Cell Mol Neurobiol (2007) 27:973–983
DOI 10.1007/s10571-007-9204-7

ORIGINAL PAPER

Effect of Fatty Acids Isolated from Edible Oils Like Mustard, Linseed or Coconut on Astrocytes Maturation

Anindita Joardar · Sumantra Das

Received: 21 June 2007 / Accepted: 11 August 2007 / Published online: 7 September 2007

Abstract The omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA, 22:6n-3) has been previously shown to facilitate some of the vital functions of astrocytes. Since some dietary oils contain α-linolenic acid (ALA, 18:3n-3), which is a precursor of DHA, we examined their effect on astrocyte development. Fatty acids (FAs) were isolated from commonly used oils and their compositions were determined by GLC. FAs from three oils, viz. coconut, mustard and linseed were studied for their effect on astrocyte morphology. Parallel studies were conducted with FAs from the same oils after heating for 72 h. Unlike coconut oil, FAs from mustard and linseed, both heated and raw, caused significant morphogenesis of astrocytes in culture. ß-AR binding was also substantially increased in astrocytes treated with FAs from raw mustard and linseed oils as compared to astrocytes grown in normal medium. The expression profile of the isoforms of GFAP showed that astrocyte maturation by FAs of mustard and linseed oil was associated with appearance of acidic variants of GFAP and disappearance of some neutral isoforms similar to that observed in cultures grown in serum containing medium or in the presence of DHA. Taken together, the study highlights the contribution of specific dietary oils in facilitating astrocyte development that can have potential impact on human health.

Keywords Glia · Astrocytes · β-Adrnergic receptors · Glial fibrillary acidic protein · 2D electrophoresis

Inroduction

Fatty acids (FAs) are a major metabolic energy source and building blocks for membrane lipids (Spector et al. 1985). The health benefits of foods rich in n-3 FAs in

A. Joardar · S. Das (✉)
Neurobiology Division, Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Kolkata 700 032, India
e-mails: sdas@iicb.res.in; sumantra00@yahoo.com

persons with cardiovascular disease and rheumatoid arthritis have been well documented (James et al. 1997; Simopoulos et al. 1997). Essential FAs (EFA) and their long-chain polyunsaturated FAs (LCPUFA) derivatives are of great importance in cell growth and development (Uauy et al. 1999). The mammalian brain is highly enriched in the polyunsaturated FA (PUFA), docosahexaenoic acid (DHA) (22:6n-3), which is essential for normal visual and cognitive development (Anderson et al. 1990). Our recent studies have demonstrated the importance of DHA in astrocyte maturation (Joardar et al. 2006).

Dietary oils serve as the main source of FAs. The FAs in commonly occurring visible fats vary according to the source and the use of any single oil does not ensure the quality of fat (individual FA levels, PUFAs, saturated FAs, and n-6/n-3 ratios) as recommended for the prevention of cardiovascular disease (Duttaroy 2003). In many countries, deep fat fried products form the major route through which oils and fats are consumed. There is a tendency for excessive accumulation of oxidized, polymeric, and cyclic compounds during heating of oils for prolonged periods of time (Billek et al. 1978). There is also formation of both volatile and nonvolatile products that may affect human health (Herzellah et al. 2005).

This study was undertaken to evaluate the effect of FAs of dietary oils on astrocyte development using parameters like their effect on cell morphology, on beta adrenergic receptor (β-AR) activity and on expression of the various isoforms of the cytoskeletal protein, glial fibrillary acidic protein (GFAP). FA composition of a variety of edible oils was initially analyzed and some of these were selected based on their unique FA compositions. Oils exposed to prolonged heat were also used in the study.

Methods

Fatty Acid Isolation from Different Edible Oils

FAs were isolated from different edible oils, namely, mustard oil, coconut oil, sunflower oil, safflower oil, ricebran oil, groundnut oil, rapeseed oil, soyabean oil, and linseed oil. FAs were isolated by stirring the oils with 2 N NaOH overnight followed by removal of the sterols, etc. by treating with chloroform. The saponified FAs were acidified with 1 N HCl and isolated with the help of chloroform, which is then dried leaving behind the FAs. These FAs were esterified to corresponding methyl esters and separated by GLC on a capillary HP_5 column to check the composition of each of the oil. The column temperature was raised from 160 to 250°C over 40 min and nitrogen served as the carrier gas. The FAs come out according to their retention time in the column. The faster moving FA comes out much earlier than the slower ones. Individual FA methyl esters were identified by comparison with known standards.

Primary Cultures

Primary cultures of glial cells from the cerebra of neonates (<24 h old) were prepared as described earlier (Joardar et al. 2006). In short, cerebra were dissected aseptically, washed, and pooled in an isotonic balanced salt solution (BSS). The cerebra were then freed of meninges and blood vessels, minced into pieces (4–5 pieces/cerebrum) and trypsinized at 37°C for 5–10 min with 0.5 mg/ml trypsin in BSS containing 0.5% BSA. Cells were then dissociated by repeated passaging with fire polished Pasteur pipettes

(1 mm diameter). The resulting cell suspension was filtered through a double-layered Nylon mesh and plated in 175 cm^2 poly-L-lysine (PLL) coated plates for 5 min for preferential attachment of neurons. Unattached cells were then pelleted down at 200×*g* for 2.5 min and were seeded into PLL (Sigma, USA) 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.

Incubation of Cultures with Fatty Acids

After culture of primary astroglial cells for 5 days, media was replaced with serum free media containing DMEM and F12 Nutrient media (1:1 ratio) (Gibco-BRL). After a further 5 days of culture, media was supplemented with 100 ng of total FAs isolated from the edible oils in ethanol as vehicle (final conc. of ethanol, 0.15%). One percent FCS and 0.1 mM ascorbic acid (as antioxidant) were added and cells were cultured for a further period of 48 h (Joardar et al. 2006). FAs from mustard, coconut, and linseed oils were supplemented in the astrocyte culture. These oils were selected on the basis of the diverse nature of FAs present in them after an initial screening of a variety of other dietary oils through GLC.

Immunocytochemistry

Cover-slip cultures were immunostained using a monoclonal antibody against the astrocyte specific marker protein GFAP, GA-5 clone (glial fibrillary acidic protein) (Sigma Chemical Co., USA) followed by FITC conjugated goat anti-mouse IgG (1:10 dilution) (Gharami et al. 2000). After mounting on glass slides in buffered glycerol (pH 7.8), the cells were examined under Leitz fluorescence microscope.

Beta-Adrenergic Receptor Binding Assay

Membranes isolated from the cultured astrocytes supplemented with FAs from selected edible oils were used for binding assays (Joardar et al. 2006). 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. The binding assay was carried out using iodinated Pindolol [(–)-PIN] (RBI, USA). PIN was iodinated to a specific activity of 2.2 Ci/μmoles by the method of Witkin and Harden (1981).

Extraction of Cytoskeletal Proteins

The cytoskeletal proteins were extracted as reported earlier (Paul et al. 1999). Astrocyte cultures were washed three times with ice-cold PBS. Cells were then scraped off from the plates with 180 μl of a hypotonic buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, pH 7.4) containing the following protease inhibitor Sigma, USA) cocktail: antipain (1 mM), aprotinin (1.4 mM), phenylmethylsulfonyl fluoride (1 mM), benzamidine (1 mM), leupeptin (10 mM) and pepstatin (1 mM). After addition of 20 μl of 5% Triton X-100, the samples were allowed to stand on ice for 5 min, and then centrifuged at 12,000×*g* for 10 min at 4°C. The resulting pellet was used for identification of the GFAP isoforms.

2D Immunoblotting

The pellets of cytoskeletal proteins were dissolved in 8 M urea, 2% Triton X-100, 2% Ampholyte (pH 3–10) and 50 mM DTT (Sigma, USA). Isoelectric focusing (IEF) was performed on a 4–7 immobilized pH gradient (IPG) gels of 7 cm length, by using an IPGphor apparatus (Amersham Biosciences, Sweden). Gels were run up to 10,000 volt-hours. Gels were equilibrated twice for 10 min each with a solution of 500 mM Tris–Cl (pH 6.8), 6 M urea, 20% (v/v) glycerol, 2% SDS. Additionally, the first step contained 2% DTT and the second step 2.5% iodoacetamide (Sigma, USA). Proteins were then separated on 10% SDS-PAGE followed by an electrotransfer to PVDF membranes (Amersham Biosciences) using a transblot apparatus (Biorad, USA). For the detection of isoforms of GFAP, polyclonal anti-GFAP antibody (Dako, USA) at 1:1,200 dilution followed by anti-rabbit IgG-HRP (Santa-Cruz, USA) at 1:5,000 dilution were used. The signals were detected using Lumiglo Reagent and Peroxide (Cell Signalling, USA). Light is captured on a film by exposure for 5 min.

Results

GLC of Fatty Acids

There are two extreme conditions under which oils are generally consumed: as salad oil and as deep-fried. Hence, experiments were carried out on both raw oil and those that are subjected to prolonged heating. Analysis of FA composition of the various dietary oils by GLC showed a marked variation in their compositions. All the dietary oils analyzed showed substantial presence of palmitic acid, stearic acid, oleic acid, and linoleic acid (Table 1A). Coconut oil, in addition, contained a good amount of the saturated FAs, lauric acid (18.9%), and myristic acid (26%). Linseed oil, on the other hand, had significant presence of the n-6 FA precursor, α-linolenic acid (34.2%) and the n-3 FA, arachidonic acid (19.9%). Analysis of mustard oil showed the presence of significant amount of eicosanoic acid (6.5%) and erucic acid (38.5%), in addition to α-linolenic acid (15.7%) and arachidonic acid (12.4%). For the unique compositions found in coconut oil, linseed oil, and mustard oil, these were used for further studies. GLC analysis of these oils upon prolonged heating for 72 h showed a marked decrease in unsaturated FAs and increase in saturated FAs (Table 1B). Interestingly, heating caused complete depletion of arachidonic acid from both mustard oil and linseed oil (Table. 1B).

Immunocytochemistry

Pimary cultures of astrocytes were maintained in medium containing normal serum for 3, 7, and 12 days and morphology of the cells were observed after immunofluorescence cytochemistry using anti-GFAP. The cells underwent progressive maturation, differentiating from radial cells at 3 days of culture (Fig. 1A) to large polygonal astrocytes at day 7 (Fig. 1B) and subsequently to process bearing stellate cells at day 12 (Fig. 1C) similar to that observed earlier (Paul et al. 1999). Another set of cells were also cultured for 12 days where the cultures were supplemented with the FAs isolated from individual oils, namely, mustard, coconut and linseed during the last 48 h of culture instead of

Table 1 GLC composition of oils

(A) Raw oils									
	Mustard	Rapeseed	Coconut	Ricebran	Linseed	Sunflower	Safflower	Soyabean	Groundnut
LaA			18.9						
MA			26.0						
PA	2.0	12.2	17.7	20.5	5.3	22.5	12.5	17.3	12.1
SA	0.6	6.3	5.8	2.1	4.1	16.9	2.6	10.3	14.2
OA	28.6	36.8	22.7	48.2	21.5	41.8	29.0	33.2	39.6
LA	8.6	43.8	12.6	27.9	14.0	18.8	52.6	37	30.9
ALA	15.7				34.2			2.3	
AA	12.4				19.9		1.6		
EA	6.5						2.4	0.6	1.8
ErA	38.5								
(B) Oils after prolong heating									
	Mustard	Coconut	Linseed						
CA		5.9							
LaA		52.7							
MA		20.8							
PA	9.3	11.8	8.1						
SA	12.2	7.3	7.0						
OA	28.3		50.4						
LA	9.8		6.9						
ALA	12.9		11.6						
EA	5.4		17.2						
ErA	32.1								

Values for individual fatty acids are expressed as percentage of total fatty acids present in the oil. Fatty acids of raw oils were analyzed by GLC (A) out of which three oils selected were heated for 72 h and also analyzed for fatty acids (B). Details of GLC analysis are described in Sect. 2. Abbreviations used are as follows: CA, capric acid; LaA, lauric acid; MA, myristic acid; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, alpha linolenic acid; AA, arachidonic acid; EA, eicosanoic acid; ErA, erucic acid

normal serum. Addition of FAs of mustard oil (Fig. 1F, G) and linseed oil (Fig. 1H, I), in their raw and heat treated forms, respectively, had a marked effect on cell morphology when the astrocytes underwent transformation into mature stellate forms with thick filaments comparable to astrocytes grown in normal serum containing media all throughout. Coconut oils failed to initiate transformation of cells when added to the culture in raw and heat treated forms, respectively (Fig. 1D, E) and most of the astrocytes remain in their immature radial glia forms.

Beta-Adrenergic Receptor Binding Assay

We had earlier observed an increased expression of β-AR in matured astrocyte cultures (Joardar et al. 2006). β-AR binding assay was, therefore, performed with astrocyte membranes after treatment of cultures with FAs isolated from the selected oils (Table 2). While specific [^{3}H]PIN binding to β-AR was significantly enhanced in cells cultured in medium containing serum compared to those cultured in medium devoid of serum, supplementation with FAs from mustard and linseed oils in their raw forms showed a further enhancement in [^{3}H]PIN binding. The [^{3}H]PIN binding, in case of, heat-treated mustard and linseed oil were not detectable. Coconut oil, which is rich in saturated FAs, did not significantly influence [^{3}H]PIN binding in raw form when compared with that observed in membranes of astrocyte cultured in medium only. The heat-treated form of the oil, instead, showed a lower binding activity.

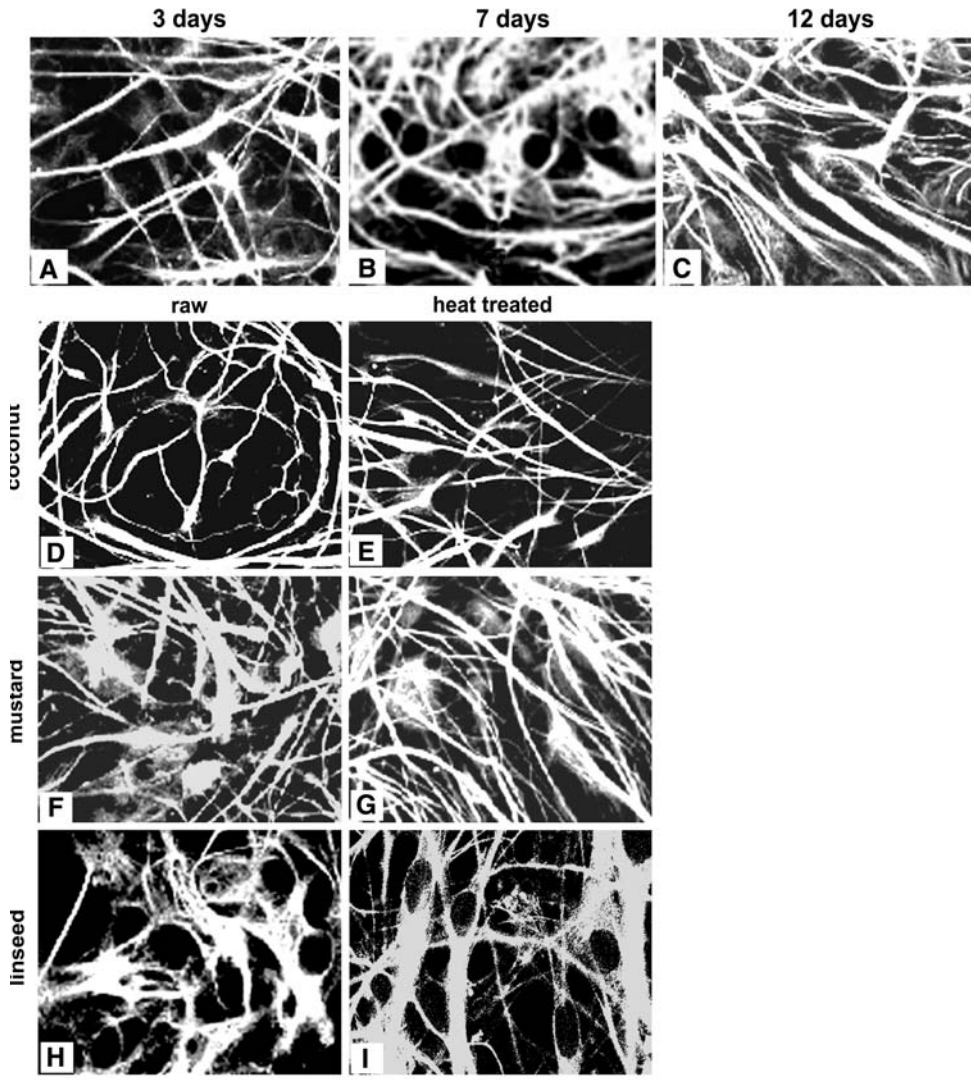


Fig. 1 Effect of supplementation of fatty acids isolated from oils on the morphology of cultured astrocytes. Cerebral astrocytes from newborn rat pups were cultured for 12 days. (**A**–**C**) represents astrocytes cultured for 3, 7, and 12 days, respectively, in normal serum. In others, 5-day-old cultures in normal serum were further cultured for 5 days in serum free media containing DMEM and F12 and then supplemented with 1% FBS and 100 nM FAs isolated from coconut oil (**D**, **E**), mustard oil (**F**, **G**) and linseed oil (**H**, **I**) for an additional 2 days. Left panel (**D**, **F**, **H**) indicate experiments with FAs from raw oil while right panel (**E**, **G**, **I**) indicate those derived from oils that were burnt. 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 Sect. 2. Photomicrographs are representatives from three separate experiments. Magnification 1,000×

2D Immunoblotting

2D electrophoresis followed by western blotting with anti-GFAP showed approximately 25 distinguishable spots in case of 7-day-old cultures, grown in normal serum (Fig. 2A).

Table 2 Specific binding of different FA supplemented astrocyte membranes to β-adrenergic receptors

FAs from edible oils supplemented in cultures	fmoles/mg of protein (I^{125}-PIN binding)
Mustard oil (R)	131.8 ± 0.013**
Mustard oil (B)	ND
Coconut oil (R)	28.4 ± 0.4
Coconut oil (B)	3.15 ± 0.39
Linseed oil (R)	324.28 ± 10.1**
Linseed oil (B)	ND
DMEM + F12	22.36 ± 0.17
DMEM + 10% sera	53.7 ± 1.38

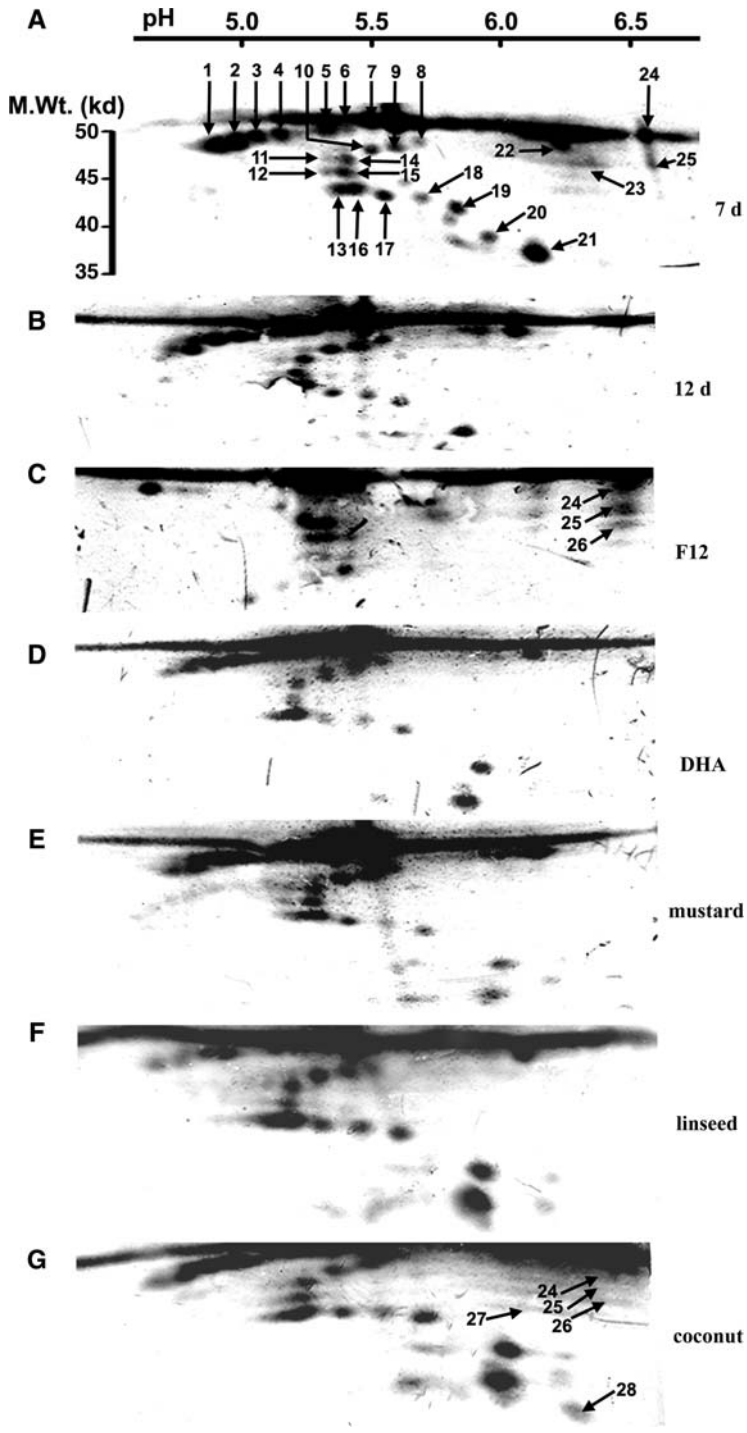
Cultured cells were treated without (control) or with 100 nM fatty acids from edible oils dissolved in 0.1% ethanol and 1% FBS for 48 h. Membrane preparation and receptor binding have been described in Sect. 2. Specific binding to β-AR using [^{125}I]pindolol (PIN). Students, *t*-test were performed. Results are expressed as mean ± S.E. of four determinations. ** $P < 0.001$ and * $P < 0.01$ from respective controls. ND, not detectable

However, at 12 days of culture, when the astrocytes underwent further differentiation, spots representing 23, 24, and 25 having isoelectric points of about 6.6 were absent (Fig. 2B). The immunoblotting pattern of cultures grown in medium (DMEM and F12) only for 12 days, devoid of serum (Fig. 2C), was somewhat similar to that observed for cultures of 7 days, except that a new isoform 26 was detected while spots representing 2, 3, 4, 18, 19, 20, and 21 were absent. Two dimensional gel electrophoresis immunoblots of astrocytes cultured in presence of DHA (Fig. 2D), FAs from mustard (Fig. 2E) and linseed oil (Fig. 2F) showed similar pattern and resembled that of cells grown in serum containing medium. In contrast, astrocytes supplemented with FAs from coconut oil showed the additional presence of the isoforms 24, 25, 26, 27, and 28 (Fig. 2G). It can be concluded that, as the astrocytes mature, a number of neutral isoforms of GFAP disappear.

Discussion

The major aim of the study was to determine the effect of edible oils of varying FA profile on the morphology and function of astroglial cells. Individually, we have observed a beneficial effect of the n-3 FA, DHA compared to the n-6 FA, arachidonic acid, mono unsaturated FA, linoleic acid or the saturated FA, stearic acid on the maturation and β-adrenergic transmission of developing astrocytes (Joardar et al. 2006). Increase in the consumption of n-3 FAs, namely, alpha-linolenic acid (ALA, 18:3n-3) and its long chain metabolites, eicosapentanoic acid (EPA, 20:5n-3), and DHA has been strongly recommended by health authorities of a number of developed countries like Canada (Scientific Review Committee 1990), the United Kingdom (The British

Fig. 2 Effect of supplementation of fatty acids isolated from oils on the expression of isoforms of GFAP ▶ by 2D electrophoresis. Astrocytes cultured under various treatment conditions as described in Fig. 1 were subjected to 2D electrophoresis followed by western blotting with GFAP. Details of the methods are described in Sect. 2. Figures are representative of atleast three experiments. A, B represents immunoblots of astrocytes cultured for 7 and 12 days, respectively, in normal serum. Astrocytes were cultured in serum free medium (**C**), or in medium supplemented 100 nM DHA (**D**), 100 ng of FAs isolated from mustard oil (**E**), linseed oil (**F**) and coconut oil (**G**) dissolved in 0.1% ethanol for an additional 2 days


A
pH
5.0
5.5
6.0
6.5
M.Wt. (kd)
50
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7 d
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mustard
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26
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coconut

Nutrition Foundation 1992) and Australia (National Health and Medical Research Council 1992). Generally fish oil and to a lesser extent, fish have been used as the source of n-3 FAs in clinical and biochemical studies (Meydani et al. 1993). ALA is present in certain vegetable oils, whereas EPA and DHA are present in fish and fish oil. Consumption of ALA leads to significant increase in tissue EPA (Mantzioris et al. 1994). However, the consumption of fish and fish oil yields higher concentrations of both EPA and DHA (Brown et al. 1990).

Out of the various oils studied, GLC analysis showed that vegetable oils, generally, did not contain EPA or DHA. FA composition of the oils tallied with published data (Niu et al. 2005). Only mustard, linseed and to some extent soyabean oil contains ALA and linoleic acid (18:2n-6) and could be potential source of n-3 FAs in our diet. Under the present experimental conditions it appeared that the FA composition of mustard oil was much more resistant to change during prolonged heating compared to coconut oil. Mustard oil has been used in the human diet over centuries and is considered to be safe. Results of the present study clearly demonstrated that FAs isolated from mustard oil, both raw and after heat treatment, was more effective than other oils in the morphogenesis in astrocytes.

Linseed oil, although edible, is not generally consumed due to its strong characteristic smell. Development of edible linseed has been jointly undertaken by CSIRO (Commonwealth Scientific and Industrial Research Organisation, Australia) and United Grain Growers Ltd of Winnipeg, Canada (UGG Ltd). This crop has been named Linola. Ground Linola seed contains mucilage, linked to reduce blood cholesterol and is a rich source of lignans, a group of anti-carcinogenic compounds (Askew 1992). Like mustard oil, addition of FAs of linseed oil also facilitated maturation of astrocytes. On the other hand, coconut oil contains a large fraction of saturated FAs whereas other oils contain a combination of both saturated as well as unsaturated FA.

Maturation of astrocytes involves contraction and extension of processes, which finally attain complex forms (Hatten 1990). Generally, differentiation of astrocytes in vivo involves changes from a flat epithelioid to a process bearing stellate morphology (Das 1976). Unlike coconut oil, the ability of FAs of mustard and linseed oil to promote maturation of astrocytes in culture, indicates an important contribution of ALA in astrocyte morphogenesis. ALA being a precursor of the n-3 FAs and in view of our previous observations of a beneficial role of DHA in astrocyte maturation (Joardar et al. 2006), it is suggestive that oils rich in ALA could be potentially suitable for astrocyte development. Although some studies indicate that the main source of DHA for the brain is uptake of the preformed DHA (Su et al. 1999), others indicate that DHA synthesis from n-3 precursors like ALA and EPA can also occur in the brain (Pawlosky et al. 1994). Furthermore, these oils also enhanced binding of [^{125}I]PIN to β-AR, which has a profound role in a number of functions in the cell. It is interesting to note that these oils in their burnt forms when supplemented in astrocytes, show no detectable binding to β-AR. The oils in their burnt forms lose arachidonic acid found in raw forms of the same oil. The depletion in binding may occur due to the loss of arachidonic acid. Since our previous work (Joardar et al. 2006) have emphasized the role of DHA in β-AR binding and cell maturation, the above observation may be due to an imbalance in the ratio of DHA and arachidonic acid. Further work remains to be done to explain the observations.

Glial fibrillary acidic protein is one of the intermediate filament (IF) family of proteins that interact with other proteins of IFs to make up the cytoskeleton of

vertebrate cells along with microfilaments and microtubules. IF cellular assembly and organization provide cells with a mechanism for resisting mechanical stress and deformation, but most importantly this facilitates the complex array of motile activities of the cytoskeleton (Helfand et al. 2003, 2004). During development, when the astrocytes undergo rapid changes in morphology, the expression of GFAP is also dynamically regulated (Pixley and de Vellis 1984; Stichel et al. 1991). GFAP is mainly a 50 kD protein but exists as various isoforms due to co-translational and post-translational modifications which include phosphorylation, sulphation, glycosylation, acetylation, oxidation etc., which may affect the molecular weight of GFAP (Noetzel 1990). Because the expression of GFAP is intimately connected with astrocyte maturation, we assessed the effect of treatment of the cultures with FAs isolated from the selected oils on the expression of GFAP isoforms in the cytoskeleton. It was observed that treatment of cultures with FAs of oils, which facilitated maturation of the cells, many of the neutral isoforms of GFAP were not expressed whereas new acidic variants were detected. The presence of GFAP isoforms with molecular masses varying between 36 and 50 kDa has been reported in glial tumors (Luider et al. 1999). Mechanisms, structures and functional consequences of modification of GFAP isoforms due to presence of FAs for different edible oils remain to be further investigated.

In summary, we show that dietary oils rich in n-3 fatty acids can be beneficial for astrocyte development and function. With the unraveling of a wide variety of functions of astrocytes, the major cells of the brain, the present understanding of the different functions of the brain has undergone a sea of change. Today, the function of the neuron cannot be considered in isolation due to its intricate interactions with astrocytes and other glial cells. The varied effect of the fatty acid constituents of the dietary oils, evaluated in the present study, on the development of functional astrocytes, signifies a closer look at other oils consumed worldwide.

Acknowledgments The Council of Scientific & Industrial Research, Govt. of India provided financial support to AJ to carry out the study. Authors would like to thank Dr. A. K. Sen for his help in analyzing the fatty acid composition of the various oils studied by GLC.

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