Docosahexaenoic acid facilitates cell maturation and β-adrenergic transmission in astrocytes

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Abstract The effects of docosahexaenoic acid (DHA; 22:6 n-3), a major ω-3 PUFA in the mammalian brain, on the structure and function of astrocytes were studied using primary cultures from rat cerebra. Gas-liquid chromatography of methyl esters of FAs isolated from cultures exposed to individual FAs, namely, stearic acid, linoleic acid, arachidonic acid, and DHA, showed alterations in the lipid profiles of the membranes, with a preferential incorporation of the FA to which the cells were exposed. Immunofluorescence studies demonstrated that unlike treatment with other FAs, after which the astrocytes remained as immature radial forms, DHA-treated astrocytes showed distinct differentiation, having morphology comparable to those grown in normal serum-containing medium. Receptor binding studies to determine the concentration of various neurotransmitter receptors showed that DHA selectively increased the number of β-adrenergic receptors (β-ARs) compared with FA-untreated controls, suggesting a greater role of DHA on β-AR expression in membranes. This was also reflected by an increase in downstream events of the β-AR pathways, such as the induction of protein kinase A and glycogen turnover by isoproterenol (ISP), a β-AR agonist in DHA-treated cells. Moreover, ISP completely transformed DHA-treated cells into mature astrocytes bearing long processes, as in cells grown under normal conditions. ⬛ Together, our observations suggest that DHA plays a unique role in facilitating some of the vital functions of astrocytes in the developing brain.—Joardar, A., A. K. Sen, and S. Das. Docosahexaenoic acid facilitates cell maturation and β-adrenergic transmission in astrocytes. *J. Lipid Res.* 2006. **47:** 571–581.

Supplementary key words β-adrenergic receptor • morphology • glycogen

The long-chain FA docosahexaenoic acid (DHA; 22:6n-3) constitutes a large proportion of membrane phospholipids in the central nervous system and is particularly concentrated in the aminophospholipids phosphatidylethanolamine and phosphatidylserine of membranes. DHA can also be synthesized from the shorter chain precursor α-linolenic acid (18:3n-3), an essential FA that cannot be synthesized de novo by animal tissues and must be obtained from the diet (1). The importance of DHA in maintaining the structural and functional integrity of membranes is highlighted by the fact that it cannot be easily depleted from the brain (2). It is now well recognized that DHA is essential for normal brain development in animals and humans (3). In the human brain, the major accumulation occurs during the last period of gestation (4). DHA deficiency causes impairment of learning and memory. Supplementation of DHA reverses the effect by increasing the number of Fos-positive neurons and promoting neurite outgrowth and the size of the cell body in the CA1 hippocampus (5, 6) and restores long-term potentiation in aged rats (7, 8). DHA plays a crucial role in membrane order (membrane fluidity), the regulation of phosphatidylserine levels (9), and the protection of neural cells from apoptotic death (10–12). Changes in energy metabolism induced by n-3 deficiency could result from functional alteration in glucose transporters (13).

Although much of our understanding of the effect of DHA on the central nervous system has involved studies of neurons, existing knowledge regarding its effect on astrocytes is scarce. Astroglial cells constitute the major cells of the adult mammalian brain, outnumbering neurons by manyfold. Like neurons, astrocytes also undergo morphogenesis both in vivo (14) and in vitro (15, 16) characterized by changes in cell shape. In addition to maintaining the microenvironment of neurons, astrocytes play a constitutive role in the formation of the blood-brain barrier (17), represent the major glycogen depots of brain (18), support immune defense by producing various immunoactive cytokines (17), and maintain the external potassium concentration (19). Additionally, astrocytes possess myriad neurotransmitter receptors (20), most of which are transmembrane in nature and belong to the G

Abbreviations: AA, arachidonic acid; β-AR, β-adrenergic receptor; DHA, docosahexaenoic acid; GFAP, glial fibrillary acidic protein; ISP, isoproterenol; LA, linoleic acid; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide; NMDA, *N*-methyl-D-aspartate; PIN, pindolol; PKA, protein kinase A; SA, stearic acid.

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protein-coupled receptor family. The presence of such receptors suggests that astrocytes have the ability to integrate information in the brain in conjunction with neuronal processes. DHA is known to affect some of these neurotransmitter receptors in the brain (21–24), although there is a dearth of information on its role in the neurotransmitter receptors of astrocytes.

Any perturbation in the lipid profile of membranes by exposing cells to various types of FAs is likely to have an impact on the structure and function of the cells. In view of the reports of beneficial effects of DHA in brain, it was considered important to study the role of DHA on astrocyte structure and some of its important functions. The results demonstrate, for the first time, that DHA treatment promotes the differentiation and maturation of astroglial cells in culture and facilitates the incorporation of β-adrenergic receptor (β-AR) into cell membranes, resulting in the potentiation of receptor-mediated downstream signaling and function.

MATERIALS AND METHODS

Materials

DMEM, F12, antibiotics, Trizol™, and FBS were obtained from Gibco-BRL, Life Technologies. Trypsin, soybean trypsin inhibitor, poly-L-lysine hydrobromide (molecular weight > 300,000), FITC-conjugated goat anti-mouse IgG, kemptide, atropine, pindolol (PIN), propranolol, isoproterenol (ISP), amylo-1,4-α-1,6-glucosidase (EC 3.2.1.3; grade IV from *Aspergillus niger*), 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide (MTT), stearic acid (SA), linoleic acid (LA), arachidonic acid (AA), DHA, and all other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). All plasticware for tissue culture was procured from Tarson (Kolkata, India). Monoclonal antibody against glial fibrillary acidic protein (GFAP; GA-5 clone) was from Chemicon International (Temecula, CA), whereas [³H]quinuclidinyl benzilate, [³H]dihydroalprenolol, [³H]MK-801, and γ[³²P]ATP were from Amersham (Buckinghamshire, UK). Taq DNA polymerase was obtained from Bangalore Genei and Platinum SYBR Green qPCR supermix-UDG from Invitrogen. [¹⁴C]glucose was obtained from BRIT (Trombay, India). All other reagents were of analytical grade and obtained locally.

Primary culture of astroglial cells and treatment with FAs

Primary cultures of glial cells from the cerebra of neonatal rats (<24 h old) were prepared as described previously (25). In short, cerebra were dissected out aseptically, freed of meninges and blood vessels, minced into pieces (four to five pieces per cerebrum), and trypsinized at 37°C for 5–10 min with 0.5 mg/ml trypsin in isotonic balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.2 mM Na_2HPO_4, 0.2 mM KH_2PO_4 5.5 mM glucose, 59 mM sucrose, and 0.4% gentamycin, pH 7.2) containing 0.5% BSA. Cells were then dissociated by repeated pipetting with fire-polished Pasteur pipettes (1 mm diameter). The resulting cell suspension was filtered through a double-layered nylon mesh to remove aggregates. Cells were resuspended in DMEM containing 10% FBS and plated on 175 cm² poly-L-lysine-coated plates for 5 min for preferential attachment of neurons. Unattached cells were then seeded onto poly-L-lysine-coated plates at 6×10^6 cells/dish. Cultures were maintained in a Forma-CO_2 Incubator (5% CO_2/95% air) at 37°C with change of medium every other day.

After 5 days, medium was replaced with serum-free medium containing DMEM and F12 (1:1 ratio). At day 10, free FAs, namely, SA (18:0), LA (18:2), AA (20:4n-6), and DHA, were added, and the cells were cultured for a further 48 h. Untreated controls received vehicle only. Ascorbic acid (40 μM) was used as an antioxidant agent. Initially, two methods were used to incorporate FA in the cultured cell membranes: *a*) treatment with FA conjugated to BSA (26), and *b*) treatment with FA solubilized in ethanol and 1% serum.

Immunocytochemistry

Coverslip cultures of astroglial cells were processed for immunofluorescent staining to study their morphological pattern. Cells were fixed in ice-cold methanol for 10 min, washed three times with PBS, and incubated with monoclonal antibody against the astrocyte-specific marker protein GFAP, at 1:10 dilution, followed by FITC-conjugated goat anti-mouse IgG (1:10 dilution). All incubations were carried out for 30–45 min at 37°C. The coverslips were mounted onto glass slides in buffered glycerol containing 0.1% *p*-phenelene diamine (pH 7.5) and examined with a Leitz fluorescence microscope or a Leica SP2 confocal spectral microscope.

Membrane preparation

Culture plates were rinsed twice with ice-cold 50 mM Tris-HCl buffer containing 0.9% saline, pH 7.4. Cells were scraped into a small volume of 50 mM Tris-HCl, pH 7.4, homogenized, and centrifuged at 40,000 *g* for 15 min. The resulting pellet was resuspended in fresh buffer and incubated for 20 min at 37°C and recentrifuged at 40,000 *g* for 15 min. The pellets containing the crude membrane were stored at −20°C.

Determination of the FA composition of membranes

Lipids were extracted from the membrane pellet using the procedure of Folch, Lees, and Sloane Stanley (27) with some minor modifications. Briefly, the lipids were extracted with a 2:1 mixture of chloroform-methanol. The lipids were present in the lower chloroform phase. The upper phase was removed without disturbing the lower phase. The chloroform phase was dried under nitrogen and resuspended in 500 μl of dry methanol. FA methyl esters were prepared from total lipids by acid-catalyzed transmethylation as described by Feiser and Feiser (28). One hundred microliters of acetyl chloride was added to 500 μl of dry methanol on ice. This was added to 500 μl of total membrane lipid extracts dissolved in dry methanol. This mix was kept at room temperature overnight to produce FA methyl esters. The esters were dried under nitrogen and resuspended in dry *n*-hexane for gas-liquid chromatography. The FA methyl esters were separated by gas-liquid chromatography (Hewlett Packard 6890+ gas chromatograph fitted with a flame ionization detector) using a fused silica HP-5 column (0.32 mm × 0.25 μm × 30 m). For best resolution, the oven temperature was programmed as follows: 160°C for 2 min, increase by 3°C/min to 220°C, hold for 18 min. Standard FA methyl esters were used for the identification of their respective FAs. For the quantification of individual peaks, a Hewlett Packard 3398A Chemstation was used.

Assessment of cell viability

Cell viability was determined by the reduction of yellow MTT into a purple formazan product by mitochondrial dehydrogenases of metabolically active cells based on the method described by Mabley et al. (29). Cells were grown on 24-well plates, and FA treatment was carried out after 10 days of culture for 48 h as described earlier. After treatment, the medium was

removed. Four hundred microliters of fresh medium was added to each well followed by 40 μl of MTT (5 mg/ml). After 4 h, the MTT solution was carefully removed, the purple crystals were solubilized in 1.4 ml of DMSO, and absorbance was measured at a test wavelength of 550 nm with a reference wavelength of 620 nm. The absorbances obtained from treated cells were expressed as percentages of the absorbances obtained from untreated cells.

Additionally, lactate dehydrogenase (LDH) assay was also used to analyze cell viability using a protocol published previously (30). Briefly, after treatment with the FAs, 10 μl of the medium was added to 190 μl of 80 mM Tris-HCl buffer, pH 7.2, containing 200 mM NaCl in the well of a microtiter plate. After addition of 200 μl of reaction mixture (80 mM Tris-HCl buffer, pH 7.2, containing 200 mM NaCl, 3.2 mM pyruvate, and 0.4 mM NADH), the decrease in the absorbance at 340 nm was recorded up to 10 min in 2 min intervals at room temperature using the microtiter plate reader. The LDH activity in an aliquot of the incubation buffer was compared with the LDH activity in incubation buffer after total lysis of the cells by incubation at room temperature (30 min) after the addition of Triton X-100 to 1% (w/v) final concentration. Zero percent viability corresponds to 100% LDH activity in the buffer.

Binding assays

The membranes of astrocytes, prepared as described above, were used for binding assays. All assays were performed in duplicate. β-AR binding was carried out using ^{125}I-PIN (2 nM) according to the method of Barovsky and Brooker (31) for 20 min at 30°C, in the absence and presence of 100 μM propranolol, to determine specific binding. Alternatively, 4 nM [^{3}H]dihydroalprenolol (specific activity, 60 Ci/mmol) was used for β-AR binding for 30 min at 37°C as performed previously in astrocyte membranes (32) in the presence and absence of 1 μM propranolol. The binding of 3 nM [^{3}H]MK-801 (specific activity, 29 Ci/mmol) to N-methyl-D-aspartate (NMDA) receptors was determined as described by Layer et al. (33) using 10 μM MK-801 to determine nonspecific binding. Specific binding of 1 nM [^{3}H]quinuclidinyl benzilate (specific activity, 25 Ci/mmol) to muscarinic cholinergic receptors was determined by the method of Suga (34) in the absence and presence of 1 μM atropine. After incubation, the reaction was terminated by the addition of 5 ml of ice-cold buffer, and the samples were filtered through GF/B glass fiber filters in a filtration manifold (Millipore) with two additional washes in the same buffer at 5 ml each. Radioactivities retained on the filters were measured with a liquid scintillation counter. (−)-PIN was iodinated to a specific activity of 2.2 Ci/μmol by the method of Witkin and Harden (35).

For saturation studies with ^{125}I-PIN, membrane proteins (100 μg) were incubated with a range of ^{125}I-PIN concentrations (0.02–0.2 pmol) in 0.05 M Tris (pH 7.5) buffer in a total volume of 250 μl for 30 min at 37°C. The samples were then rapidly filtered on Whatman GF/B filters and washed with ice-cold assay buffer. Specific binding was determined from the difference between counts of ^{125}I-PIN binding performed in the absence and presence of 25 μM of the specific antagonist, propranolol. For determination of the maximum number of binding sites and affinity values, the resulting data were subjected to Scatchard plot analysis.

RNA extraction and real-time PCR

Total RNA from cultured astroglial cells was isolated using Trizol™ according to Gibco-BRL instructions. Briefly, the astrocyte cells were lysed with 1 ml of Trizol and the lysate was passed several times through a 1 ml syringe. After addition of 200 μl of chloroform, the mixture was shaken vigorously and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred into a fresh tube, 500 μl of isopropyl alcohol was added, and the mixture was kept at −20°C overnight and then centrifuged again at 12,000 g for 15 min at 4°C. The RNA in the pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. Total RNA was dissolved in 50 μl of RNase-free water and quantified by UV absorption. The RNA samples were kept frozen until use.

For reverse transcription, 3 μg of total RNA was denatured for 5 min at 65°C and incubated for 52 min at 42°C in the presence of 400 μM deoxynucleoside triphosphate (dNTP), 5 pM oligo(dT), and 200 units of RT per reaction with a reaction buffer containing 50 mM Tris-HCl (pH 8.3 at 25°C), 3 mM MgCl$_2$, 75 mM KCl, and 10 mM DTT. The mixture was heated to 70°C for 15 min at the end of the reaction.

For PCR analysis, the primers used for β$_1$-AR were 5′-ATG TGT GAC GGC CAG CAT CG-3′ (sense) and 5′-AAG CGG CGC TCG CAG CTG TC-3′ (antisense); the primers used for β$_2$-AR were 5′-GAG ACC CTG TGC GTG ATT GC-3′ (sense) and 5′-GAG GAC CTT CGG AGT CCG TG-3′ (antisense). The reaction mixture consisted of 2 μl of cDNA, 1 unit of Taq DNA polymerase, 0.2 mM deoxynucleoside triphosphate, and 2 mM MgCl$_2$. Denaturation was carried out at 94°C for 30 s (5 min in the first cycle) followed by an annealing step at 58.5°C or 57.5°C (depending on the primer) for 30 s and an extension step at 72°C for 30 s (10 min in the last cycle) for a total of 35 cycles of β$_1$-AR and 30 cycles for β$_2$-AR. The PCR products were electrophoresed on a 1% agarose gel in 1× Tris acetate EDTA containing 0.2% ethidium bromide along with a 100 bp ladder.

To quantitate the levels of mRNA, they were analyzed by real-time PCR using the Bio-Rad iCycler system. PCR was carried out with Platinum SYBR Green qPCR supermix-UDG using 1 μl of RT product in 20 μl of final reaction mixture according to the manufacturer's protocol. PCR cycling parameters were as described above. The mRNA levels for each sample were normalized against GAPDH mRNA using the manufacturer's software included in the unit. For PCR of GAPDH, the primers used were 5′-TGG GGT GAT GCT GGT GCT GAG-3′ (sense) and 5′-GGT TTC TCC AGG CGG CAT GTC-3′ (antisense).

Assay of cAMP-dependent protein kinase or protein kinase A

Cells after appropriate treatment were washed with TBS and harvested in extraction buffer containing 20 mM Tris-HCl (pH 7.2), 1 mM EGTA, 2 mM EDTA, 50 mM β-mercaptoethanol, 0.5 mM theophylline, 2 mM PMSF, and protease inhibitors. Lysates were centrifuged at 100,000 g for 30 min, and the supernatant was used for protein kinase A (PKA) assay. Briefly, activity was measured at 37°C in a 50 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 50 mM NaF, 50 μM vanadate, 20 mM p-nitrophenyl phosphate, 1 mM PMSF, 10 mM Mg-acetate, 5 mM β-mercaptoethanol, 20 μM ATP containing 1 μCi [γ-^{32}P] ATP/assay, and 100 μM kemptide as substrate, with or without 10 μM cAMP, based on the method of Glass et al. (36). The reaction was started by the addition of 10 μg of cytosolic protein as an enzyme source; after 15 min, the reaction was stopped by the addition of 5 μl of 75 mM phosphoric acid and cooled on ice for 30 min. Then, 10 μl was spotted on P81 paper and washed three to four times in 75 mM phosphoric acid. Dried papers were counted for radioactivity, and the results are expressed as ratios of the two PKA activities in the absence and presence of 10 μM cAMP.

Incorporation of ^{14}C-labeled glucose into glycogen

Medium was replaced with minimal essential medium (for 100 ml, 20 mg of anhydrous CaCl$_2$, 40 mg of KCl, 19.7 mg of

MgSO$_4$.7H$_2$O, 680 mg of NaCl, 14 mg of NaH$_2$PO$_4$.H$_2$O, 90 mg of glucose, 11 mg of sodium pyruvate, pH 7.2, by NaHCO$_3$) containing 1 µCi/ml [^{14}C]glucose; specific activity was 140 mCi/ mmol in the presence and absence of ISP, and cultures were incubated for 1 h. Medium was removed and cells were rinsed twice with 150 mM NaCl before extraction in 0.1 M NaOH. Glycogen and protein were determined after sonication of cell extract. Protein was determined by the method of Lowry et al. (37), and extracts containing equal amounts of protein was deproteinized with 8% (w/v) TCA (final concentration) containing glycogen carrier. The glycogen in the supernatant was precipitated with ethanol (63% (v/v) final concentration]. The precipitate was dissolved in water and reprecipitated in ethanol, and radioactivity was determined (38).

Glycogen assay

The glycogen content of the cultures was determined by the method of Cambrey-Deakin et al. (39) with some minor modifications. One set of cultures was incubated with 1 mM ISP for 1 h. The cultures were washed twice in ice-cold 0.9% NaCl and then harvested into 800 µl of 0.1 M NaOH. The resulting suspension was heated to 80°C for 20 min. Aliquots were then taken for protein content determination (37). NaOH insoluble material was removed by centrifugation (12,400 g for 10 min), and the supernatant was supplemented with 1.6 ml of 95% ethanol to precipitate the glycogen, which was pelleted by centrifugation (1,500 g for 10 min). After removal of the supernatant, the pellet was resuspended in 100 µl of 0.1 mM sodium acetate buffer (pH 4.7) and reprecipitated as before. The final glycogen pellet was resuspended in 100 µl of acetate buffer and degraded to glucose by incubation with 5 µl of 20 mM Tris-HCl (pH 7.5) containing 0.02% BSA (fraction V) and 5 µg of amylo-1,4-α-1,6-glucosidase (EC 3.2.1.3; grade IV from *A. niger*) at 37°C for 1 h. The resulting glucose was assayed using the glucose-oxidase method.

RESULTS

FA incorporation in astrocyte membranes

There are mainly two approaches for the selective incorporation of FAs into cell membranes described in the literature, as discussed in Materials and Methods. We evaluated the efficacy of both procedures in incorporating DHA into astrocyte membranes in 10 day old astrocyte cultures. Some variations in the proportion of the components and treatment time were also investigated. The results indicated that all of the treatment conditions caused increases in the DHA content of astrocytic membranes compared with untreated controls (**Fig. 1**). Of the various protocols using DHA conjugated to BSA, it was observed that treatment of cultured cells with 50 µM DHA conjugated to 15% or 50% BSA for 48 h followed by an additional incubation with fresh BSA conjugate for 24 h caused the maximum incorporation of DHA (6.3%), compared with 1.8% in untreated controls. On the other hand, in cultures treated with the same amount of DHA (50 µM) dissolved in 0.1% ethanol and 1% FBS for 48 h, the incorporation of DHA in cells was greatest, being 10% of total FAs. Serum was omitted initially from the treatment to avoid DHA from being diluted by the FAs of the serum, but it was observed that the presence of 1% FBS increased the incorporation of DHA in the membrane. This procedure of supplementation of DHA dissolved in 0.1% ethanol and 1% FBS was also used for other FAs, namely, SA, LA, and AA, and showed significant incorporation of the individual FA into the membranes of astrocytes (data not shown).

Morphology of astrocytes on FA supplementation

In primary culture, astrocytes maintained in normal serum-containing medium undergo progressive maturation involving two distinct stages of morphological differentiation (16). At the initial stages, the cells remain as the immature radial glia characterized by a small cell body with three to four long processes. With time, the radial glia differentiates into flat polygonal cells with epithelioid morphology and then to mature process-bearing cells with stellate morphology. We examined the morphology of the 12 day old cultured cells when supplemented with
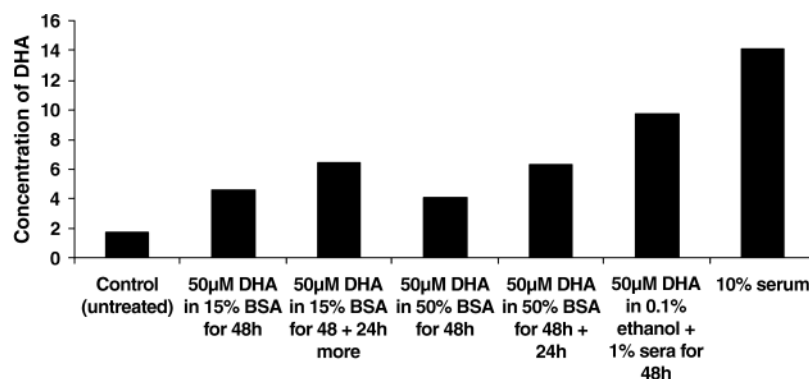

Fig. 1.   Selective incorporation of docosahexaenoic acid (DHA) in astrocyte membranes. Cerebral astrocytes from newborn rat pups were cultured for 10 days (last 5 days in serum-deficient DMEM/F12). The cultures were then supplemented with DHA using various protocols, and the amounts of DHA in membranes were estimated. Astrocytes cultured in serum-deficient medium as well as in 10% serum were also studied for comparison. Culture conditions, FA isolation, and estimation are described in Materials and Methods. Results are expressed as percentages of total FAs in cell membranes, and values represent means of two determinations.
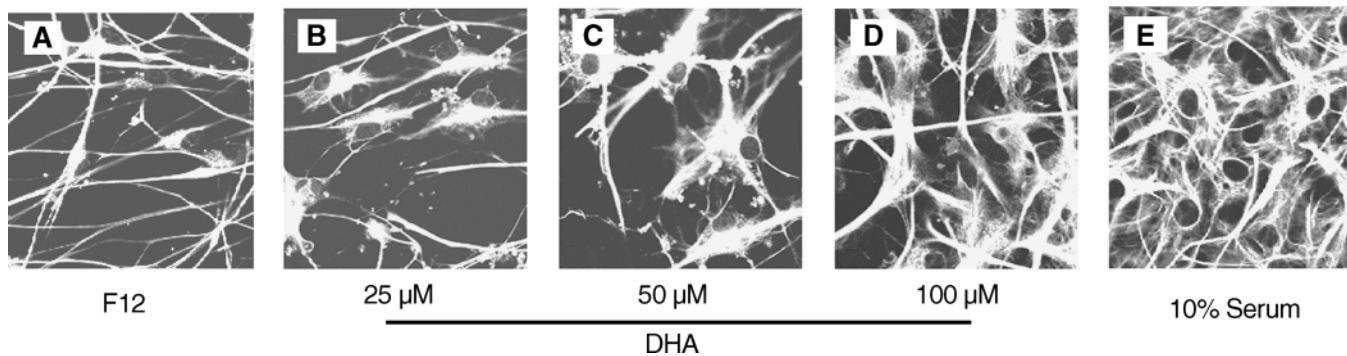
A   B   C   D   E

F12   25 μM   50 μM   100 μM   10% Serum

DHA

Fig. 2. Comparison of the effects of different concentrations of DHA on astrocyte morphology. Ten day old astrocytes grown in DMEM/F12 were treated with 25, 50, and 100 μM DHA in 0.1% ethanol and 1% FBS for an additional 2 days (B, C, D, respectively). A shows cells grown under serum-deficient conditions in DMEM/F12, and E shows cells cultured in 10% normal serum throughout the culture. Cells were immunofluorescence stained with glial fibrillary acidic protein (GFAP) and observed with a confocal microscope to ascertain their morphology. Details of the methods are described in Materials and Methods. Photomicrographs are representatives from three separate experiments. Magnification, ×1,000.

different concentrations of DHA (25, 50, and 100 μM) dissolved in 0.1% ethanol and 1% FBS during the last 48 h for maximum incorporation of DHA in membranes (**Fig. 2B–D**). Compared with serum-deficient controls (Fig. 2A), DHA supplementation, at all concentrations, caused the cells to undergo transformation into flat polygonal cells with thick filaments, with a maximum effect at 100 μM (Fig. 2D), which was comparable to astrocytes grown in normal serum-containing medium (Fig. 2E).

We also examined the effect of supplementation of other FAs on astrocyte morphology. Control cells, grown in serum-deficient DMEM/F12, failed to differentiate and remained in radial form (**Fig. 3A**). Supplementation of the cell with LA or AA also did not initiate differentiation of the radial astrocytes (Fig. 3C, D). Treatment of the cells with SA, however, caused most of the cells to differentiate poorly (Fig. 3B).

Effect of fatty acid supplementation on the cell viability of cultured astrocytes

The failure of some of the FAs to induce the differentiation of astrocytes prompted us to evaluate the effect
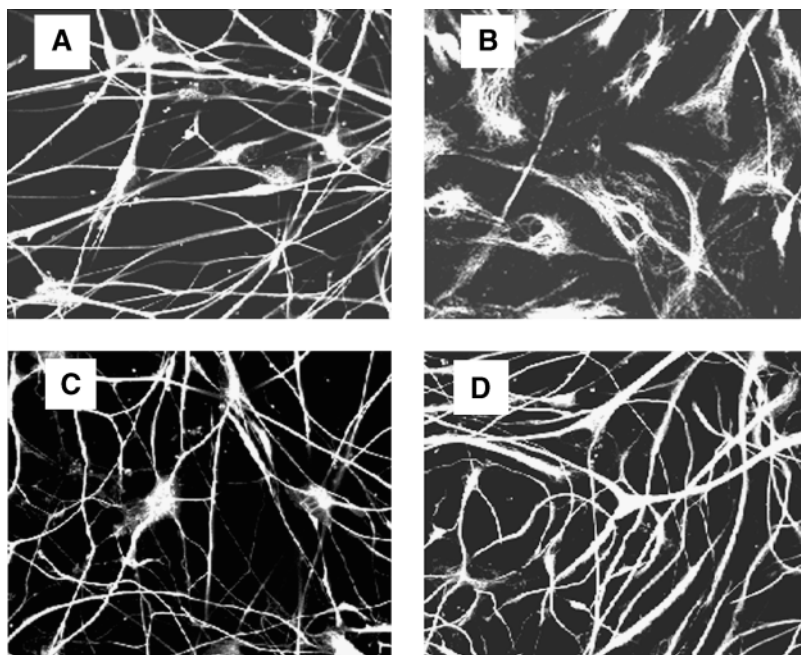

Fig. 3. Effects of FA supplementation on the morphology of cultured astrocytes. Ten day old astrocytes grown in DMEM/F12 were supplemented with 1% FBS and 100 μM of the individual FAs stearic acid (B), linoleic acid (C), and arachidonic acid (D) dissolved in 0.1% ethanol for an additional 2 days. Control cells received only medium containing 0.1% alcohol and 1% FBS (A). Details of culture and anti-GFAP immunostaining are described in Materials and Methods. Photomicrographs are representatives from three separate experiments. Magnification, ×400.
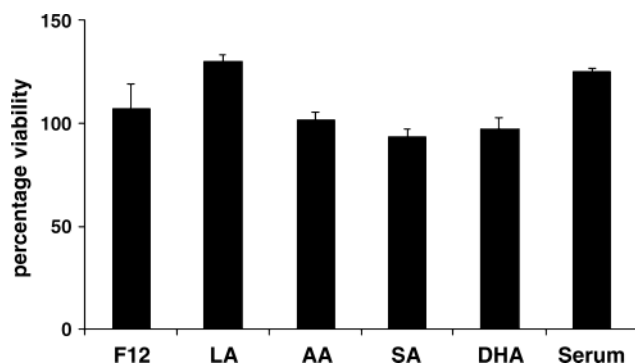
Fig. 4. Effects of FA supplementation on the cell viability of cultured astrocytes using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) assay. Cerebral astrocytes from newborn rat pups were cultured for 10 days (last 5 days in serum-deficient DMEM/F12). The cultures were then supplemented with different fatty acids for an additional 2 days. Details of culture and MTT assay are described in Materials and Methods. Results are expressed as percentages of viable cells as means ± SEM of three determinations. AA, arachidonic acid; LA, linoleic acid; SA, stearic acid.

of the FAs on the viability of the cultured cells. Using the MTT assay, it was observed that there was no significant difference in the cell viability of cultures treated with individual FAs during the last 48 h compared with astrocytes cultured under serum-deficient conditions (**Fig. 4**). Total LDH activity in medium was also negligible compared with cellular LDH activity, being 0.9, 0.05, 1.0, 1.1, 0.08, and 1.2% of total LDH activity of cells treated in serum, F12, DHA, AA, LA, and SA, respectively.

Effect of DHA on neurotransmitter receptors

We investigated whether the alteration of membrane lipid profiles by FA treatment affected any of these receptors. **Table 1** depicts the effect of different FA treatments on NMDA, β-AR, and muscarinic cholinergic receptors. No significant change was observed in [3H]MK801 binding to NMDA receptors in the astrocytes supplemented with DHA and LA. However, binding was decreased significantly in cells treated with AA and SA. The effect of FAs

on the binding of [3H]quinuclidinyl bezilate to muscarinic cholinergic receptors of astrocytes shows significant decreases in binding in AA- and SA-supplemented cells. However, the other two FAs supplemented showed no significant differences in binding. On the other hand, a significant increase in the specific binding of [3H]dihydroalprenolol as well as 125I-PIN to β-ARs was observed in cells treated with DHA. Treatment with other FAs did not produce any significant effect on the binding to β-AR compared with serum-deficient cultures.

Effect of DHA supplementation on β-AR

We investigated the effect of varying concentrations of DHA on β-AR by determining the specific binding of 125I-PIN to the membranes of astrocytes supplemented with 25, 50, or 100 µM DHA (**Fig. 5A**). Compared with FA-untreated astrocytes, the binding of 125I-PIN to β-AR increased dose-dependently with DHA concentration (Table 1).

To study whether the observed increase in specific 125I-PIN binding during DHA supplementation was attributable to changes in the number or affinity of β-AR, both the FA-untreated (control) astrocytes and the astrocytes supplemented with DHA were subjected to Scatchard plot analysis using 125I-PIN (Fig. 5B). There was a significant increase in the maximum number of binding sites in the DHA-supplemented cells compared with the controls. The affinity of these receptors to the ligand did not show any significant difference between the two treatment conditions.

To ascertain whether the increase in the number of β-AR receptors in the astrocyte membrane was attributable to an effect at the transcriptional level, we quantitated the mRNA of β_1-AR and β_2-AR in the astrocytes treated without (control) or with DHA by real-time PCR. It was observed that mRNA levels of both β_1- and β_2-AR are greater in control cells than in DHA-treated cells (Fig. 5C).

An increase in the number of β-ARs in the membrane, as observed in DHA-supplemented cells, would be expected to facilitate the downstream effectors of β-AR-mediated signaling in the cell. Activated PKA being one such effector, we monitored both basal and 1 µM ISP-induced (a β-AR agonist) PKA activity in DMEM/F12-treated astrocyte cultures (control) as well as in the DHA-

TABLE 1. Specific binding of different FA-supplemented astrocyte membranes to neurotransmitter receptors

Sample	Specific Binding			
	[3H]MK801	125I-pindolol	[3H]dihydroalprenolol	[3H] quinuclidinyl bezilate
	fmol/mg membrane protein			
Control	45.1 ± 4.5	19.6 ± 0.5	15.3 ± 1.9	97.2 ± 6.4
Stearic acid	15.2 ± 2.3[a]	17.0 ± 0.3	9.5 ± 1.5	69.0 ± 4.0[b]
Linoleic acid	24.7 ± 2.7	12.1 ± 0.4	12.2 ± 2.7	95.7 ± 1.0
Arachidonic acid	16.6 ± 3.1[a]	15.8 ± 0.2	15.9 ± 1.7	50.0 ± 8.1[a]
Docosahexaenoic acid	50.0 ± 4.4	48.4 ± 0.5[a]	22.7 ± 0.4[a]	106.0 ± 5.0
Normal serum	259.0 ± 6.1	ND	89.6 ± 8.6	198.6 ± 8.9

Ten day old cultures were exposed to 100 µM of the individual fatty acids for 48 h, and membranes prepared from the cells were used for binding studies as described in Materials and Methods. Results are expressed as means ± SEM of four determinations. Student's *t*-tests were performed. ND, experiment not done.
[a] $P < 0.001$ compared with corresponding controls.
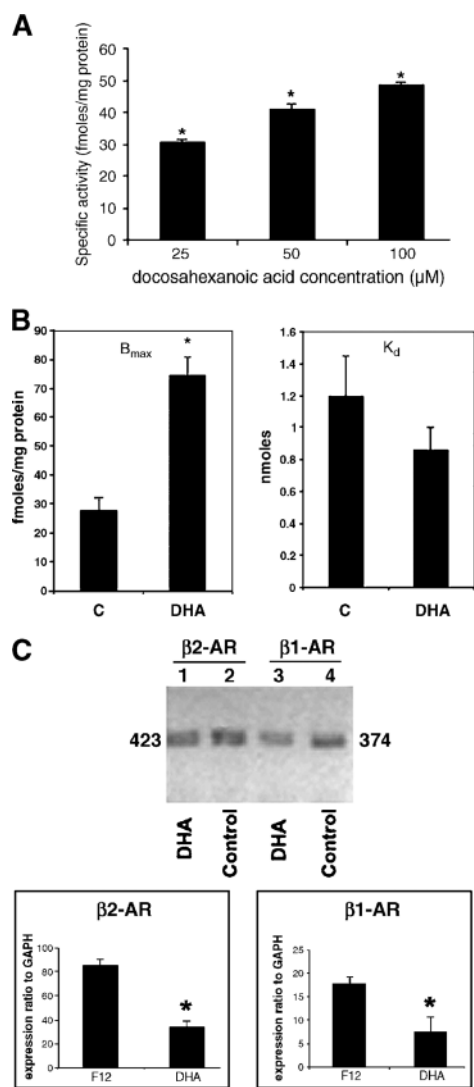[b] $P < 0.01$ compared with corresponding controls.

Fig. 5. Effects of DHA supplementation on the β-adrenergic receptor (β-AR) system of cerebral astrocyte cultures. A, B: ^{125}I-pindolol (PIN) binding to astrocyte membranes. C: Real-time analysis of β_1-AR and β_2-AR in total RNA. Ten day old astrocytes grown in DMEM/F12 were treated with vehicle or 100 μM DHA in 0.1% ethanol and 1% FBS for an additional 2 days. Details of the methods used are described in Materials and Methods. A: Effects of ^{125}I-PIN binding to membranes prepared from astrocyte cultures treated with the indicated concentrations of DHA. Results are means ± SEM of three different experiments. The specific binding in the presence of DMEM + 10% serum is 52 ± 0.6. * Significantly different ($P < 0.001$) from control. B: Binding parameters B_{max} (maximum number of binding sites) and K_d (affinity) were derived from Scatchard analysis and expressed in fmol/mg protein and nM respectively. Comparisons among tests were done by Student's t-test. Values represent means ± SEM of four determinations. * Significantly different ($P < 0.001$) from corresponding normal [control (C)] values. C: RNA was isolated from cultures and analyzed by RT-PCR using specific oligonucleotides as primers. PCR products were analyzed by electrophoresis on a 1% agarose gel. Lanes 1 and 2, PCR products of β_2-AR gene; lanes 3 and 4, PCR products of β_1-AR gene. Lanes 1 and 3, RT products from control astrocytes; lanes 2 and 4, RT products from DHA-supplemented astrocytes (top). The relative gene expression for β_1-AR and β_2-AR levels in F12 and DHA-supplemented astrocytes was also quantitated by real-time PCR by normalizing against GAPDH mRNA (bottom). Results are expressed as means ± SEM of three determinations. * Significantly different ($P < 0.001$) from control.

supplemented cells. The results showed that PKA activity significantly increases on ISP treatment in DHA-containing cells, thereby confirming the role of β-AR present in the membrane (**Fig. 6**).

Studies on glycogen storage and incorporation into glycogen

The β-AR system is an important intrinsic regulator of the glycogen turnover of mammalian brain. We determined the glycogen content in specific FA-enriched astrocytic cells under normal and ISP-treated conditions (**Fig. 7A**). Compared with untreated controls, supplementation of SA and AA had no effect on the glycogen content of the cells, whereas LA and DHA caused significant increases in total glycogen, with average increases of ∼250% and 760%, respectively. When the cells were exposed to ISP, there was significant breakdown in total glycogen content in all FA-treated cells compared with corresponding cells in which no ISP was added. Here also, the maximum breakdown of glycogen by ISP occurred in DHA-supplemented cells, which decreased to the level observed in FA-deficient astrocytes.

Because the observed 7.5-fold increase in total glycogen content in DHA-supplemented cells could be attributable to an effect on the synthesis rate of glycogen, we evaluated the amount of incorporation of [^{14}C]glucose into glycogen in these cells in the presence and absence of ISP (Fig. 7B). In the absence of ISP, treatment with DHA caused an ∼50% decrease in the newly synthesized glycogen compared with FA-deficient control cells. However, on ISP treatment, there was a 10-fold increase in the incorporation of [^{14}C]glucose into glycogen in the DHA-supplemented astrocytes compared with the ISP-treated control cells.

Effect of ISP on the morphology of astrocytes supplemented with FAs

In view of the upregulation of β-AR in astrocytes enriched with DHA, we investigated whether ISP had any effect on the morphology of astrocytes supplemented with the various FAs. Unlike control, SA-, LA-, or AA-treated cells, in which ISP failed to induce further differentiation of the astrocytes (data not shown), DHA supplementation followed by exposure to ISP (1 μM) for 2 days resulted in complete transformation into mature astrocytes with stellate morphology (**Fig. 8**).

DISCUSSION

Based on previous reports, a protocol has been developed for the significant incorporation of FAs in membranes of cultured astrocytes. It has been observed that supplementation of DHA and/or AA in medium containing a small amount of serum helps to alter the membrane phospholipid composition in astrocyte cultures (40), which is supported by our observations. Although most studies have used dietary supplementation as a means of DHA enrichment in the brain, this study provides an
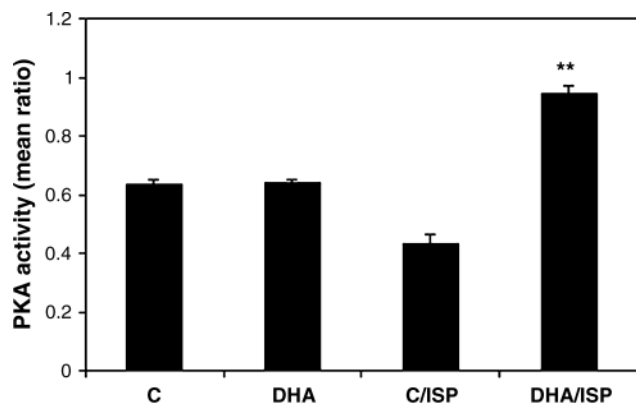
Fig. 6. Effects of DHA on the induction of protein kinase A (PKA) activity by isoproterenol (ISP) in astrocyte cultures. Ten day old astrocytes grown in DMEM/F12 were treated with or without DHA for an additional 2 days. Cells were exposed to vehicle or 1 μM ISP for 30 min and then assayed for PKA activity. Details of the methods used are described in Materials and Methods. Mean ratio refers to PKA activity in the absence of cAMP (intrinsic activity) divided by activity in the presence of cAMP (total activity). Values shown are means ± SEM of at least four sets per group. Comparisons among tests were done by Student's *t*-test. ** $P < 0.001$ compared with the untreated group. C, control.

alternative of using a single FA to selectively increase its level in membranes of cultured cells, with the purpose of studying its effect on cell morphology and function in vitro.

The morphology of astroglial cells changes dramatically during neural tissue development (15), and these changes in cell shape are important for the morphogenesis and function of the central nervous system (41). Although the immature radial glia, during early development, helps in guiding neuronal migration, laminar pattern formation, and axonal guidance (42–44), mature astrocytes are required for various supporting functions. Results from this study demonstrate that the normal morphology observed

in cultures grown in normal serum-containing medium is mimicked to a great extent in cultures grown in serum-deficient conditions supplemented with DHA.

There are very few reports on the effect of PUFAs on the neurotransmitters and their downstream regulators in astrocytes. Enrichment of the astrocytes with ω-6 PUFAs did not alter basal levels of cAMP, nor did it affect the amounts of cAMP formed in response to forskolin, ISP, adenosine, or histamine (45). Uptake of [³H]glutamate was not altered in the ω-6 PUFA-enriched astrocytes, although the uptake of [³H]adenosine was modified. The choice of the neurotransmitter receptors, with the exception of β-AR, which has an established role in astrocyte differentiation, for binding studies was rationalized as follows. Muscarinic cholinergic agonists have a profound role in astrocyte proliferation (46); glutamate is a physiological activator of phospholipase D, which is known to be a differential but important participant in regulating the signaling of mitosis and differentiation in astrocytes during development (47). In this study, DHA supplementation significantly increased β-AR concentrations in the cultured astrocytes without affecting the affinity of the receptors. The effect of DHA appeared to be specific for β-AR, because binding of [³H]QNB at the muscarinic cholinergic receptor and [³H]MK801 at the NMDA was unaffected in cells treated with DHA. However, the increase in β-AR receptors in DHA-supplemented cells was not attributable to an effect at the transcriptional level, because the levels of both β₁- and β₂-AR mRNA were considerably less in these cells compared with control cells cultured in DMEM/F12. It has been observed that dietary supplementation of long-chain PUFAs causes a differential effect on hippocampal muscarinic 1 and serotonergic 1A receptor density, and the reason has been attributed to a biochemical regulation of receptor sensitivity besides a change in membrane fluidity (23). It remains to be determined how DHA supplementation facilitates the upregulation of β-AR in astrocyte membranes.
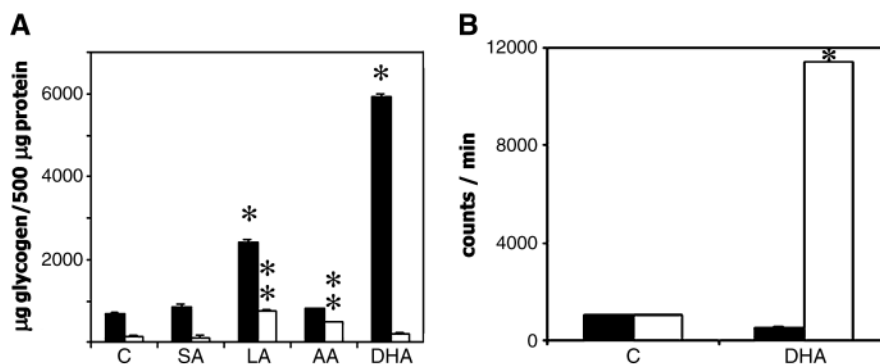

Fig. 7. Effects of FA supplementation on glycogen regulation in astrocytes. A: Total glycogen concentration in the various FA-treated astrocytes in the absence (closed bars) and presence (open bars) of 1 μM ISP. Control (C) cells were FA-deficient, cultured in DMEM/F12 medium. B: [¹⁴C]glucose incorporation as glycogen in control and DHA-supplemented cells without (closed bars) and with (open bars) 1 μM ISP. Culture conditions, estimation of glycogen, and [¹⁴C]glucose incorporation are described in Materials and Methods. Results are means ± SEM of four separate determinations. * $P < 0.001$, ** $P < 0.01$ compared with corresponding controls.
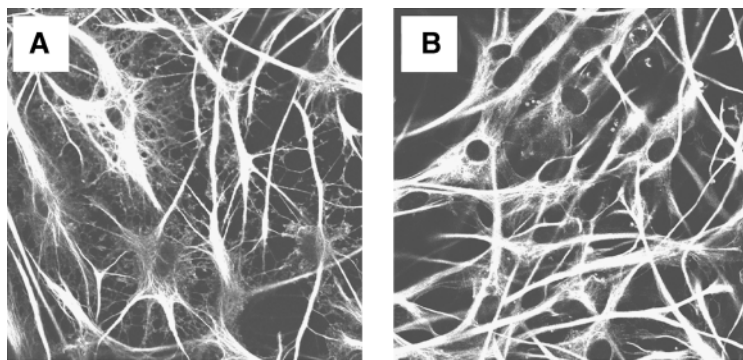
Fig. 8. Effects of ISP on the morphological differentiation of astrocytes supplemented with DHA. Ten day old cerebral astrocyte cultures were supplemented with 100 μM DHA and 1% FBS for an additional 2 days. Cells were treated without (A) or with 1 μM ISP (B) for 48 h. Details of culture, GFAP immunofluorescence staining, and confocal microscopy are described in Materials and Methods. Representative cells from three experiments are displayed. Magnification, ×1,000.

Of the neurotransmitter receptors present in astrocytes, β-ARs occupy a special place, because unequivocal evidence suggests that astroglial cells may be the targets of the central noradrenergic system (48). These receptors have multifarious functions that include regulating astroglial glycogen metabolism (41), stimulating the expression of cytokines (49), inducing gene expression of neurotrophic factors (50), modulating glial inwardly rectifying potassium channels (51), and regulating the extracellular concentration of adenosine (52). Additionally, the β-AR system has a profound effect on the differentiation and maturation of astrocytes (53, 54). The observation of increased PKA activity by ISP in DHA-supplemented astrocytes compared with that in FA-deficient cells suggests that the observed upregulation of β-AR in astrocyte membranes during DHA supplementation also facilitated downstream effectors as well.

The function of brain glycogen, present predominantly in astrocytes, is not very clear. A principal role as an energy reserve, analogous to its role in the periphery, is questionable because of its relatively low abundance. However, recent studies involving continuous in vivo measurements using NMR spectroscopy (55) as well as studies supporting a prominent physiological role of brain glycogen as a provider of supplemental energy substrate during periods of increased energy demand (56–58) have prompted a closer look at the role of glycogen in the brain. It is postulated that the majority of glucose reaches the neuron via the astrocytic intracellular space and the extracellular fluid. Our studies demonstrate that DHA supplementation in astrocytes has a positive effect on the total glycogen content of cells. Surprisingly, this increased accumulation is not attributable to an increase in the rate of glycogen synthesis, which was found to be significantly lower compared with FA-deficient control astrocytes. The β-AR agonist ISP not only helps in the transportation of glucose into cells and the synthesis of glycogen (59, 60) but also helps in the conversion of glycogen to glucose. In glioma cell lines, β-AR stimulation results in an increase in intracellular cAMP, which in turn induces a breakdown of glycogen (61). ISP induced glycogen breakdown in both FA-deficient and FA-supplemented cells, in confirmation of previous reports (61). A higher induction of glycogen breakdown by ISP in DHA-supplemented cells can be logically attributed to a preponderance of membrane β-ARs in these cells. The β-AR pathway is also involved in glycogen synthesis, because noradrenaline induces glycogen synthesis in astrocytes (59). Similar observations in our studies of ISP-induced [^{14}C]glucose incorporation into glycogen with a greater prevalence in DHA-treated astrocytes support the involvement of β-AR in the process.

ISP also induces glial cell differentiation both in primary astrocyte cultures (62) and in astroglial cell lines (63). The ability of DHA-supplemented cells to undergo differentiation in a manner similar to that observed for serum-supplemented cells, coupled with the ability of ISP to induce complete stellation of the DHA-treated cultures, gives credence to a vital role of DHA in the morphological differentiation of astrocytes.

The major nutritional problem of developing and underdeveloped countries is chronic energy deficiency associated with low fat intake. FAs present in fats and oils not only contribute toward energy demand but are vitally important for the structure and function of membranes, thereby facilitating cellular signaling processes as well as gene expression. The choices of fats and oils vary considerably among populations. So also does the FA composition of each source of oils and fats. This varied dietary intake of FAs suggests a closer look at the role of FAs in the developing brain, which requires specialized lipids for proper function. Both ω-3 and ω-6 FAs are considered to be essential because the organism cannot synthesize them and they must be obtained by alimentation. With the increasing information on the beneficial effects of these FAs, there is an inclination for their inclusion in dietary oils. The importance of ω-3 and ω-6 FAs and their intake ratio are being addressed. The results of the present study suggest a greater role of ω-3 FAs in the structure and function of developing astrocytes.

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