Assessment of the role of sphingosine 1-phosphate and its receptors in high-density lipoprotein-induced stimulation of astroglial cell function

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It has been suggested that lipoproteins in the central nervous system are involved in the regulation of several neural functions independent of cholesterol metabolism as well as those related to lipid metabolism. We recently demonstrated that lipoproteins are carriers for sphingosine 1-phosphate (S1P). This raised the possibility that S1P mediates the neural cell functions induced by lipoproteins. In the current study, we examined the effects of plasma high-density lipoprotein (HDL) on astroglial cell functions, focusing especially on the role of the lipoprotein-associated S1P. In rat type I astrocytes or C6 glioma cells, similar to S1P, HDL stimulated DNA synthesis and mRNA expression of fibroblast growth factor-2, a potent neurotrophic factor, which was associated with the activation of extracellular signal-regulated kinase (ERK) in a pertussis toxin-sensitive manner. The data from fractionation studies of HDL indicated that S1P may

be a major component for the activation of ERK. In C6 glioma cells, HDL also induced phospholipase C-dependent intracellular Ca^{2+} mobilization. Desensitization of the C6 glioma cells with S1P abolished these HDL-induced actions. Furthermore, over-expression of S1P receptors in C6 glioma cells led to a significant enhancement of HDL-induced ERK activation and Ca^{2+} mobilization. Thus, at least some HDL-induced actions may be mediated by cell-surface S1P receptors in astroglial cells. These results imply that S1P might partially mediate lipoprotein-induced cholesterol metabolism-independent neural cell functions in the central nervous system.

Key words: central nervous system, extracellular signal-regulated kinase, fibroblast growth factor-2, lipoprotein, sphingosine 1-phosphate receptor.

INTRODUCTION

Recent studies have shown that sphingosine 1-phosphate (S1P) functions as a potent regulator in neural cells; for example, oligodendrocytes [1], astrocytes [2–4], C6 glioma cells [2,3,5] and cultured neuronal cells [6–8]. Five subtypes of S1P-specific receptor, i.e. S1P₁/Edg-1, S1P₂/Edg-5, S1P₃/Edg-3, S1P₄/Edg-6 and S1P₅/Edg-8, have been isolated so far [9–18]. In neural cells, these receptors are expressed in a cell-specific manner [8,19]. In PC12 and N1E115 cells, S1P induced a rapid retraction of neurites and the transient rounding of the cell body [6,7]. This action of S1P is likely to be mediated through S1P₂ [8]. In astrocytes and C6 glioma cells, S1P induced stimulation of the expression of fibroblast growth factor-2 (FGF-2; basic FGF), which was associated with extracellular signal-regulated kinase (ERK) activation and early growth response-1 (Egr-1) expression [3,5]. FGF-2 is required for the differentiation and survival of neuronal cells [20,21]. S1P also induced activation of phospholipase C (PLC) and Ca^{2+} mobilization, resulting in the activation of phospholipase D [5], which may be important for cell proliferation [22,23]. These S1P-induced actions may also be mediated by S1P receptors; S1P₁ is more important than S1P₂ for the stimulation of the ERK/FGF-2 system, whereas S1P₂ may be responsible for the activation of the PLC/ Ca^{2+} system [19]. Thus, S1P and its receptors may play important roles in maintaining the functions of the central nervous system (CNS).

It has been suggested that the lipoproteins in the CNS are involved in the regulation of several neural functions independent of cholesterol transport as well as those related to lipid metabolism [24–27]. We recently demonstrated that S1P is concentrated in lipoproteins, such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL), in circulating blood [28] and that it mediates lipoprotein-induced cell survival actions in endothelial cells [29,30]. Thus, lipoproteins seem to serve as carriers for extracellular S1P in circulating blood. In pathological conditions such as brain trauma and haemorrhage [31], S1P is released from platelets during blood clotting, and plasma lipoproteins or the lipoprotein-associated S1P may contact neural cells. Under normal or physiological conditions as well, a high concentration of lipoproteins, including HDL and LDL, has been shown to be present in the CNS [32–34]: the transport of these lipoproteins across the blood/brain barrier has been suggested [35,36]. Furthermore, it has been reported that apolipoprotein A-I, a major apolipoprotein in plasma HDL, has also been synthesized in brain endothelial cells [37]. These results suggest that lipoproteins are carriers for extracellular S1P in the CNS as well and thereby exert some neural cell functions independent of cholesterol metabolism through S1P and its receptors.

In the present study, we examined the effects of HDL on astroglial cell functions, including proliferation, FGF-2 expression, ERK activation and Ca^{2+} mobilization, focusing especially on the role of lipoprotein-associated S1P. We found that HDL

Abbreviations used: BrdU, 5-bromo-2'-deoxy-uridine; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; CHO, Chinese hamster ovary; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; Egr-1, early growth response-1; ERK, extracellular signal-regulated kinase; FGF-2, fibroblast growth factor-2; fura-2/AM, fura-2/acetoxymethylester; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; LPC, L- α -lysophosphatidylcholine palmitoyl; LSF, lysosulphatides; PAF, platelet-activating factor; PLC, phospholipase C; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; VLDL, very-low-density lipoprotein.

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stimulated these responses; some of these actions were sensitive to S1P receptor desensitization and/or pertussis toxin (PTX), as was the case for S1P. The data from fractionation studies of HDL indicated that S1P might be a major component for the activation of ERK. Furthermore, overexpression of S1P₁ or S1P₂ enhanced these responses to HDL. Thus, S1P and its receptors may be involved in the plasma HDL-induced activation of rat type I astrocytes and C6 glioma cells.

EXPERIMENTAL

Materials

S1P was purchased from Cayman Chemical Co.; Hepes and fura-2/acetoxymethylester (fura-2/AM) were from Dojindo (Tokyo, Japan); [methyl-³H]thymidine (20 Ci/mmol) was from American Radiolabelled Chemicals; [α -³²P]dATP (3000 Ci/mmol), [γ -³²P]ATP (3000 Ci/mmol) and the p42/p44 mitogen-activated protein kinase (ERK1/2) enzyme assay kit were from Amersham Biosciences; and an ERK-specific antibody (K-23; amino acids 305–327 of rat ERK1 which recognizes both ERK1 and ERK 2) was from Santa Cruz Biotechnology. 1-Oleoyl-*sn*-glycero-3-phosphate (lysophosphatidic acid; LPA), L- α -lysophosphatidylcholine palmitoyl (LPC; C_{16:0}), L- α -lysophosphatidylcholine β -acetyl- γ -O-alkyl (platelet-activating factor; PAF) and lysosulphatides (LSF) were from Sigma-Aldrich; DL-*threo*-dihydrosphingosine, *N,N*-dimethylsphingosine, sphingosylphosphorylcholine (SPC) and PD98059 were from Biomol Research Labs; and FuGene 6 transfection reagent was from Roche Molecular Biochemicals.

S1P was routinely dissolved as 1 mM in 10 mM NaOH and stored at –20 °C; LPA was used at 2 mM in distilled water. For each experimental use, S1P and LPA solutions were diluted in isotonic buffers or media for cell culture containing 0.1 % (w/v) BSA. Plasmid pTB784 containing a rat FGF-2 cDNA [38] was provided generously by Dr T. Kurokawa (Takeda Chemical Industries, Tsukuba, Ibaraki, Japan). The sources of all other reagents were described previously [3,5,7,19,28,29,39].

Preparation of plasma lipoproteins

Human plasma was collected from normal healthy volunteers. Plasma lipoproteins were isolated from fresh human plasma by NaBr density-gradient centrifugation. Very low density lipoprotein (VLDL; density < 1.019 g/ml), LDL (1.019–1.063 g/ml) and HDL (1.063–1.21 g/ml) were separated by sequential ultracentrifugation as described previously [28]. After extensive dialysis against saline/PBS (9:1), lipoproteins were stored on ice and used for experiments within 2 weeks. For preparation of charcoal-treated lipoproteins, plasma lipoproteins (1 mg in 1 ml) were treated with 100 mg of activated charcoal, which was prepared by mixing with 5 % (w/v) BSA and subsequent washing with PBS. Lipoproteins were then recovered by centrifugation and filtration (0.22 μ m pore size).

Extraction of active components from HDL

Extraction of active components of HDL was performed as described previously [29]. In brief, the components of HDL (about 2–5 mg in 2 ml) were separated into three fractions: fraction a, which contains the majority of phospholipids and neutral lipids; fraction b, which contains S1P and other lipids soluble under an alkaline aqueous solution; and fraction c, which contains substances soluble in aqueous solution. Fraction b was further processed by a silica gel high-performance TLC. The chromatography was performed using two kinds of solvent

system, chloroform/methanol/20 % NH₄OH (60:35:8, by vol.) for TLC 1 and butan-1-ol/acetic acid/water (3:1:1, by vol.) for TLC 2. The silica gel with the resolved lipids (about 1.5 cm length each) was scraped off to obtain lipids covering the entire area of migration. Lipids were then eluted with chloroform/methanol/HCl (100:100:1, by vol.) and dried by evaporation. All fractions thus separated were dissolved in PBS containing 0.1 % BSA and were used at a final concentration corresponding to 100–300 μ g/ml HDL for stimulation of ERK1/2 in astrocytes. The content of S1P and LPA in fraction b was estimated as shown below.

Cell culture and transfection

C6 glioma cells, a rat glioma cell line, obtained from the Japanese Research Resources Bank (JCRB9041), were grown in Ham's F10 medium supplemented with heat-inactivated horse serum (15 %, v/v) and fetal bovine serum (2.5 %, v/v). The cells were plated on 10 cm dishes for the measurement of cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_i), and on 6 cm dishes for other experiments including extraction of total RNA and measurement of ERK1/2 activity. When the cells had become 50–60 % confluent, the culture medium was changed to fresh Ham's F10 medium containing 0.1 % (w/v) BSA to make them quiescent and the cells were further cultured for 2 days. PTX (100 ng/ml) was added to serum-free medium 24 h before experiments. S1P receptor (S1P₁ or S1P₂)-transfected C6 cells were maintained in the Ham's F10 medium with 100 μ g/ml G418 as described in [19]. To construct an LPA-receptor expression plasmid, the cDNAs for LPA₁/Edg-2 (GenBank accession number Y09479) from a human cDNA library were cloned by reverse transcriptase PCR, and the entire coding region of the LPA₁ (1095 bp) was subcloned into the *Eco*RI site of the pEFneo eukaryotic expression vector [5]. Transfections were carried out by the lipofection technique with FuGene 6 transfection reagent (Boehringer Mannheim) as described in [19]. The LPA₁-transfected cells were selected with neomycin (G418 at 1 mg/ml) and maintained in the Ham's F10 medium with 100 μ g/ml G418. Human 1321N1 astrocytoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10 %, v/v). S1P-receptor-transfected Chinese hamster ovary (CHO) cells were cultured in DMEM with fetal bovine serum (10 %, v/v) [13], and LPA-receptor-transfected RH7777 cells were cultured in minimal essential medium with fetal bovine serum (10 %, v/v). PC12 cells and HL-60 cells were cultured as described in [7,40]. Preparation of type I astrocytes was carried out as described in [3]. Briefly, the cerebral cortex was removed from 1–2 day-old rat and minced. The tissue samples were digested with 0.25 % (w/v) trypsin at 30 °C for 20 min. The digested tissues were dispersed mechanically with a disposable pipette in 25 mM Hepes-buffered DMEM supplemented with 10 % (v/v) fetal bovine serum. The dissociated cells were collected by centrifugation, re-suspended in the same medium and placed in a 75 cm² flask at a density of 1.5 $\times$ 10⁶ cells/flask. After a 24 h incubation, the medium was changed and the dissociated cells were cultured until 70–80 % confluent. The cultures were shaken vigorously to remove non-adherent cells in the flask. The adherent cells (type I astrocytes) were dispersed in DMEM containing 10 % (v/v) fetal bovine serum and were placed in a new flask. The cells were subsequently cultured for a few days by removing non-adherent cells to concentrate type I astrocytes. The cells were plated on 6 cm plates for extraction of total RNA and measurement of ERK1/2 activity, and put into 12-well dishes for experiments of thymidine incorporation. When the cells had become 80–90 % confluent, the culture medium was changed to fresh DMEM

containing 0.1% (w/v) BSA to make them quiescent. Where indicated, PTX treatment of the cells was performed by adding the toxin (100 ng/ml) to the medium 24 h before experiment.

Measurement of ERK 1/2 activity

The cells were washed once, pre-incubated for 15 min at 37 °C in a Hepes-buffered medium composed of 20 mM Hepes, pH 7.4, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2 mM CaCl_2 , 2.5 mM NaHCO_3 , 5 mM glucose and 0.1% (w/v) BSA (fraction V), and then incubated with test agents for various lengths of time at 37 °C. For the desensitization experiments with S1P, the cells were pretreated with or without S1P (3 μM) for 1 h prior to incubation with test agents. The enzyme activity was then measured as described previously [5]. The kinase activity was determined with an assay kit (Amersham Corp.) that measures the incorporation of [γ - ^{32}P]ATP into a synthetic peptide (KRELVEPLTPAGEAPNQALLR) as a specific substrate. The protein concentration of the supernatant was determined with the BCA protein assay (Pierce, Rockford, IL, U.S.A.). The same supernatant as that used for ERK activity was also analysed by Western blotting with an ERK-specific antibody [5].

Measurement of $[\text{Ca}^{2+}]_i$

The cells were washed twice with Ca^{2+} - and Mg^{2+} -free PBS containing 1 mM EGTA, warmed in the same medium for 5 min at 37 °C, and gently harvested from dishes with a transfer pipette. The cells were sedimented by centrifugation at 250 *g* for 5 min, suspended in Ham's F10 medium containing 0.1% (w/v) BSA and incubated with fura-2/AM for 20 min at 37 °C. The cells were washed twice to eliminate excess fura-2/AM by repeating the sedimentation at 250 *g* for 5 min and resuspended in the Hepes-buffered medium. The change in $[\text{Ca}^{2+}]_i$ was estimated from the change in the fluorescence of fura-2-loaded cells as described previously [39].

Estimation of S1P and LPA content in HDL

To estimate the content of S1P and LPA in the fraction b of HDL as shown above, two sensitive and specific bioassays were used based on their ability to stimulate S1P_3 or LPA_1 . S1P activity was estimated by its ability to produce inositol phosphate in human S1P_3 -expressed CHO cells [13] and LPA activity was estimated by its ability to inhibit cAMP accumulation in mouse LPA_1 -expressed RH7777 cells (a generous gift from Professor Kevin R. Lynch, University of Virginia School of Medicine, Charlottesville, VA, U.S.A.). Vector-transfected CHO cells and RH7777 cells were unable to exert a significant effect on the production of inositol phosphate and the inhibition of cAMP accumulation in response to S1P and LPA, respectively. By using these bioassays, we detected S1P activity (1–100 nM) and LPA activity (0.1–3 nM). Measurement of inositol phosphate production was performed as described previously [28,29]. In brief, the [^3H]inositol-labelled CHO cells were harvested from the 10 cm dishes with trypsin (0.05% in PBS containing 0.53 mM EDTA). The cells were washed by sedimentation (250 *g* for 5 min) and re-suspension in the Hepes-buffered medium. The washing procedure was repeated, and the cells were finally re-suspended in the same medium. The cells (about 5×10^6 cells) were pre-incubated for 10 min with 10 mM LiCl in polypropylene vials (20 ml) in a final volume of 1.04 ml. The samples, including S1P (0.26 ml), were then added to the medium, and the cells were further incubated for 1 min. The cell suspension (0.5 ml) in

duplicate was transferred to tubes containing 1 ml of chloroform/methanol/HCl (100:100:1, by vol.). ^3H -Labelled inositol polyphosphates (mainly InsP_2 and InsP_3) were separated. The results were normalized to 10^5 d.p.m. of the total radioactivity incorporated into the cellular inositol lipids. The radioactivity of the 5% trichloroacetic acid-insoluble fraction was measured as the total radioactivity. Measurement of cAMP accumulation was performed as described previously [13]. The cells were washed twice and preincubated for 10 min at 37 °C in Hepes-buffered medium supplemented with 10 μM forskolin and 0.5 mM isobutylmethylxanthine. After a 10 min incubation, the reaction was terminated by adding 100 μl of 1 M HCl. cAMP in the acid extracts was measured.

Northern blot analysis

For analysis of FGF-2 mRNA, total RNA isolates were prepared from astrocytes and C6 cells [treated for 6 h with agonists in serum-free medium containing 0.1% (w/v) BSA] according to the manufacturer's instructions for Trizol reagent (Life Technologies, Grand Island, NY, U.S.A.). Northern blot analysis was performed as described previously [3]. For detection of S1P receptors, Northern blot analysis was performed with cDNA probes for S1P receptors as described previously [5,16]. The cDNAs for S1P_3 (GenBank® accession number AF233649) were cloned from a rat brain cDNA library by reverse transcriptase PCR.

DNA synthesis

Incorporation of [^3H]thymidine into the DNA fraction was measured using a modification of the method reported in [4]. Briefly, astrocytes treated with or without PTX were further incubated for 24 h in DMEM containing 0.1% (w/v) BSA and the agents to be tested. [^3H]Thymidine (1 μCi in 1 ml) was added during the last 18 h of incubation. The cells were washed with ice-cold PBS, followed by two washes with 5% (w/v) trichloroacetic acid. The trichloroacetic acid-insoluble material was solubilized with a mixture containing 2% (w/v) Na_2CO_3 , 0.1% (w/v) SDS and 0.1 M NaOH. Radioactivity was measured in a liquid scintillation counter. Another proliferation assay of astrocytes was carried out by the 5-bromo-2'-deoxy-uridine (BrdU) incorporation method with a Cell Proliferation Kit (Amersham Biosciences). Astrocytes grown to subconfluency on microscope coverslips were incubated in fresh DMEM containing 0.1% (w/v) BSA for 24 h before each experiment. The cells were stimulated by the agents to be tested for a further 24 h. BrdU was added during the last 1 h of incubation according to the manufacturer's instructions. The cells containing BrdU were immunolabelled with monoclonal antibody against BrdU in the Cell Proliferation Kit, and then visualized using the Vectastain ABC Kit (Vector Laboratories). The counterstaining was carried out with Diff-Quik Solution II (International Reagents Corp.). The cells in three places were photographed at $\times 100$ magnification under a microscope. The number of cells that were brown (responsive) or blue (non-responsive) were counted, and the proliferation activity was expressed as a percentage of the brown cells in the total cells.

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as means $\pm$ S.E.M. or as representative results from more than three different batches of cells, unless otherwise stated.

RESULTS

Plasma HDL as well as S1P stimulates the proliferation and expression of FGF-2 mRNA in rat type I astrocytes

S1P has been shown to be involved in the regulation of proliferation in many types of cells. In astrocytes as well, S1P stimulated DNA synthesis, as shown by an increase in the incorporation of BrdU (Figure 1A) and thymidine (Figure 1B). HDL also increased DNA synthesis, although to a lesser extent than S1P. These responses to S1P and HDL were almost completely suppressed by PTX treatment, suggesting an involvement of PTX-sensitive G_i/G_o -proteins in the signalling pathways of HDL as well as S1P (Figure 1B). The effect of PTX seems to be specific to HDL and S1P because fetal bovine serum-induced DNA synthesis was insensitive to the toxin.

We previously reported that S1P, as well as endothelin, platelet-derived growth factor and PMA, induced FGF-2 mRNA expression in rat astrocytes [3]. In Figure 1(C), we found that HDL also increased the FGF-2 mRNA expression in a PTX-sensitive manner, although HDL was less effective than S1P (Figure 1D, upper panel). In the previous study, we showed that ERK activation may link to the S1P-induced FGF-2 expression in C6 cells [3]. In astrocytes as well, PD98059, an ERK kinase inhibitor, suppressed FGF-2 mRNA expression (Figure 1D, lower panel). In fact, HDL as well as S1P partially induced the shift in the mobility of both ERK1/2 on SDS gel, probably reflecting phosphorylation, which was associated with an activation of the enzyme (Figure 1E). The enzyme activation was maintained for at least 30 min. We also found that PTX treatment of the cells decreased the ERK activation by HDL (Figure 1F, upper panel) and PD98059 suppressed the HDL-induced ERK activation (Figure 1F, lower panel). Thus, HDL-induced FGF-2 expression may be mediated by a PTX-sensitive G_i/G_o -protein and the subsequent ERK activation, as was the case for S1P.

S1P may be a major component of HDL-induced ERK activation in rat type I astrocytes

If S1P mediates the HDL-induced action, HDL should contain enough S1P to exert action comparable with that of the lipoprotein. To assess this, we examined the correlation in the dose-response for S1P- and HDL-induced ERK activation (Figure 2A). On the basis of the S1P content in HDL being roughly 150 pmol/mg of protein (Figure 2B), which is consistent with a previous result [28], the dose-response curve of S1P was overlaid on the dose-response curve of HDL in Figure 2(A). In this experiment, we employed 0.03–1 mg/ml HDL, which corresponds to 4.5–150 nM S1P. The two dose-response curves could be almost completely superimposed.

Several bioactive lipids, such as S1P, LPA, SPC, PAF, LPC and LSF, have been suggested to be concentrated in lipoproteins [29,30,41–43]. In Figure 2(C), we examined the effects of these bioactive lipids on ERK activity. Among these lipids, S1P was most effective. LPA and SPC also significantly activated the enzyme activity, but to a lesser extent than S1P; the potency of LPA and SPC was at least an order of magnitude less than that of S1P. The other lipids, PAF, LPC and LSF, were ineffective, at least at concentrations employed in the present study.

To further examine the involvement of lipid species in the HDL-induced action, lipid fractions were prepared as described previously [29]. Components of HDL were separated into three fractions; fraction a, the lipid fraction containing the majority of lipids, such as fatty acids, neural lipids and phospholipids, including SPC and LPC; fraction b, lipids soluble in alkaline aqueous solution, such as S1P and LPA; and fraction c,

Figure 1 HDL as well as S1P stimulates proliferation and FGF-2 mRNA expression in rat type I astrocytes

(A) BrdU incorporation. Astrocytes were grown to subconfluency on microscope coverslips and incubated in fresh DMEM containing 0.1% (w/v) BSA for 24 h before the experiment. The cells were stimulated by 3 μ M S1P, 300 μ g/ml HDL or 5% (v/v) fetal bovine serum (FBS) for 24 h. Cells containing BrdU were visualized with monoclonal antibody against BrdU. The labelling index was expressed as a percentage of positive cells among total cells and is shown in parentheses in each panel. Data are means from two separate experiments. (B) [3 H]Thymidine incorporation and PTX effect. The cells treated with (closed column) or without (open column) PTX (100 ng/ml) were further incubated for 24 h in DMEM containing 0.1% (w/v) BSA together with 3 μ M S1P, 300 μ g/ml HDL, 5% (v/v) fetal bovine serum or vehicle (None). [3 H]Thymidine (1 μ Ci/ml) was added during the last 18 h of incubation. The trichloroacetic acid-insoluble material was solubilized, and radioactivity was measured in a liquid scintillation counter. Results are expressed as percentages of the basal value. The asterisk indicates statistically significant differences from the vehicle (* P < 0.05 and ** P < 0.01). (C) The cells were cultured in fresh DMEM containing 0.1% (w/v) BSA for 6 h and then incubated for another 6 h with 3 μ M S1P, 1 mg/ml HDL or vehicle. Total RNAs (20 μ g) were electrophoresed for the measurement of the mRNA content of FGF-2 and GAPDH. Representative results from Northern blot analysis are shown. (D) Effect of PTX (upper panel) and PD98059 (lower panel) on HDL- or S1P-induced expression of FGF-2 mRNA. PD98059 (10 μ M) was added to the medium 1 h before the addition of the test agents during the serum-deprivation period. The cells treated with (closed column) or without (open column) PTX or PD98059 (PD) were stimulated with 3 μ M S1P, 1 mg/ml HDL or vehicle for 6 h. The expression of FGF-2 and GAPDH mRNA was quantified by densitometry, and results are expressed as percentages of the basal value. Data are means from two separate experiments. (E) Time course of ERK activation by HDL or S1P. The serum-starved cells were stimulated by 1 μ M S1P (■), 300 μ g/ml HDL (●) or vehicle (○) for the times indicated. The lysates were used for the measurement of ERK1/2 activity. Data are means $\pm$ S.E.M. from three separate experiments. In the inset, the lysates at 5 min were used for Western blot analysis of ERK 1/2. Data are representative of more than three separate experiments. (F) Effect of PTX (upper panel) or PD98059 (lower panel) on ERK activation by HDL or S1P. The astrocytes were pre-treated as described above and then stimulated by 1 μ M S1P, 300 μ g/ml HDL or vehicle for 5 min. The lysates were used for the measurement of ERK1/2 activity. Results are expressed as pmol/min per mg and are means $\pm$ S.E.M. from three separate experiments.

Figure 2 Ability of exogenous lipids and lipid components of HDL to activate ERK in rat type I astrocytes

(A) Dose-dependent effect of ERK activation by HDL and S1P. The serum-starved astrocytes were incubated for 5 min with indicated dose of HDL (●) and S1P (○). The lysates were used for the kinase assay. Results are expressed as percentages of the basal value. The basal value (pmol/min per mg) without agonist was 85 ± 8 . Data are the means $\pm$ S.E.M. from five separate experiments. The two dose-response curves were overlaid on the same figures on the basis of information about the S1P content in HDL, estimated in (B). See the text for more details. (B) S1P and LPA contents of HDL were estimated by two sensitive and receptor-mediated bioassays as described in the Experimental section. Results are expressed as S1P or LPA content (pmol) in HDL (1 mg of protein). Data are means $\pm$ S.E.M. from five separate experiments. (C) Dose-dependent effect of ERK activation by various lipids. The serum-starved astrocytes were incubated for 5 min with the indicated doses of S1P (●), LPA (■), SPC (▲), PAF (○), LPC (□) or LSF (△). Results are expressed as percentages of the maximal response obtained by $10 \mu\text{M}$ S1P (264 ± 18 pmol/min per mg). The basal value of ERK activity is 95 ± 5 pmol/min per mg. Data are the means $\pm$ S.E.M. from three separate experiments. (D) Effect of fractions (a, b and c) on ERK activity. Each fraction was prepared from HDL as described in the Experimental section. The serum-starved astrocytes were incubated for 5 min with each fraction corresponding to $300 \mu\text{g/ml}$ HDL. Results are expressed as the increment above the basal value of ERK activity (168 ± 4 pmol/min per mg). Data are representative of three separate experiments. (E) Effect of TLC fractions from fraction b of HDL on ERK activity. The fraction b was further processed for TLC as described in the Experimental section. Chromatography was performed using two kinds of solvent system, chloroform/methanol/20% NH_4OH (60:35:8, by vol.) for TLC 1 or butan-1-ol/acetic acid/water (3:1:1, by vol.) for TLC 2. The serum-starved astrocytes were incubated for 5 min with each fraction corresponding to $300 \mu\text{g/ml}$ HDL. Results are expressed as the increment above the basal value of ERK activity (167 ± 4 pmol/min per mg). Data are representative of two separate experiments.

substances soluble in aqueous solution. The ERK-activating activity of HDL was recovered in the S1P- and LPA-rich fraction b but not in other fractions (Figure 2D).

The lipid components of fraction b were further separated by two kinds of TLC (Figure 2E). The activity for stimulation of ERK in fraction b was mostly recovered in fraction 4 of TLC 1 and in fractions 4 and 5 of TLC 2. In these fractions, both S1P and LPA are migrated, suggesting that S1P and/or LPA are possible lipid mediators responsible for HDL-induced ERK activation. As shown in Figure 2(C), LPA was at least one order of magnitude less potent than S1P. This means that at least a 10 times higher concentration of LPA than S1P would be necessary to induce the same activity as that induced by S1P. However, LPA content in HDL was measured to be about one-fifth of that of S1P (Figure 2B). Thus, LPA might be a possible mediator of

HDL, but its contribution in the lipoprotein action might be much less than that of S1P. Actually, based on the content of LPA in HDL being 25 pmol/mg (Figure 2B), the LPA concentration can be estimated to be 25 nM when 1 mg/ml HDL was used. This concentration of LPA is too low to exert a significant effect on ERK activation (Figure 2C). Taken together, these results suggest that S1P may be a major component of HDL-induced ERK activation.

Astroglial cells express a variety of S1P receptor subtypes

In Figure 3, we examined the expression patterns of S1P receptor mRNAs in astroglial cells including rat type I astrocytes, human 1321N1 astrocytoma cells and C6 glioma cells. As a positive control, we also examined mRNA expression of S1P receptor

Figure 3 Northern blot analysis of S1P receptors

Expression of S1P receptors. Total RNAs (20 μ g) were prepared from rat brain on embryonic day 17 (E17), postnatal days 1 (P1) and 21 (P21), rat type I astrocytes, C6 glioma cells, 1321N1 astrocytoma cells, PC12 cells and HL60 cells. The RNAs were then electrophoresed to analyse the expression of mRNAs of S1P₁/Edg-1 (A), S1P₃/Edg-3 (B), S1P₂/Edg-5 (C), S1P₄/Edg-6 (D), S1P₅/Edg-8 (E) and GAPDH (F) as described in the Experimental section.

subtypes in rat brain, PC12 and HL60 cells. We have previously reported that both S1P₁ and S1P₂ mRNAs were expressed in rat C6 glioma cells [5]. Here, we also detected S1P₅ mRNA expression in rat astrocytes and C6 cells, although the approx. 2.5 kb major band observed in adult rat and PC12 cells [17] was marginal or undetected. In rat astrocytes, S1P₁, S1P₂ and S1P₃ were expressed in addition to S1P₅; on the other hand, in 1321N1 astrocytoma cells, S1P₁ and S1P₃ but not S1P₂ or S1P₅ were expressed. S1P₄ was not detected in either these astroglial cell types or rat brain, confirming that the expression of this S1P receptor is restricted to lymphatic and haematopoietic tissues or related cells [15].

Plasma HDL mimics the increase in expression of FGF-2 mRNA, ERK activation and $[Ca^{2+}]_i$ induced by S1P in C6 cells

Consistent with the results in astrocytes, HDL as well as S1P increased by about two times the expression of FGF-2 mRNA in C6 cells (Figure 4A). The increase in FGF-2 mRNA expression was associated with ERK activation, as shown in Figure 4(B), although the time course of the activation was transient and sustained in C6 cells and astrocytes, respectively. The PTX treatment almost completely inhibited the ERK activation induced by both HDL and S1P (Figure 4C), suggesting a mediation of the lipoprotein and lipid-induced enzyme activation by G_i/G_o-proteins in C6 cells as well as astrocytes. Hence we used C6 cells for further characterization of HDL-induced actions.

Figure 4 HDL as well as S1P induced FGF-2 mRNA expression and ERK activation in C6 glioma cells

(A) Northern blot analysis of FGF-2 mRNA. The serum-starved cells were incubated with 300 μ g/ml HDL (closed column), 1 μ M S1P (hatched column) or vehicle (open column) for 6 h. Other experimental conditions were the same as those for Figure 1(C). (B) Time course of ERK activation. Serum-starved cells were stimulated by 1 μ M S1P (■), 100 μ g/ml HDL (●) or vehicle (○) for the times indicated. Data are expressed as pmol/min per mg and are the means $\pm$ S.E.M. from three separate experiments. In the inset, Western blot analysis of ERK 1/2 at 2.5 min is shown. Data are representative of more than three separate experiments. (C) Effect of PTX on HDL-induced ERK activation. The cells were pre-treated with or without PTX and then stimulated for 2.5 min with vehicle (open column), 1 μ M S1P (hatched column) and 100 μ g/ml HDL (closed column) to measure ERK activity. Data are shown as the means $\pm$ S.E.M. from four separate experiments.

S1P has recently been shown to activate PLC and subsequently increase $[Ca^{2+}]_i$ in several types of cell [40,44,45], although PTX sensitivity varied from cell to cell. In C6 cells, S1P induced a

Figure 5 Ca^{2+} response to HDL, S1P or LPA in C6 cells

(A) Representative traces of $[Ca^{2+}]_i$ change by 1 μ M S1P, 1 μ M LPA and 100 μ g/ml HDL. (B) Effect of PTX treatment on $[Ca^{2+}]_i$ increase stimulated by 100 μ g/ml HDL, 100 nM S1P and 100 nM LPA. $[Ca^{2+}]_i$ was estimated from the change in the fluorescence of the fura-2-loaded cells. Data are expressed as the difference between peak and basal values and shown by the means $\pm$ S.E.M. from three separate experiments.

transient $[Ca^{2+}]_i$ increase in association with the activation of PLC [5]. Similarly, HDL increased $[Ca^{2+}]_i$ within 30 s (Figure 5A). However, the increases in $[Ca^{2+}]_i$ induced by HDL and S1P were not inhibited by PTX treatment of the cells (Figure 5B). In contrast, the LPA-induced $[Ca^{2+}]_i$ change in PTX-treated cells was decreased to more than half of the initial value. Thus in contrast to ERK activation, PTX-sensitive G_i/G_o -proteins may not be involved in a $[Ca^{2+}]_i$ increase induced by HDL and S1P.

Apolipoproteins do not seem to be important for HDL- and other lipoprotein-induced ERK activation and $[Ca^{2+}]_i$ increase

HDL might induce ERK activation and $[Ca^{2+}]_i$ increase through its specific pathways. One candidate is a lipoprotein receptor specific to HDL. To see whether a signal pathway specific for HDL is involved in the actions, we examined the effect of plasma lipoproteins other than HDL (Figure 6). LDL and VLDL contain apolipoprotein B and apolipoprotein C as a major apolipoprotein, respectively. Both lipoproteins also stimulated ERK 1/2 activity (Figure 6A) and increased $[Ca^{2+}]_i$ (Figure 6B). Thus, the difference in apolipoproteins may not be reflected in the lipoprotein-induced actions. Charcoal is sometimes used to remove low-molecular-mass compounds that are associated with pro-

Figure 6 Effects of LDL and VLDL in C6 glioma cells

(A) ERK activation. The serum-starved cells were stimulated with 100 μ g/ml LDL, 100 μ g/ml VLDL or vehicle (None) for 2.5 min. The lysates were used for Western blot analysis of ERK 1/2 (upper panel). Data are representative of more than three separate experiments. Effect of charcoal-treated lipoproteins on ERK activity (lower panel). Charcoal-treated lipoproteins were prepared as described in the Experimental section. The cells were stimulated with charcoal-treated or -untreated LDL or VLDL (each at 100 μ g/ml) or vehicle for 2.5 min. The lysates were used for the measurement of ERK1/2 activity. Data are expressed as pmol/min per mg and are the means $\pm$ S.E.M. from three separate experiments. (B) $[Ca^{2+}]_i$ increase. Representative traces of $[Ca^{2+}]_i$ change by 100 μ g/ml LDL and 100 μ g/ml VLDL (upper panel). Effect of charcoal-treated lipoproteins on $[Ca^{2+}]_i$ increase (lower panel). The cells were stimulated with charcoal-treated or -untreated LDL or VLDL (each at 100 μ g/ml). Data are expressed as the difference between peak and basal value.

teins. Actually, we previously reported that activated charcoal adsorbs about 80 % of S1P from the LDL and VLDL particles, but only about 20 % in the case of HDL, which is probably due to the tight binding of the lipid to the HDL particle [28]. The charcoal treatment of LDL and VLDL caused a marked attenuation of the responses to these lipoproteins (lower panels in Figure 6). In the case of HDL, as expected, the charcoal treatment hardly affected the lipoprotein-induced actions (results not shown). Thus, at least in the cases of LDL and VLDL, charcoal-adsorbed components may be important for the induction of ERK activation and the Ca^{2+} response. Taken together, these results suggest that the HDL- and other lipoprotein-induced actions seem not to be mediated through the lipoprotein-specific receptors.

HDL-induced activation of ERK/FGF-2 and PLC/ Ca^{2+} systems may be mediated by S1P receptors

On the basis of studies with sphingosine kinase inhibitors, i.e. DL-threo-dihydrosphingosine and N,N-dimethylsphingosine, it has been proposed that S1P may act on the cells intracellularly to activate ERK [10]. However, these inhibitors at 30 μ M, the concentration usually used to inhibit the enzyme, hardly affected the HDL-induced activation of ERK in C6 cells (results not shown). This is a negative result for the role of S1P as an intracellular signalling molecule, at least for the activation of ERK by HDL.

To assess the primary sites of HDL for the induction of ERK activation and an increase in $[Ca^{2+}]_i$, we have performed

Figure 7 Desensitization of HDL-induced ERK1/2 activation and $[Ca^{2+}]_i$ increase by S1P pre-treatment in C6 cells

(A) Serum-starved cells were incubated with (S1P pre-treatment) or without (control) 3 μ M S1P for 1 h before experiments and then stimulated by vehicle (open columns), 300 μ g/ml HDL (closed columns), 1 μ M S1P (hatched columns) or 1 μ M LPA (shaded columns) for 2.5 min to detect the activity of the enzymes. Data are means $\pm$ S.E.M. from three separate experiments. (B) After pre-treatment with 1 μ M S1P or 1 μ M LPA for 3 min, the cells were stimulated by 100 μ g/ml HDL (closed columns), 1 μ M S1P (hatched columns) and 1 μ M LPA (shaded columns) to monitor $[Ca^{2+}]_i$ change. Data are expressed as the difference between peak and basal value and shown by the means $\pm$ S.E.M. from three separate experiments.

desensitization experiments as shown in Figure 7. HDL- and S1P-induced ERK activation peaked at 2.5 min and gradually decreased to the basal level at 10 min in C6 cells (Figure 4B). A similar time course was observed for LPA (results not shown). Pre-treatment of the cells with S1P, however, led to no significant response to the subsequently applied HDL or S1P, while LPA-induced ERK activation was hardly affected by the S1P pre-treatment (Figure 7A). In the case of the $[Ca^{2+}]_i$ change, the HDL-induced action was abolished after pre-treatment of the cells with S1P for 3 min. In contrast, the LPA-induced Ca^{2+} response was hardly affected by S1P pre-treatment of the cells. As expected, LPA pre-treatment exhibited no significant effect on the $[Ca^{2+}]_i$ change induced by S1P or HDL (Figure 7B). This result suggests that the HDL actions are mediated by S1P receptor-dependent mechanisms.

To further confirm the involvement of S1P receptors in HDL-induced actions, we used C6 cells transfected with S1P receptors [19]. Overexpression of the target S1P receptor is a useful approach for confirming the involvement of S1P receptors. Overexpression of S1P₁ and S1P₂ led to the enhancement of S1P-

induced actions in C6 cells; S1P₁ was more important for the S1P-induced stimulation of the ERK/FGF-2 system, while S1P₂ was responsible for the S1P-induced activation of the PLC/ Ca^{2+} system [19]. If S1P receptors were responsible for the HDL-induced actions, the responses to HDL would also be enhanced in the cells overexpressing these S1P receptors. Indeed, the overexpression of S1P₁ and S1P₂ led to the significant enhancement of HDL-induced ERK activation (Figure 8A) and an increase in $[Ca^{2+}]_i$ (Figure 8C), as was the case for S1P. In contrast, LPA-induced actions were hardly affected. Overexpression of G-protein-coupled receptors might result in a non-specific enhancement of the signalling pathways. However, the overexpression of LPA₁, one of the LPA-specific receptors, led to the significant enhancement of ERK activation induced by LPA but not by HDL and S1P (Figure 8B). Taken together, these results suggest that HDL may act at least partly through cell-surface S1P receptors.

DISCUSSION

In the present study, we showed that plasma HDL stimulated DNA synthesis, the expression of FGF-2 mRNA, ERK activity and Ca^{2+} mobilization, probably through a mechanism involving S1P and the lipid receptors in rat astrocytes or C6 cells. HDL is composed of several apolipoproteins, including apolipoprotein A-I as a major protein, and several lipid components, including cholesterol, phospholipids and S1P [28,29]. In the present study, we employed 0.03–1 mg/ml HDL, which corresponds to 4.5–150 nM S1P based on the S1P content in HDL being roughly 150 pmol/mg of protein [28]. These concentrations of S1P are high enough to stimulate several astroglial cell functions including ERK activation in astrocytes (Figure 2A) and ERK activation (Figure 8), Ca^{2+} mobilization (Figure 8), FGF-2 expression [3] and Egr-1 expression [3] in C6 cells. HDL could be internalized via receptor-mediated endocytosis through the LDL receptor family of lipoprotein receptors or scavenger receptors. Thus, HDL-associated S1P might enter the cells together with the lipoprotein and function as a second messenger for the activation of cellular events. Alternatively, HDL might stimulate sphingosine kinase activity, resulting in the increase in intracellular S1P concentration, although this lipoprotein has been reported to inhibit the enzyme activity in endothelial cells [46]. However, under conditions in which intracellular S1P was accumulated by incubating C6 cells with sphingosine as an S1P precursor, there was no detectable effect on ERK activation, as shown previously [5]. In addition, inhibitors for sphingosine kinase did not appreciably affect the HDL-induced ERK activation in C6 cells (results not shown). Thus, change in the intracellular S1P content, even if it occurred, may not be an important signal for the HDL-induced stimulation of astroglial cell functions. Recent studies suggest that the LDL receptor family of lipoprotein receptors, such as an apolipoprotein E receptor-2 and LDL receptor-related protein, may play a role in ligand-mediated signal transductions [24,25]. Thus, these receptors link to intracellular signalling pathways, resulting in neurite outgrowth or neuronal apoptosis depending on the ligand apolipoprotein E isoforms [26,27]. However, the participation of such lipoprotein receptors may also be ruled out, at least for HDL-induced ERK activation and Ca^{2+} mobilization, because the difference in the apolipoprotein components was not reflected in the lipoprotein-induced actions (Figure 6). Furthermore, fractionation experiments showed that most of the ERK-activating activity was recovered in lipid fractions (Figure 2).

The following observations support the involvement of cell-surface G-protein-coupled S1P-receptors. First, PTX treatment

Figure 8 Enhancement of HDL-induced ERK-activation and $[Ca^{2+}]_i$ increase in $S1P_1$ - or $S1P_2$ -transfected C6 cells

(A) Overexpression of $S1P_1$ leads to an enhancement of ERK activity in response to HDL and S1P. Vector (○)- and $S1P_1$ (●)-transfected cells were stimulated with the indicated concentrations of HDL, S1P or LPA for 2.5 min at 37 °C. The lysates were used for the kinase assay. Results are expressed as percentages of the basal value. The basal value without agonist was 69 ± 6 pmol/min per mg for vector-transfected cells and 78 ± 10 pmol/min per mg for $S1P_1$ cells. Data are the mean $\pm$ S.E.M. from three separate experiments. (B) Effect of LPA₁ overexpression on HDL-, S1P- and LPA-induced ERK activation. Vector (○)- and LPA₁ (■)-transfected cells were used for the stimulation of ERK activity by indicated concentrations of HDL, S1P or LPA for 2.5 min at 37 °C. The basal value obtained in the absence of agonist was 69 ± 19 pmol/min per mg for vector-transfected cells and 79 ± 14 pmol/min per mg for LPA₁ cells. (C) Overexpression of $S1P_2$ leads to an enhancement of Ca^{2+} response to HDL and S1P. Vector (○)- and $S1P_2$ (▲)-transfected cells were used for the detection of $[Ca^{2+}]_i$ change induced by the indicated concentrations of HDL, S1P and LPA. Results are expressed as the difference between peak and basal value. The asterisk indicates statistically significant differences from vector-transfected cells in the respective experiment (* $P < 0.05$).

inhibited several HDL- and S1P-induced actions including DNA synthesis, FGF-2 mRNA expression and ERK activation, suggesting an involvement of G_i/G_o -proteins and hence the G-protein-coupled receptors. Second, ERK activation and the Ca^{2+} increase induced by HDL as well as S1P were lost in the cells where the S1P receptor was homologously desensitized. Third, overexpression of S1P receptors $S1P_1$ and $S1P_2$ caused the enhancement of $S1P_1$ -mediated ERK activation and $S1P_2$ -mediated Ca^{2+} mobilization as induced by HDL and S1P but not by LPA. Thus, we propose that S1P and its receptors may be involved in the HDL-induced activation of astroglial cell functions. It should be noted, however, that there are many kinds of lipid components other than S1P in association with lipoproteins, which may be removed by charcoal. For example, LPA, LPC and PAF are usually present in LDL at a low level, but their concentration increases in response to oxidative stress [41–43], while S1P content decreases during oxidation [29,30]. Therefore, we could not rule out the possibility of the involvement of LPA or other lipid mediators in the activation of ERK and $[Ca^{2+}]_i$ increase by LDL and VLDL, whereas the involvement of these lipids may be negligible in the case of HDL actions. Actually, LPA can induce ERK activation (Figure 2) and $[Ca^{2+}]_i$ increase (Figures 7 and 8) and may be removed by activated charcoal (Figure 6).

Five specific receptors for S1P, $S1P_1$ – $S1P_5$, have been identified so far. $S1P_1$, $S1P_2$ and $S1P_3$ seem to be expressed in several tissues, whereas others are expressed in specific tissues or cell types, i.e. $S1P_5$ in the adult brain and $S1P_4$ in lymphoid cells. As indicated [4], $S1P_1$ and $S1P_3$ are expressed in mouse astrocytes. In rat astrocytes, we also detected $S1P_2$ and $S1P_5$ in addition to $S1P_1$ and $S1P_3$ by Northern blot analysis. In C6 cells, $S1P_2$ was detected as a major band and $S1P_1$ and $S1P_5$ as minor bands (Figure 3). We speculated that $S1P_1$ is more important than $S1P_2$ for the stimulation of the ERK/FGF-2 system, while $S1P_2$ may be responsible for the activation of the PLC/ Ca^{2+} system [19]. In any event, the expression pattern of S1P receptors, even within astroglial cell types, seems to be different. However, $S1P_2$ and $S1P_3$ seem to couple to similar types of G-proteins [14] and signalling pathways [13]. Thus, $S1P_2$ and $S1P_3$ might share the same signalling pathways, i.e. the stimulation of the PLC/ Ca^{2+} system, and $S1P_1$ might be responsible for the G_i/G_o -protein-mediated stimulation of the ERK/FGF-2 system in rat astrocytes. The role of $S1P_5$, however, remains uncharacterized.

In the nervous system, FGF-2 is synthesized by astroglial cells and influences the proliferation and differentiation of the same cells as an autocrine factor [20,21]. The expression of the growth factor mRNA was induced by S1P in astrocytes and C6 glioma cells [3], although we have not succeeded in detecting the

expression of FGF-2 itself. Thus, S1P may act on astroglial cells in an indirect manner through the expression of FGF-2. S1P may also act on astroglial cells in a direct manner, resulting in the stimulation of proliferation [4] and induction of morphological change [2]. In the present study, we also showed that plasma HDL as well as S1P stimulates proliferation and FGF-2 mRNA expression in astrocytes (Figure 1). Thus, HDL and S1P may indirectly and directly act on astroglial cells and thereby activate cell functions.

One of the major roles of lipoproteins is the transport of cholesterol from cell to cell [32–34]. However, recent experimental evidence suggests that actions other than cholesterol transport may also be involved in maintaining neural cell functions by lipoproteins [24–27]. The present results suggest that S1P as a lipoprotein-associated lipid component may mediate some lipoprotein-induced actions, which are not directly related to the cholesterol metabolism in neural cells. Although it has not been demonstrated, we can readily speculate that lipoprotein particles present in the CNS contain S1P. In plasma lipoproteins, S1P is accumulated in not only HDL but also LDL and VLDL more than in lipoprotein-deficient plasma fractions, which contain mainly albumin [28]. Thus, the interaction of S1P with lipoprotein particles does not seem to be directly relevant to the nature of the apolipoprotein. Brain is one tissue in which S1P is abundantly present at a level of about 4 nmol/g of wet weight [47], which corresponds roughly to 4 μ M, if S1P is evenly distributed throughout the CNS. Thus, S1P in the CNS may interact with apolipoproteins or lipoproteins, which are newly synthesized in astrocytes [48–50] or transcytosed across the blood/brain barrier from plasma [35,36]. In any event, it will be necessary to demonstrate the presence of the lipoprotein-associated S1P at a practical level in the CNS to further characterize the role of lipoproteins as carriers for extracellular S1P in the CNS under normal or physiological conditions. Nevertheless, if the integrity of the blood/brain barrier is destroyed by traumatic injury, haemorrhage or neuronal diseases, plasma lipoproteins could enter the CNS and act directly on neural cells including astrocytes and neurons. Under such pathological conditions, S1P might mediate some lipoprotein-induced actions, i.e. activation of astrocytes, through the lipid receptors, as demonstrated in the present study.

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