ABC transporters in Japanese has been constructed and identified 67 SNPs at the ABCA7 locus [14]. No association has been indicated between variations and pathological phenotypes to date.

Analysis of human ABCA7 gene structure revealed that the ABCA7 gene localizes in tandem with the minor histocompatibility antigen HA-1 on the same strand [15]. Studies on SNPs in the HA-1 gene demonstrated that the incidence of an allele (168 His) was lower in the patients with Sjögren's syndrome than controls [16]. In addition, SS-N is a part of the first extracellular domain of ABCA7 [13] and an antibody against this domain reacted to infiltrating plasma cells in salivary glands from the patients of this disease, but not in those from non-specific sialoadenitis patients [17]. The pathophysiological significance of these observations is unclear to date.

A homozygous ABCA7 knockout mouse was found embryonic lethal [7], but one other group reported generation of ABCA7 null mice [8]. No remarkable phenotype was observed in these animals, except that white adipose tissue mass was about 50% less and the serum HDL cholesterol level was somewhat but significantly lower than that of the wild type control only in the female mice at the age of 10-weeks. No such change was detected in the male knockout mice [8]. No difference was shown for apoA-I and apoE protein levels in the HDL or serum free fatty acid profile [8]. Production of the homozygous mouse by intercrossing of the heterozygotes was at the expected rate, and its development, including feeding behavior and weight gain up to 10 weeks, was normal [8]. These results indicate that ABCA7 deficiency in human might be also asymptomatic and no patient may have been found even if present.

There are no data available for hormone-dependent events and fertility of the knockout mouse. Analysis of gonadal steroid hormone would be helpful to address these issues.

4. Expression pattern

4.1. Tissue distribution

The ABCA7 gene is widely expressed both in adult and embryonic tissues in human [5,10], mouse [11], and rat [12]. The tissue distribution profile of ABCA7 is different from that of ABCA1. While the latter is ubiquitously expressed and especially abundant in the liver [2], RNA dot blot analysis of human tissues demonstrated the highest expression of ABCA7 in the thymus and predominant expression in other immune and hematopoietic tissues (spleen, lymph node, peripheral leukocytes, and bone marrow) [10]. In addition to these tissues, Northern blot analysis [5] showed high-level mRNA expression in the brain. In mouse, a similar expression pattern was observed by Northern blotting showing the highest mRNA levels in myelolymphatic tissues [11]. mRNA was also detected in the brain, adrenal glands, and uterus [11]. In the brain, neuronal staining was demonstrated by in situ hybridization analysis [8].

Data are limited for the tissue distribution of ABCA7 protein in human, but the expression pattern of ABCA7 protein in mouse and rat is almost identical to the mRNA expression. In mouse, the ABCA7 protein level is high in the spleen, lung, adrenal glands, and brain, moderate in the peripheral macrophages and lymphocytes, and low in the liver [4]. High-level expression of ABCA7 protein was also detected in platelets [4], erythrocytes [4], and in the white adipose tissue [8]. ABCA7 protein was highly expressed in platelets in rat, and much lower in lymphocytes, erythrocytes, brain, and ovary [12]. Analysis of the ABCA7 expression in human peripheral blood cells gave controversial results. One article reported expression of ABCA7 in lymphocytes, granulocytes, and monocytes [10] but the other claimed the failure of detection of ABCA7 protein in peripheral blood cells in spite of the mRNA expression [5].

4.2. Subcellular localization

Little is known concerning the subcellular localization of endogenous ABCA7 protein. Immunofluorescence confocal microscopy with rabbit anti-mouse ABCA7 antibody detected no signal at the plasma membrane but in the intracellular space in peritoneal macrophages [7]. The same authors described positive staining of the brush border membrane in proximal tubules of the kidney [7], but the background in the control staining was significant [7] and expression levels of ABCA7 mRNA and protein were both reportedly low in the kidney [4,8,11,12].

ABCA7 protein resulting from transfected cDNA (wild type, GFP-tagged, and Flag-tagged) was localized predominantly in the plasma membrane but was also detected in the intracellular membranes [4–7,12]. When expressed in CHO cells, rat ABCA7 with no tag and that with GFP tag at the N-terminus were both detected mainly in the plasma membrane, while ABCA7 with GFP fusion to the C-terminus was localized in the perinuclear membrane as well being probed by the ABCA7 epitope and GFP fluorescence [12]. Cell surface biotinylation [4] and immunofluorescence microscopy analysis of non-permeabilized cells with the antibody against the first putative extracellular domain of human ABCA7 (amino acids 45–549) [5] confirmed the cell surface expression of the ABCA7 protein. The latter experiment also revealed that this domain is exposed to the outside of the cell indicating the same topological arrangement as ABCA1 [5,13,18]. Most of type II ABCA7 protein was found intracellularly localized when expressed in HEK 293 cells [5] indicating that the N-terminal domain is important for sorting ABCA7 protein to the plasma membrane.

5. Regulation

5.1. Transcriptional regulation

The predicted promoter regions of human and mouse ABCA7 conserved two modules containing putative transcription factor-binding sites. The sites are targets for either ubiquitous transcription factors or liver- or lymphoid-specific transcription factors [11]. No date are available on the contribution of these regions to the promoter activity.

ABCA7 is assumed to be a sterol-regulated gene, because mRNA and protein levels of human monocyte-derived macrophages were reportedly increased by adding acetyl-low-density lipoprotein to the medium and decreased by HDL3 [10]. The liver X receptor (LXR) and the retinoid X receptor (RXR) are major transcriptional regulators of the ABCA1 gene [2]. However, the ABCA7 mRNA level in mouse peritoneal macrophages was not affected by an LXR agonist with and without an RXR agonist [4]. The ABCA7 protein level of the

mouse bone marrow-derived macrophages was also unaffected by stimulation with free cholesterol and LXR/RXR agonists [8].

We have recently found that ABCA7 mRNA level is regulated by sterol responsive/regulatory element binding protein 1a and 2 in human and mouse fibroblasts (Iwamoto et al., unpublished data). These findings should provide a new clue to understand regulatory mechanism of gene expression and biological roles of ABCA7.

5.2. Posttranscriptional regulation

Nothing has been reported for posttranscriptional regulation of endogenous ABCA7. In the transfected cells, apoA-I and protease inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) increased the steady state level of human ABCA7-GFP protein in HEK293 cells [6]. We have shown that apoA-I and ALLN upregulate cellular ABCA1 protein level through inhibiting a thiol protease, most likely calpain [19,20]. Therefore, there might be a similar regulatory mechanism for ABCA7 protein metabolism. Phorbol 12-myristate 13-acetate (PMA) treatment of the same cells resulted in a slight increase of the ABCA7 protein but apoA-I-mediated cholesterol and phospholipid release was significantly inhibited. In contrast, in the ABCA1-expressing cells, both ABCA1 protein level and apoA-I-mediated lipid release were significantly upregulated by PMA. Gö6976, an inhibitor of Ca^{2+}-dependent protein kinase C isoform(s), reversed all these effects of PMA. Thus, protein kinase C is likely involved in posttranscriptional regulation of both ABC proteins, and it seems to modulate the specific activity of ABCA7 for release of cellular lipid and generation of HDL in vitro [6].

6. Functions

6.1. HDL generation

The physiological function of ABCA7 is unknown. Because of the structural homology to ABCA1, and because of the fact that many ABC transporters act as lipid transporters [13,18,21], the study on the function of ABCA7 has been focused on cellular lipid metabolism. ABCA1 was identified as a causative gene for familial HDL deficiency (Tangier disease) and other genetic HDL deficiencies [22–26] resulting in the lack of the apolipoprotein-mediated HDL generation from the cellular lipid [27,28]. Thus ABCA7 was investigated for the analogy to this type of reaction in the transfected cells [4–9] (Table 1).

All the results agree that ABCA7 supports apolipoprotein-mediated phospholipid release and that phospholipids are the primary substrate for this ABCA7-mediated reaction. ABCA7 expressed in several cell lines promoted phospholipid release by lipid-free apolipoproteins such as apoA-I and apoA-II in a time- and dose-dependent manner, similarly to ABCA1. As was reported in ABCA1 [29], HEK293 cells expressing mouse ABCA7 showed increased specific binding of apoA-I, and covalent cross-linking studies revealed complex formation of ABCA7 and apoA-I [4]. These data suggested that apoA-I binds ABCA7 directly, or localizes very close to ABCA7 on the cell surface. After normalization of cell surface protein expression with Flag-tag, mouse ABCA7 was shown to have the same "phospholipid transporter" activity [7] as ABCA1, or 10–50% of the ABCA1 activity [8].

ABCA7 mediates cholesterol release much less than phospholipid, though we claim it is low but positive by using human ABCA7 without or with GFP-tag expressed in several types of cell lines even in the absence of ABCA1 [5,6,9] and others report negative [4,7] or no [8] data (Table 1). It is noteworthy that type II ABCA7 protein, very little is localized in plasma membrane, mediates neither cholesterol nor phospholipid release [5].

According to our data, the released cholesterol/phospholipid ratio from ABCA1-expressing cells is much higher than that from ABCA7-expressing cells [6,9]. We have developed a HEK 293 cell system with ecdysone-inducible human ABCA1 and ABCA7. While the cholesterol/phospholipid ratio in the apolipoprotein-released lipid increased along with the increase

Table 1
ApoA-I-mediated lipid release by ABCA7

Cell	cDNA	Tag	Expression[d]	Additional treatment	Ch release[e]	PL release[e]	Reference
HEK293	Human	–	T	–	+	+	[6]
		–	T	–	–	+	[7]
		GFP	T	–	+	+	[5]
		GFP	T	–	+	+	[6]
		GFP	S	–	+	+	[6]
HEK293 ECR[a]	Human	GFP	I	–	+	+	[9]
HEK293	Human type II	GFP	T	–	–	–	[5]
L929[b]	Human	GFP	SM	–	+	+	[6]
HEK293	Mouse	–	T	–	–	+	[4]
		–	T	–	–	+	[7]
		–	T	–	ND	+	[8]
293-EBNA-T	Mouse	–	T	–	ND	+	[8]
HEK293	Mouse	Flag	T	–	ND	+	[4]
		Flag	T	–	ND	+	[8]
		–	T	Ch loading	–	ND	[4]
		–	T	SR-BI expression	–	ND	[4]
CHO[c]	Mouse	–	T	–	–	ND	[4]

[a]HEK293 cells expressing ecdysone receptor.
[b]Parent cells are positive for apoA-I-mediated phospholipid release but negative for cholesterol release.
[c]Parent cells are positive for apoA-I-mediated cholesterol and phospholipid release.
[d]I, S, SM, and T denote inducible, stable, mixture of stable clones, and transient, respectively.
[e]ND: not described.

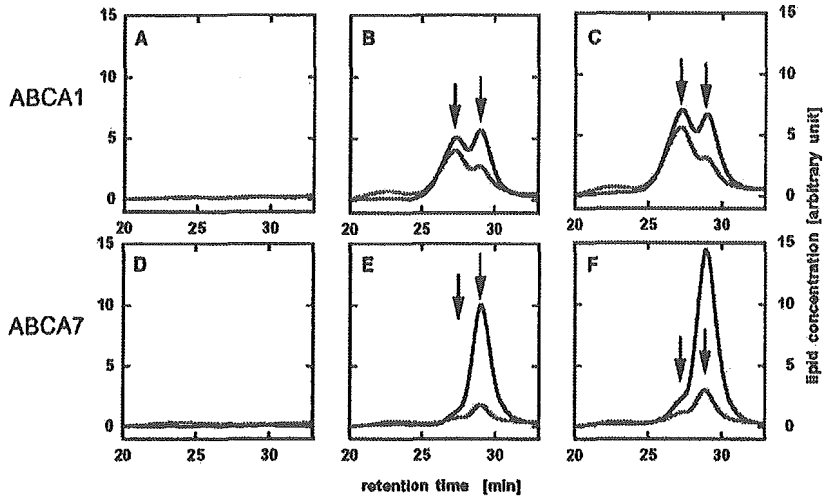

Fig. 1. Lipoprotein analysis of the HDL particles generated from the ABCA1- and ABCA7-induced cells (taken from Ref. [9]). Ecdysone receptor-expressing HEK293 cells with inducible human ABCA1-GFP (A–C) and inducible human ABCA7-GFP (D–F) were incubated without (A,D), with low dose (B,E), and with high dose (C,F) of ponasterone A for induction. After 24-h incubation with 10 μg/ml apoA-I, medium was collected for HPLC analysis. The concentration of cholesterol (red lines) and phospholipid (blue lines) in each fraction was measured.

of ABCA1 protein level by ecdysone, the ratio remained constant when the ABCA7 protein level was increased [9]. Analyses of size distribution and lipid composition of the generated HDL particles by a gel permeation HPLC system revealed that the ABCA1-mediated reaction produced two types of HDL particles, large particles with a high cholesterol/phospholipid ratio and small particles with a low cholesterol/phospholipid ratio (Fig. 1B). The ABCA7-mediated reaction generated mostly the small particles (Fig. 1E). Induction of the ABC transporters by ecdysone caused a dominant increase of the large particles in the ABCA1-expressing cells and only the small particles in the ABCA7-expressing cells (Fig. 1C and F). Despite all these in vitro findings on the ABCA7-mediated generation of HDL, it is clear that ABCA1, not ABCA7, is essential to maintain the plasma HDL concentration from the studies on Tangier disease patients and ABCA1 knockout mice [2]. In addition, apoA-I-mediated cellular cholesterol and phospholipid release were all unaffected in bone marrow-derived macrophages prepared from ABCA7 knockout mice [8], peritoneal macrophages from ABCA7 heterozygous mice [7], and normal peritoneal macrophages treated with ABCA7 siRNA [7]. Therefore, the ABCA7-mediated lipid release may not significantly contribute to a source of plasma HDL. However, it may still play an important role in cellular cholesterol homeostasis in certain particular tissues including macrophages.

6.2. Others

ABCA7 is also expressed in several cells other than the reticuloendothelial system. Platelets express high-levels of ABCA7 at least in mouse and rat [4,12]. As platelets secrete various lipid mediators including sphingosine 1-phosphate and lysophosphatidic acid, possible functional integration of ABCA7 in the secretion mechanism of the mediators was suggested [12].

HaCaT cells, a spontaneously immortalized human keratinocyte cell line cells, and primary cultured normal human epidermal keratinocytes (NHEK cells) can be differentiated by increased Ca^{2+} concentration in the medium. During this differentiation period, the cellular ceramide concentration increases. RT-PCR analysis of the cells found that ABCA7 mRNA increased 3 times from the undifferentiated to the differentiated stage of NHEK cells [30]. Expression of human ABCA7 in HeLa cells resulted in an increase of intracellular phospholipids compared to the mock-transfected cells (to 135%, 130%, and 115% in ceramide, phosphatidylserine, and phosphatidylcholine, respectively) and an increase of cell surface ceramide expression [30]. From these data, a potential role of ABCA7 in epidermal lipid reorganization was proposed.

The ABCA7 protein level is high in mouse white adipose tissue and is upregulated during differentiation of the mouse preadipocyte cell line 3T3L1 to adipocytes, suggesting its potential role in lipid transport in these cells [8]. In the differentiated 3T3L1 cells, some ABCA7 protein is shown colocalized with perilipin in the lipid droplet and microsomal fractions, being different from ABCA1 [8].

7. Conclusions and perspectives

ABCA7 is a protein that has the highest homology to ABCA1. ABCA7 mediates apolipoprotein-derived generation of HDL similarly to ABCA1, suggesting that it may compensate the function of ABCA1 for release of cellular lipid in a certain condition(s). However, it is obvious that ABCA7 does not rescue the plasma HDL deficiency caused by the defect of ABCA1 function, in either man or mouse. In addition, differences between ABCA1 and ABCA7, especially in tissue distribution profile and transcriptional regulatory mechanism, implicate that ABCA7 may have a more specific role(s) than mimicking ABCA1. Studies on similarity and difference between ABCA1 and ABCA7 would provide us with more information to address the questions for understanding functions of these transporters.

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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-microgram 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.

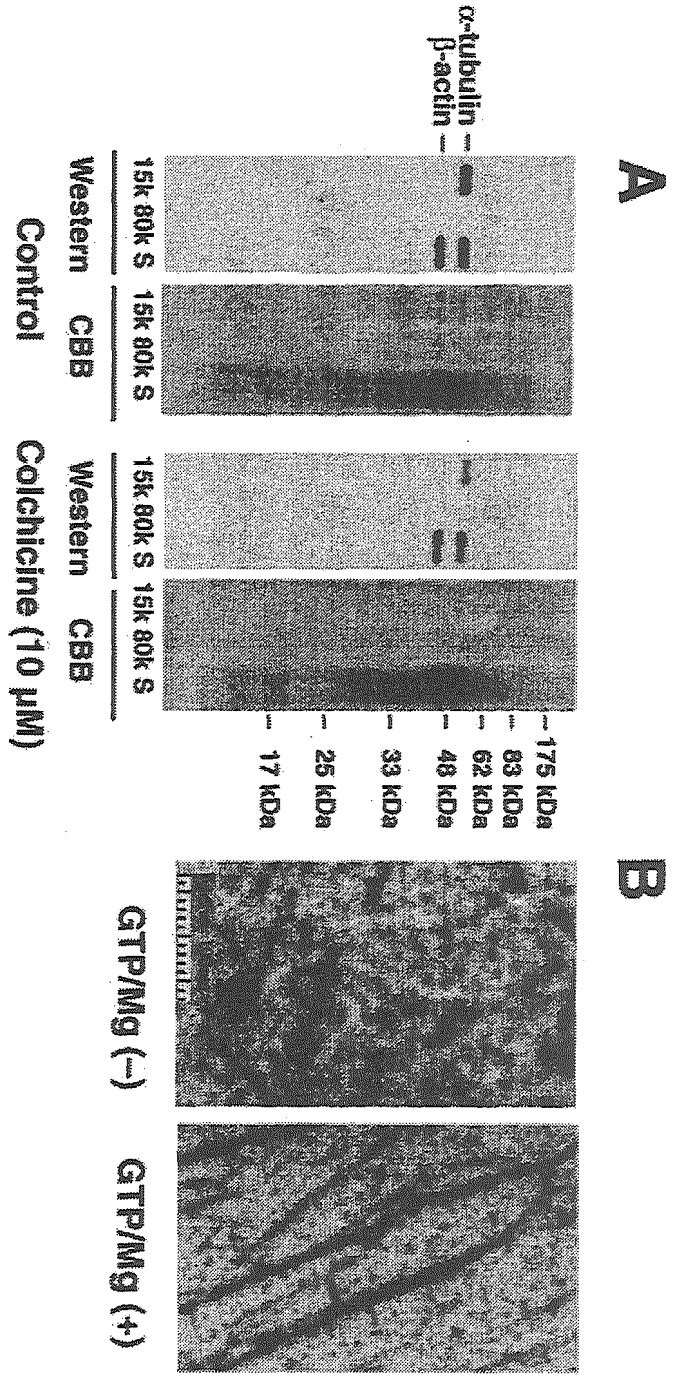
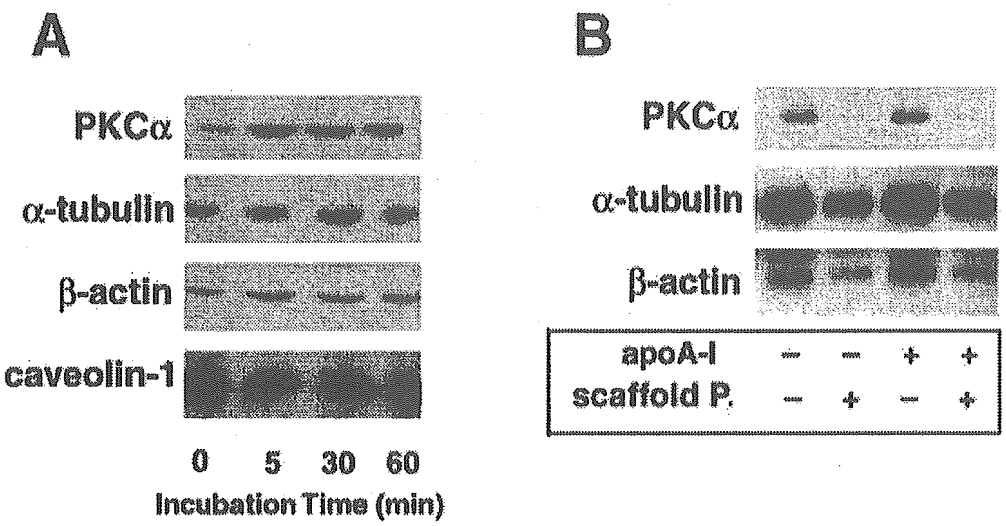


Figure 1, J. Ito et al.

Figure 2. J. Ito et al.



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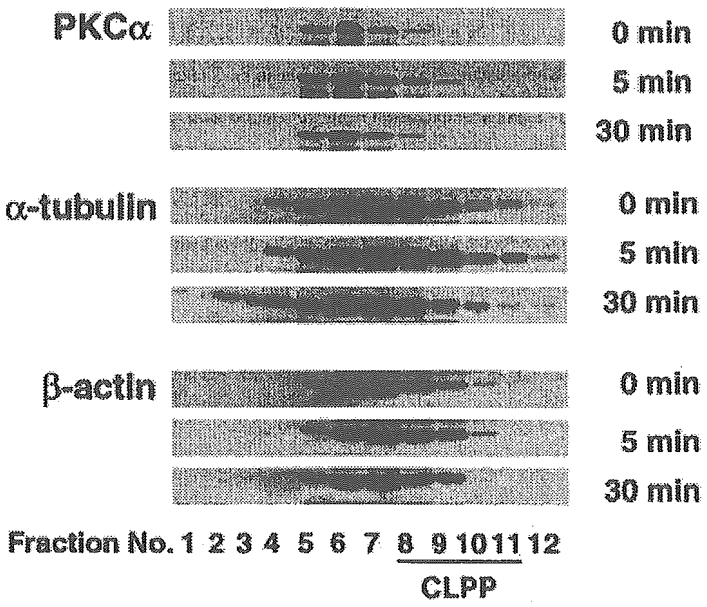


Figure 4. J. Ito et al.

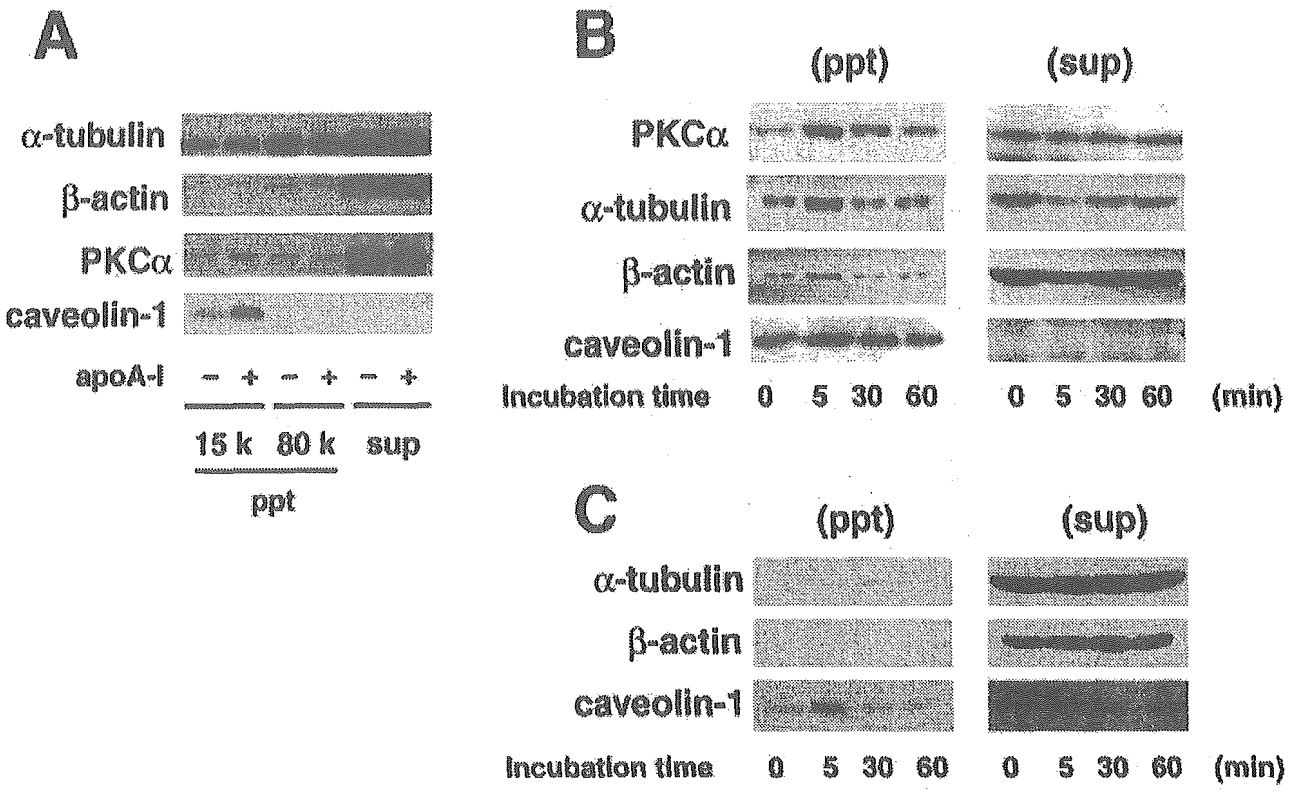


Figure 5. J. Ito et al.

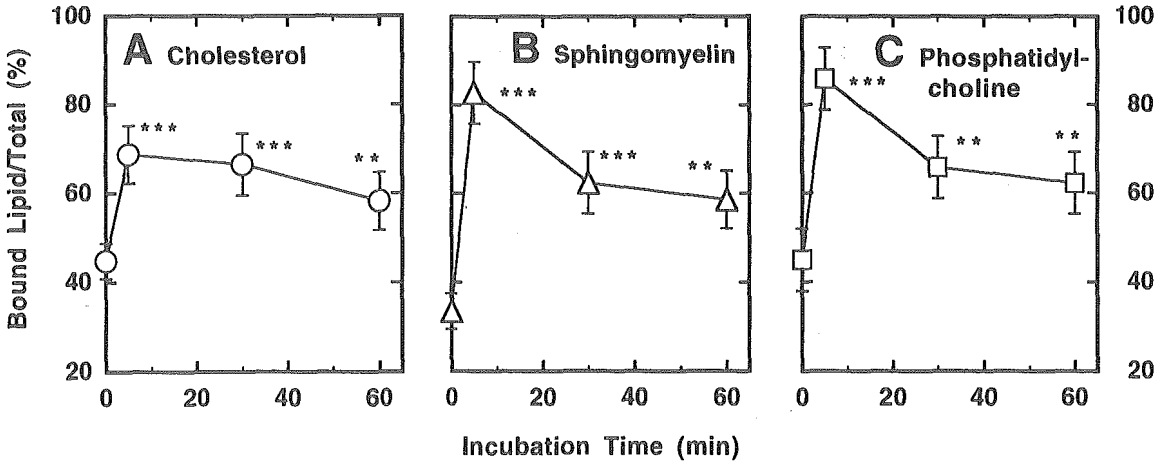


Figure 6A, Ito et al.

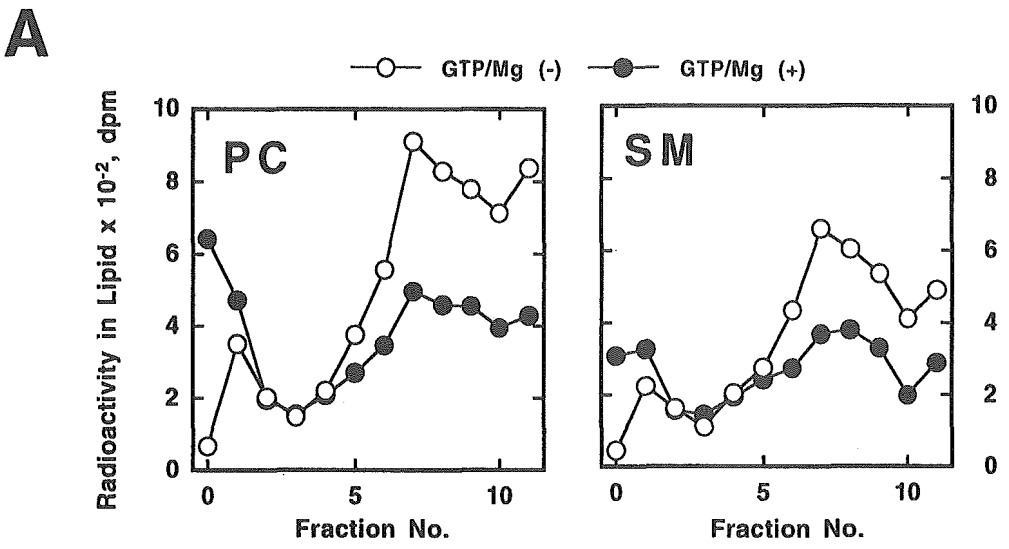


Figure 6B, Ito et al.

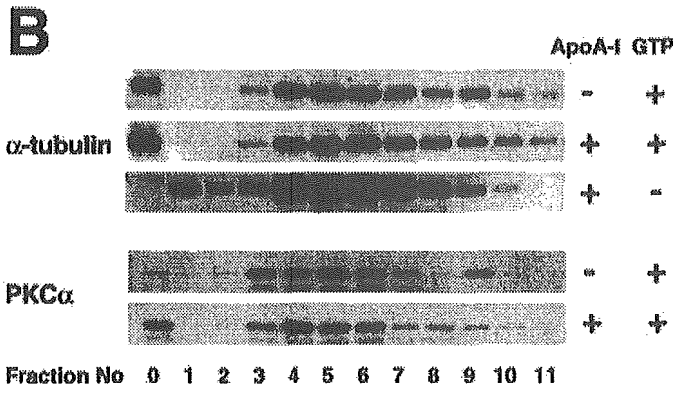
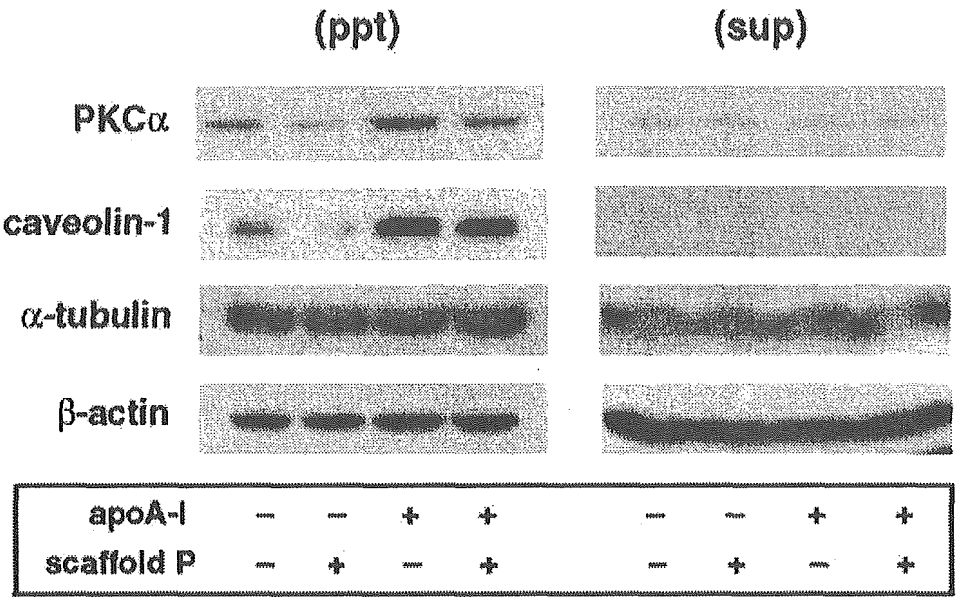


Figure 7A. J. Ito et al.



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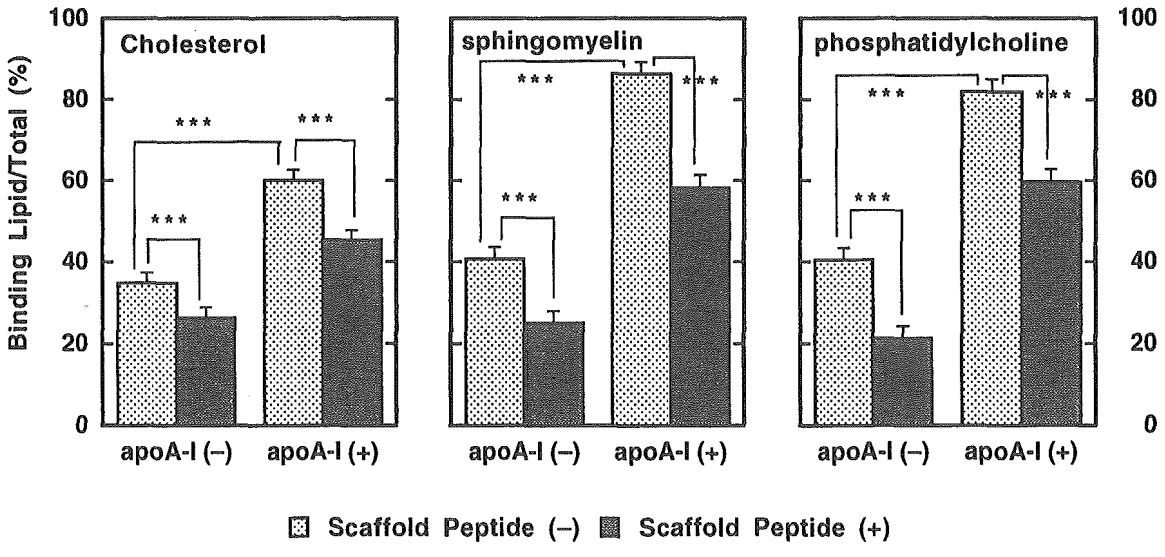

Figure 8. J. Ito et al.

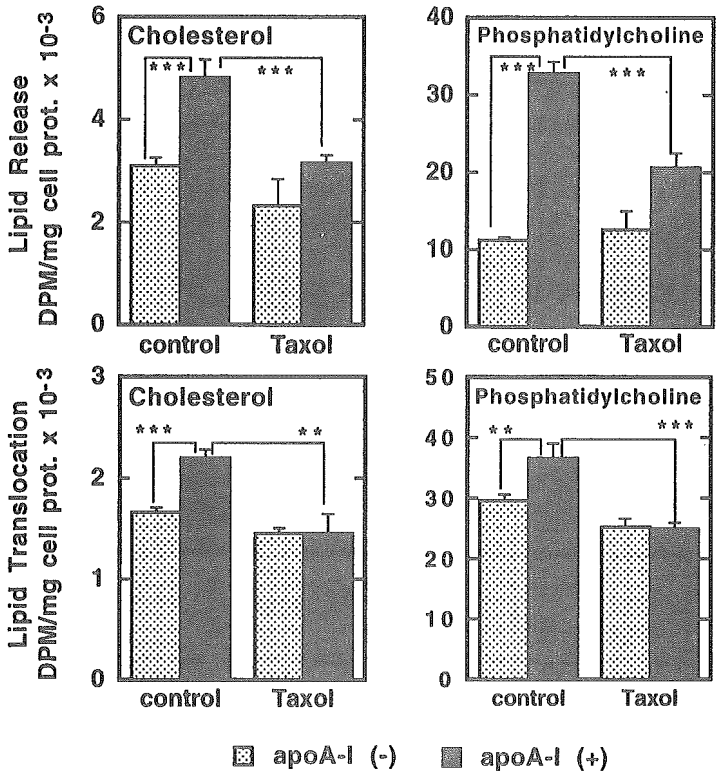

Figure 9. J. Ito et al.