Mechanism for FGF-1 to regulate biogenesis of apoE-HDL in astrocytes

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Abstract Fibroblast growth factor-1 (FGF-1) is secreted by astrocytes and stimulates apolipoprotein E (apoE)-HDL biogenesis by an autocrine mechanism to help in recovery from brain injury. In apoE-deficient mouse astrocytes, FGF-1 stimulated cholesterol biosynthesis without enhancing its release, indicating a signaling pathway independent of apoE biosynthesis upregulation. SU5402, an inhibitor of FGF receptor, inhibited FGF-1-induced phosphorylation of MEK, ERK, and Akt, as well as all the apoE-HDL biogenesis-related events in rat astrocytes. LY294002, an inhibitor of phosphatidylinositide 3-OH kinase (PI3K) and of Akt phosphorylation, inhibited apoE-HDL secretion but not cholesterol biosynthesis, whereas U0126, an inhibitor of MEK and of ERK phosphorylation, inhibited cholesterol biosynthesis but not apoE-HDL secretion. Increase of apoE-mRNA by FGF-1 was not influenced by either inhibitor. When rat apoE/pcDNA3. his was transfected to transformed rat astrocyte GA-1 cells that otherwise do not synthesize apoE (GA-1/25), FGF-1 did not influence apoE-mRNA, but did increase the apoE secretion and Akt phosphorylation that were suppressed by LY294002. Lipid biosynthesis was increased by FGF-1 in GA-1/25 cells and suppressed by U0126. FGF-1 upregulates apoE-HDL biogenesis by three independent signaling pathways. The PI3K/Akt pathway upregulates secretion of apoE/apoE-HDL, the MEK/ERK pathway stimulates cholesterol biosynthesis, and an unknown pathway enhances apoE transcription.—Ito, J-i., Y. Nagayasu, K. Okumura-Noji, R. Lu, T. Nishida, Y. Miura, K. Asai, A. Kheirollah, S. Nakaya, and S. Yokoyama. **Mechanism for FGF-1 to regulate biogenesis of apoE-HDL in astrocytes. *J. Lipid Res.* 2007.** 48: **2020–2027.**

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Neuronal cells in the central nervous system (CNS), such as neurons, oligodendroglias, and astrocytes, all appear in unique shapes, with large cell surfaces, and accordingly contain large amounts of cholesterol. Cholesterol content in the CNS could thus account for 25–30% of total body cholesterol in humans (1, 2). Cholesterol plays many key roles in the CNS, including roles in neurite outgrowth (3, 4) and synapse formation (5).

Cholesterol homeostasis in animals is maintained by intra- and extracellular regulation of its metabolism (6), and extracellular transport of cholesterol in vertebrates is carried by the plasma lipoprotein system. However, the blood-brain barrier prevents the CNS from accessing this system, so the CNS operates as a unique and independent CNS-specific lipoprotein system for extracellular cholesterol transport. HDL is a lipoprotein found exclusively in cerebrospinal fluid that contains mainly apolipoprotein E (apoE) and apoA-I (7). Although apoA-I is not synthesized by neural cells, and its origin is uncertain (8, 9), apoE is known to be synthesized, at least in astrocytes and microglias, to generate apoE-HDL (10, 11). Many reports suggest that apoE-HDL is a key lipoprotein in the delivery of cholesterol to the neural cells, and this lipoprotein seems to increase in the lesions of injury of brain and nerves (12–21).

We reported that astrocytes produce cholesterol-rich HDL with endogenously synthesized apoE and cellular lipid, and that they generate cholesterol-poor HDL with exogenous apoA-I (22). Astrocytes synthesize and release fibroblast growth factor-1 (FGF-1), and it stimulates the cells in an autocrine manner to produce apoE-HDL when the cells are cultured for longer times, such as 1 month primary and 1 week secondary culture (23, 24). FGF-1 is produced prior to apoE production in the astrocytes in the peri-injury regions after cryo-injury of mouse brain, and healing of the injury was much delayed in the apoE-deficient mice, although FGF-1 appeared at the same post-

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Abbreviations: apoE, apolipoprotein E; CNS, central nervous system; DPBS, Dulbecco's phosphate-buffered saline; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FGF-1, fibroblast growth factor-1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositide 3-OH kinase; PKBα, protein kinase-Bα.

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injury time (25). On the basis of these findings, we propose that FGF-1 is a key factor in triggering the recovery process in brain injury by stimulating production of apoE-HDL by an autocrine/paracrine mechanism.

FGF-1 enhances transcription of apoE, cholesterol biosynthesis, and secretion of apoE and cholesterol in its stimulation of apoE-HDL biogenesis in astrocytes. We investigated the mechanism through which FGF-1 stimulates each component reaction for apoE-HDL biogenesis by focusing on intracellular signal transduction pathways in astrocytes. FGF-1 was shown to initiate differential signal transductions for stimulation of cholesterol biosynthesis, apoE transcription, and a posttranscriptional stage for apoE secretion.

MATERIALS AND METHODS

Reagents

LY294002, an inhibitor of phosphatidylinositide 3-OH kinase (PI3K) and U0126, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), were purchased from Calbiochem. Inhibitors of FGF receptor (SU5402, SU4984), vascular endothelial growth factor receptor (SU5614), epidermal growth factor receptor (AG490), and platelet-derived growth factor receptor (AG17) (26–29) were also obtained from Calbiochem.

Cell culture

Astrocytes were prepared from 17 day fetal brain of Wistar rats, C57BL/6 wild-type mice, and apoE-deficient C57BL/6 mice according to the method previously described (30). After 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) for rat astrocytes or 10% FCS/DMEM for mouse astrocytes at 37°C for 1 week as primary culture. After treatment with 0.1% trypsin/DPBS/G containing 1 mM EDTA, the cells were transferred to a fresh plate and cultured in 10% FCS/F-10 or 10% FCS/DMEM for 1 week as secondary culture. Transformed rat astrocyte cell line GA-1 cells (31) were cultured in 10% FCS/F-10.

Construction of expression vectors for apoE and its transfection

Total RNA was extracted from rat astrocyte primary cultures, and the first-strand cDNA was prepared by SuperScript II reverse transcriptase and random hexamer primers (Gibco BRL). The apoE cDNA of rat astrocytes was amplified by PCR with the primers: sense, 5′-TGGGAAGATGAAGGCTCTGT-3′, and antisense, 5′-GGCGTAGGTGAGGGATGAT-3′ designed from the sequence of rat apoE mRNA (32), J700705, residues at 17–36 for sense and at 959–977 for antisense primer, by using *pfu* DNA polymerase and Taq polymerase. The products were cloned into pGEM-T Easy vector (Promega). We confirmed the insert DNA encoding APE_RAT (apoE precursor) by GenBank database analysis. After digestion by *Eco*RI (Takara) and electrophoresis in 1% agarose gel, the insert DNA was extracted and cloned into mammalian expression vector pcDNA3.1His (Invitrogen). The expression vector and control mock vector were transfected to GA-1 cells, transformed rat astrocytes that produce no apoE,

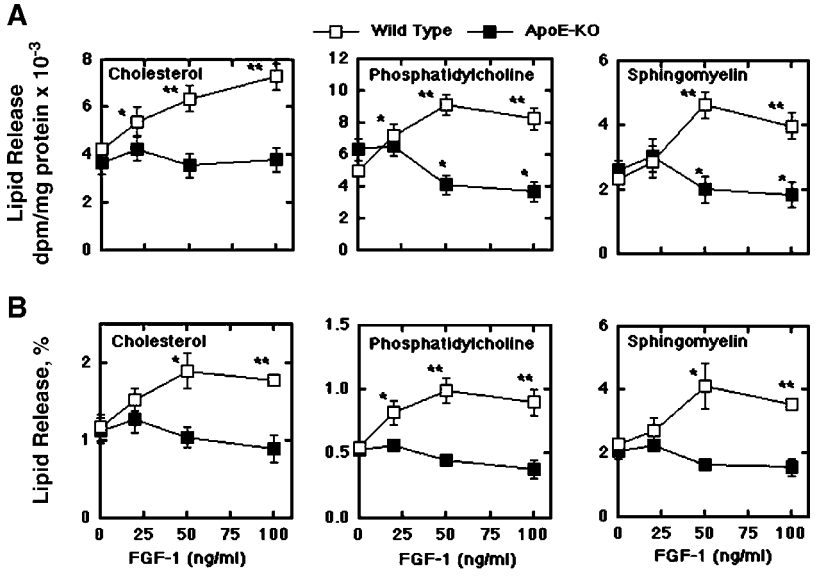

Fig. 1. Effect of fibroblast growth factor-1 (FGF-1) on cholesterol release from wild-type and apolipoprotein E (apoE)-deficient mouse astrocytes. After washing with Dulbecco's phosphate-buffered saline (DPBS) three times and replacement with 0.1% BSA/DMEM, astrocytes prepared from wild-type (open symbols) and apoE-deficient (closed symbols) mice were stimulated with FGF-1 for 24 h and washed with DPBS two times. The cells were incubated with [^{3}H]acetate (20 μCi/ml) for 18 h in 0.02% BSA/DMEM, washed, and incubated in fresh 0.02% BSA/DMEM for 7 h. Lipid was extracted from the cells and the conditioned medium and analyzed by TLC for determination of radioactivity in cholesterol, phosphatidylcholine, and sphingomyelin. The results of cellular lipid release were expressed as radioactivity per cell protein (A) and as a percentage of cellular lipid (B). Data represent the average and standard error for triplicate samples. Significance of change from the non-FGF-1 control is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

by using the low-toxicity polyamine transfection reagent, *Trans*IT-LT (Mirus Corp.). Transfected cells were selected by culturing in 10% FCS/F-10 containing 800 μg/ml aminoglycoside antibiotics, G-418. The most highly apoE-positive clone was selected as the GA-1/25 cell. Mock vector (pcDNA3.1His)-transfected GA-1 cells were used as control GA-1 cells, and lack of apoE expression was confirmed for the mRNA and protein.

Preparation of cytosol and membrane fractions

Cytosol and membrane fractions of rat astrocytes and GA-1/25 cells were prepared according to the method previously described (33). Briefly, cell pellet was obtained by centrifugation at 1,000 rpm for 10 min after washing with DPBS four times and harvesting with a rubber policeman. The cell pellet was treated with cold hypotonic buffer, 0.02 M Tris-HCl, pH 7.5, containing a protease inhibitor cocktail (Sigma), for 15 min with strong Vortex mixing 25 times for 10 s every 5 min. The cell suspension was centrifuged at 5,000 rpm (1,000 *g*) for 20 min for preparation of denuclear supernatant fraction, and it was further centrifuged at 90 krpm (367,000 *g*) for 30 min at 4°C in a Hitachi S100AT6 rotor to obtain the cytosol as a supernatant and the membrane fraction as a pellet (34).

Analysis of proteins by Western blotting

The conditioned medium was treated with 10% TCA and centrifuged at 15,000 rpm for 20 min to obtain protein pellet after removal of cell debris by centrifugation at 15,000 rpm for 30 min. Proteins in the cell or in the conditioned medium were separated in 0.5% sodium dodecylsulfate/12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Sequi-BlotTM PDVF membrane (Bio Rad). The membrane was immunostained with specific antibodies for proteins to be analyzed: rabbit antibodies against MEK1/2 and phospho-MEK 1/2 (Ser 217/221, P-MEK) (Cell Signaling); rabbit antibodies against p44/42 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and phospho-p44/42 MAPK (Thr202/Tyr204, P-ERK) (Cell Signaling); mouse antibody against PKBα (Akt) (BD Transduction); rabbit antibody against phospho-Akt (Thr308, P-Akt) (Cell Signaling); rabbit antibody against Raf-1 (BioVision); mouse antibody against phospho-Raf-1 (ser338) (Upstate); and rabbit antibody against rat apoE (a generous gift from Dr. Jean Vance, University of Alberta). Immunoreactive bands were visualized as previously described and semiquantified by digital scanning using Adobe Photoshop software (23). Data demonstrated represent a typical set out of several experiments repeated in each series.

RT-PCR

Total cellular RNA was extracted from rat astrocytes and GA-1/25 cells with ISOGEN (Wako Life Science), and reverse transcribed to cDNA using a Super Script Preamplification System (Gibco BRL). The resulting cDNA was subjected to PCR by using the DNA primers for rat apoE-mRNA, as described in a previous paper (23). After the electrophoresis of the products, agarose gel was stained with freshly prepared SYBR Gold nucleic acid gel stain solution. Bands were detected by using an ultraviolet transilluminator (UVP NLM-20E) at 302 nm. The apoE primer pairs were 5′-AAGCCCGTCGGTGTCCATGG as sense and 5′-GATGGCACAGTGGATGGGAC as antisense, and the ABCA1 primer pairs were 5′-CATCGGCATCAATGGTAGTG -3′ and 5′-CCCAAGAGAGTGGAGAGACG -3′ (24).

Biosyntheses and secretion of cholesterol and phospholipid

Procedures for analysis of cellular lipid metabolism were described in our previous publication (24). After washing with DPBS four times, rat astrocytes or rat GA-1/25 cells at the con-

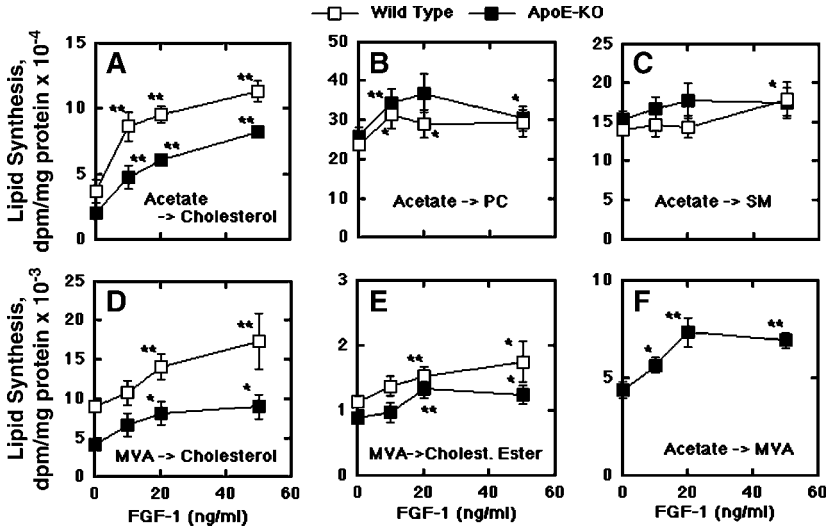

Fig. 2. Effect of FGF-1 on lipid biosynthesis in astrocytes. Astrocytes prepared from wild-type and apoE-deficient mice were stimulated with FGF-1 in 0.1% BSA/DMEM for 24 h and then washed. The cells were incubated with [^{3}H]acetate (20 μCi/ml) (A–C) for 2 h or [^{3}H]mevalonate (MVA) (4 μCi/ml) (D, E) for 4 h in 0.02% BSA/DMEM. After washing, lipid was extracted from the cells and analyzed by TLC for cholesterol, phosphatidylcholine (PC), sphingomyelin (SM), and cholesteryl ester. To analyze mevalonate (MVA) synthesis, the apoE-deficient astrocytes were incubated with 500 μl/well of 0.2 N HCl at 37°C for 15 min after incubation with FGF-1 for 24 h and then [^{3}H]acetate (20 μCi/ml) for 2 h (F). Radioactivity of mevalonolactone was determined after lipid extraction from the cell suspension and separation by TLC. Data represent the average and standard error of the triplicate samples. Significance of change from the non-FGF-1 control is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

fluent cell density were incubated for 24 h in 0.1% BSA/F-10, or mouse astrocytes in 0.1% BSA/DMEM. To measure secretion of cholesterol and phospholipid, the cells were incubated with [^{3}H]acetate (20 μCi/ml; New England Nuclear) or [^{14}C]acetate (2 μCi/ml; New England Nuclear) in fresh 0.02% BSA/F-10 or 0.02% BSA/DMEM for the indicated times (usually 16 h), washed three times with cold DPBS, and incubated in fresh 0.02% BSA/F-10 (or DMEM) for the indicated times (4–6 h). Lipid was extracted from the cells and conditioned medium with hexane-isopropanol (3:2; v/v) and chloroform-methanol (2:1; v/v), respectively, and analyzed by TLC on Silica Gel-60 plates (Merck). The radioactivity in cholesterol, phosphatidylcholine, and sphingomyelin was determined with a liquid scintillation counter. To measure the rate of lipid biosynthesis, the cells were incubated with [^{3}H]acetate for 2 h. Lipid was extracted and analyzed by TLC for radioactivity in cholesterol, phosphatidylcholine, and sphingomyelin. Alternatively, cholesterol synthesis was measured by using [^{3}H]mevalonolactone (5 μCi/ml in 100 μM mevalonolactone; Wako Chemicals). To measure the rate of mevalonic acid synthesis, the cells were incubated with [^{3}H]acetate, rinsed three times with cold DPBS, and incubated with 0.25 N HCl at 37°C for 15 min to allow lactonization of the [^{3}H]mevalonic acid produced during the incubation. The cells were scraped with a rubber policeman and sonicated. Radioactivity in mevalonolactone was analyzed by TLC after lipid extraction from the cell suspension with chloroform-methanol (2:1; v/v). Cellular protein was measured by the Lowry method using BSA as standard for standardization of the lipid data.

RESULTS

FGF-1 enhanced release of cholesterol and choline-phospholipid from mouse astrocytes, whereas it failed to increase the lipid release from apoE-deficient mouse astrocytes (**Fig. 1**). In contrast, FGF-1 enhanced cholesterol biosynthesis as well as phospholipid biosynthesis from acetate, in both wild-type and apoE-deficient mouse astrocytes (**Fig. 2**). Cholesterol biosynthesis was enhanced at the steps both prior to and after mevalonic acid biosynthesis (Fig. 2). The results suggest that upregulation of lipid biosynthesis is independent of apoE-HDL biogenesis but that the increase of lipid release by FGF-1 is dependent on production of apoE.

Signaling pathways by FGF-1 stimulation were investigated by using rat astrocytes. **Figure 3A** demonstrates phosphorylation of the signal proteins MEK, ERK, and Akt by incubation of the cells with FGF-1. Figure 3B shows that

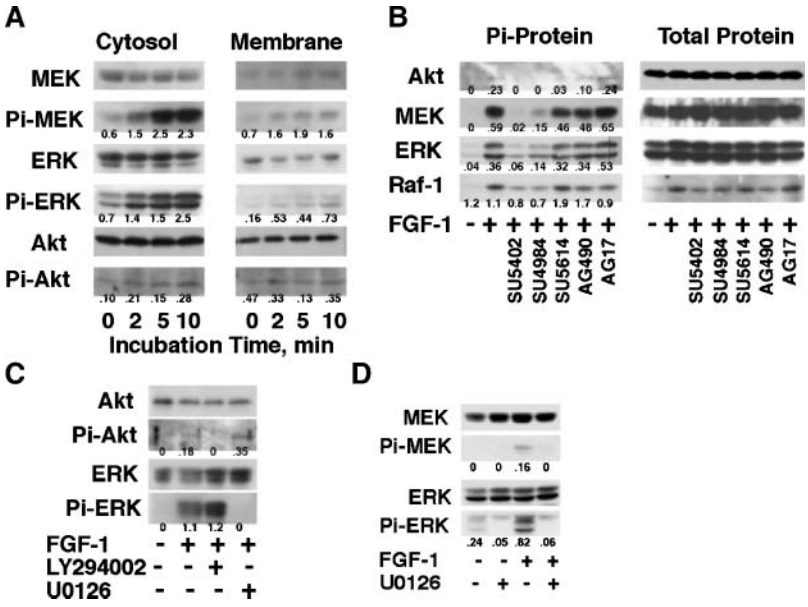

Fig. 3. Intracellular signal production induced by FGF-1 in rat astrocytes. A: Phosphorylation of signal proteins by FGF-1. Rat astrocytes were stimulated with 50 ng/ml of FGF-1 in 0.02% BSA/F-10 for the indicated time. The cytosol fraction and membrane fraction were prepared from the cells as described in Materials and Methods. After precipitation with 10% TCA, the protein of both fractions (70 μg each) was analyzed by immunoblotting for MEK, phospho (Pi)-MEK, ERK, Pi-ERK, Akt, and Pi-Akt by using their respective specific antibodies. B: Inhibition of the FGF-1-induced reactions by the FGF receptor inhibitors. The cells were stimulated with FGF-1 for 5 min as above after pretreatment with the inhibitors of FGF-1 receptor (SU5402, SU4984), vascular endothelial growth factor (SU5614), epidermal growth factor (AG490), and platelet-derived growth factor (AG17) (10 μM, respectively) for 1 h. The whole cells (100 μg proteins/lane) were applied on SDS-PAGE and analyzed by Western blotting for MEK, Pi-MEK, ERK, Pi-ERK, Akt, and Pi-Akt. C, D: Inhibition of phosphorylation of signal proteins by specific signal inhibitors. Rat astrocytes were pretreated with 10 μM LY294002, a phosphatidylinositide 3-OH kinase (PI3K) inhibitor, or 10 μM U0126, an MEK inhibitor, for 1 h in 0.1% BSA/F-10, followed by incubation with FGF-1 (0 or 50 ng/ml) for 5 min. After washing with DPBS, the cytosol fraction (70 μg protein) was prepared and analyzed by immunoblotting for Akt, Pi-Akt, ERK, Pi-ERK, MEK, and Pi-MEK. Numbers indicate relative phosphorylation of each protein in arbitrary units based on digital scanning of bands.

phosphorylation of all these proteins was inhibited by the inhibitors of the FGF receptors (SU5402 and SU4984) but not by those of other receptors (SU5614, AG490, and AG17), showing that these reactions are mediated by FGF-1 and its receptors. Akt phosphorylation by FGF-1 was selectively inhibited by a PI3K inhibitor, LY294002, and phosphorylation of MEK and ERK was inhibited by an MEK inhibitor, U0126 (Fig. 3C, D). The receptor inhibitor SU5402 inhibited the FGF-1-induced reactions for apoE-HDL biogenesis, such as increase of cholesterol release and induction of cholesterol biogenesis (**Fig. 4**), showing that these reactions are all dependent on the FGF-1 receptors. Cells could not tolerate long-term exposure to the receptor inhibitor, so apoE secretion was unable to be examined because it requires long-term incubation.

A PI3K inhibitor, LY294002, but not the MEK inhibitor, U0126, inhibited the increase by FGF-1 of apoE secretion from astrocytes, as 1.0 and 2.9 times increase, respectively, in comparison to 5.6 times increase in control, based on quantification of immunoreactive bands (24). Being consistent with this result, increase of cellular cholesterol release by FGF-1 was inhibited by LY294002 but not by U0126 (**Fig. 5A**). In contrast, stimulation of cholesterol biosynthesis by FGF-1 was canceled by U0126 but not by LY294002 (Fig. 5B).

The effects of FGF-1 on apoE expression and its secretion were further examined in detail. Rat apoE was transfected with a vector promoter in the form of apoE/pcDNA3.his into rat astrocyte cell line GA-1 cells that otherwise do not produce apoE (GA-1/25). The effect of FGF-1 on this cell was examined for apoE biosynthesis

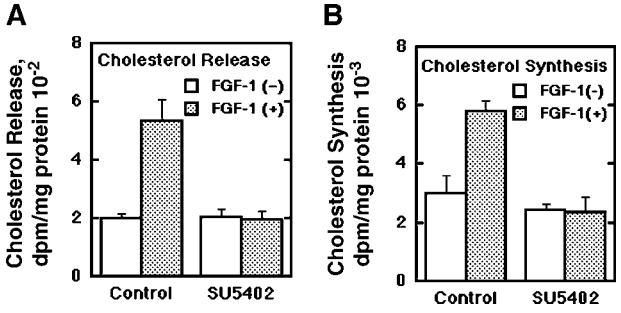

Fig. 4. Effect of the FGF receptor inhibitors on the FGF-1-induced cellular events. A: Cholesterol release. The cells were incubated with [^{14}C]acetate (4 μCi/ml) for 16 h in the presence or absence of FGF-1 (50 ng/ml) and SU5402 (5 μM) in 0.1% BSA/DMEM after washing with DPBS three times and replacement with 0.1% BSA/DMEM. After washing with DPBS, the cells were further incubated for 6 h in 0.02% BSA/F-10. Lipid was extracted from the conditioned medium and separated by TLC to determine radioactivity in cholesterol. B: Cholesterol biosynthesis. The cells were stimulated with FGF-1 (0 or 50 ng/ml) for 24 h in the presence or absence of SU5402 (5 μM). After washing with DPBS, the cells were incubated with [^{14}C]acetate (4 μCi/ml) in fresh 0.02% BSA/F-10 for 2 h. Lipid was extracted from the cells and separated by TLC to determine radioactivity in cholesterol. Data represent the average and standard error of the triplicate samples. Significance of change by FGF-1 is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

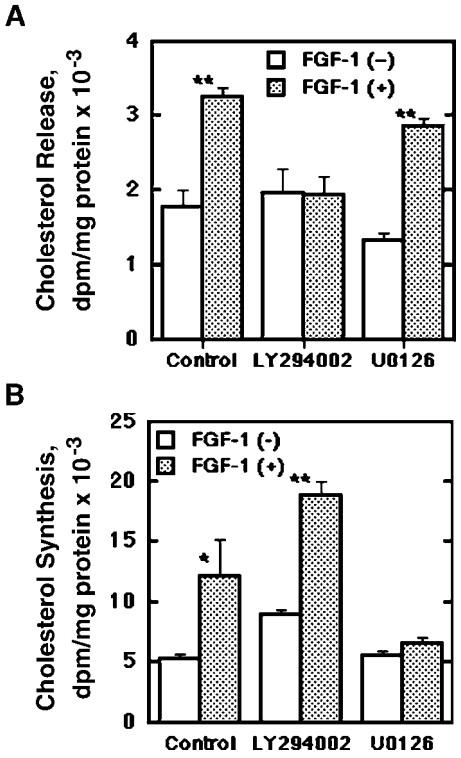

Fig. 5. Effect of specific signal inhibitors on the FGF-1-induced cellular events. A: Cholesterol release. Rat astrocytes were stimulated with or without FGF-1 (50 ng/ml) for 24 h in the presence of [^{14}C]acetate (3 μCi/ml) in 0.1% BSA/F-10. After washing and the replacement with fresh 0.02% BSA/F-10, the cells were incubated with or without 10 μM LY294002 or 10 μM U0126 for 5 h. Lipid was extracted from the conditioned medium and analyzed by TLC for determination of cholesterol radioactivity. B: Cholesterol biosynthesis. The cells were stimulated with FGF-1 (0 or 50 ng/ml) for 24 h in the presence or absence of LY294002 or U0126. After washing with DPBS, the cells were incubated with [^{14}C]acetate (4 μCi/ml) in fresh 0.02% BSA/F-10 for 2 h. Lipid was extracted from the cells and separated by TLC to determine radioactivity in cholesterol. Data represent the average and standard error of the triplicate samples. Significance of change by FGF-1 is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

and secretion. **Figure 6A** demonstrates phosphorylation of Akt, MEK, and ERK by FGF-1 and inhibition of Akt phosphorylation by LY294002, showing that FGF-1 receptor and subsequent signaling pathways normally function in this cell line. Figure 6B shows that FGF-1 increases apoE mRNA in rat astrocytes, as well as TO901317, an agonist of LXR. In contrast, neither of these compounds influenced the apoE mRNA level in GA-1/25 cells as expected. The increase of mRNA by FGF-1 in astrocytes was not influenced by LY294002 or U0126, indicating that signaling to upregulate apoE transcription is independent of either the P13K/Akt or the MEK/ERK pathway (Fig. 6C). Interestingly, apoE secretion was increased by FGF-1 and reversed by LY29002, despite of the lack of effect of FGF-1 and LY29004 on the apoE transcription step (Fig. 6D). Because the change of apoE level in the medium and cell is reciprocal, the effect of FGF-1 seems to be on the apoE transport/secretion rather than its translation. Consistent

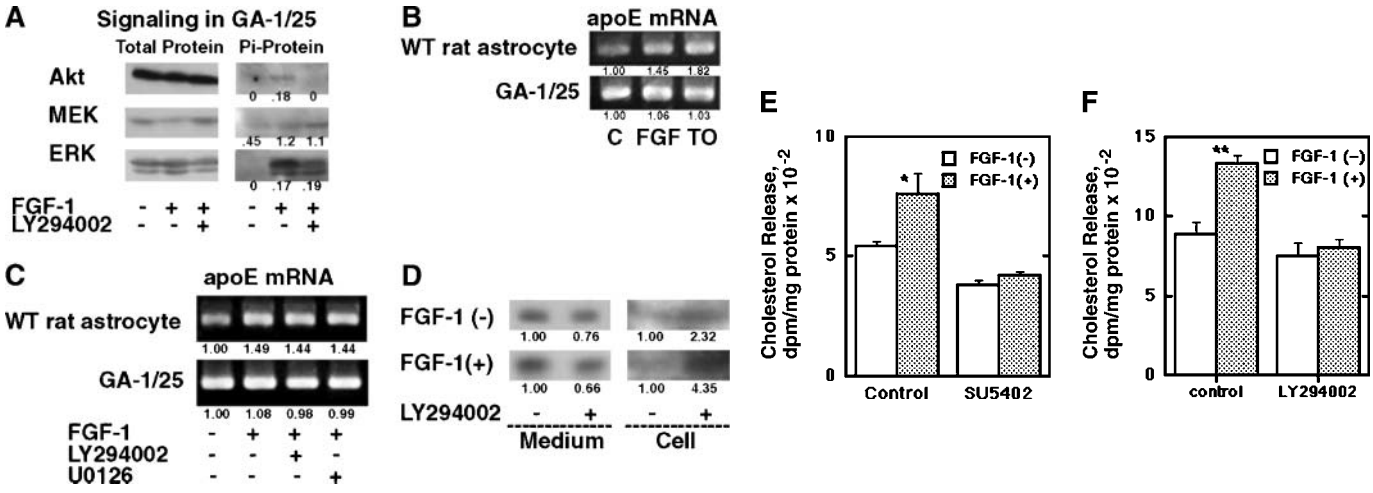

Fig. 6. Effect of FGF-1 on secretion of apoE that is transfected. A: Rat apoE/pcDNA3.his was transfected to rat astrocyte cell line GA-1 cells as described in the text (GA-1/25 cells). GA-1/25 cells were stimulated with FGF-1 (50 ng/ml) for 5 min in 0.02% BSA/F-10 after pretreatment with or without 10 μM LY294002 for 1 h. The cytosol protein (100 μg/lane) was analyzed by immunoblotting for Akt, phospho (Pi)-Akt, MEK, Pi-MEK, ERK, and Pi-ERK after precipitation with 10% TCA treatment. Numbers indicate relative phopshorylation of each protein in arbitrary units based on digital scanning of the bands. B: Analysis of apoE mRNA in GA-1/25 cells. Total RNA (3 μg) was prepared from wild-type rat astrocytes and from GA-1/25 cells after stimulation of the cells with FGF-1 (50 ng/ml) or TO901317 (10 μM) for 16 h, and apoE mRNA was analyzed by RT-PCR as described in the text. Numbers indicate relative increase of mRNA by FGF-1 based on digital scanning of bands. Numbers indicate relative increase by FGF-1 or by TO901317. C: Effect of signal inhibition on the apoE mRNA expression by FGF-1. Rat astrocytes and GA-1/25 cells were incubated with FGF-1 (50 ng/ml) for 16 h in the presence or absence of LY294002 (10 μM) or U0126 (10 μM) in 0.1% BSA/F-10. ApoE-mRNA was analyzed by RT-PCR as described in the text. Numbers indicate relative increase by FGF-1. D: GA-1/25 cells were stimulated with FGF-1 (50 ng/ml) in 0.02% BSA/F-10 for 24 h and washed with DPBS, after washing and incubation in 0.1% BSA/F-10 for 24 h. The cells were incubated with or without 10 μM LY294002 in fresh 0.02% BSA/F-10 for 8 h. The apoE in conditioned medium and in the cells (100 μg cell proteins) was analyzed by immunoblotting by using rabbit anti-apoE antibody. Numbers indicate relative change by LY294002. E, F: Effect of FGF-1 on cell cholesterol release and its inhibition by signaling inhibitors in GA-1/25 cells. The cells were incubated with FGF-1 (50 ng/ml) in the presence of SU5402 (10 μM) (E) or LY294002 (10 μM) (F) in the presence of [^{14}C]acetate (3 μCi/ml) in 0.1% BSA/F-10 for 16 h. After washing, the cells were incubated in fresh 0.02% BSA/F-10 for 6 h. The lipid was extracted from the conditioned medium and analyzed by TLC for the radioactivity of cholesterol after the removal of cell debris. Data represent the average and standard error of the triplicate samples. Significance of change by FGF-1 is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

with these results, both the FGF-1 receptor inhibitor and the P13K inhibitor inhibited the increase of cellular cholesterol release by FGF-1 in GA-1/25 cells (Fig. 6E, F).

It was also shown in GA-1/25 cells that inhibition of an MEK/ERK pathway resulted in suppression of the FGF-1-induced lipid biosynthesis (**Fig. 7**), again showing differential signal transduction for the apoE/HDL release by a P13K/Akt pathway and lipid biosynthesis by an MEK/ERK pathway.

DISCUSSION

FGF-1 is a stimulant for proliferation and cell differentiation of mesodermal and neuroectodermal cells such as fibroblasts and astrocytes (35). Most studies of this factor have focused on its activity in promoting cell growth and differentiation. We recently reported that FGF-1 is produced by long-cultured rat astrocytes and stimulates syntheses and secretion of apoE-HDL, perhaps by an autocrine mechanism (24). We here characterized the FGF-1 function to stimulate the apoE-HDL secretion in astrocytes with respect to differential signal transduction pathways for various component reactions of apoE-HDL biogenesis. The results are summarized as follows: *1*) FGF-1 enhances the reactions for apoE-HDL biogenesis via the FGF-1 receptors; *2*) FGF-1 stimulates cholesterol biosynthesis independent of apoE production; *3*) a PI3K inhibitor, LY294002, inhibited the FGF-1-stimulated release of cholesterol and apoE without influencing apoE mRNA expression, and an MEK inhibitor, U0126, inhibited cholesterol biosynthesis stimulated by FGF-1. Thus, we conclude that FGF-1 upregulates apoE-HDL biogenesis by using at least three independent signaling pathways, a P13K/Akt pathway for transport/secretion of apoE, an MEK/ERK pathway for cholesterol and lipid biosynthesis, and perhaps an independent pathway for apoE transcription. All of these pathways are probably initiated by the interaction of FGF-1 with its receptors. The pathway to stimulate transcription of the apoE gene is being investigated in detail by using reporter genes.

The FGF-1-initiated pathways should be important reactions for astrocytes to generate apoE-HDL in response to brain injury for recovery. This hypothesis is supported by our own findings that the healing of cryo-injury is substantially delayed in the apoE-deficient mouse brain, and production of FGF-1 in astrocytes in the peri-injury area was detected prior to the production of apoE in these cells (25). FGF-1 production was shown in the apoE-deficient

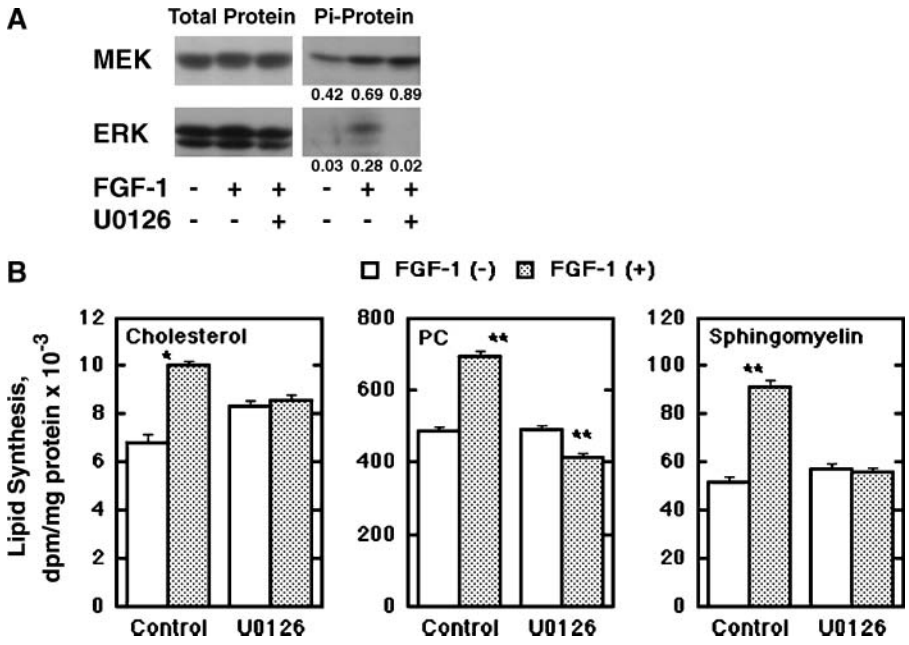

Fig. 7. Inhibition of MEK cancels the FGF-1-induced increase of lipid biosynthesis in GA-1/25 cells. A: Phosphorylation of signal proteins. The cells were incubated with or without U0126 (10 μM) for 55 min and then stimulated with FGF-1 for 5 min in 0.02% BSA/F-10. The cytosol protein (80 μg) was analyzed by immunoblotting for MEK, phospho (Pi)-MEK, ERK, and Pi-ERK after precipitation with 10% TCA. Numbers indicate relative phosphorylation of each protein. B: Lipid synthesis. GA-1/25 cells were stimulated with FGF-1 (50 ng/ml) in 0.1% BSA/F-10 for 3 h after pretreatment with U0126 (10 μM) for 1 h. After washing and replacement with 0.02% BSA/F-10, the cells were incubated with [^{3}H]acetate (20 μCi/ml) for 2 h. The lipid was extracted from the cells and analyzed by TLC after washing the cells. Significance of change by FGF-1 is indicated by * as $P < 0.05$ and ** as $P < 0.01$.

astrocytes at the same timing as the wild-type mouse brain after the brain injury (25). There is also a report that the FGF-1-induced signal to activate protein kinase B/Akt contributes to neuroprotection by acting against glutamate toxicity through suppression of glycogen synthase kinase-3β activity (36).

Independent regulation of cholesterol and lipid biosynthesis by FGF-1 through an MEK/ERK pathway may be related to a more general function of FGF-1. FGF-1 stimulates the cell division of astrocytes, and ERK/MAP kinase regulates the cell cycle of astrocytes (37). Therefore, the increase by FGF-1 of cholesterol biosynthesis may be more directly related to cell proliferation.

Identification of the trigger for astrocytes to produce and release FGF-1 is under investigation, especially in relation to brain injury. The FGF-1-transfected cells release FGF-1 under heat shock conditions (38). Thus, astrocytes may cause the increase of production and release of FGF-1 under a certain stress. The cellular environment in long-term culture in vitro may be one of these conditions. Brain injury could cause various types of stress around the lesions, such as hypoxia, oxidative stress, or other conditions that may trigger production of FGF-1 in astrocytes. It is important to identify specific stress conditions under which astrocytes will react in such a manner to establish the pathophysiological relevance of these reactions. Understanding the details of the mechanism for triggering apoE-HDL biogenesis in brain damage will lead to the development of technology to assist the healing process in brain injury, either acute or chronic, such as cerebral infarction and Alzheimer's disease.

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REFERENCES

1. Dietschy, J. M., and S. D. Turley. 2001. Cholesterol metabolism in the brain. *Curr. Opin. Lipidol.* **12:** 105–112.
2. Dietschy, J. M., and D. S. Turley. 2004. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J. Lipid Res.* **45:** 1375–1395.
3. Posse De Chaves, E. I., D. E. Vance, R. B. Campenot, R. S. Kiss, and J. E. Vance. 2000. Uptake of lipoproteins for axonal growth of sympathetic neurons. *J. Biol. Chem.* **275:** 19883–19890.
4. Hayashi, H., R. B. Campenot, D. E. Vance, and J. E. Vance. 2004. Glial lipoproteins stimulate axon growth of central nervous system neurons in compartmented cultures. *J. Biol. Chem.* **279:** 14009–14015.
5. Mauch, D. H., K. Nagler, S. Schumacher, C. Goritz, E. C. Muller, A. Otto, and F. W. Pfrieger. 2001. CNS synaptogenesis promoted by glia-derived cholesterol. *Science.* **294:** 1354–1357.

6. Ito, J., and S. Yokoyama. 2004. Roles of glia cells in cholesterol homeostasis in the brain. *In* Non-Neural Cells of the Nervous System: Function and Dysfunction. L. Hertz, editor. Elsevier, Amsterdam. 519–534.
7. Koch, S., N. Donarski, K. Goetze, M. Kreckel, H-J. Stuerenburg, C. Buhmann, and U. Beisiegel. 2001. Characterization of four lipoprotein classes in human cerebrospinal fluid. *J. Lipid Res.* **42:** 1143–1151.
8. Weiler-Guttler, H., M. Sommerfeldt, A. Papandrikopoulou, U. Mischek, D. Bonitz, A. Frey, M. Grupe, J. Scheerer, and H. G. Gassen. 1990. Synthesis of apolipoprotein A-I in pig brain microvascular endothelial cells. *J. Neurochem.* **54:** 444–450.
9. Mockel, B., H. Zinke, R. Flach, B. Weis, H. Weiler-Guttler, and H. G. Gassen. 1994. Expression of apolipoprotein A-I in porcine brain endothelium in vitro. *J. Neurochem.* **62:** 788–798.
10. Pitas, R. E., J. K. Boyles, S. H. Lee, D. Foss, and R. W. Mahley. 1987. Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim. Biophys. Acta.* **917:** 148–161.
11. Nakai, M., T. Kawamata, T. Taniguchi, K. Maeda, and C. Tanaka. 1996. Expression of apolipoprotein E mRNA in rat microglia. *Neurosci. Lett.* **211:** 41–44.
12. Boyles, J. K., R. E. Pitas, E. Wilson, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.* **76:** 1501–1513.
13. Muller, H. W., P. J. Gebicke-Harter, D. H. Hangen, and E. M. Shooter. 1985. A specific 37,000-dalton protein that accumulates in regenerating but not in nonregenerating mammalian nerves. *Science.* **228:** 499–501.
14. Dawson, P. A., N. Schechter, and D. L. Williams. 1986. Induction of rat E and chicken A-I apolipoproteins and mRNAs during optic nerve degeneration. *J. Biol. Chem.* **261:** 5681–5684.
15. Ignatius, M. J., P. J. Gebicke-Harter, J. H. P. Skene, J. W. Schilling, K. H. Weisgraber, R. W. Mahley, and E. M. Shooter. 1986. Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc. Natl. Acad. Sci. USA.* **83:** 1125–1129.
16. Snipes, G. J., C. B. McGuire, J. J. Norden, and J. A. Freeman. 1986. Nerve injury stimulates the secretion of apolipoprotein E by nonneuronal cells. *Proc. Natl. Acad. Sci. USA.* **83:** 1130–1134.
17. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science.* **240:** 622–630.
18. Harel, A., M. Fainaru, Z. Shafer, M. Hernandez, A. Cohen, and M. Schwartz. 1989. Optic nerve regeneration in adult fish and apolipoprotein A-I. *J. Neurochem.* **52:** 1218–1228.
19. Graham, D. I., K. Horsburgh, J. A. Nicoll, and G. M. Trasdale. 1999. Apolipoprotein E and the response of the brain to injury. *Acta Neurochir. Suppl. (Wien).* **73:** 89–92.
20. Haasdijk, E. D., A. Vlug, M. T. Mulder, and D. Jaarsma. 2002. Increased apolipoprotein E expression correlates with the onset of neuronal degeneration in the spinal cord of G93A–SOD1 mice. *Neurosci. Lett.* **335:** 29–33.
21. Aoki, K., T. Uchihara, N. Sanjo, A. Nakamura, K. Ikeda, K. Tsuchiya, and Y. Wakayama. 2003. Increased expression of neuronal apolipoprotein E in human brain with cerebral infarction. *Stroke.* **34:** 875–880.
22. Ito, J., Y. L. Zhang, M. Asai, and S. Yokoyama. 1999. Differential generation of high-density lipoprotein by endogenous and exogenous apolipoproteins in cultured fetal rat astrocytes. *J. Neurochem.* **72:** 2362–2369.
23. 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.
24. Ito, J., Y. Nagayasu, R. Lu, A. Kheirollah, M. Hayashi, and S. Yokoyama. 2005. Astrocytes produce and secrete FGF-1, which promotes the production of apoE-HDL in a manner of autocrine action. *J. Lipid Res.* **46:** 679–686.
25. 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.
26. Duhrsen, U., T. Martinez, G. Vohwinkel, S. Ergun, L. Sun, G. McMahon, J. Durig, D. Hossfeld, and W. Fiedler. 2001. Effects of vascular endothelial and platelet-derived growth factor receptor inhibitors on long-term cultures from normal human bone marrow. *Growth Factors.* **19:** 1–17.
27. Meydan, N., T. Grunberger, H. Dadi, M. Shahar, E. Arpaia, Z. Lapidot, J. Leeder, M. Freedman, A. Cohen, A. Gazit, et al. 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature.* **379:** 645–648.
28. Mohammadi, M., G. McMahon, L. Sun, C. Tang, P. Hirth, B. K. Yeh, S. R. Hubbard, and J. Schlessinger. 1997. Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science.* **276:** 955–960.
29. Narita, M., A. Usui, M. Narita, K. Niikura, H. Nozaki, J. Khotib, Y. Nagumo, Y. Yajima, and T. Suzuki. 2005. Protease-activated receptor-1 and platelet-derived growth factor in spinal cord neurons are implicated in neuropathic pain after nerve injury. *J. Neurosci.* **25:** 10000–10009.
30. Lim, R., K. Misunobu, and W. K. Li. 1973. Maturation-stimulating effect of brain extract and dibutyryl cyclic AMP on dissociated embryonic brain cells in culture. *Exp. Cell Res.* **79:** 243–246.
31. Zhang, L-Y., J. Ito, T. Kato, and S. Yokoyama. 2000. Cholesterol homeostasis in rat astrocytoma cells GA-1. *J. Biochem. (Tokyo).* **128:** 837–845.
32. McLean, J. W., C. Fukazawa, and J. M. Taylor. 1983. Rat apolipoprotein E mRNA. Cloning and sequencing of double-stranded cDNA. *J. Biol. Chem.* **258:** 8993–9000.
33. Thom, D., A. J. Powell, C. W. Lloyd, and D. A. Rees. 1977. Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.* **168:** 187–194.
34. 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.
35. Perraud, F., F. Besnard, B. Pettmann, M. Sensenbrenner, and G. Labourdette. 1988. Effects of acidic and basic fibroblast growth factors (aFGF and bFGF) on the proliferation and the glutamine synthetase expression of rat astroblasts in culture. *Glia.* **1:** 124–131.
36. Hashimoto, M., Y. Sagara, D. Langford, I. P. Everall, M. Mallory, A. Everson, M. Digicaylioglu, and E. Masliah. 2002. Fibroblast growth factor 1 regulates signaling via the glycogen synthase kinase-3beta pathway. Implications for neuroprotection. *J. Biol. Chem.* **277:** 32985–32991.
37. Asada, S., Y. Kasuya, H. Hama, T. Masaki, and K. Goto. 1999. Cytodifferentiation potentiates aFGF-induced p21ras/Erk signaling pathway in rat cultured astrocytes. *Biochem. Biophys. Res. Commun.* **260:** 441–445.
38. Jackson, A., S. Friedman, X. Zhan, A. K. Engleka, R. Forough, and T. Maciag. 1992. Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells. *Proc. Natl. Acad. Sci. USA.* **89:** 10691–10695.