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

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

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

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

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

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

Manuscript received 14 June 2004 and in revised form 20 August 2004.

Published, JLR Papers in Press, September 17, 2004.
DOI 10.1194/jlr.M400222-JLR200

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

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

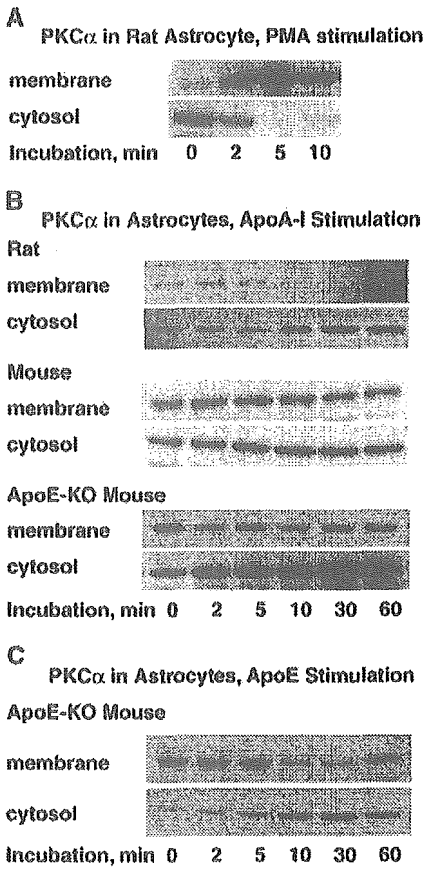

Fig. 1. Redistribution by apolipoprotein A-I (apoA-I) of protein kinase Cα (PKCα) in astrocytes. A: Rat astrocytes were treated with 200 nM phorbol 12-myristate 13-acetate (PMA). The membrane fraction protein (15 μg/lane) and the cytosol protein (50 μg/lane) were analyzed for protein kinase Cα by immunoblotting.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

MATERIALS AND METHODS

Materials

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

Cell culture

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

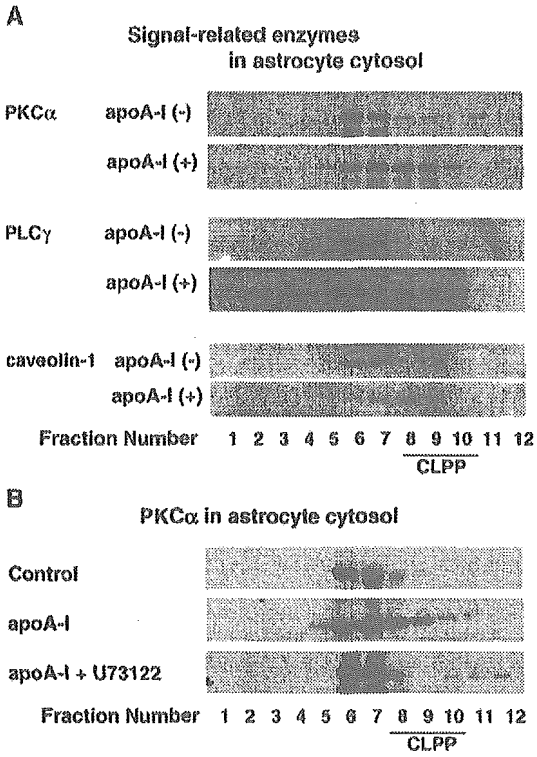

Fig. 2. Redistribution by apoA-I of protein kinase Cα (PKCα) and phospholipase Cγ (PLCγ) in cytosol of mouse astrocytes. A: After washing and medium replacement with 0.02% BSA/DMEM, apoE-KO mouse astrocytes were incubated with or without apoA-I (5 μg/ml) for 5 min. The cytosol (350 μg protein/7 ml) was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

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

Cytosol preparation and density gradient ultracentrifugation analysis

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

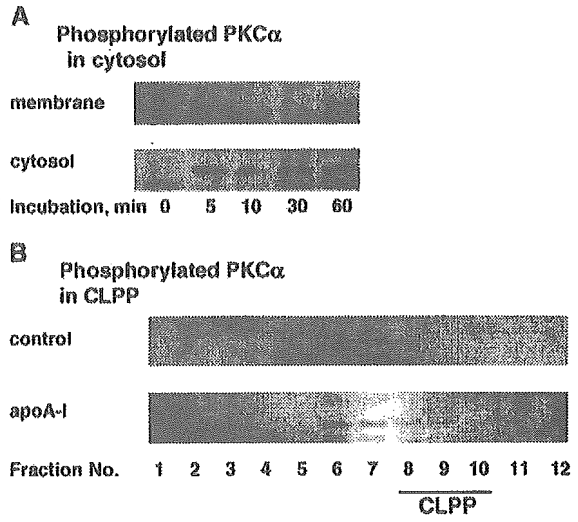

Fig. 3. Phosphorylation of protein kinase Cα (PKCα) in apoA-I-stimulated mouse astrocytes. A: The cytosol and membrane fractions were prepared from mouse astrocytes pretreated with 5 μg/ml apoA-I for the indicated periods of time in fresh 0.02% BSA/DMEM. Each sample was analyzed by SDS-PAGE (40 μg/lane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

Caveolae/rafts preparation from the membrane fraction

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

Western blotting

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

De novo syntheses and release of lipid

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

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

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

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

RESULTS

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

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

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

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

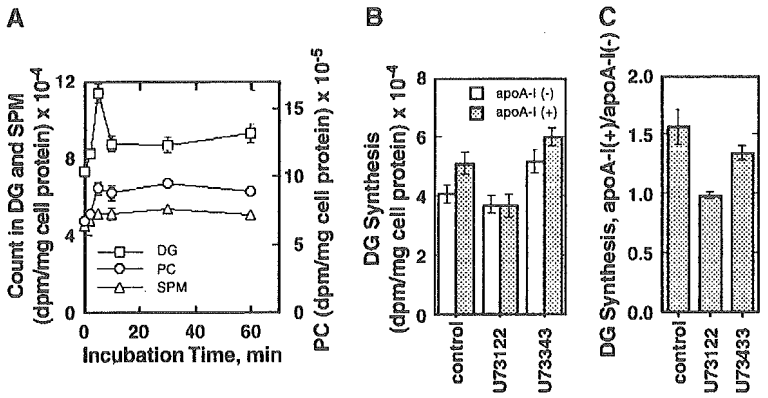

Fig. 4. Increase of diacylglyceride (DG) production by apoA-I and the effect of U73122 in mouse astrocytes. A: Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of DMEM medium containing 0.02% BSA (0.02% BSA/DMEM). After three complete washes with Dulbecco's phosphate-buffered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

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

DISCUSSION

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

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

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

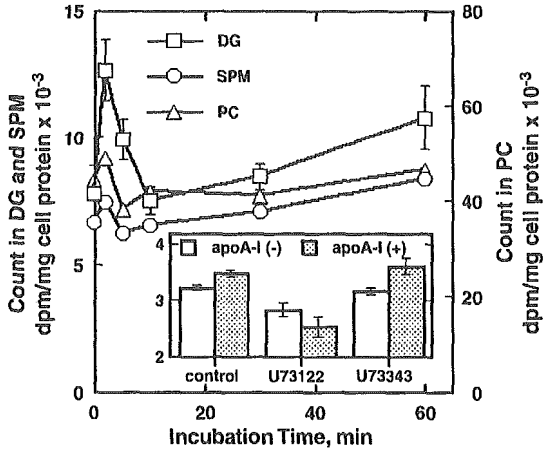

Fig. 5. Increase of DG production by apoA-I and the effect of U73122 on DG production in WI-38 cells. WI-38 cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM. The cells were incubated with apoA-I (5 μg/ml) for 0, 2, 5, 10, 30, and 60 min as described for Fig. 4A.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

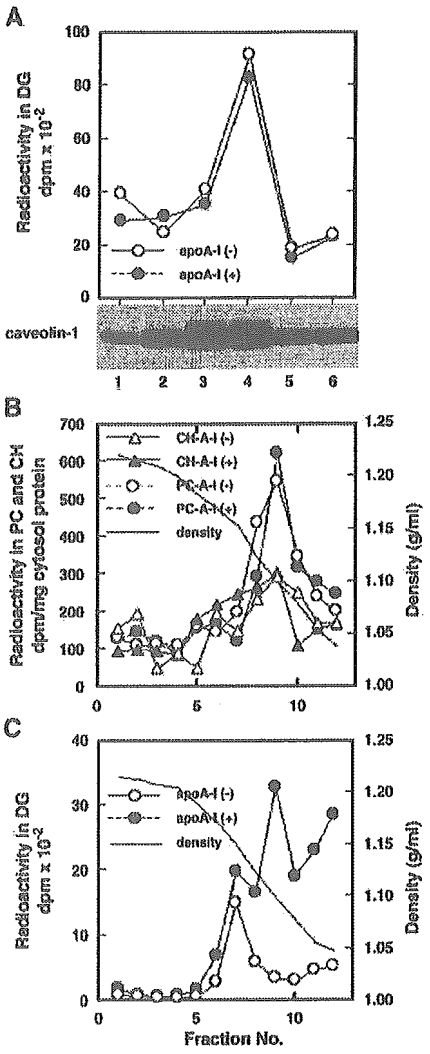

Fig. 6. Increase of DG by apoA-I in the cytosol of astrocytes. Mouse astrocytes were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM and then treated with (closed symbols) or without (open symbols) 5 μg/ml apoA-I for 5 min after washing and medium replacement with fresh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

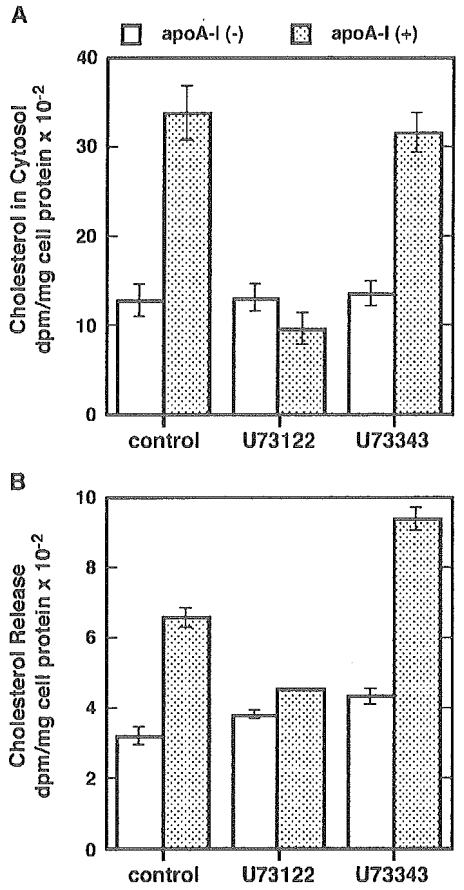

Fig. 7. Effects of U73122 on cholesterol trafficking in mouse astrocytes. A: The cells were pulse-labeled for 3 h with 20 μCi of [^{3}H]acetate in 1 ml of 0.02% BSA/DMEM followed by washing and medium replacement with fresh 0.02% BSA/DMEM containing 1 mM sodium acetate. The cells were treated with (dotted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

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

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

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

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

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

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare and the Ministry of Education, Science, Culture, and Sports and by an International HDL Research Award.

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

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

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

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

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

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

Manuscript received 12 October 2004.

Published, JLR Papers in Press, November 1, 2004.
DOI 10.1194/jlr.M400402-JLR200

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

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

MATERIALS AND METHODS

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

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

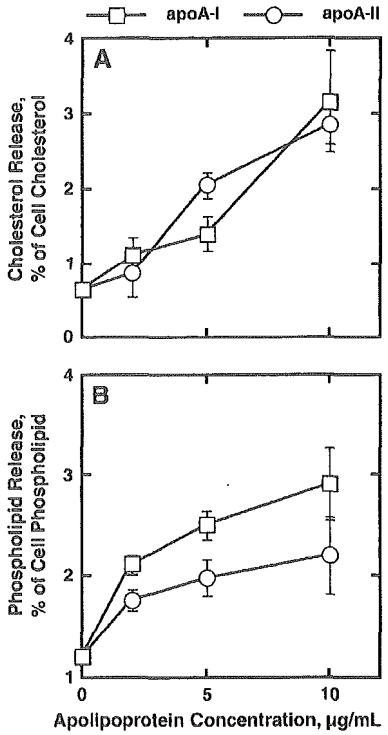

Fig. 1. Apolipoprotein-mediated lipid release from HepG2 cell. Cells were incubated for 16 h with minimum essential medium Eagle α modification (MEM-α) containing 0.02% BSA and the indicated concentration of apolipoprotein A-I (apoA-I) or apoA-II. Cellular lipid released into the conditioned medium was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

HepG2 cells

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

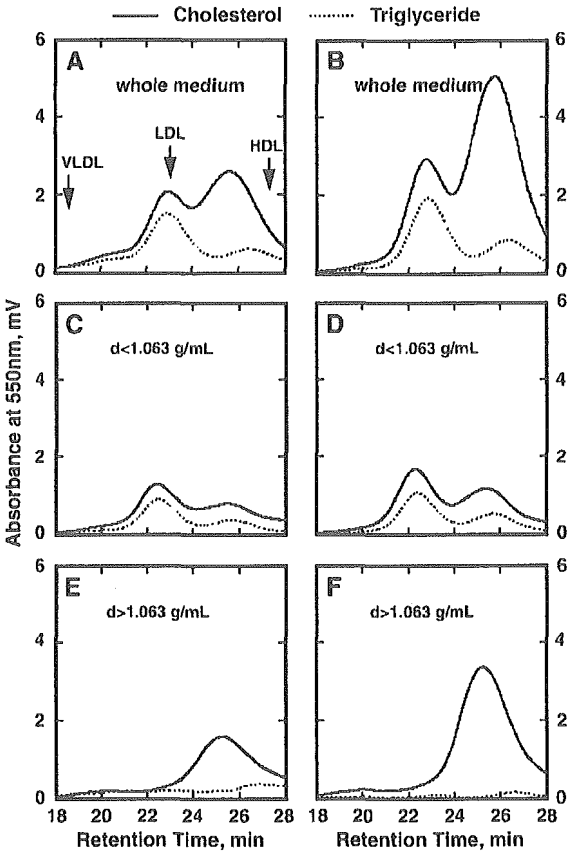

Fig. 2. Lipoprotein analysis of the culture medium of HepG2 cells by HPLC. Cells were incubated with MEM-α containing 0.02% BSA with (B, D, F) or without (A, C, E) 10 μg/ml human apoA-I for 16 h. The conditioned medium of HepG2 cells (100 μl) was analyzed by the HPLC lipoprotein analysis system using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

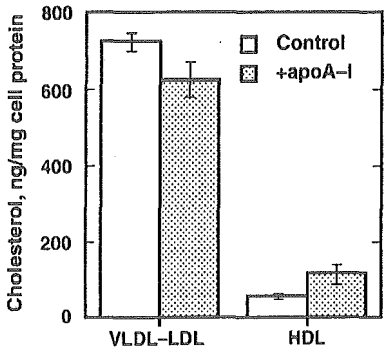

Fig. 3. Analysis of lipoprotein generated by mouse primary culture hepatocytes. Primary culture hepatocytes of C57Bl/6 mice were harvested using the Hanks'-EDTA and collagenase two-medium method described in Materials and Methods. The cells were incubated in DMEM (high glucose)/BSA medium with or without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

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

Mouse primary hepatocytes

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

Lipoprotein analysis in the conditioned medium

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

Apolipoprotein analysis

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

TABLE 1. Chemical compositions of cellular lipids in HepG2 cells

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

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

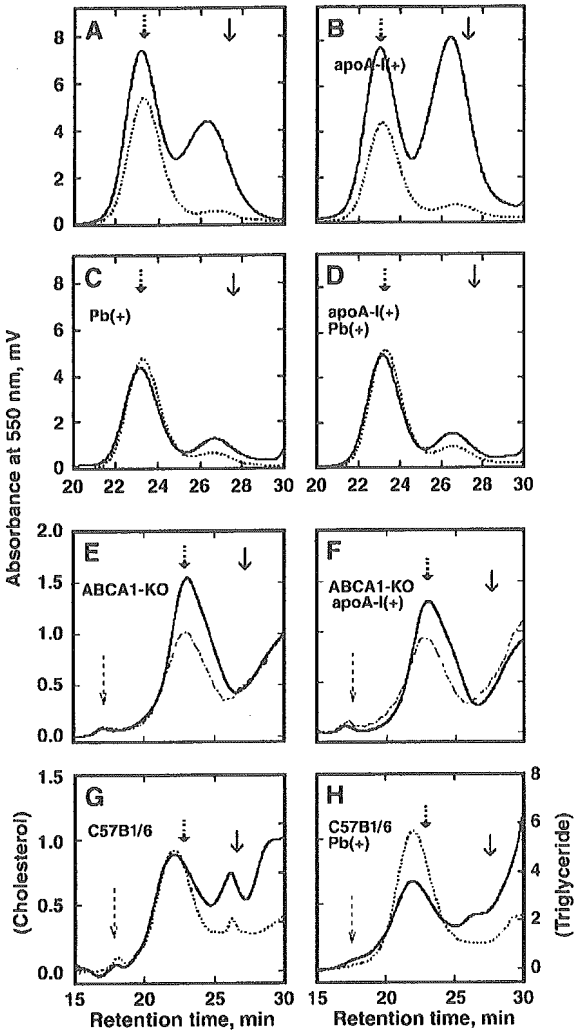

Fig. 4. The effect of ABCA1 inactivation on the generation of HDL by hepatocytes as analyzed by HPLC. A–D: HepG2 cells. A: The conditioned medium of HepG2 cells after 16 h. B: The conditioned medium incubated with apoA-I (10 μg/ml) for 16 h. C: The conditioned medium of probucol-loaded HepG2 cells after 16 h. D: The conditioned medium of probucol-loaded HepG2 cells with apoA-I (10 μg/ml) for 16 h. E–F: Primary cultured hepatocytes. E: The conditioned medium of the hepatocytes isolated from an ABCA1-deficient mouse after 16 h of incubation. F: The conditioned medium of the hepatocytes of an ABCA1-deficient mouse after incubation with apoA-I (10 μg/ml) for 16 h. G: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with control chow after 16 h of incubation. H: The conditioned medium of the hepatocytes obtained from C57Bl/6 mice fed with 0.5% probucol-containing chow. The medium was analyzed by HPLC using two tandem gel-permeation columns (Lipopropak XL; 7.8 mm × 300 mm; Tosoh) to yield the lipoprotein profiles as described in the text. Solid lines represent cholesterol, dotted lines represent triglyceride, and dot-and-broken lines (in E and F) represent choline-phospholipid. Arrows with a solid line, a thick broken line, and a thin broken line indicate the eluting positions of plasma HDL, LDL, and VLDL, respectively. Pseudopeaks generated by the color of the medium caused baseline shifts in the analysis of the primary culture medium as the sensitivity of the monitoring lipid was increased. KO, knockout.

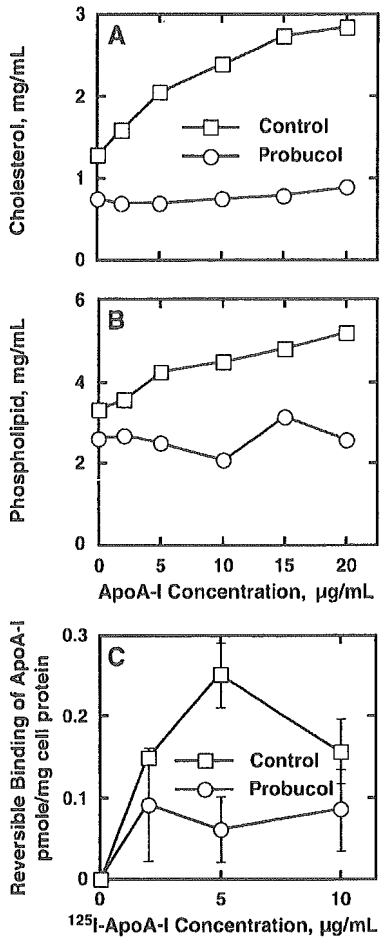

Fig. 5. Effect of probucol loading on apoA-I-mediated cellular lipid release from HepG2 cells and exchangeable binding apoA-I to HepG2 cells. A and B: Lipid release. HepG2 cells were incubated with either control LDL or probucol-containing LDL for 24 h. Cellular cholesterol and phospholipid release were measured after incubation for 16 h with various amounts of apoA-I. HDL-cholesterol (A) and HDL-phospholipid (B) were determined by an HPLC system. Open squares, control LDL-loaded cells; open circles, probucol-LDL loaded cells. C: ApoA-I binding. The indicated amounts of ^{125}I-apoA-I-incubated HepG2 cells were preloaded with control LDL or probucol-containing LDL for 2 h at 4°C. The cells were washed two times with ice-cold PBS, and the bound ^{125}I-apoA-I was displaced two times by 50 μg/ml nonlabeled apoA-I for 9 h. The difference in the total radioactivity after the first washing and after the second displacement was calculated as displaceable or exchangeable binding of apoA-I to the cells. Squares, binding to the cells loaded with control LDL; circles, binding to the cells loaded with probucol-containing LDL. Error bars indicate ± SEM.

of acetone and dissolved in 10 μl of sample buffer for SDS-polyacrylamide electrophoresis, incubated at 100°C for 5 min, and 5 μl of 9 M urea was added. The electrophoresis was performed on a polyacrylamide gel with a gradient of 4–20%. The conditioned media and fractionated media by ultracentrifugation were directly analyzed by immunoblotting against apoA-I. Anti-human albumin IgG fraction of rabbit antiserum was purchased from Sigma-Aldrich Inc. and used as a secondary antibody. Blot-

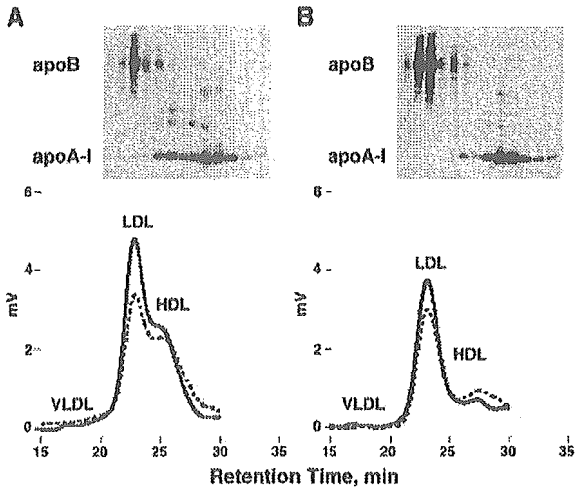

Fig. 6. Analysis of apolipoproteins in the HPLC fractions of the HepG2 conditioned medium. The conditioned medium of HepG2 cells (A) and probucol-loaded HepG2 cells (B) was tested after 16 h of incubation. The medium was analyzed by HPLC and fractionated as described in the text. The upper panels show immunoblot analysis of the fractions for apoB and apoA-I, and the lower panels represent HPLC elution profiles of lipids (solid lines, cholesterol; dotted lines, choline-phospholipid). Positions of lanes in the immunoblot analysis are adjusted for the HPLC elution times. VLDL, LDL, and HDL indicate the eluting positions for plasma lipoproteins.

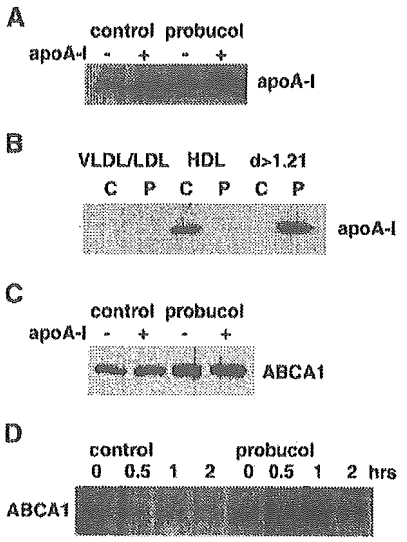

Fig. 7. Immunoblot analysis of apoA-I and ABCA1. A: ApoA-I in the conditioned medium of HepG2 cells was analyzed after preincubation with control LDL and probucol-containing LDL and subsequent incubation in the presence and absence of 10 μg/ml apoA-I for 16 h. B: Density distribution of apoA-I in the medium. ApoA-I was analyzed in density subfractions of the conditioned medium of HepG2 cells preincubated with 50 μg/ml control LDL (C) or probucol-containing LDL (P) for 24 h and then incubated for 16 h. The medium was fractionated by sequential ultracentrifugation for the VLDL/LDL fraction ($d < 1.063$ g/ml), the HDL fraction ($1.063 < d < 1.21$ g/ml), and the bottom fraction ($d > 1.21$ g/ml). Each fraction, corresponding to 10 μl of the original medium, was dialyzed and analyzed for apoA-I. C: ABCA1 in the plasma membrane of HepG2 cells prepared as described for A. D: Clearance of ABCA1 in HepG2 cells. The cells were preloaded with control or probucol-containing LDL, and ABCA1 was examined after incubation with 71 μM cycloheximide.

ted bands were visualized by the chemiluminescence method (ECL Western Blotting Detection System; Amersham Pharmacia Biotech).

Clearance rate of ABCA1

The clearance rate of ABCA1 was analyzed by immunoblotting of the protein in the bulk cellular membrane fraction (21). HepG2 cells were loaded with control and probucol-containing LDL for 2 h and incubated in the presence of 71 μM cycloheximide to inhibit the synthesis of new protein. The cells were harvested and applied for ABCA1 immunoblotting using a specific antibody raised against a C-terminal peptide of human ABCA1 (21).

Other methods

Cellular lipid was analyzed as described previously (20). The sample was also used for probucol analysis using an HPLC system (22). Protein was measured with the bicinchoninic acid method using BSA as a standard.

RESULTS

Apolipoprotein-induced cellular lipid release from HepG2 cells is shown in **Fig. 1**. There was a significant spontaneous release of cholesterol and choline-phospholipid by HepG2 cells (indicated at zero concentration of apolipoproteins). Both apoA-I and apoA-II, when exogenously added to the culture medium, induced further release of the lipids in a dose-dependent manner. The medium was analyzed for lipoprotein particles using gel-permeation HPLC (**Fig. 2**). Two major peaks were identified corresponding to LDL and HDL sizes by human plasma reference, whereas no significant peak was detected at the position of VLDL (Fig. 2A). The peaks were indeed verified to correspond to density classification of lipoprotein as fractions $d < 1.063$ and $d > 1.063$ (Fig. 2C, E). The conditioned medium incubated with 10 μg/ml human apoA-I showed an increase of the HDL peak (Fig. 2B, D, F). Thus, increase of the release of cholesterol and phospholipid by apolipoprotein was shown to be attributable to the additional production of the HDL-size particles. Generation of lipoprotein was also examined for primary cultured mouse hepatocytes (**Fig. 3**). The cells were incubated with and without exogenous 10 μg/ml human apoA-I for 24 h, and the conditioned medium was fractionated by ultracentrifugation. Cholesterol content in the HDL fraction ($d > 1.063$) was increased by 2-fold, and the VLDL-LDL fraction ($d < 1.063$) was insignificantly decreased. These results indicated that the system is functional both in HepG2 cells and in mouse hepatocytes for generation of the HDL-size lipoprotein by helical apolipoprotein.

To examine the contribution of ABCA1 to this system, the effect of an inactivator of ABCA1, probucol, was analyzed in those cells. Probucol was loaded to HepG2 cells, and the culture medium was analyzed by HPLC after 16 h of incubation with and without 10 μg/ml apoA-I. The

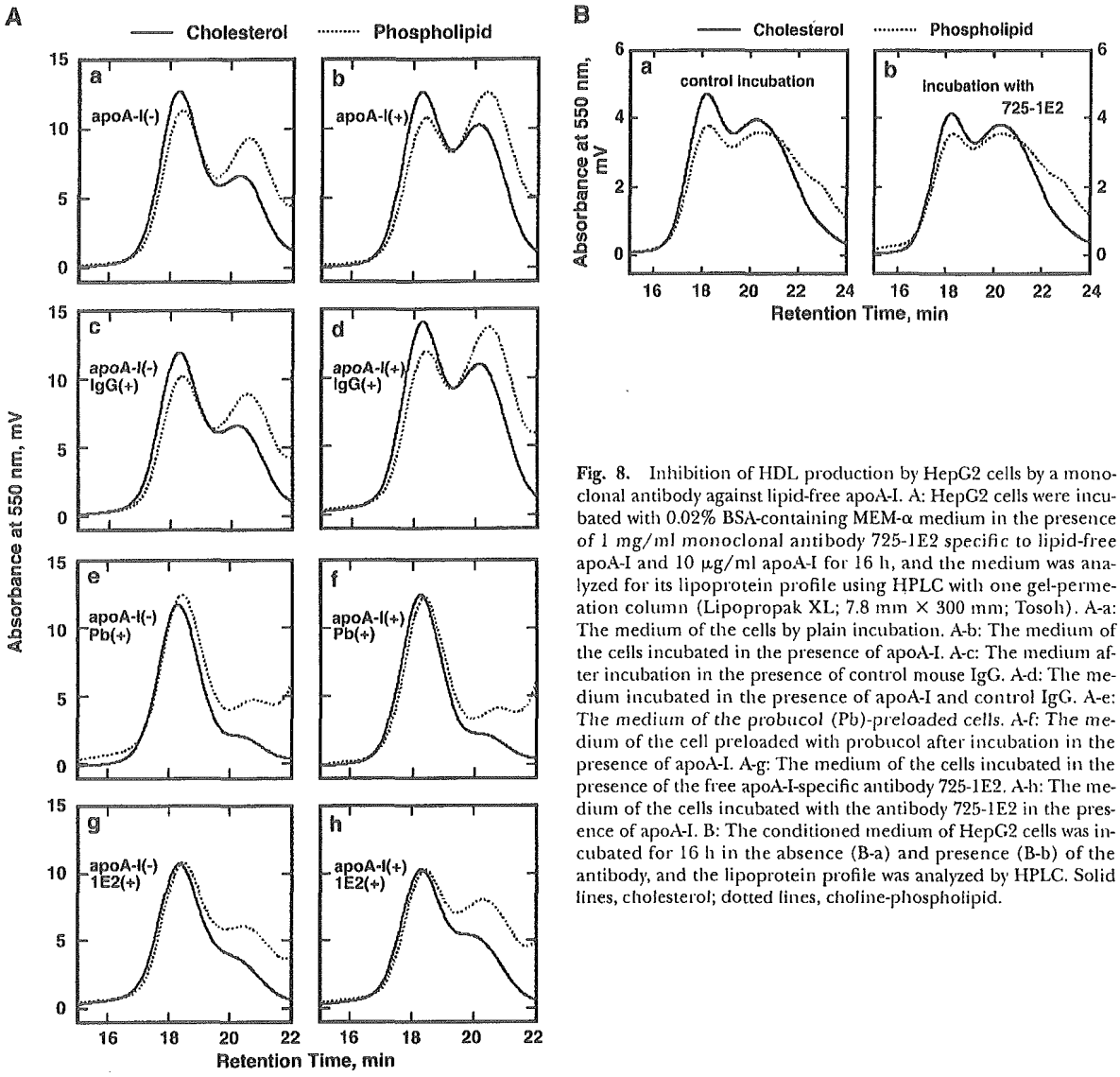

Fig. 8. Inhibition of HDL production by HepG2 cells by a monoclonal antibody against lipid-free apoA-I. A: HepG2 cells were incubated with 0.02% BSA-containing MEM-α medium in the presence of 1 mg/ml monoclonal antibody 725-1E2 specific to lipid-free apoA-I and 10 μg/ml apoA-I for 16 h, and the medium was analyzed for its lipoprotein profile using HPLC with one gel-permeation column (Lipopropak XL; 7.8 mm × 300 mm; Tosoh). A-a: The medium of the cells by plain incubation. A-b: The medium of the cells incubated in the presence of apoA-I. A-c: The medium after incubation in the presence of control mouse IgG. A-d: The medium incubated in the presence of apoA-I and control IgG. A-e: The medium of the probucol (Pb)-preloaded cells. A-f: The medium of the cell preloaded with probucol after incubation in the presence of apoA-I. A-g: The medium of the cells incubated in the presence of the free apoA-I-specific antibody 725-1E2. A-h: The medium of the cells incubated with the antibody 725-1E2 in the presence of apoA-I. B: The conditioned medium of HepG2 cells was incubated for 16 h in the absence (B-a) and presence (B-b) of the antibody, and the lipoprotein profile was analyzed by HPLC. Solid lines, cholesterol; dotted lines, choline-phospholipid.

data for cellular lipid and probucol are listed in **Table 1**. There was no difference in lipid composition between the control and probucol-loaded cells. The latter cells contained probucol at ~1 μg/mg cell proteins, which was equivalent to our previous data with mouse peritoneal macrophages to which probucol was delivered by acetylated LDL (9). LDL- and HDL-size particles were identified in the medium, and the HDL fraction was increased by apoA-I (**Fig. 4A, B**). Probucol markedly decreased the HDL peak with both spontaneous production or exogenously added apoA-I (Fig. 4C, D). This finding was confirmed by electrophoretic analysis of the medium on an agarose gel (data not shown). The medium of mouse primary hepatocytes was also examined. The hepatocytes isolated from ABCA1-deficient mice produced no HDL, and apoA-I did not induce the production of HDL either (Fig. 4E, F). The effect of probucol was examined in hepatocytes prepared from C57Bl/6 mice by feeding control chow or 0.5% probucol-containing chow. The HDL peak decreased by probucol feeding (Fig. 4G, H). **Figure 5** shows the effect of probucol on the apoA-I-induced lipid release from HepG2 cells. The results indicated again that probucol inhibits the lipid release for both the baseline and an additional part induced by apoA-I. The cell-specific (reversible) binding of apoA-I was examined using ^{125}I-labeled apoA-I for HepG2 cells. Probucol inhibited the binding of apoA-I (Fig. 5C).

ApoB and apoA-I were analyzed in the HPLC fractions of the HepG2-conditioned medium (**Fig. 6**). ApoB was detected in the LDL fractions, and the probucol treatment did not alter this distribution. ApoA-I in the HDL fractions was markedly decreased by the probucol treatment. ApoA-I was also present in the free protein fractions, and this is

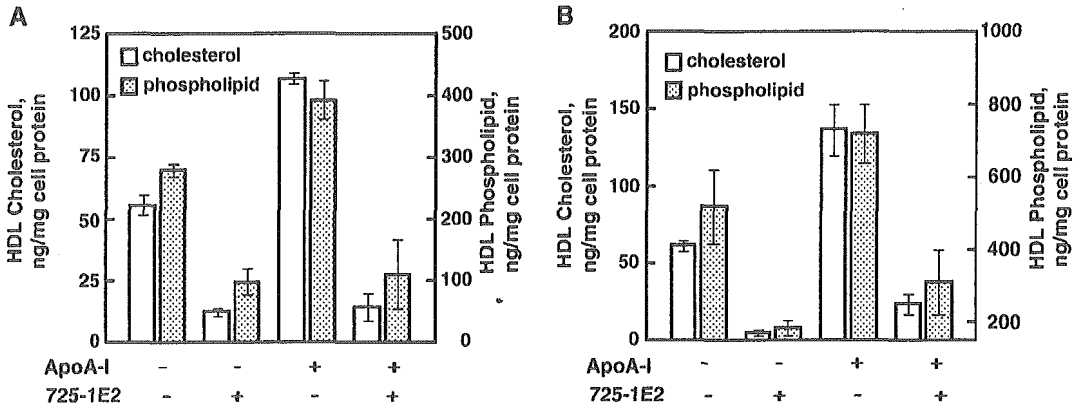

Fig. 9. Effect of free apoA-I-specific monoclonal antibody on HDL production by HepG2 cells. A: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on HPLC analysis. The data represent means ± SEM of triplicate assays. B: Cholesterol and phospholipid in HDL in the medium of HepG2 cells incubated in the presence of the antibody 725-1E2 and apoA-I, based on ultracentrifugal analysis. Data represent means ± SEM of triplicate assays.

consistent with the findings from the analysis of human plasma by HPLC in the diluted condition equivalent to the culture medium (×250) (data not shown). Although this HPLC method was thoroughly validated for lipoprotein lipid elution profile by its excellent correlation with the other gel-permeation method with a Superose column, apoA-I may dissociate from HDL particles during the analysis, presumably as a result of the use of 0.005% Brij-35 and extreme dilution of lipoprotein (17, 19).

Because of this limitation of the HPLC method, the medium was analyzed by ultracentrifugation to identify the status of apoA-I in the medium. When ABCA1 was inactivated by probucol in HepG2 cells, overall secretion of apoA-I was not influenced, as demonstrated in **Fig. 7A**. However, although apoA-I was localized in the HDL fraction ($1.063 < d < 1.21$ g/ml) in the control conditioned medium, it was found exclusively in the $d > 1.21$ g/ml fraction in the medium of probucol-loaded cells (Fig. 7B). Thus, apoA-I is secreted by the cells mostly in its free form when ABCA1 is inactivated.

Stabilization and increase of ABCA1 by additional apoA-I was not observed in this cell line, presumably because it is already affected by endogenously secreted apolipoproteins (Fig. 7C). However, ABCA1 was resistant against degradation in the probucol-loaded HepG2 cells (Fig. 7C, D) in the same manner as we demonstrated in fibroblasts (11).

Finally, we attempted to inactivate free apoA-I in the medium by trapping it using a monoclonal antibody to lipid-free apoA-I, 725-1E2 (15). As shown in **Fig. 8**, production of the HDL-size particles by both endogenous and exogenous apoA-I was effectively suppressed when this antibody was present in the medium during the incubation of HepG2 cells (Fig. 8Ag, Ah), whereas control IgG did not influence the profile (Fig. 8Ac, Ad). On the other hand, this antibody did not change lipoprotein profile when incubated with the HepG2-conditioned medium after removal of the cells (Fig. 8Ba, Bb). These results are quantitatively illustrated in **Fig. 9A**. Alternatively, the medium was analyzed by ultracentrifugation after the same experiments, and these results are shown in Fig. 9B. Similar to the results of the HPLC analysis, HDL production was selectively suppressed by the lipid-free apoA-I monoclonal antibody 725-1E2.

DISCUSSION

The results of this work are summarized as follows. *1*) HepG2 cells and mouse primary cultured hepatocytes produce HDL with endogenously synthesized apolipoprotein as well as with exogenously added apolipoprotein. *2*) Production of HDL by both pathways is inhibited by the inactivation of ABCA1, but secretion of endogenous apoA-I was not decreased as much. ApoA-I is secreted as a free form when ABCA1 is inactivated. *3*) A monoclonal antibody against lipid-free apoA-I, 725-1E2, suppresses the production of HDL by HepG2 cells but has no effect on lipoprotein when directly incubated with the HepG2-conditioned medium. Based on these observations, we conclude that the majority of HDL produced by hepatocytes are generated by the interaction of apoA-I that is secreted in a free or lipid-poor form with ABCA1 of the hepatocytes in an autocrine-like manner.

Many reports have characterized the HDL-like particles secreted in the culture medium by HepG2 cells and other liver cell lines (23–25). We essentially confirmed these findings with HepG2 cells and, in addition, demonstrated the increase of production of such particles by exogenously added apoA-I and apoA-II. Thus, the HepG2 cells have the same pathway to generate HDL as many other cells upon interaction with helical apolipoproteins, presumably dependent on ABCA1 (26).

Probucol has been shown to inhibit the apolipoprotein-cell interaction and accordingly to suppress the generation of HDL (9, 10). We recently demonstrated that this compound inactivates ABCA1 in the plasma membrane with respect to its activity as well as its calpain-mediated degradation (11). Probucol inhibited the production of

HDL, whether by endogenous or exogenous apolipoprotein, in hepatocytes. These findings suggested that endogenous and exogenous apolipoprotein, mainly apoA-I, generate HDL by a common mechanism, most likely by the ABCA1 pathway. Under this condition, the amount of the secreted apoA-I did not change, and it was secreted mostly in its free form (Fig. 7A, B). Therefore, the interaction of apoA-I with ABCA1 does not influence the rate of its secretion, but most apoA-I remains in a free form in the medium when it is unable to assemble HDL.

A monoclonal antibody raised against apoA-I, 725-1E2, has been characterized to be selective for lipid-free apoA-I (15). By using this antibody, we attempted to alter the production of HDL by HepG2 cells. In the presence of this antibody in the culture medium of HepG2 cells, the production of HDL markedly decreased. This finding strongly suggested that most apoA-I is secreted in its free form and trapped by the antibody, resulting in a decrease of HDL production. This view was supported by the finding that the incubation of the antibody with the HepG2-conditioned medium did not cause a change of the lipoprotein profile. Therefore, the effect of the antibody was not likely caused by the reaction with HDL after its generation by the cells.

It was reported that a certain portion of apoA-I (20%) was intracellularly lipidated in HepG2 cells and in primary cultured hepatocytes (8, 27), and some of the lipidation may not be mediated by ABCA1 (8). However, the present results indicated that the majority of HDL particles are produced by a common mechanism for endogenous and exogenous apolipoproteins, both of which can be inhibited by probucol. Also, most of the HDL production was inhibited by lipid-free apoA-I-specific antibody, indicating an autocrine mechanism: apoA-I is secreted as a free form and then interacts with hepatocytes to generate HDL. These findings by no means exclude the possibility of intracellular lipidation of apoA-I and the presence of the ABCA1-independent pathway as a minor source of HDL production.

ApoA-I is secreted as pro-apoA-I and converted to a mature form by a metalloprotease in blood plasma (28). Both pro-apoA-I and mature apoA-I were identically capable of generating HDL by reacting with ABCA1 (15), so perhaps there is no need to consider apoA-I maturation for this autocrine mechanism.

The authors thank Michi Hayashi for her preparation of apolipoproteins. This work was supported in part by grants-in-aid from the Ministry of Science, Education, Culture, and Sports and the Ministry of Health, Welfare, and Labor. It was also partly sponsored by a research grant from the Pharmaceuticals and Medical Device Agency.

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Astrocytes produce and secrete FGF-1, which promotes the production of apoE-HDL in a manner of autocrine action

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Abstract The astrocytes prepared by 1 week secondary culture after 1 month primary culture of rat brain cells (M/W cells) synthesized and secreted apolipoprotein E (apoE) and cholesterol more than the astrocytes prepared by conventional 1 week primary and 1 week secondary culture (W/W cells) (Ueno, S., J. Ito, Y. Nagayasu, T. Furukawa, and S. Yokoyama. 2002. 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. *Biochim. Biophys. Acta.* 1589: 261–272). M/W cells also highly expressed fibroblast growth factor-1 (FGF-1) mRNA. FGF-1 was identified in the cell lysate of both cell types, but M/W cells released more of it into the medium. Immunostaining of FGF-1 and apoE revealed that both localized in the cells that produce glial fibrillary acidic protein. The conditioned media of M/W cells and FGF-1 stimulated W/W cells to release apoE and cholesterol to generate more HDL. Pretreatment with a goat anti-FGF-1 antibody or heparin depleted the stimulatory activity of M/W cell-conditioned medium. The presence of the anti-FGF-1 antibody in the medium suppressed apoE secretion by M/W cells. Differential inhibition of signaling pathways suggested that FGF-1 stimulates apoE synthesis via the phosphoinositide 3-OH kinase for PI3K/Akt pathway. Thus, astrocytes release FGF-1, which promotes apoE-HDL production by an autocrine mechanism. These results are consistent with our in vivo observation that astrocytes produce FGF-1 before the increase of apoE in the postinjury lesion of the mouse brain (Tada, T., J. Ito, M. Asai, and S. Yokoyama. 2004. Fibroblast growth factor 1 is produced prior to apolipoprotein E in the astrocytes after cryo-injury of mouse brain. *Neurochem. Int.* 45: 23–30).—Ito, J., Y. Nagayasu, R. Lu, A. K. M. Hayashi, and S. Yokoyama. Astrocytes produce and secrete FGF-1, which promotes the production of apoE-HDL in a manner of autocrine action. *J. Lipid Res.* 2005. 46: 679–686.

Supplementary key words apolipoprotein E • fibroblast growth factor-1 • high density lipoprotein • brain damage • cholesterol

The brain cells are segregated from lipoproteins in the systemic circulation by the blood-brain barrier, so that cholesterol homeostasis in the brain is dependent on its specific extracellular lipid transport system by apolipoproteins and lipoproteins (1–5). Helical apolipoproteins such as apolipoprotein E (apoE), apoA-I, apoD, apoA-IV, and apoJ have been identified in the cerebrospinal fluid as components of HDL, but the main apolipoproteins are apoE and apoA-I (6–10). The phenotype of human brain apoE does not change after liver transplantation, so that brain apoE is mostly produced in the brain (11).

It is known that apoE is produced mainly by astrocytes and partly by microglia in the brain, suggesting that astrocytes play an important role in cholesterol homeostasis in the central nervous system (5, 10, 12–16). Astrocytes produce cholesterol-rich HDL with cellular lipid by autologously synthesized apoE (8, 17). They also react with exogenous apoA-I to generate cholesterol-poor HDL through a unique system for intracellular cholesterol transport (18–20). Like plasma HDL, ATP binding cassette transporter A1 supports such production of brain HDL, although other pathways may also function as backup systems (21, 22). These lipoproteins are thought to play important roles in intercellular lipid transport in the brain. It has been noted that apoE synthesis is upregulated in the brain during development and after injury (13, 23–31), and this reaction is likely to be involved in the healing process of the injury (32–35).

Four types of apoE binding receptor are identified in the brain: very low density lipoprotein receptor, low density lipoprotein receptor, low density lipoprotein receptor-related protein, and apoE receptor-2; thus, apoE is thought to function as a recognition site of lipoproteins for lipid

Abbreviations: apoE, apolipoprotein E; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; FGF-1, fibroblast growth factor-1; GFAP, glial fibrillary acidic protein; PI3K, phosphoinositide 3-OH kinase; TBS, salined 0.02 Tris-Hcl buffer.

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Manuscript received 17 August 2004.
Published, JLR Papers in Press, January 1, 2005.
DOI 10.1194/jlr.M400313-JLR200

delivery among brain cells (36, 37). However, some of them are also likely to mediate signals for the migration of brain cells during the developmental integration of the brain (38).

We reported previously that astrocytes prepared by 1 month primary culture of rat fetal brain cells and subsequent 1 week secondary culture (M/W cells) synthesized and secreted apoE and cholesterol more actively than astrocytes prepared according to the conventional method of 1 week primary and 1 week secondary culture (W/W cells) (39). A fibroblast growth factor-1 (FGF-1)-like factor is secreted by the long-cultured rat fetal brain cells, and their conditioned media stimulated W/W astrocytes for the secretion of apoE. We also found that FGF-1 is produced by astrocytes adjacent to the cryoinjury lesions of mouse brain before the increase of apoE synthesis in vivo (40). We identified a promoter polymorphism of FGF-1 related to risk for Alzheimer's disease (41). Thus, we hypothesize that FGF-1 is a trigger stimulant of apoE synthesis and generation of HDL in the postinjury brain, presumably by an autocrine mechanism. In the present work, we attempted to identify the cells that secrete FGF-1 in the culture system and demonstrate an autocrine mechanism for this factor to stimulate apoE-HDL production. This is an important process to identify the triggering mechanism for the production of apoE and its HDL in the postinjury brain for recovery from damage.

MATERIALS AND METHODS

Preparation of fetal rat astrocytes

Astrocytes were prepared from the 17 day fetal brain of Wistar rats according to the method previously described (42). After removal of the meninges, the brain 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 pellet by centrifugation at 1,000 rpm for 3 min was cultured in F-10 medium containing 10% fetal calf serum (10% FCS/F-10) at 37°C for 4 weeks as a primary culture. After treatment with 0.1% trypsin/DPBS/G containing 1 mM ethylenediaminetetraacetic acid, the cells were cultured in 10% FCS/F-10 for 1 week as a secondary culture (M/W cells). Alternatively, astrocytes were prepared by a conventional method of 1 week primary and subsequent 1 week secondary culture (W/W cells). Both preparations contained 95% astrocytes [glial fibrillary acidic protein (GFAP)-positive], 0.3% oligodendroglias (anti-myelin basic protein-positive), and 3% microglias (ED-1-positive) (39).

Synthesis and release of cellular cholesterol

Rat astrocytes at a confluent stage were washed with DPBS four times and incubated in 0.1% BSA/F-10 for 24 h. The cells were incubated with [^{3}H]acetate (New England Nuclear) in fresh 0.02% BSA/F-10 for certain periods of time. For the lipid-release experiments, the cells were washed three times with cold DPBS and further incubated in fresh 0.02% BSA/F-10 in the presence of 1 mM acetate. Cholesterol was extracted from the cells and the conditioned medium with hexane-isopropanol (3:2, v/v) and chloroform-methanol (2:1, v/v), respectively, and separated by TLC on Silica Gel-60 plates (E. Merck, Darmstadt, Germany). Radioactivity in the cholesterol fraction was counted (43). The medium was also analyzed by density gradient ultracentrifugation as described previously (17). After removing cell debris by centrifugation, the medium (8 ml) was overlaid on the sucrose solution (d = 1.175; 17 ml) and centrifuged at 1×10^5 *g* for 48 h. Samples were fractionated and analyzed for cholesterol mass by the enzymatic colorimetric method (44) and for apoE by Western blotting (see below).

Analysis of cell and medium protein by Western blotting

The cells were harvested with a rubber policeman after washing four times with DPBS. The cell pellet by centrifugation at 1,000 rpm for 10 min was treated with cold and salined 0.02 M Tris-HCl buffer, pH 7.5 (TBS), containing the protease inhibitor cocktail (Sigma) for 10 min with 25 agitations for 10 s every 5 min. The suspension was centrifuged at 3,000 rpm for 10 min for removal of nuclei and cell debris. The supernatant was sonicated and centrifuged at 370,000 *g* for 30 min to obtain supernatant as a cell protein extract fraction. Cell debris was removed from the conditioned medium by centrifugation at 15,000 rpm for 30 min. Protein in the cell extract or in the conditioned medium was precipitated by 10% trichloroacetate and centrifugation at 15,000 rpm for 20 min, separated by SDS-PAGE, and transferred to a Sequi-Blot™ polyvinylidene difluoride membrane (Bio-Rad). The membrane was immunostained with a goat anti-FGF-1 antibody (Santa Cruz Biotechnology) and a rabbit antibody against rat apoE, a generous gift from Dr. Jean Vance (University of Alberta).

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from rat astrocytes with Isogen (Wako Life Science) and reverse-transcribed to generate cDNA in a SuperScript Preamplification System (Gibco BRL). The cDNA was subjected to PCR using the DNA probes for rat apoE mRNA and FGF-1 mRNA as described in the previous paper (39). After electrophoresis of the products, an agarose gel was stained with freshly prepared SYBR Gold nucleic acid gel stain solution. The band was detected by an ultraviolet transilluminator (UVP NLM-20E) at 302 nm. The apoE primer pairs were 5′-GCGCACCTCCTC-CATCTCCTC-3′ (sense) and 5′-AGGATCTATGCAACCGACTCG-3′ (antisense). The FGF-1 primers were 5′-AAGCCCGTCGGTGT-CCATGG-3′ and 5′-GATGGCACAGTGGATGGGAC-3′.

Immunocytostaining of astrocytes

Astrocytes on a tissue culture chamber/slide (Mikes Scientific) were washed with DPBS and fixed with 100% methanol at −20°C for 30 min. The cells were treated with 1% Triton X-100 in 0.02 M phosphate buffered saline at room temperature for 2 min after washing with DPBS. The cells were washed with DPBS again, treated with goat anti-FGF-1 antibody, or rabbit anti-rat apoE antibody, at room temperature for 60 min and washed. After incubation with biotin-conjugated anti-goat IgG, or anti-rabbit IgG antibody (Histofine) for 30 min at room temperature, the cells were washed, treated with peroxidase-conjugated streptavidin (Histofine) for 15 min, and then washed. The cells were stained by reaction with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Dojindo)/0.03% H_2O_2/0.05 M Tris buffer, pH 7.5, for 5 min at room temperature.

Alternatively, M/W astrocytes were fluorescence immunostained after being fixed in organic solution composed of methanol, chloroform, and acetic acid (6:3:1) at −20°C for 3 h. After washing with cold TBS, the cells were reacted with either goat anti-FGF-1 or goat anti-rat apoE antibody (Santa Cruz Biotechnology) and mouse anti-GFAP antibody (BD Transduction Laboratories) in TBS containing 3% donkey serum and 3% horse serum at room temperature for 1 h. The cells were reacted with rhodamine-conjugated donkey anti-goat IgG antibody (Chemicon International) or fluorescein-conjugated horse anti-mouse IgG antibody (Vector Laboratories) in the presence of 3% donkey or