Apolipoprotein A-I induces translocation of protein kinase Cα to a cytosolic lipid-protein particle in astrocytes

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

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

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

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

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

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

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

MATERIALS AND METHODS

Materials

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

Cell culture

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

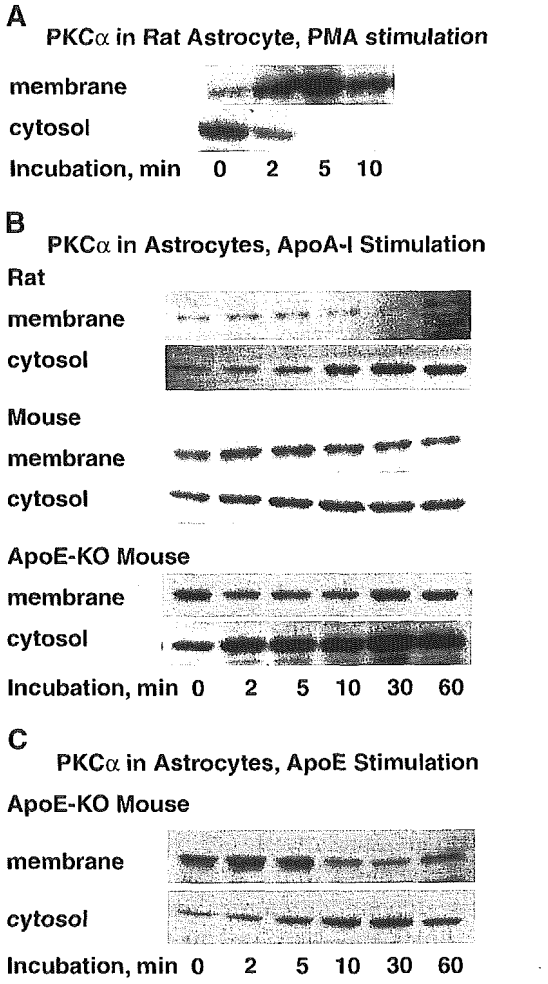

Fig. 1. Redistribution by apolipoprotein A-I (apoA-I) of protein kinase Cα (PKCα) in astrocytes. A: Rat astrocytes were treated with 200 nM phorbol 12-myristate 13-acetate (PMA). The membrane fraction protein (15 μg/lane) and the cytosol protein (50 μg/lane) were analyzed for protein kinase Cα by immunoblotting.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

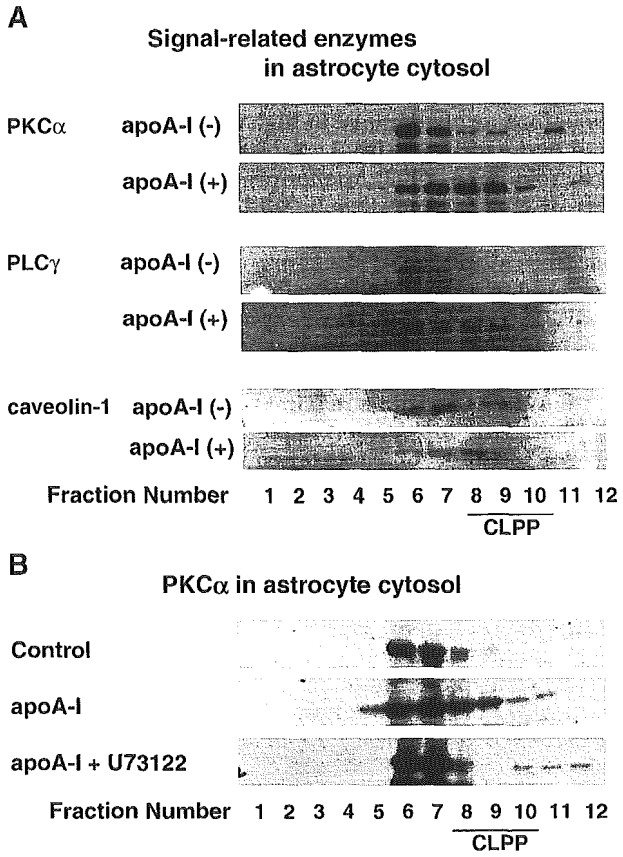

Fig. 2. Redistribution by apoA-I of protein kinase Cα (PKCα) and phospholipase Cγ (PLCγ) in cytosol of mouse astrocytes. A: After washing and medium replacement with 0.02% BSA/DMEM, apoE-KO mouse astrocytes were incubated with or without apoA-I (5 μg/ml) for 5 min. The cytosol (350 μg protein/7 ml) was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

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

Cytosol preparation and density gradient ultracentrifugation analysis

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

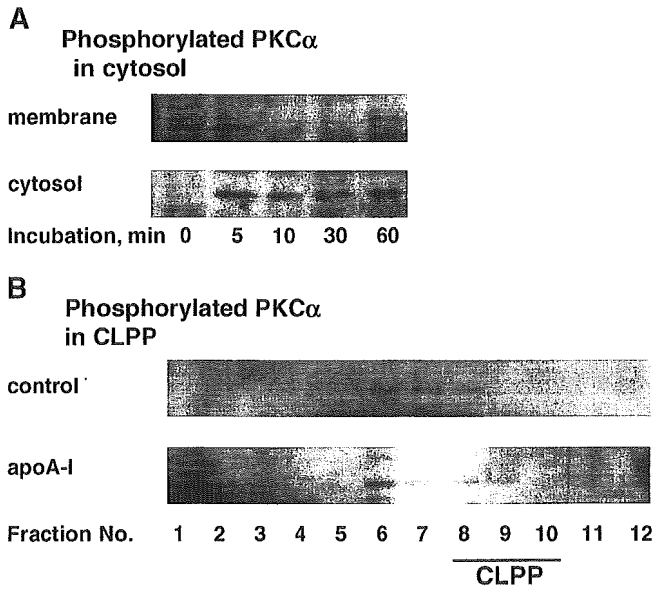

Fig. 3. Phosphorylation of protein kinase Cα (PKCα) in apoA-I-stimulated mouse astrocytes. A: The cytosol and membrane fractions were prepared from mouse astrocytes pretreated with 5 μg/ml apoA-I for the indicated periods of time in fresh 0.02% BSA/DMEM. Each sample was analyzed by SDS-PAGE (40 μg/lane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

Caveolae/rafts preparation from the membrane fraction

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

Western blotting

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

De novo syntheses and release of lipid

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

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

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

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

RESULTS

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

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

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

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

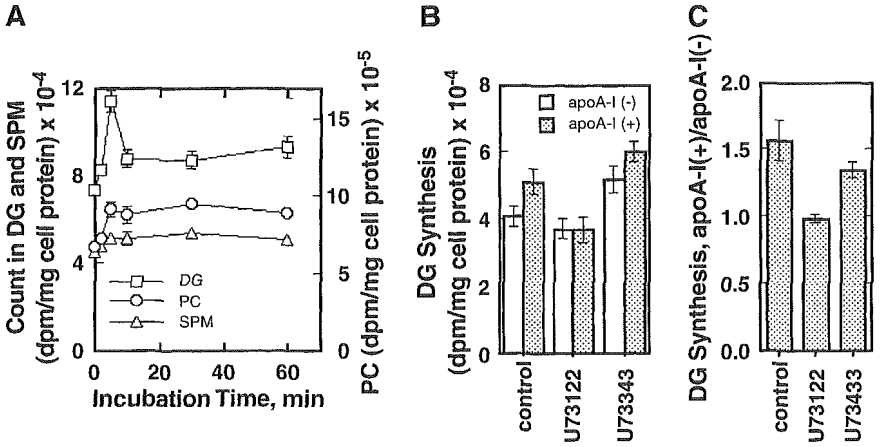

Fig. 4. Increase of diacylglyceride (DG) production by apoA-I and the effect of U73122 in mouse astrocytes. A: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of DMEM medium containing 0.02% BSA (0.02% BSA/DMEM). After three complete washes with Dulbecco's phosphate-buffered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

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

DISCUSSION

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

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

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

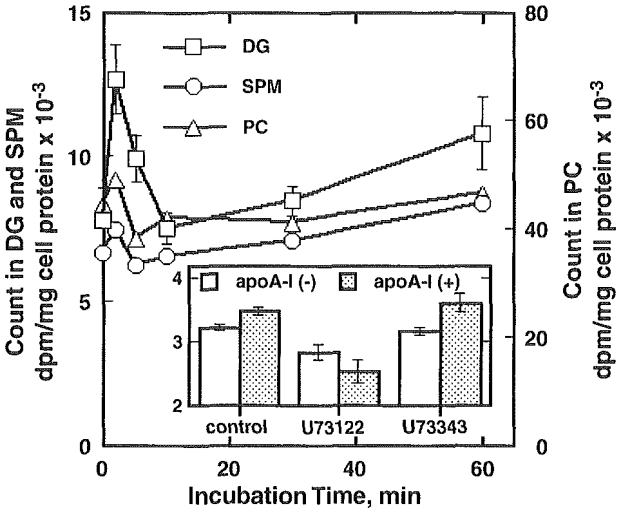

Fig. 5. Increase of DG production by apoA-I and the effect of U73122 on DG production in WI-38 cells. WI-38 cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM. The cells were incubated with apoA-I (5 μg/ml) for 0, 2, 5, 10, 30, and 60 min as described for Fig. 4A.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

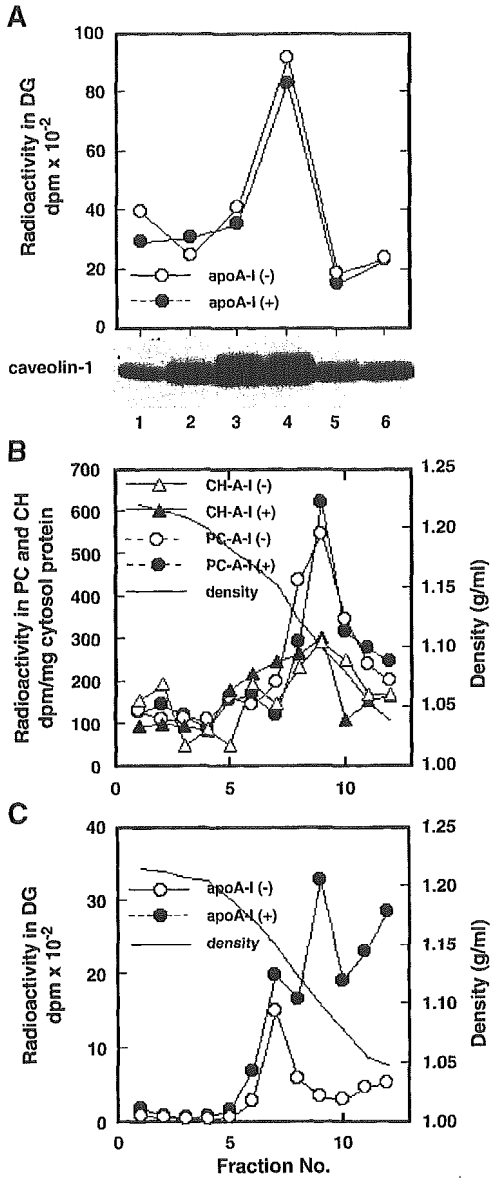

Fig. 6. Increase of DG by apoA-I in the cytosol of astrocytes. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM and then treated with (closed symbols) or without (open symbols) 5 μg/ml apoA-I for 5 min after washing and medium replacement with fresh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

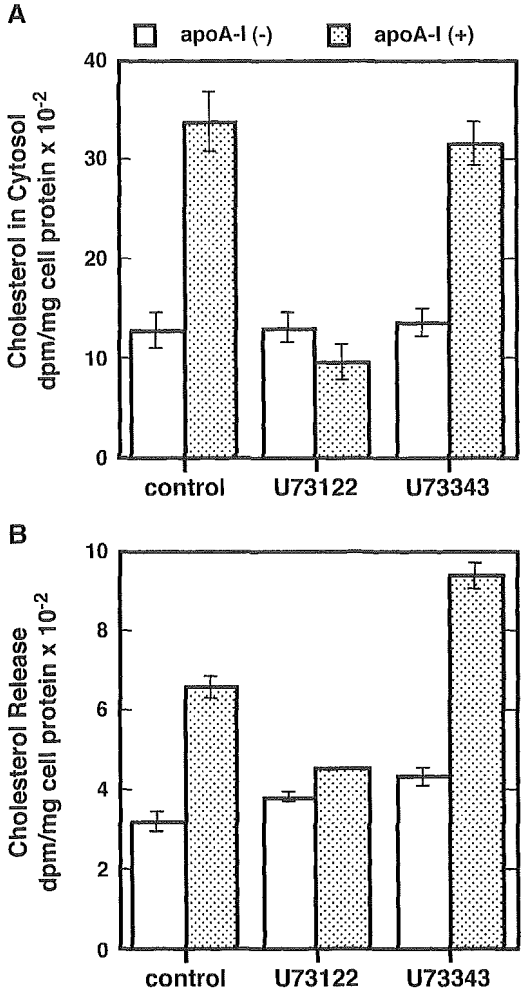

Fig. 7. Effects of U73122 on cholesterol trafficking in mouse astrocytes. A: The cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM followed by washing and medium replacement with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were treated with (dotted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

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

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

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

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

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

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Promoter polymorphism in fibroblast growth factor 1 gene increases risk of definite Alzheimer's disease

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Abstract

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease (AD) and HIV encephalitis. The FGF1 gene is therefore a strong candidate gene for AD. Using the promoter polymorphism of the FGF1 gene, we examined the relationship between AD and the FGF1 and apolipoprotein E (APOE) genes in 100 Japanese autopsy-confirmed late-onset AD patients and 106 age-matched non-demented controls. The promoter polymorphism (−1385 A/G) was significantly associated with AD risk. The odds ratio for AD associated with the GG vs non-GG genotype was 2.02 (95% CI = 1.16–3.52), while that of ε4 vs non-ε4 in APOE4 gene was 5.19 (95% CI = 2.68–10.1). The odds ratio for APOEε4 and FGF1 GG carriers was 20.5 (95% CI = 6.88–60.9). The results showed that the FGF1 gene is associated with autopsy-confirmed AD.

Keywords: Definite Alzheimer's disease; Fibroblast growth factor 1 gene; Promoter polymorphism; Association study; APOE; Risk factor

Alzheimer's disease (AD; MIM#104300) is the most common cause of dementia in mid- to late-life. Studying the factors that influence the risk of developing AD may lead to the identification of those at high risk for developing it, strategies for prevention or intervention, and clues to the cause of the disease. Both genetic and environmental factors have been implicated in the development of AD [1], but the cause of AD remains unknown, and no cure or universally effective treatment has yet been developed [2]. Even the diagnosis is difficult. A definitive diagnosis depends on analysis of neuritic plaques and neurofibrillary tangles found in brain tissue [3]. Given the recognition that AD constitutes a heterogeneous disorder, identification of established risk factors would be difficult using conventional methods.

Fibroblast growth factor 1 (FGF1, also known as acidic FGF) is a member of the fibroblast growth factor family that possesses broad mitogenic and cell survival activities and is involved in a variety of biological processes [4]. FGF1 protects selective neuronal populations against neurotoxic effects such as those in Alzheimer's disease [5,6] and HIV encephalitis [7]. Immunohistochemical examination of postmortem brain tissue of AD revealed that FGF1 was specifically expressed in a subpopulation of reactive astrocytes surrounding senile

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plaques. Such upregulation of FGF1 expression might be related to the presence of reactive astrocytes rather than β-amyloid protein deposition [8,9]. Recent studies suggest that FGF1 upregulates APOE synthesis and subsequently HDL production in reactive astrocytes in an autocrine or paracrine manner, and exerts its effect after central nervous system (CNS) damage through APOE secretion [10,11]. Besides, the fact that FGF1 expression is lower in the hippocampal formation than in motoneurons suggests that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [6,12]. The FGF1 gene is therefore a strong candidate gene for AD. However, there are no reports regarding the association of FGF1 gene polymorphism with AD. Therefore, we investigated whether FGF1 gene polymorphism could contribute to risk in a limited subgroup of AD (autopsy-confirmed AD).

Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected based on the NINCDS-ADRDA criteria for definite AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination [3,13]. Brain and blood samples were obtained with informed consent from subjects in the Chubu and Kansai areas of Japan. A total of 100 unrelated late-onset AD (LOAD) patients had been diagnosed previously, and 106 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence of the patients as described elsewhere [14,15]. The mean age ± SD (years) at the time of this study was as follows: 85.3 ± 6.0 for LOAD, 83.0 ± 4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol–chloroform method [16].

During screening for FGF1 gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of −1385 G/A (C/T) (rs34011) in the promoter region. This polymorphism could easily be detected by PCR-RFLP using the restriction enzyme *Hha*I, where G and A, with respective frequencies of 0.65 and 0.35, were observed in our Japanese control population. The polymorphic region was amplified by PCR with the primers FGF1-F (5′-TCAAGC AATTCTCCTGCCTT-3′) and FGF1-R (5′-CCACTTCAAGGGATT ATGGTG-3′). PCR was carried out in a 25-μl reaction volume containing standard reaction buffer (1.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3), 200 μM each dNTP, 5 μM each primer, 0.5 U *Taq* DNA polymerase and 50 ng genomic DNA as a template with 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR product size was 355 bp, and the G allele was digested by *Hha*I to 53 + 141 + 161 bp, and the A allele to 53 + 302 bp. DNA was electrophoresed on 2% agarose gels and visualized with ethidium bromide staining under UV light (Fig. 1). To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and normal control subjects. Because APOEε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously. Allelic and genotypic distribution were analyzed by the usual χ^2 test of association. The genotypic frequencies were compared by χ^2 test with the values predicted by the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p < 0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals.

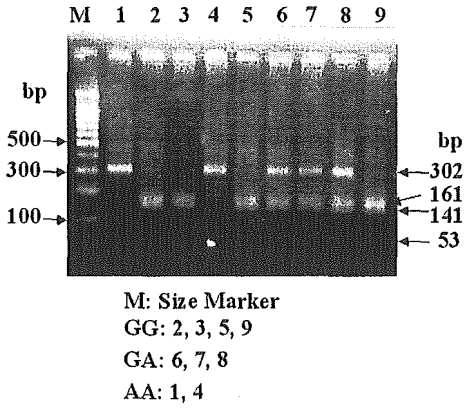

Fig. 1. Promoter polymorphism of FGF1. After amplification, PCR products were digested with *Hha*I and DNA was detected after electrophoresis on 2% agarose gels. Three genotypes of −1385 G/A (*Hha*I polymorphism) are shown: genotypes GG (lanes 2, 3, 5, and 9), GA (lanes 6–8), and AA (lanes 1 and 4).

Results

The PCR results were scored by two independent investigators who did not know whether each sample was from a case patient or a control. No intraobserver variability was found on repeated readings of the same gel, and the interobserver variability was less than 1%. All ambiguous samples were analyzed a second time.

The distribution of the three genotypes (GG, GA, and AA) reached Hardy–Weinberg equilibrium. The G allele was found in 75% of the 100 LOAD patients and 63% of the 106 control subjects. A significant association was observed between the −1385 G/A polymorphism and LOAD ($p < 0.03$; Table 1). We then examined the GG genotype as a risk factor for AD, considering the APOE status. As expected, APOEε4 conferred an increased risk for AD [odds ratio (OR) = 5.19]. OR in homozygotes for the G allele was 2.02 [95% confidence interval (CI) = 1.16–3.52]. However, the risk-increasing effect was smaller for −1385 G than for APOEε4 (Table 2). Four categories were defined by the presence (+) or absence (−) of a ε4 or GG genotype. The GG genotype alone showed an increased risk (95% CI: 1.81–7.69), and OR for APOEε4 and the GG genotype was 20.5 (95% CI: 6.88–60.9).

Discussion

To date, some polymorphisms of the FGF1 gene have been reported to associate with intracranial aneurysm [17]. However, functional role of the haplotype in its pathophysiology remains unclear. As the FGF1 gene contains alternative 5′-untranslated exons, the transcription is controlled by at least four distinct promoters in a tissue-specific manner [18–20]. Payson et al. [19] have reported that the sequence from −1614

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in promoter of FGF1

Group	Genotype (frequency)				Allele (frequency)	
n	AA	GA	GG	AA + GA	G	A
LOAD (100)	6 (0.06)	38 (0.38)	56 (0.56)*	44 (0.44)**	150 (0.75)	50 (0.25)***
Control (106)	14 (0.13)	51 (0.48)	41 (0.39)	65 (0.61)	133 (0.63)	79 (0.37)

LOAD, late-onset AD.
* $p < 0.03$.
** $p < 0.02$.
*** $p < 0.01$.

Table 2
Relative risk for interaction between APOEε4 and −1385 GG

		LOAD cases	Controls	Odds ratio	95% CI
	−1385 G/A				
	non-GG	44	65	Reference	
	GG	56	41	2.02	1.16–3.52
APOEε4					
−		52	90	Reference	
+		48	16	5.19	2.68–10.1
APOEε4	−1385 GG				
−	−	17	58	Reference	
−	+	35	32	3.73	1.81–7.69
+	−	18	11	5.58	2.21–14.1
+	+	30	5	20.5	6.88–60.9

APOEε4 (+), one or two copies of ε4: APOEε4 (−), no copies of ε4, 95% CI, confidence interval at 95% level.

to the FGF1 start site is sufficient to stimulate promoter activity. Therefore, it is reasonable to think that −1385 G/A polymorphism in the FGF1 promoter region can contribute the promoter activity. We performed an association study of the promoter polymorphism of the FGF1 gene.

We have evaluated definite LOAD as a relatively homogeneous case group. Our preliminary data suggest that the FGF1 gene, or a nearby gene, is an additional risk factor, independent of the APOE gene. Association studies often produce conflicting results. There are three possible reasons. First, this might be due to a type I statistical error, where there is a weak association between the polymorphism and the disease. Second, it might arise from the difference in genetic background between the American, French, Asian, and Japanese populations. In some studies, the AD group was made up of a mixture of familial and sporadic patients. We therefore tried to choose homogeneous subjects (autopsy-confirmed and late-onset AD) as much as possible. A third possibility could be linkage disequilibrium with other causative polymorphisms.

Patients with the GG genotype in this study had a higher risk of AD than those with the A allele. This indicates that the GG genotype in the promoter may influence the expression of FGF1 and could be involved in the selective vulnerability of neurons in AD. The results of this study support the hypothesis that FGF1 contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of FGF1 immunoreactivity may play an important role in the pathophysiological processes of AD [11,6,12]. This hypothesis should be further examined by functional analysis of FGF1 polymorphisms.

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Apolipoprotein A-I Induces Translocation of Cholesterol, Phospholipid, and Caveolin-1 to Cytosol in Rat Astrocytes*

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Intercellular cholesterol transport in the brain is carried by high density lipoprotein (HDL) generated *in situ* by cellular interaction with the apolipoprotein apoE, which is mainly synthesized by astrocytes, and with apoA-I secreted by cells such as endothelial cells. Rat astrocytes in fact generate HDL with extracellular apoA-I in addition to releasing HDL with endogenously synthesized apoE, seemingly by the same mechanism as the HDL assembly for systemic circulation. Relating to this reaction, apoA-I induced translocation of newly synthesized cholesterol and phospholipid to the cytosol prior to extracellular assembly of HDL, accompanied by an increase of caveolin-1 in the cytosol, activation of sterol regulatory element-binding protein, and enhancement of cholesterol synthesis. The lipid translocated into the cytosol was recovered in the fraction with a density of 1.09–1.16 g/ml as well as caveolin-1 and cyclophilin A. Cyclosporin A inhibited these apoA-I-mediated reactions and suppressed apoA-I-mediated cholesterol release. The findings suggest that such translocation of cholesterol and phospholipid into the cytosol is related to the apo A-I-mediated HDL assembly in astrocytes through functional association with caveolin-1 and a cyclosporin A-sensitive cyclophilin protein(s).

The central nervous system (CNS)[1] is sheltered from interaction with the lipoproteins of the systemic circulation by the blood-brain barrier. Therefore, extracellular cholesterol transport in the CNS is mediated by its own lipoprotein system, consisting mainly of the particles equivalent to plasma HDL (1). The main apolipoproteins are apoE produced by astrocytes (2), microglias (3), and apoA-I from an unknown source but reportedly secreted by brain endothelial cells (4). The astrocytes were shown to generate HDL, not only with endogenously synthesized apoE but also with exogenous apoE and apoA-I (5).

The apolipoprotein-cell interaction that generates HDL is common for many somatic cells from various origins and is distinct from the diffusion-mediated cholesterol efflux from the cell surface (6). The reaction is a main source of plasma lipoprotein (7) and is also one of the major pathways for cholesterol release from the cells (8). Generation of HDL with cellular phospholipid seems to require a cellular interaction site with apolipoprotein. An intracellular cholesterol trafficking system linked to such interaction is responsible for incorporation of cholesterol into the HDL (9, 10). Mutations in the ABCA1 transporter protein were identified in patients with plasma HDL deficiency who lack the ability to generate HDL by this reaction (11–13). Thus, the reaction depends on the cellular system to export materials. A specific intracellular cholesterol transport system is important to make the HDL cholesterol-rich. In macrophages and smooth muscle cells, protein kinase C was shown to be involved in this trafficking (9, 10).

It is not known whether the mechanism for generation of HDL in somatic cells is different from that for the HDL assembly in the CNS. There are, however, a few unique findings concerning HDL generation by CNS cells. We have reported in a previous study (5) that the cultured rat astrocytes produce cholesterol-poor and phosphatidylcholine-rich HDL with exogenous apoA-I, whereas the HDL formed with endogenous apoE is cholesterol-rich. Digestion of sphingomyelin with the extracellular enzyme enhances the exogenous apoA-I-mediated cholesterol release to make the HDL cholesterol-rich, but not the endogenous apoE-mediated pathway (14). This result indicated that the behavior of cholesterol molecules in the astrocytes upon HDL assembly with extracellular apolipoprotein is influenced by interaction with sphingomyelin. Thus, intracellular trafficking of cholesterol to link to the exogenous apoA-I-mediated cholesterol release and assembly of HDL might undergo the restriction by sphingomyelin in these cells.

The plasma membrane organizes unique cholesterol/sphingomyelin-rich domains called rafts or caveolae (15). Such domains have indeed been implicated as sites where cellular cholesterol might be leaving the cells (14, 16). Caveolin-1, a 22-kDa protein, is one of the major components of rafts/caveolae that holds a high affinity for cholesterol and sphingomyelin molecules (15, 17). This protein has been shown to be involved in intracellular cholesterol trafficking. It directly binds to cholesterol (18, 19) and induces caveolae domains and their cholesterol enrichment (20). It is also translocated between caveolae and the endoplasmic reticulum by cholesterol oxidation and its removal (21, 22). Caveolin-1 reportedly forms a chaperone complex with heat-shock protein and cholesterol in cytosol to transport newly synthesized cholesterol (19, 23). A more specific role for this protein was demonstrated in the cholesterol transport system to incorporate cholesterol molecules into the HDL generated by apolipoprotein-cell interaction (24, 25).

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[1] The abbreviations used are: CNS, central nervous system; HDL, high density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin; HSP, heat-shock protein; SREBP, sterol regulatory element-binding protein; DRM, detergent-resisting membrane; Cs-A, cyclosporin A.

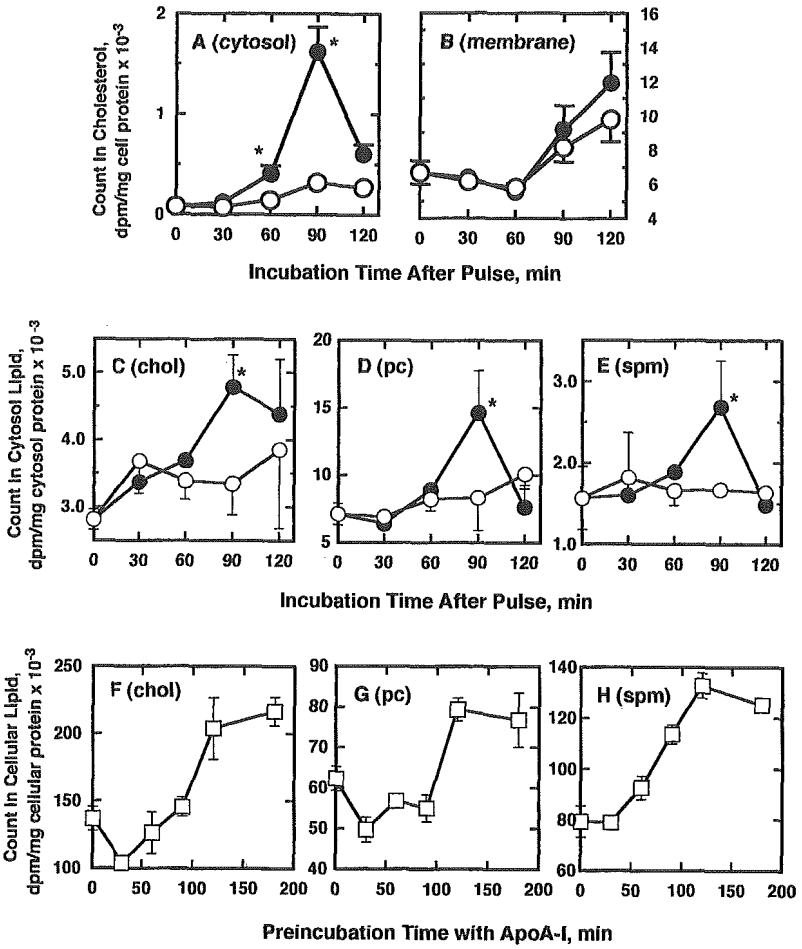

FIG. 1. **Synthesis and translocation to the cytosol of cholesterol and phospholipid in the rat astrocytes treated with apoA-I.** *A* and *B*, translocation of the lipid. Rat astrocytes were pulse-labeled for 30 min with 40 μCi of [^{3}H]acetate in 1 ml of F-10 containing 0.02% BSA for 30 min. After washing three times with buffer A (see "Experimental Procedures"), the cells were incubated with (*closed circles*) or without (*open circles*) 5 μg/ml apoA-I in 0.02% BSA/F-10 containing 1 mM sodium acetate for the indicated time. Lipid was extracted with the chloroform/methanol (2:1) or the hexane/isopropyl alcohol (3:2) solution from the cytosol fraction (*A*) or the membrane fraction (*B*), respectively. The radioactivity of cholesterol was determined after separation by TLC. *C–E*, rat astrocytes were pulse-labeled with 40 μCi/ml of [^{3}H]acetate for 120 min. The cells were incubated with (*closed circles*) or without (*open circles*) apoA-I (5 μg/ml) and were chased with 1 mM cold acetate for the indicated time. Lipid was extracted from the cytosol, and radioactivity was determined. *C* (*chol*), cholesterol; *D* (*pc*), phosphatidylcholine; *E* (*spm*), sphingomyelin. *F–H*, the rat astrocytes were pretreated with apoA-I (5 μg/ml) for the indicated time (0–180 min), washed, and incubated with 40 μCi/ml [^{3}H]acetate for 60 min. Lipid was extracted from the whole cells after washing four times. *F* (*chol*), cholesterol; *G* (*pc*), phosphatidylcholine; *H* (*spm*), sphingomyelin. The data represent the average ±S.E. of triplicate assays. *, $p < 0.01$ from the control by Student's *t* test.

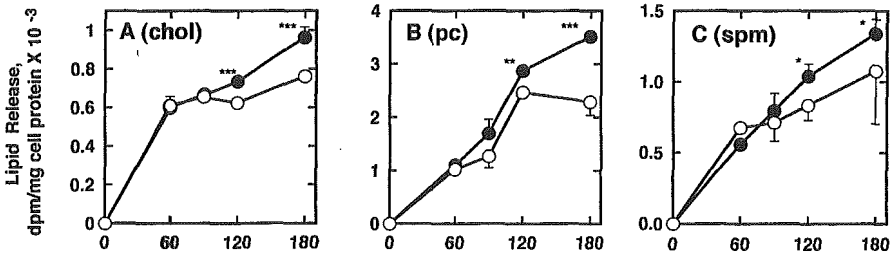

FIG. 2. **Release by apoA-I of newly synthesized lipid from rat astrocytes.** Rat astrocytes were pulse-labeled with 40 μCi/ml [^{3}H]acetate for 120 min. The cells were immediately washed, chased, and treated with (*closed circles*) or without (*open circles*) apoA-I (5 μg/ml) for the indicated time. Lipid was extracted from the conditioned medium after the removal of cell debris by centrifugation at 15,000 rpm for 1 h. Radioactivity was counted in cholesterol (*A*, *chol*), phosphatidylcholine (*B*, *pc*), and sphingomyelin (*C*, *spm*) after separation by TLC. Each data point represents the average ±S.E. of the triplicate samples. *, $p < 0.1$, **, $p < 0.05$, ***, $p < 0.01$, significantly different from the control by Student's *t* test.

Based on these findings, we investigated the mechanism by which caveolin-1 mediates cholesterol trafficking to the cellular surface stimulated by the apolipoprotein-cell interaction to generate HDL in rat astrocytes. We discovered that newly synthesized cholesterol was translocated to the cytosol fraction prior to its appearance in the generated HDL, apparently forming a complex with phospholipid accompanied by the increase of caveolin-1 in the cytosol.

EXPERIMENTAL PROCEDURES

Materials—ApoA-I was isolated from freshly prepared human HDL by delipidation and anion-exchange chromatography according to a previously described method (26). Astrocytes were prepared from the brain of day-17 Wistar rat fetus according to the method previously described (5, 27, 28). The cells were seeded and cultured in 10% fetal calf serum/F-10 medium for 1 week.

Radioisotope Labeling of Cellular Cholesterol and Phospholipid—The rat astrocytes at the confluent stage were pulse-labeled with [^{3}H]acetic acid (PerkinElmer Life Sciences) for 30 or 120 min and chased with a medium containing 1 mM cold acetic acid and 0.02% bovine serum albumin (BSA). Alternatively, cellular cholesterol was labeled by incubating with low-density lipoprotein containing [^{3}H]cholesteryl ester for 24 h as previously described (5).

Cytosol Preparation and Density Gradient Ultracentrifugation Analysis—The cytosol of rat astrocytes was prepared according to the method of Thom *et al.* (29). Briefly, the cells pretreated with or without apoA-I in F-10 medium containing 0.02% BSA were harvested with a rubber policeman after washing with 53.6 mM sodium phosphate-bicarbonate buffer, pH 7.4, containing 135 mM NaCl, 2.7 mM KCl, and 5.5 mM glucose (buffer A) four times. The cell pellet from centrifugation at 1,000 rpm for 10 min was treated with cold extract solution composed of 0.02 M boric acid, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine, pH 10.0, for 15 min with 25 agitations for 10 s every

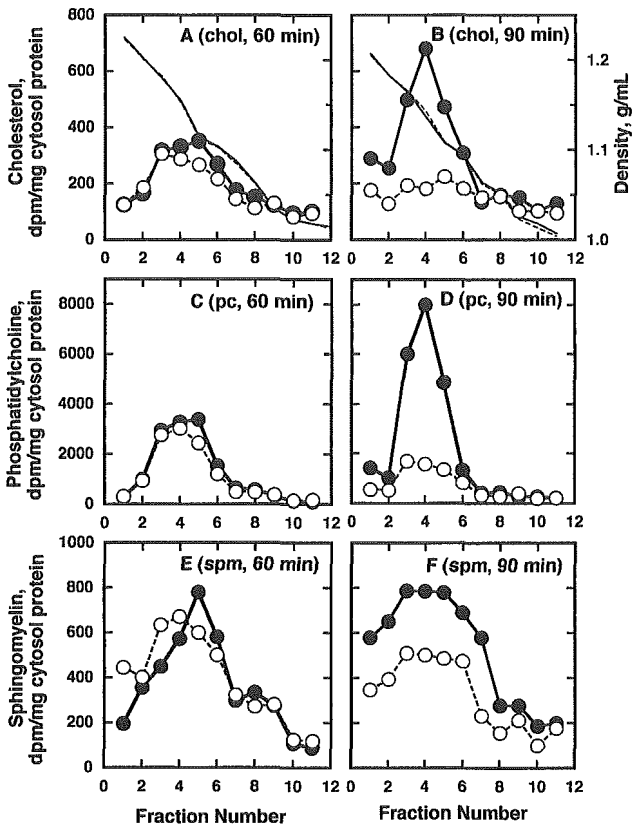

FIG. 3. **Density gradient analysis of the cytosol lipid of the rat astrocytes.** The rat astrocytes were labeled with 40 μCi/ml [^{3}H]acetate for 2 h. The cells were treated with (*closed circles, solid lines*) or without (*open circles, broken lines*) apoA-I (5 μg/ml) for 60 min (*A, C,* and *E*) or 90 min (*B, D,* and *F*) after washing and medium replacement with fresh 0.02% BSA/F-10. The cytosol fraction of each cell was centrifuged at 49,000 rpm for 48 h in sucrose density gradient between 1.20 g/ml and 1.07 g/ml using a Hitachi RP50T rotor. The sample was collected in 12 fractions from bottom to top in a centrifuge tube. Lipid was extracted from each fraction and separated by TLC. Radioactivity was determined for cholesterol (*chol*), phosphatidylcholine (*pc*), and sphingomyelin (*spm*).

5 min. The suspension was neutralized with 0.5 N HCl and was then centrifuged at 3,000 rpm for 10 min. After removal of nuclei and cell debris, the suspension was centrifuged at 300,000 × *g* for 30 min at 4 °C to obtain supernatant as the cytosol fraction. For standard analysis, the cytosol (9 ml) was overlayered on top of the sucrose solutions with a density of 1.20 g/ml (bottom, 8 ml) and of 1.07 g/ml (middle, 8 ml) and centrifuged at 49,000 rpm for 48 h at 4 °C by using a Hitachi RP50T rotor. The solution in the centrifuged tube was collected from the bottom as 12 fractions (8). Alternatively, the cytosol fraction (7 ml) was overlayered on the sucrose solution of 1.18 g/ml (18 ml) and centrifuged under the same conditions in order to achieve better separation in the fractions of higher density.

Analysis of Lipid—After cellular lipid was labeled, lipid was extracted from the whole cell with hexane/isopropyl alcohol (3:2, v/v) and from various cell fractions and culture medium with chloroform/methanol (2:1). Extraction efficiency of the lipid was estimated as 0.895 ± 0.015 and 0.926 ± 0.015 from the cell and its fractions and from the medium, respectively, by using internal standards. The extract was separated by thin layer chromatography (TLC), and radioactivity in each fraction, such as cholesterol, phosphatidylcholine, and sphingomyelin, was determined (5, 8). For mass assay, total and free cholesterol and triglyceride were directly determined by enzymatic assay methods. For choline phospholipid, the extracted lipid was separated by TLC with a developing solvent of chloroform/methanol/acetic acid/water (25:15:4:2) and was then enzymatically determined. Phosphatidylcholine (egg, Avanti Polar Lipids, 0–20 μg/spot) and sphingomyelin (bovine brain, Sigma-Aldrich, 0–10 μg/spot) were applied and separated on the same plate as the standards, for chromatography and quantitative assay. Fractions were scraped, and lipid was extracted from each sample with 2 ml of chloroform/methanol solution (2:1, v/v) five times for enzymatic assay of choline phospholipid (5, 8).

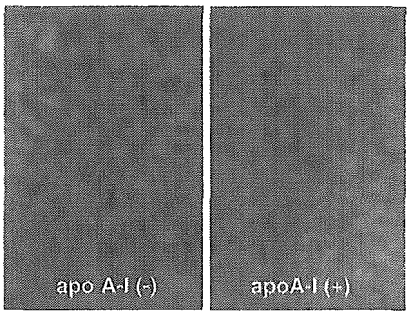

FIG. 4. **Negatively stained electron micrograph of the lipid fraction isolated from the cytosol.** The lipid fraction of the cytosol was prepared by two-step ultracentrifugation. The cytosol fraction prepared from the cells treated with (5 μg/ml) and without apoA-I for 90 min was overlaid on the sucrose solutions of density 1.20 g/ml (bottom) and 1.07 g/ml (middle) and centrifuged at 49,000 rpm for 48 h. The fraction of the density 1.09–1.16 g/ml was recovered, adjusted to 1.18 g/ml with sucrose, and centrifuged at 49,000 rpm for 48 h by using an RP55T rotor (Hitachi). The top fraction (500 μl) was recovered and dialyzed against 10 mM ammonium bicarbonate, pH 7.9 at 4 °C overnight with a formvar-coated grid in the solution. The specimen adsorbed on the grid was negatively stained with 0.5% uranium acetate and examined in a Hitachi 7100 electron microscope.

Immunoblot Analysis—For the analysis of cytosol, protein was precipitated with 10% trichloroacetic acid from the cytosol aliquot (700 μl) of rat astrocytes and analyzed by polyacrylamide gel electrophoresis in the presence of SDS. Protein was then transferred to polyvinylidene difluoride membrane (Bio-Rad) and visualized by an immunoblotting technique using rabbit anti-caveolin 1 (N-20) and goat anti-histone H1 polyclonal antibodies (Santa Cruz Biotechnology), rabbit anti-cyclophilin A antibody (Affinity BioReagents), mouse anti-EEA1, anti-GM130, anti-Bip/GRP78, anti-heat-shock proteins (HSP) 70, 90, and 110, and anti-flotillin 1 monoclonal antibodies (BD Transduction Laboratories). For analysis of activation of sterol regulatory element-binding proteins (SREBP), its increase in the nuclear extracts was detected as described (30). Briefly, the cell pellet by centrifugation at 1,000 rpm for 5 min was strongly agitated by vortexing for 10s times 25, repeating four times with a 5-min interval in between, in a cold extract solution of 0.02 M boric acid, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 m benzamidine, pH 10.0. The suspension was neutralized with 0.5 N HCl and centrifuged at 3,000 rpm for 15 min in order to pellet the crude nuclear fraction. Polyclonal antibodies were raised in rabbits against recombinant human SREBP2 (1–481) with a His tag (RS004) (30) and recombinant SREBP1 (1–487) with a His tag (RS005) and used for detecting the mature SREBPs in the nuclear fractions (30).

Detergent-resisting Membrane (DRM)—The plasma membrane fraction was treated in 1% Triton X-100, and the insoluble fraction was obtained as described previously (14). The sample solution was made to 2 ml in 30% sucrose, overlayered by 1.5 ml of Tris-buffered saline, and centrifuged at 90,000 rpm for 1 h in a Hitachi CS 120GX centrifuge using a S100AT6 rotor. Twelve fractions were collected from the bottom, and each fraction was analyzed for cholesterol content and protein by enzymatic measurement and for caveolin-1 and flotillin 1 by immunoblotting.

Negative-staining Electron Micrograph—The lipid fraction of the cytosol was dialyzed against 10 mM ammonium bicarbonate, pH 7.9 at 4 °C overnight with a formvar-coated grid in the solution. The specimen adsorbed on the grid was negatively stained with 0.5% uranium acetate and examined in a Hitachi 7100 electron microscope.

RESULTS

The cells were metabolically pulse-labeled by incubating with [^{3}H]acetate for 30 min or 120 min and then incubated with apoA-I. Radioactivities in cholesterol, phosphatidylcholine, and sphingomyelin were monitored in the cytosol and membrane fractions. After 30 min of pulse-labeling, the radioactivity profile of cholesterol in the cytosol fraction demonstrated that the transient increase has a peak at 90 min in the apoA-I-treated astrocytes (Fig. 1*A*). On the other hand, the increase of choles-

TABLE I
Lipid Composition of the Lipid-containing Cytosol Fraction

The fraction was isolated as the density 1.09–1.16 g/ml and then as a floating fraction in the density 1.18 g/ml from the cytosol of the astrocytes preincubated with and without apoA-I (5 μg/ml) for 90 min. Lipid was analyzed by using enzymatic assay systems. Total and free cholesterol, choline-phospholipid, and triglyceride were directly determined for the whole preparation. Cholesteryl ester was estimated by subtracting free cholesterol from total cholesterol. Lipid was extracted and separated by TLC, and choline-phospholipid was measured for the TLC fractions of phosphatidylcholine and sphingomyelin by referring to the internal standard. The values represent the average ± S.E. of the triplicate samples, standardized as μg of lipid per mg of original cytosol protein.

	PC[a]	SPM[b]	FC[c]	CE[d]	TG[e]
apoA-I(−)	4.93 ± 1.25	0.87 ± 0.22	0.70 ± 0.17	ND[f]	0.54 ± 0.07
apoA-I(+)	5.63 ± 0.43	0.93 ± 0.07	0.83 ± 0.09	ND	0.29 ± 0.08

[a] PC, phosphatidylcholine.
[b] SPM, sphingomyelin.
[c] FC, free cholesterol.
[d] CE, cholesteryl ester.
[e] TG, triglyceride.
[f] ND, not detected.

terol radioactivity in the membrane fraction started with a 90-min incubation and continued to 120 min or longer (Fig. 1*B*). The radioactivity profile of phosphatidylcholine and sphingomyelin in the cytosol of the apoA-I-stimulated cells also showed a peak at 90 min simultaneously with cholesterol when pulse-labeled for 120 min (Fig. 1, *C–E*). Biosyntheses of cholesterol, phosphatidylcholine, and sphingomyelin were monitored by incorporation of [^{3}H]acetate into each lipid in the cell for 120 min after preincubation with apoA-I for various periods of time. Synthesis was stimulated when the apoA-I treatment was longer than 90 min (Fig. 1, *F–H*). The release of labeled cholesterol, phosphatidylcholine, and sphingomyelin into the medium from pulse-labeled cells was increased when the apoA-I pretreatment was longer than 120 min (Fig. 2). These findings indicate that when the cells interact with apoA-I, translocation of cholesterol and phospholipid to the cytosol takes place prior to incorporation of these lipids into HDL and prior to the increase of lipid biosynthesis.

The cytosol was analyzed by density gradient ultracentrifugation to examine the status of lipid translocated to the cytosol by the apoA-I stimulation. When the cells pulse-labeled for 120 min were incubated with apoA-I, only a low lipid count was detected in fractions 3–6 at around 1.12 g/ml (at 60 min) without a significant difference with or without apoA-I. At 90 min, a substantial increase in the count was induced by apoA-I in cholesterol, phosphatidylcholine, and sphingomyelin at the same position of density (Fig. 3, *B, D,* and *F*). Fractions 3–6 with a cytosol density of 1.09–1.16 g/ml were collected and examined with an electron microscope using a negative-staining technique. As shown in Fig. 4, somewhat amorphous lipid particles were observed regardless of the incubation with and without apoA-I. The average diameters of the particles were 17.7 ± 7.0 nm and 17.1 ± 6.8 nm with and without apoA-I, respectively. This cytosol fraction was further analyzed for chemical composition of lipid (Table I). Major lipid components were phosphatidylcholine, sphingomyelin, and cholesterol, but a small amount of triglyceride was also detected. The results show that small lipid particles are present in the cytosol, regardless of the stimulation by apoA-I. Translocation of the newly synthesized lipid to these particles was induced by the reaction to extracellular apoA-I. The lipid particles were likely to be complexed with protein as judged by the density.

Fig. 5 demonstrates the increase of the activated SREBP1 and SREBP2 in the nuclear fraction by apoA-I. SREBP1 increased somewhat earlier than SREBP2. Translocation of newly synthesized cholesterol is thus associated with stimulation of cholesterol biosynthesis and other sterol-relating metabolic pathways via the SREBP-mediated mechanism.

The cytosol preparation was analyzed for protein in density gradient ultracentrifugation (Fig. 6). Regardless of the stimulation by apoA-I, caveolin-1 and cyclophilin A were recovered in the

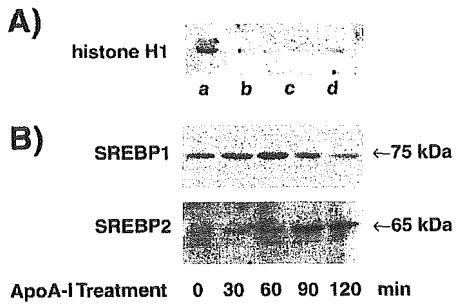

FIG. 5. **Activation of SREBP by apoA-I.** Astrocytes were incubated in fresh F-10 medium containing 0.02% BSA and 50 μM *N*-acetyl-Leu-Leu-norleucinal (Calbiochem) for 4 h. Then, the cells were incubated with 5 μg/ml apoA-I for the indicated periods of time, and the nuclei were prepared from each cell preparation. *Panel A*, histone H1 was estimated by immunoblotting in the nuclear fraction prepared; *a*, the nuclear fraction by 1000 × *g* for the 20-min pellet; *b*, the plasma membrane and inner membrane fraction by 300,000 × *g* for the 30-min pellet; *c*, the mitochondria fraction by 5,000 × *g* for the 30-min pellet; *d*, the cytosol fraction by 300,000 × *g* for the 30-min supernatant. Ten micrograms of protein were analyzed for each fraction. *Panel B*, the nuclear protein (20 μg, *fraction a* in *panel A*) of each cell preparation was analyzed by immunoblotting, using the antibodies against SREBP1 and SREBP2.

fractions with densities of 1.103–1.148 g/ml (Fig. 6*A*), equivalent to those of lipid (Fig. 3) and were separated from Bip/GRP78 (an endoplasmic reticulum marker protein). The increase of caveolin-1, but not cyclophilin A, was demonstrated in these fractions by stimulation of the cells with apoA-I (Fig. 6*A*). Cytosolic caveolin-1 (1.102–1.146 g/ml) was also separated from organelle membrane marker proteins EEA1 (an early endosome marker protein) and GM130 (a Golgi apparatus protein) (Fig. 6*B*). Heat-shock proteins, HSP 70, 90, and 110 were found in the fractions with higher density. A time-dependent increase of caveolin-1 in the cytosol was synchronized with the translocation of the lipids, reaching a maximum at 60–90 min, whereas cyclophilin A did not show a time-dependent change (Fig. 6*C*).

To examine whether the cytosol particles identified are distinct from the DRM fraction rich in caveolin-1, DRM and the cytosol particles were directly compared (Fig. 7). The density range of DRM was 1.02–1.10 g/ml where the main protein peak, cholesterol, caveolin-1, and flotillin (another DRM marker) were simultaneously recovered. The cytosol lipid particles were recovered at 1.10–1.16 g/ml, with caveolin-1 separated from the main cytosol protein peak (Fig. 7, *A* and *B*). The lipid particles in the cytosol were sensitive to Triton X-100, apparently with loss of their original integrated structure, suggesting that the particles were not derived from the DRM domain of the plasma membrane (Fig. 7*C*).

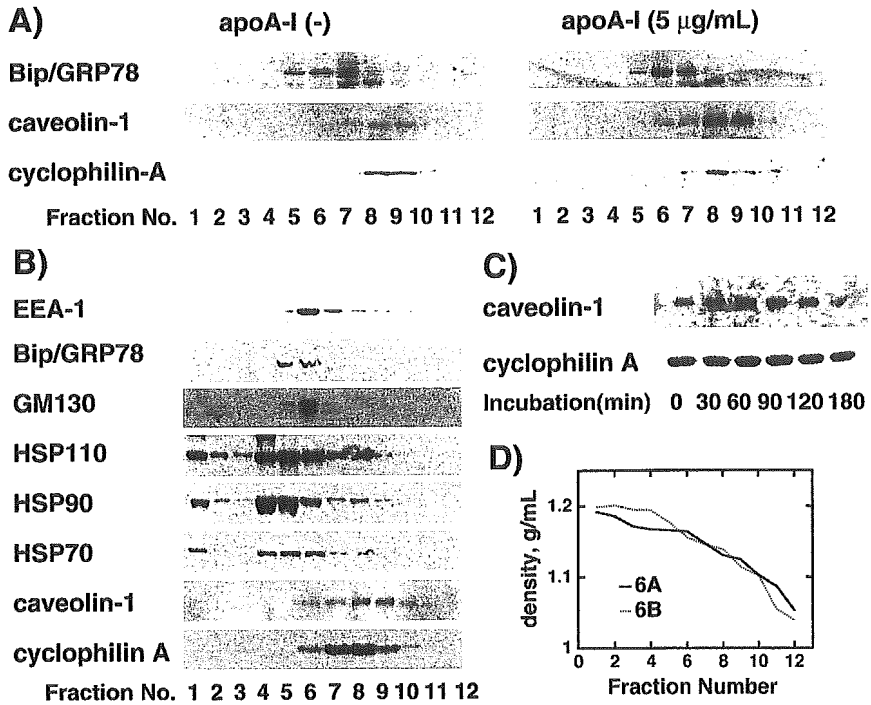

FIG. 6. **Increase of caveolin-1 in the cytosol by apoA-I.** *A*, density distribution of cytosol protein. Astrocytes were treated with (5 μg/ml) and without apoA-I for 90 min, and the cytosol fraction (0.7 mg of protein/7 ml) was collected and analyzed by density gradient ultracentrifugation by overlaying the 7-ml cytosol fraction on top of 18 ml of sucrose solution of density 1.18 g/ml. The samples were collected from the bottom for 12 fractions, and each fraction was analyzed by immunoblotting by using specific antibodies against rat caveolin-1 (N-20), cyclophilin A, and Bip/GRP78. *B*, density gradient analysis of the cytosol of the untreated cells. The cytosol was prepared (1.5 mg of protein/7 ml) and analyzed by the same density gradient ultracentrifugation. Each of the 12 fractions was analyzed for EEA1, Bip/GRP78, GM130, HSP110, HSP90, HSP70, caveolin-1, and cyclophilin A. *C*, time-dependent change in caveolin-1 in the cytosol. After incubation of the cells with 5 μg/ml of apoA-I for the indicated time, the cells were washed, and the cytosol was collected. The proteins (30 μg/lane) were analyzed by immunoblotting. *D*, density gradient profiles for the experiments shown in *A* and *B*.

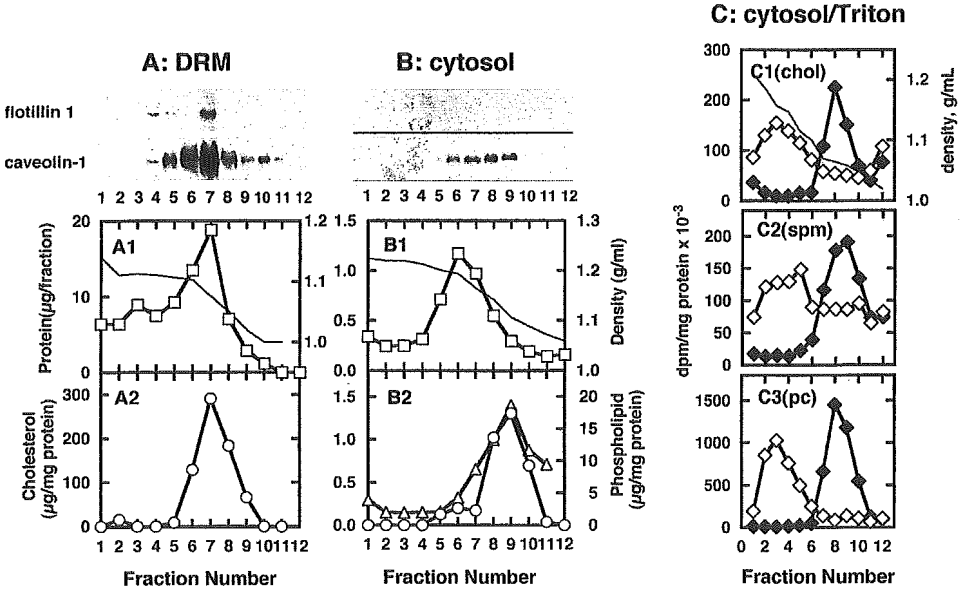

FIG. 7. **Density gradient ultracentrifugation analysis of the cytosol in comparison to DRM.** *A*, DRM from the astrocytes stimulated by apoA-I (5 μg/ml) for 90 min. After washing, DRM was prepared from the membrane fraction and analyzed by density gradient ultracentrifugation (see text) for caveolin-1 and flotillin 1 (a DRM marker), protein (*A1*), and cholesterol (*A2*). *B*, the cytosol from the astrocytes pretreated with apoA-I. Caveolin-1 and flotillin 1, protein (*B1*), cholesterol (*circles*) and phospholipid (*triangles*)(*B2*) were analyzed for each fraction by density gradient ultracentrifugation (see "Experimental Procedures"). *C*, detergent sensitivity of the lipid fraction of the cytosol. Rat astrocytes were incubated with 20 μCi/ml [^{3}H]acetate for 16 h. The cells were treated with apoA-I (5 μg/ml) for 90 min after washing. The cytosol fraction was prepared, neutralized with 0.5 N HCl, and incubated with (*closed diamonds*) or without (*open diamonds*) 1% Triton X-100 at 0 °C for 20 min. The samples were analyzed by density gradient ultracentrifugation, and 12 fractions were collected from bottom to top. The lipids were extracted from each fraction and analyzed by TLC.

When Cs-A, a ligand to cyclophilin proteins, was added to the cells, the increase of caveolin-1 in the cytosol by apoA-I was completely suppressed (Fig. 8*A*). The effects of Cs-A were also examined with regard to cholesterol synthesis, its translocation into the cytosol, and lipid release into the medium by apoA-I. As demonstrated in Fig. 8*B*, all of the intracellular reactions induced by apoA-I were completely inhibited by Cs-A, and the cholesterol release was substantially suppressed. The results thus suggested that the apoA-I-mediated intracellular events are seemingly related to cholesterol transport to the HDL assembly site.

DISCUSSION

The results in rat astrocytes are summarized in the following statements. 1) ApoA-I causes translocation of newly synthesized cholesterol and phospholipid to the cytosol to form the lipid-protein complex particles (density of 1.09–1.16 g/ml) prior to their appearance in the HDL assembled by apoA-I and to the increase of their biosynthesis. 2) ApoA-I also induces the in-

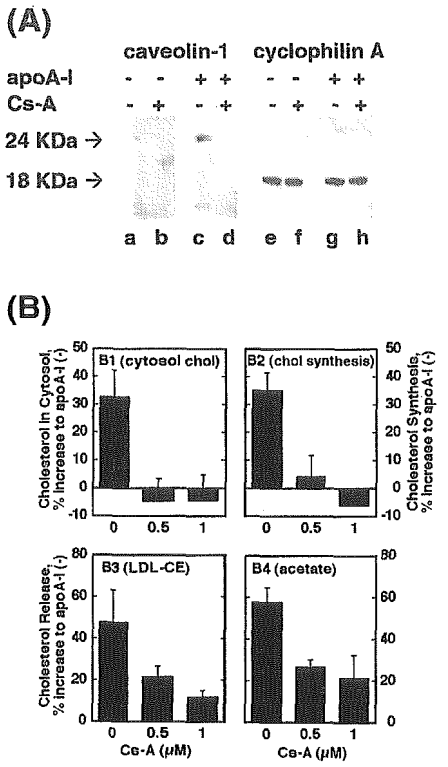

FIG. 8. **Effects of Cs-A on the apoA-I-mediated reactions in the cells.** *A*, caveolin-1 and cyclophilin A in the cytosol. Rat astrocytes were incubated with (5 μg/ml) and without apoA-I in the presence and absence of Cs-A (1 μM) for 90 min. The cytosol fraction was analyzed by immunoblotting for caveolin-1 and cyclophilin A. *B1*, translocation of cholesterol into the cytosol. Rat astrocytes were incubated with 40 μCi/ml [^{3}H]acetate for 2 h and incubated with and without apoA-I in the presence of Cs-A (0, 0.5, and 1 μM) for 90 min after washing. Lipid was extracted from the cytosol, and radioactivity in cholesterol was determined after separation by TLC. *B2*, cholesterol biosynthesis. The cells were incubated with apoA-I for 2 h in the presence of Cs-A (0, 0.5, or 1 μM). After washing, the cells were incubated with 40 μCi/ml [^{3}H]acetate for 1 h in the presence of Cs-A at the same concentration as the pretreatment. Lipid was extracted from the whole cell, and radioactivity in cholesterol was determined. *B3*, the apoA-I-mediated release of cholesterol originating in cholesteryl ester in low-density lipoprotein given to the cells. The cells were incubated with low-density lipoprotein containing [^{3}H]cholesteryl ester at 25 μg of protein/ml in 0.02% BSA/F-10 medium for 24 h and washed. The cells were pretreated with Cs-A (0, 0.5, and 1 μM) for 1 h and incubated with apoA-I for 7 h in the presence of Cs-A at the same concentration. Lipid was extracted from the medium, and radioactivity in cholesterol was determined. *B4*, release of newly synthesized cholesterol by apoA-I. The cells were labeled by incubating with 40 μCi/ml [^{3}H]acetate for 2 h, then treated with Cs-A (0, 0.5, and 1 μM) for 1 h in the presence of 1 mM acetate after washing. ApoA-I was added and incubated an additional 2 h. Radioactivity of cholesterol in the medium was determined. All of the results in *series B* were expressed as a percent increase of apoA-I with respect to each control without apoA-I. The data represent mean ±S.E. for triplicate samples.

crease of caveolin-1 in the cytosol that associates with lipid to form a density peak of 1.10–1.15 g/ml. 3) Cyclophilin A also associates with lipid in the cytosol found in the fractions with roughly similar density to caveolin-1, and its inhibitor Cs-A suppressed all of the apoA-I-mediated reactions including the enhancement of cholesterol biosynthesis, its translocation to the cytosol, and its incorporation into the generated HDL, as well as the appearance of caveolin-1 in the cytosol. The results are thus consistent with the hypothesis that apoA-I stimulates translocation of the newly synthesized lipids to the cytosol as an initial event in cholesterol trafficking for the assembly of HDL.

The translocated lipids to the cytosol appear to form particles having a density of the lipid-protein complex. Caveolin-1 and cyclophilin A also seem to associate with lipid in the cytosol, being distinct from the endosome and the microsome derived from the endoplasmic reticulum/Golgi apparatus. EEA1, GM130, and Bip/GRP78 were recovered in higher density fractions than the lipid-protein complex particles as well as the HSPs (Fig. 6). It is also obvious that the particles are not derived from the DRM domain or caveolae because of their sensitivity to Triton X-100 (Fig. 7). Biosynthesis of cholesterol and phospholipids appears to be enhanced after this translocation, presumably triggered by the decrease of cholesterol in the SREBP-sensitive compartment. The labeled lipids appear in the HDL extracellularly assembled by apoA-I after their translocation into the cytosol.

Because caveolin-1 has been proposed to mediate intracellular cholesterol transport to the cellular surface, which is linked to cholesterol release by apoA-I, it is conceivable that this particle may function to carry cholesterol and other lipids to the cell surface site where HDL assembly takes place. It has been reported that the movement of newly synthesized cholesterol to the plasma membrane is accompanied by the formation of the protein complex composed of caveolin-1, cyclophilin A, cyclophilin 40, and heat-shock protein 56 in the cytosol of NIH 3T3 cells and also lymphoid cell line L1210-JF transfected by caveolin-1 (23). In the case of rat astrocytes, however, cyclophilin 40 was recovered in the membrane fraction and the higher density protein fraction of the cytosol, but not in the lipid-caveolin-1 fraction.[2] Whereas such a proposed protein complex reportedly contains only cholesterol as a lipid component, the lipid-protein complexed particles identified in the rat astrocyte cytosol in this study contained cholesterol, phosphatidylcholine, and sphingomyelin. ApoA-I induced incorporation of newly synthesized cholesterol and phospholipid into these particles. Although both caveolin-1 and cyclophilin A seem to be associated with these particles in a density gradient analysis, an anti-caveolin-1 antibody failed to coprecipitate cyclophilin A with caveolin 1 from the rat astrocytes cytosol.[2]

Intracellular cholesterol distribution has been shown to be greatly dependent on the function of caveolin-1 and caveolae. Expression of caveolin-1 is closely related to cellular cholesterol levels (24, 31). Exposure of human fibroblasts to cholesterol oxidase rapidly allows caveolin-1 to redistribute from the plasma membrane to the endoplasmic reticulum and then to the Golgi complex (21). The newly synthesized cholesterol was first moved from the endoplasmic reticulum to caveolae linked to the caveolin-1 movement and then to the noncaveolae domain of the plasma membrane (32). Cholesterol transport from the endoplasmic reticulum to the plasma membrane is greatly enhanced in caveolin-1-transfected lymphocytes (23). Down-regulation of caveolin-1 reduced the incorporation of cholesterol into HDL assembled by the cell-apoA-I interaction (24, 25). Thus, it may not be irrelevant to conceive that the cytosolic caveolin-lipid complex in rat astrocytes may function as a vehicle for transport of cholesterol and phospholipid from the endoplasmic reticulum to the DRM domain of the plasma membrane.

ABCA1 plays a key role in the apolipoprotein-mediated HDL assembly (11–13), but an exact mechanism must still be determined. Physical interaction of apolipoprotein with the cell surface seems to require ABCA1 (33, 34), and the reaction primarily mediates assembly of HDL with cellular phospholipid (25, 35, 36). Cholesterol enrichment in the HDL, thus generated, is regulated in a relatively independent manner from the assembly of HDL particles (9), and caveolin-1 seems involved in this step (25). This part is also dependent on sphingomyelin-choles-

[2] J. Ito and S. Yokoyama, unpublished data.

terol interaction in DRM in astrocytes (14) so that intracellular cholesterol transport by caveolin-1 may enrich this domain with cholesterol to lead to its incorporation into the HDL. More studies are required to prove this hypothesis.

Anderson and co-workers (37) have reported that exocrine cells that synthesize both apoA-I and apoE secreted caveolin-1 along with these apolipoproteins as a lipoprotein particle. They have suggested that caveolin-1 with apolipoproteins are present as a lipoprotein in the endoplasmic reticulum/Golgi compartment and are transported in the secretory pathway. In contrast, neither caveolin-1 was detected in the HDL fraction in the medium of the rat astrocytes pretreated with apoA-I nor was endogenous apoE in the caveolin-lipid complex of the cytosol fraction of the rat astrocytes. Therefore, cholesterol trafficking for its release in the HDL assembly reaction with extracellular apolipoprotein seems essentially different from what was observed in the exocrine cells. The absence of apoE in the cytosolic fraction excludes the possibility that the HDL particles in the secretory pathway, if any, contaminated the isolated fraction. More recently, the same group demonstrated the presence of caveolin-1 and cyclophilin A in an HDL density fraction of the cytosol of human fibroblasts (38), so the present findings may not be specific for astrocytes but rather more generally applicable for the HDL assembly system by apolipoprotein and cellular lipid.

The results of inhibition of the apoA-I-mediated cholesterol release and other cellular reactions by Cs-A support the hypothesis that the translocation of lipid and caveolin-1 to the cytosol is associated with lipid release and HDL assembly by apoA-I. The results also suggest that the reactions are dependent on the function of cyclophilin proteins.

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An acidic fibroblast growth factor-like factor secreted into the brain cell culture medium upregulates apoE synthesis, HDL secretion and cholesterol metabolism in rat astrocytes

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Abstract

Production and release of apolipoprotein (apo) E and cholesterol were highly upregulated in the astrocytes prepared by 1-week secondary culture after 1-month primary culture of rat fetal brain cells (M/W cells) in comparison to the cells prepared by a conventional method of 1-week primary and 1-week secondary culture (W/W cells). Both cell preparations were mostly composed of astrocytes with small population of other glial cells, except that type-2 astrocyte-like cells accounted for 5–15% of M/W cells indicating more activated and/or matured status. The conditioned medium of the 1-month primary culture stimulated W/W cells to increase the release of apoE and cholesterol into the medium. The treatment of W/W cells by acidic fibroblast growth factor (aFGF) similarly upregulated biosyntheses and release of apoE and cholesterol. The effect of the conditioned medium was completely inhibited by pretreatment with an anti-aFGF antibody. The increase of the aFGF message was demonstrated in the brain cells after 1-month primary culture. The findings suggested that an aFGF-like trophic factor upregulates biosynthesis and secretion of apoE-high density lipoprotein (HDL) in astrocytes probably by autocrine stimulation in this culture system. Since this cytokine is highly expressed in the development or post-injury period of the brain, it putatively activates intercellular cholesterol transport to support construction or recovery of the brain.

Keywords: HDL; ApoE; aFGF; Astrocyte; Cholesterol; Brain

1. Introduction

More than 25% of total body cholesterol in human is found in the central nervous system (CNS) and it accounts for 10% of the brain dry mass. While extracellular cholesterol transport is generally carried by the plasma lipoprotein system as an important part of cholesterol homeostasis in mammalian bodies, the cells in the CNS are segregated from the lipoproteins in the systemic circulation by the blood brain barrier and use their own lipoprotein system distinct from plasma lipoproteins. Lipoproteins found in cerebrospinal fluid are all high density lipoproteins (HDL) that contain apolipoprotein(apo) E, A-I, A-IV or J [1], and are believed to compose a specific system for regulation of cholesterol homeostasis in the CNS.

Apo E, a glycosylated 34-kDa apolipoprotein, is a major apolipoprotein in cerebrospinal fluid. It is synthesized and secreted by astrocytes and microglias being complexed with phospholipid and cholesterol as HDL particle [2–6]. Synthesis of apoE by astrocytes largely depends on the stage of cellular differentiation and many astrocytoma cells do not synthesize apoE [7,8]. However, the mechanism is unknown for assembly and secretion of HDL particles by astrocytes with endogenously synthesized apoE, and an exact function of such apoE-HDL is unclear in cholesterol homeostasis among glias and neurons in the CNS. We have reported that the astrocytes secrete apoE along with cellular cholesterol and phospholipid to generate cholesterol-rich HDL while

Abbreviations: CNS, central nervous system; HDL, high density lipoprotein; Apo, apolipoprotein; LDL, low density lipoprotein; aFGF, acidic fibroblast growth factor; FCS, fetal calf serum; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; HMG-CoA, hydroxymethylglutaryl-CoA; TLC, thin layer chromatography; SDS-PAGE, polyacrylamide gel electrophoresis; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcription-polymerase chain reaction

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