa protein in the pellet apparently decreased in the presence of colchicine (Figure 1A). These results all indicated that the pellet fraction mainly contains tubulin. Formation of the filament-like structure was confirmed in electron-micrograph shown in Figure 1B.

De novo synthesis of lipid and lipid release

After washing with DPBS four times and incubation in 0.1 % bovine serum albumin (BSA)/F-10 for 24 h, rat astrocytes at a confluent cell density were incubated with [^{14}C]acetate (3 μCi/mL, PerkinElmer) in a fresh 0.02 % BSA/F-10 for 16 h for bio-labeling of cholesterol and phospholipid. After washing three times with cold DPBS, the cells were incubated with apoA-I for various periods of time in 0.02 % BSA/F-10

(Ito et al. 1999). Lipid was extracted from the conditioned medium, cytosol fraction and rMT with chloroform:methanol (2 : 1, v/v) mixture, and analyzed by thin layer chromatography (TLC) on Silica Gel-60 plates (E. Merck, Darmstad, Germany) according to the method previously described in order to detect radioactivity in cholesterol, phosphatidylcholine and sphingomyelin (Ito et al. 1999; Ito et al. 2000).

Negative staining and electron microscope observation

The cytosol fraction treated with and without 100 μM GTP and 2 mM $MgCl_2$ was laid on the grid and negatively stained with 0.5 % uranium acetate and observed in a Hitachi 7100 electron microscope in order to confirm formation of rMT.

Protein Phosphorylation

Immunoprecipitation was carried out from the cytosol fraction with anti-caveolin-1 antibody, and the precipitated fraction was incubated with 5 μCi of [γ-^{32}P]ATP (PerkinElmer) in 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at room temperature for 10 min. The protein fraction was analyzed by SDS-PAGE and autoradiography.

Results

Association of the CLPP-related proteins with the components of microtubules was examined in the condition that microtubules are depolymerized (described in the Method section). Caveolin-1 is persistently present on CLPP though it increases by translocation of the additional amount by incubation of the cells with apoA-I (Ito et al. 2002b), so that an immunoprecipitation technique with this protein was employed for this purpose. Figure 2A shows the analysis of the co-precipitated protein with an anti-caveolin-1 antibody after astrocytes are stimulated by apoA-I. Protein kinase Cα increased in the caveolin-1-associated fraction after 5 min of the stimulation, and α-tubulin and β–actin also increased at the same timing. The interaction of these proteins with caveolin-1 decreased in the presence of a scaffolding domain peptide of caveolin-1 (Figure 2B), suggesting that the association of the cytoskeletal components with the

CLPP-related proteins is dependent on this domain of caveolin-1. When the cytosol was analyzed by density-gradient ultracentrifugation in sucrose (Figure 3), the increase of protein kinase Cα and α–tubulin was found in the CLPP fractions (Fraction No. 8 – 11) at 5 min of apoA-I stimulation. Such change was not obvious in density distribution of β-actin.

In order to investigate whether the association of CLPP with the microtubule components indicates its interaction with cytoskeleton, binding of CLPP to the microtubules was examined by using technique of rMT formation as described in the Method section. The CLPP-related proteins, protein kinase Cα and caveolin-1 increased in the 15 k-pellet fraction when the cells were stimulated by apoA-I (Figure 4A) being consistent with their increase in CLPP by apoA-I stimulation (Ito et al. 2004). Caveolin-1 disappeared in the supernatant when rMT is formed, and a large portion of caveolin-1 was recovered in the 15 k rpm-centrifuged pellets associated with rMT. Figure 4B shows time-dependent change of the proteins found in the rMT fraction by the apoA-I stimulation. Association of protein kinase Cα and caveolin-1 with the rMT fraction increased by stimulation with apoA-I for 5 min. It was noticed that caveolin-1 was somewhat recovered in the "polymer fraction" by the apoA-I-stimulation only at the 5-min timing even without GTP and $MgCl_2$ for an unknown reason. This effect may have influenced the apparent greater increase of caveolin-1 in the rMT at the 5-min timing (Figure 4B). Association with rMT of the cytosolic lipid, cholesterol, sphingomyelin and phosphatidylcholine, was also increased by apoA-I for 5 min (Figure 5). These findings suggested that apoA-I induces association between microtubules and the CLPP-related lipids and proteins.

Interaction of CLPP with rMT was supported by the finding that density shift of the CLPP-associated molecules was induced by the apoA-I stimulation. Figure 6A shows density profile of phospholipids of the cytocol stimulated by apoA-I, with and without formation of rMT by the treatment with $MgCl_2$ and GTP. The newly

synthesized lipid transferred to CLPP by apoA-I was decreased when rMT was formed, and the radioactivity increased in the pellet fraction. Figure 6B shows formation of rMT by Mg/GTP as α-tubulin is recovered in the pellet fraction. ApoA-I decreased the CLPP-associated α-tubulin and increased it in the pellet. In the condition that rMT is formed, protein kinase Cα, that otherwise increases in the CLPP fraction by apoA-I (Ito et al. 2004), apparently decreased in the CLPP fraction and increased in the pellet fraction by stimulation of the cells by apoA-I.

The association of protein kinase Cα and caveolin-1 with rMT was suppressed by the presence of a scaffolding domain-peptide of caveolin-1 (Figure 7A), similarly to the findings with association of the CLPP-related proteins with the component proteins of microtubules (Figure 2B). The association of the cytosolic lipid with rMT was also suppressed by the peptide (Figure 7B). Thus, this domain of caveolin-1 regulates the interaction of CLPP with the cytoskeletons.

The findings above strongly suggested that the interaction between CLPP and cytoskeletons is involved in the process of the HDL assembly in astrocytes mediated by ATP-binding cassette transporter A1. Therefore, it was examined whether interference with dynamic microtubule organization is involved in the apoA-I-mediated HDL assembly with rat astrocytes. The rat astrocytes were treated with paclitaxel (taxol), a reagent that stabilizes micotubules. The apoA-I-induced lipid translocation to the cytosol and cellular lipid release by apoA-I were determined (Figure 8). The treatment decreased the translocation and release of lipids, cholesterol and phosphatidylcholine. The results indicated that the microtubules play a significant role in the intracellular cholesterol trafficking related to the apoA-I-mediated cellular lipid release and HDL assembly.

Protein phosphorylation was examined among the proteins immuno-precipitable with anti-caveolin-1 antibody (Figure 9). The immuno-precipitated fraction of the cytosol was incubated with phospho-labeled ATP as described above.

The 52 kDa protein was phosphorylated to the most extent, and it was markedly suppressed by BIM. The pretreatment of the cells with apoA-I resulted in the increase of this phosphorylation. The results implied the involvement of the caveolin-1-associated protein kinase Cα in "para"-phosphorylation of the caveolin-1-associated proteins, especially the 52 kDa protein.

Discussion

In this work, we investigated the interaction between CLPP and cytoskeletons, especially microtubules, mediated by caveolin-1 on CLPP. The experimental results are summarized as follows. 1) ApoA-I induced the cytosolic condition in which association of the CLPP-related lipids and proteins with microtubules was increased. 2) This association was interfered by a scaffolding domain-peptide of caveolin-1. 3) Paclitaxel inhibited the apoA-I-mediated intracellular translocation and release of lipids from rat astrocytes. 4) "Para"-phosphorylation of the 52 kDa protein by protein kinase C associated with caveolin-1 was enhanced by pretreatment of the cells with apoA-I.

We found a unique lipid-protein particle in the cytosol of rat astrocytes (CLPP, cytosolic lipid protein particle), which has been characterized as having a density of 1.09 – 1.16 g/mL and a diameter of 17 – 18 nm (Ito et al. 2002b). The main part of cytosolic lipids such as cholesterol, phosphatidylcholine and sphingomyelin are recovered in the CLPP fraction. Transient translocation of *de novo*-labeled cholesterol and phospholipid to the CLPP fraction is induced along with the same translocation of caveolin-1 at 60 – 90 min after the stimulation of rat astrocytes with exogenous apoA-I, shortly prior to the appearance of the *de novo*-labeled lipids in the culture medium. Therefore, it can be hypothesized that the lipids translocated to CLPP are precursors of those released and assembled to HDL by apoA-I. If so, CLPP could be involved in lipid trafficking, especially of cholesterol, for the extracellular HDL assembly by apolipoprotein. As caveolin-1 is believed to play a role in intracellular cholesterol

trafficking (Fielding and Fielding 1997), we focused our experimental scope on this protein to investigate a function of CLPP in intracellular cholesterol trafficking in relation to the apoA-I-mediated cellular lipid release.

Nofer et al. reported that apoA-I promotes formation of cytoskeletons by enhancement of the stress fiber formation of microfilaments in human fibroblast (Nofer et al. 2003). Reconstitutional polymerization of α-tubulin and β-actin in the cytosol fraction *in vitro* may in fact be increased when rat astrocytes were pretreated with apoA-I for 5 min (Figure 4B), suggesting that apoA-I potentially induces the condition that promotes the formation of microtubules. The CLPP-related lipids and proteins including caveolin-1 and protein kinase Cα apparently associated with rMT and its components α-tubulin and β-actin, and the association increased when the cells were pretreated with apoA-I. The interactions were suppressed by a scaffolding domain-peptide of caveolin-1, indicating that this domain of caveolin-1 is involved in the interaction. Conrad et al. observed that the caveolin transport between plasma membrane and Golgi complex is suppressed by nocodazole, which depolymerizes microtubules, in cholesterol oxidase-treated human fibroblasts, implying that intracellular transport of chaveolin-1 is dependent on microtubules (Conrad et al. 1995). Thus, the findings in the present work can be consistent with the view that apoA-I promotes microtubules-CLPP association mediated by cavolin-1. Its scaffolding domain plays a critical role in this interaction.

Interaction of some lipid molecules with cytoskeletons has been reported. Phosphatidylinositol 4, 5-bisphosphate (PIP2), a substrate of phospholipase Cγ, binds to various microfilament-binding proteins such as α–actinin, gelsolin and profilin, and regulates organization of microfilaments (Dadabay et al. 1991; Goldschmidt-Clermont et al. 1991; Fukami et al. 1994; Yamamoto et al. 2001). Recently it has been also observed that PIP2 directly binds to tubulins (Popova et al. 2002; Chang et al. 2005). We have shown that apoA-I induces the translocation of phospholipase Cγ to the CLPP

fraction and production of diacylglycerol in CLPP, as well as the transfer of protein kinase Cα to CLPP (Ito et al. 2004). Together with the results in the present paper that apoA-I induces association of CLPP components with microtubules in rat astrocytes, these findings indicate that apoA-I may assemble the players on the microtubules for signal initiation and transduction, perhaps for cholesterol trafficking in relation to its release by biogenesis of HDL. Indeed, U73122, a phospholipase C inhibitor, suppressed apoA-I-mediated cholesterol release (Ito et al. 2004).

Paclitaxel, a stabilizer of microtubules (Schiff and Horwitz 1980), suppressed the apoA-I-mediated release of lipids and translocation of *de novo* synthesized lipid to CLPP in rat astrocytes. These are additional supporting evidence for the hypothesis that apoA-I-mediated cholesterol release and HDL assembly from astrocytes require the function of cytoskeletons.

ApoA-I apparently enhanced protein kinase C-dependent phosphorylation of the caveolin-1-coimmunoprecipitable proteins, and phosphorylation of the 52 kDa protein was greatly suppressed by the protein kinase C inhibitor. This seems to be para-phosphorylation by protein kinase Cα translocated to CLPP. Identification of the 52 kDa protein is under investigation in our laboratory. In addition to microtubules, astrocytes have two other types of cytoskeletons such as microfilaments and glia filaments. We intend to study the interaction between CLPP and these filaments by using reconstitution systems.

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Figure Legends

Figure 1. Reconstitution of microtubule-like filaments. The cytosol fraction was prepared by treatment of rat astrocytes with strong mixing in the cold hypotonic buffer and centrifugation at 90 krpm (367,000 x g, HITACHI himac CP85β) for 30 min at 4 °C as described in Methods section. The supernatant cytosol (150 μg protein/2 mL) that contains depolymerized microtubule components was incubated with 100 μM GTP and 2 mM $MgCl_2$ at room temperature for 20 min for repolymerization of microtubules. A: Analysis of the filament component proteins. The repolymerization procedure described above was carried out in the presence or absence of 10 μM colchicine. These cytosol preparations were centrifuged at 15 k rpm (10,000 x g) at 20°C for 30 min, and the supernatant was collected and further centrifuged at 80 k rpm (290,000 x g) at 20 °C for 30 min. The pellets of the first and second centrifugation are supposed to contain longer reconstituted microtubule-like filaments (rMT) and shorter rMT, respectively. The rMT fractions were analyzed in SDS-PAGE and Western blotting for α-tubulin and β-actin, as well as protein in the supernatant of the second centrifugation after its precipitation with 10 % trichloroacetic acid (the pellets of the first centrifugation, 15k; the pellets of the second centrifugation, 80k; the supernatant protein, S). Western and CBB represent Western blotting and Coomassie Brilliant Blue staining, respectively. B: Electronmicrograms of rMT and control cytosol incubated without GTP. The preparations were put on the grid, negatively stained with 0.5 % uranium acetate and examined with a Hitachi 7100 electron microscope.

Figure 2. Caveolin-1-associated proteins in cytosol. The cytosol fraction was prepared from rat astrocytes pretreated with or without 5 μg/mL of apoA-I for indicated periods of time. A: The cytosol fraction (250 μg protein/3 mL) was incubated with rabbit anti-caveolin-1 antibody and Protein G-Sepharose at 4°C for 2 hr. After washing, cytosolic protein bound to Sepharose was analyzed on SDS-PAGE and

Western blotting by using anti-protein kinase Cα (PKCα), anti-α-tubulin, anti-β-actin and anti-caveolin-1 antibodies. B: The cytosol fraction was prepared from rat astrocytes preincubated with or without apoA-I (5 μg/mL) for 5 min. The cytosol fraction (300 μg protein/3 mL) was incubated with anti-caveolin-1 antibody and Protein G-Sepharose in the presence or absence of the peptide representing a scaffolding domain of caveolin-1 (1 μM) (scaffold P.) at room temperature for 2 hr. Protein bound to Sepharose was analyzed by SDS-PAGE and Western blotting by using anti-protein kinase Cα (PKCα), anti-α-tubulin and anti-β-actin antibodies.

Figure 3. Analysis of the cytosol proteins by density gradient ultracentrifugation. The cytosol fraction was prepared from rat astrocytes pretreated with apoA-I (5 μg/mL) for 0, 5 and 30 min. The cytosol (300 μg protein/7 mL) was ultracentrifuged in density gradient by sucrose, collected into the 12 fractions from the bottom of the tube, and analysed by SDS-PEGE and Western blotting using anti-protein kinase Cα (PKCα), anti-α-tubulin and anti-β-actin antibodies, as described in Methods. CLPP indicates the CLPP fractions identified by the presence of the lipid peak at the density of 1.09 - 1.16 g/ml (data not shown)(Ito et al. 2002b)

Figure 4. Analysis of the rMT fractions prepared from rat astrocytes pretreated with apoA-I. A: The cytosol fraction was prepared in the cytoskeketon-deplymerizing condition from rat astrocytes pretreated with or without 5 μg/mL of apoA-I for 5 min. After forming rMT by incubating with 100 μM GTP and 2 mM $MgCl_2$ at room temperature for 20 min, the sample was centrifuged at 15 k rpm and then at 80 k rpm, and each pellet and the final supernatant were analyzed in SDS-PAGE and Western blotting for protein kinase Cα (PKCα), α-tubulin, β-actin and caveolin-1. B: The cytosol fraction was prepared as above from rat astrocytes pretreated with 5 μg/mL of apoA-I for 0, 5, 30 or 60 min. The cytosol fraction (160 μg protein/2 mL) was

incubated to form rMT with 100 μM GTP and 2 mM $MgCl_2$ at room temperature for 20 min, and the pellet and supernatant of centrifugation at 80 k rpm (290,000 x g) for 30 min were analyzed by SDS-PAGE and Western blotting by using antibodies as above. C: Control experiment to those in B, without the treatment with GTP and $MgCl_2$.

Figure 5. Binding of cytosolic lipids to rMT. Rat astrocytes were incubated with 3 μCi/mL of [^{14}C]acetate for 16 h in 0.1 % BSA/F-10. After complete washing 3 times with DPBS containing 0.15 % glucose, the cells were incubated for 60 min in a fresh 0.02 % BSA/F-10. ApoA-I (5 μg/mL) was added to the medium at the timing of 0, 30, 55 and 60 min after the start of the incubation in 0.02 % BSA/F-10 to make the incubation periods with apoA-I 60, 30, 5 and 0 min. After forming rMT as described in Figures 3 and 4, lipid was extracted from the rMT and the cytosol with a solvent mixture of chloroform : methanol (2 : 1, v/v) and analyzed by TLC. Total radioactivity of each lipid did not change significantly by the incubation with apoA-I. The radio-activity of cholesterol, sphingomyelin and phosphatidylcholine recoverd in the rMT fraction was displayed as a percentage to the total count of each lipid. The data points represent the mean ± SE of the triplicate measurement. Significance of the increase from the zero-time incubation is indicated as *** for $p < 0.01$ and ** for $p < 0.05$.

Figure 6. Density shift of the CLPP-related molecules by association with rMT. A: After washing and the medium replacement with 0.1 % BSA/F-10, rat asatrocytes were incubated with ^{14}C-acetate (3 μCi/ml) for 16 h and then with 5 μg/ml of apoA-I for 5 min. The cytosol (14 ml) was prepared from the cells according to the Experimental Procedure. Seven ml of cytosol was treated with or without 100 μM GTP and 2 mM $MgCl_2$ for 20 min at room temperature. Each cytosol sample was centrifuged on 1.17 g/ml sucrose solution (18 ml) at 49 krpm for 48 h at 15 ℃ and recovered into 12 fractions from the bottom as well as the pellet (fraction 0). The fraction 12 was

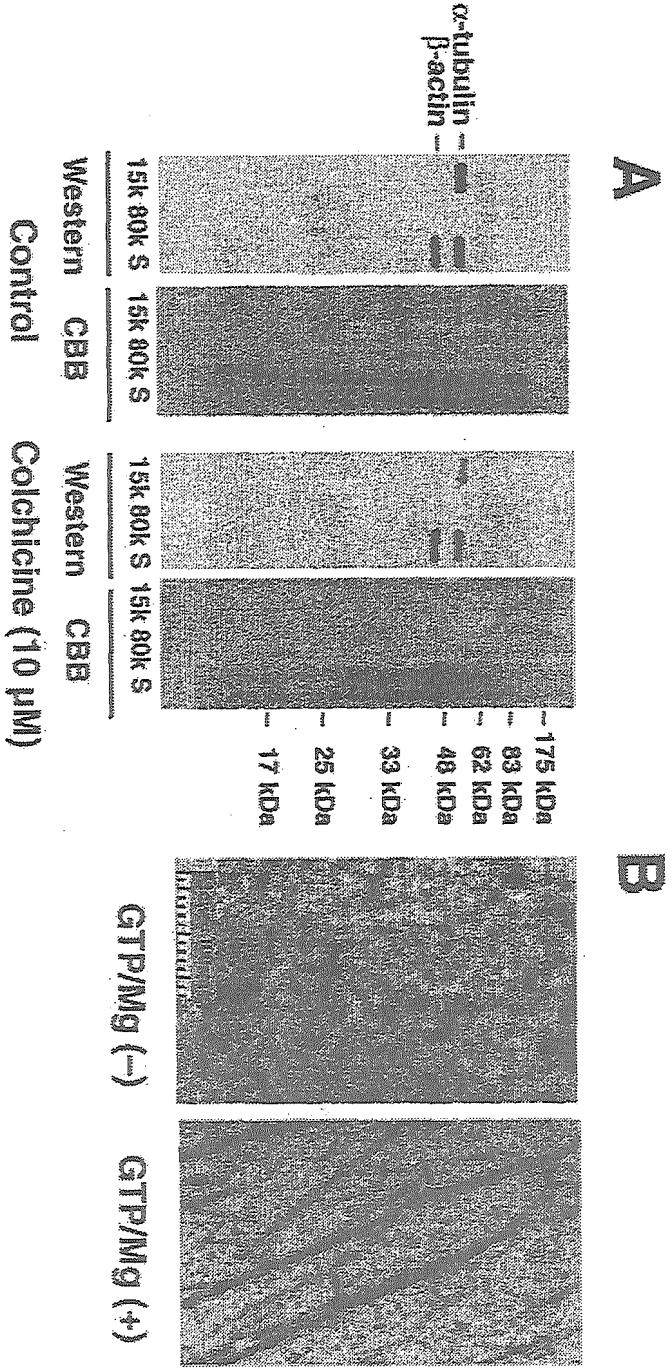
omitted from the analysis because of inevitable contamination of the pellet. Lipid was extracted from the fractions and analyzed by TLC for counting radioactivity in phosphatidylcholine and sphingomyeline. B: The cells were incubated with and without 5 μg/ml of apoA-I for 5 min. The cytosol (14 ml) was prepared from the cells and 7 ml of cytosol were treated with 100 μM GTP and 2 mM $MgCl_2$ for 20 min at room temperature. Each cytosol was analyzed in density gradient ultracentrifugation and fractionated as above. The protein in each cytosol fraction was precipitated with 10 % TCA and analyzed by SDS-PAGE and Western blotting by using anti-α-tubulin and anti-protein kinase Cα antibodies.

Figure 7. Effects of a scaffolding domain-peptide of caveolin-1 on the interaction of the CLPP-related protein and lipid with rMT. A: The effect on the CLPP-related proteins. In the condition for depolymerization of microtubules, the cytosol was prepared from rat astrocytes prestimulated with and without apoA-I (5 μg/mL) for 5 min. The cytosol (200 μg protein/2 mL) was incubated for formation of rMT in the presence or absence of 1 μM a scaffolding domain-peptide of caveolin-1 (scaffold P). The rMT fraction was analyzed by SDS-PAGE and Western blotting for protein kinace Cα (PKCα), caveolin-1, α-tubulin and β-actin as shown in Figure 4. B: The effect on cytosolic lipid. Rat astrocytes were incubated with 3 μCi/mL of [^{14}C]acetate for 16 hr in 0.1 % BSA/F-10. After washing 3 times with DPBS containing 0.15 % glucose and replacement with 0.02 % BSA/F-10, the cells were incubated with or without apoA-I (5 μg/mL) for 5 min and forming of rMT was induced in the presence or absence of a scaffolding domain-peptide 1 μM as described above. Lipid was extracted from the rMT and cytosol fractions and analyzed by TLC. Radioactivity of the lipid associated with the rMT fraction was determined for cholesterol, sphingomyelin and phosphatidylcholine. Each data point, representing mean ± SE of the triplicate samples, is expressed as percentage of the total radioactivity in the cytosol, which showed no

significant change by apoA-I. Significance of the change from the apoA-I (-) data is indicated as *** for $p < 0.01$ and ** for $p < 0.05$.

Figure 8. Effect of paclitaxel on the apoA-I-mediated release (A) and intracellular translocation (B) of lipid. A: Rat astrocytes were incubated with 3 μCi/mL of [^{14}C]acetate for 16 hr in 0.1 % BSA/F-10. After washing 3 times with DPBS containing 0.15 % glucose and replacement with 0.02 % BSA/F-10, the cells were incubated with or without apoA-I (5 μg/mL) for 3 hr in the presence or absence of paclitaxel (10 μM). Lipid was extracted from the conditioned medium and analyzed by TLC after the removal of cell debris by centrifugation at 15 krpm for 1 hr. Radioactivity was determined for cholesterol and phosphatidylcholine. B: The rat astrocytes were incubated with 4 μCi/ml of [^{14}C]acetate for 3 h. The cells were incubated with apoA-I (0, 5 μg/ml) in the presence or absence of paclytaxel (10 μM) for 90 min after washing. Lipid was extracted from the cytosol fraction and analyzed by TLC. Data represent the average and SE of the triplicate measurement. Significance of the change from the control data is indicated as *** for $p < 0.01$ and ** for $p < 0.05$.

Figure 9. Phosphorylation of the caveolin-1-associated proteins in the cytosol *in vitro*. The cytosol fraction was prepared from rat astrocytes pretreated with or without 5 μg/mL of apoA-I for 5 min. The caveolin-1-associated proteins in the cytosol fraction were immunoprecipitated by using anti-caveolin-1 antibody and Protein G-Sepharose. After washing, the Protein G-Sepharose suspension in 100 μL of Tris buffered saline/protease inhibitors was incubated with 5 μCi of [γ-^{32}P]ATP (PerkinElmer) in 1 mM $CaCl_2$ and 1 mM $MgCl_2$ in the presence or absence of 10 μM BIM at room temperature for 10 min and then washed again. The Protein G-Sepharose-bound and -unbound protein was analyzed in SDS-PAGE and autradiography.



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