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Effect of arachidonic, eicosapentaenoic and docosahexaenoic acids on the oxidative status of C6 glioma cells

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Abstract

$n-3$ polyunsaturated fatty acids (PUFAs) have been described to have beneficial effects on brain development and in the prevention and treatment of brain damage. C6 glioma cells were incubated with 100 μM of either C20:4$n-6$ (ARA), or C20:5$n-3$ (EPA), or C22:6$n-3$ (DHA) for different time periods to assess whether these acids altered the cellular oxidative state. The ARA and EPA were promptly metabolised to C22:4$n-6$ and C22:5n − 3, respectively, whereas DHA treatment simply increased the amount of DHA in the cells. Cell viability was not affected by ARA, while a cytotoxic effect was observed 72 h after $n-3$ PUFAs supplementation. The levels of reactive oxygen species and thiobarbituric acid-reactive substances were significantly higher in DHA-treated cells than in EPA- and ARA-treated groups. This modification in the oxidative cellular status was also highlighted by a significant increase in catalase activity and a decrease in glutathione content in DHA-supplemented cells. Glucose-6-phosphate dehydrogenase activity, an enzyme involved in redox regulation, and $O_2^{\bullet -}$ release were significantly increased both in EPA and DHA groups. The effect of DHA was more severe than that of EPA. No significant changes were observed in the ARA group with respect to untreated cells. These data show that EPA and DHA induce alterations in the oxidative status that could affect the glial function.

Keywords: *Reactive oxygen species, anion superoxide, thiobarbituric acid-reactive substances,* n − *3 polyunsaturated fatty acids, antioxidant enzymes*

Abbreviations: *ARA, arachidonic acid; CAT, catalase; CL, cardiolipin; DCF, 2′,7′-dichlorofluorescein; DHA, docosahexaenoic acid; DTPA, diethylentriaminopentacetic acid; EPA, eicosapentaenoic acid; FACS, fluorescence-activated cell sorting; G6PDH, glucose-6-phoshate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPX, glutathione peroxidase; LDH, lactate dehydrogenase; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances*

Introduction

There are quite a few instances in the literature describing the beneficial effects of polyunsaturated fatty acids (PUFAs) and their metabolites in the prevention of ischemic brain damage [1] and on various brain dysfunctions such as epileptic seizures, depression or bipolar diseases [2–5]. Owing to the multiplicity of double bonds, PUFAs are putative targets for free radical propagation, during which lipid peroxides are generated. Lipid peroxides are considered to be deleterious to tissues [6]. Elevating the concentration of PUFAs in brain lipids could make them particularly susceptible to oxidative damage, considering the high oxygen consumption of this organ and the modest antioxidant defences [7]. Evidence has accumulated that suggests a relationship between oxidative stress and neurodegenerative disorders [8]. Recent findings [9] provide evidence that the massive neurodegeneration in early age of familial Alzheimer's disease could be a result of an increased vulnerability of neurons through

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the activation of different apoptotic pathways as a consequence of elevated levels of oxidative stress. Oxidative stress would contribute to the cascade leading both to dopamine cell degeneration in Parkinson's disease [10,11] and demyelination in multiple sclerosis [12].

It is well known that the composition of brain fatty acids is affected by dietary lipids[13]. Our previous studies demonstrated that diets rich in either long-chain $n-3$ polyunsaturated fatty acids (fish oil), or *n*-6 fatty acids (soybean oil), or saturated fatty acids (coconut oil) affected the fatty acid composition of myelin and synaptosomes in growing rats [14]. Furthermore, our results indicated that the membranes rich in $n-3$ fatty acids are more susceptible to oxidative stress. These observations are supported by *in vitro* studies on the oligodendroglia-like cell line (OLN 93) [15]. The long-term supplementation of these cells with DHA resulted in enhanced levels of thiobarbituric acid-reactive substances (TBARS) in the culture medium, and a greater percentage of apoptotic cell death following exposure to H_2O_2-induced oxidative stress with respect to untreated cells. DHA supplementation promoted cellular damage even in the absence of genotoxic stress [15] since the enriched OLN 93 cells showed a 35% reduction of mitochondrial activity. On the other hand, a four-day exposure of C6 cells to sub-lethal concentrations of gamma-linoleic acid or eicosapentaenoic acid (EPA) did not significantly increase reactive oxygen species (ROS) [16]. This suggests that the cellular antioxidant defence could be modulated differently by various PUFAs.

An up-regulation of genes involved in the antioxidant defence system has been recently shown in the liver of mice fed a diet rich in fish oil for 3 months [17]. However, *in vivo* studies are not suitable for analysing the specific role of individual PUFAs in oxidative stress.

To understand the effects of different PUFAs on the oxidative status and antioxidant capacity of brain cells, C6 glioma cells were grown in the presence of either arachidonic acid (ARA; C20:4n − 6) or eicosapentaenoic acid (EPA; C20:5n − 3) or docosahexaenoic acid (DHA; C22:6n − 3). C6 cells have been widely used as an *in vitro* model for the study of glial cell properties, since they retain some functional and morphological properties of astrocytes and oligodendrocytes [18,19]. Lipid peroxidation and ROS were determined at different times of fatty acid exposure. The cellular redox state was also studied by measuring glutathione content, and catalase (CAT) and glutathione peroxidase (GPX) activity. The activity of glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49), a key enzyme of the pentose phosphate pathway [20], was also determined in order to assess changes in its capacity to generate NADPH involved in the regulation of the intracellular redox state of the cell [47]. The aim of this study was to investigate the risks posed by exposing the brain to high doses of exogenous PUFAs.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin and Triton were purchased from Gibco-Invitrogen Ltd. (Paisley, UK). Metanolic HCl was obtained from Supelco (Bellefonte, PA, USA). Isopropanol, methanol, chloroform, hexane, cytocrome *c* were purchased from Carlo Erba (Italy). Sodium arachidonate, eicosapentaenate, docosahexaenate, diethylentriaminopentacetic acid (DTPA), superoxide dismutase (SOD), *o*-phthaldialdehyde were provided by Sigma (St Louis, MO, USA). All fatty acids were dissolved in DMEM and stored in nitrogen at − 80°C until use.

Pyruvic acid sodium salt, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate(NADH), β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$) and β-D-glucose 6-phosphate sodium salt were purchased from Fluka (Buchs, Switzerland). All other reagents utilised were of the highest grade available.

Cell culture and treatment

Rat C6 glioblastoma cells obtained from American Type Culture Collection (ATCC) were grown to confluence in DMEM supplemented with 10% heat-inactivated FBS, penicillin (50 U/ml), streptomycin (150 μg/ml) and fungizone (100 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Upon reaching confluence, C6 cells were plated at a density of 2.5×10^5/ml in a 6-well plate and the following day the cultures were incubated in growth medium supplemented with a sodium salt solution of different fatty acids to 100 μM final concentration for 24, 48 and 72 h at 37°C. Controls were treated similarly, but fatty acids were omitted.

Cell viability assay

Cell viability was assessed by measuring intracellular lactate dehydrogenase (LDH) release into the culture medium, and expressed as percentage of total cellular activity. LDH is a cytosolic enzyme that damaged cells release in the culture medium. LDH activity was determined at 30°C as the change in absorbance at 340 nm, using 0.18 mM NADH and 0.72 mM pyruvate as substrates in 50 mM K-phosphate buffer, pH 7.4, on culture medium and cell lysates (0.5% Triton X-100 in 0.1 M PBS pH 7.4) [21].

Lipid extraction and analysis

Cells were washed twice in DMEM and scraped into PBS. Lipids were extracted from cells by isopropanol. After centrifugation, lipids were dried under a nitrogen stream, and redissolved in 1 ml of chloroform/methanol (2:1 v/v). Samples were transmethylated by methanolic HCl overnight at 70°C. The fatty acids methyl esters were analysed by a gas-chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a fused silica capillary column of Supelcowax 30 m × 0.53 mm i.d. (Supelco). Peaks were identified by a commercial standard mixture (Supelco). Results were expressed as means ± SE over a minimum of three samples. The unsaturation index was calculated as the sum of the percentages of individual fatty acids per number of double bonds.

Fluorescence-activated cell sorting (FACS) analysis

The level of ROS was determined in the control and fatty acids-treated cells by labelling with cell-permeable 2′,7′-dichlorofluorescein diacetate (DCF-DA) as previously described [22]. Once diffused into the cells, DCF-DA is hydrolysed to cell-impermeable non-fluorescent 2′,7′-dichlorofluorescein (DCF) and trapped within the cells. DCF is oxidized by intracellular oxidants to highly fluorescent DCF, providing a quantitative determination of the intracellular level of oxidant species.

In brief, the cells were incubated with 3 μM DCF-DA (Molecular Probes) for 20 min at 37°C, then pelletted by centrifugation and washed with PBS, pH 7.2. The cells were resuspended in PBS, pH 7.2, containing iodide propidium to exclude dead cells, and then kept on ice before measuring fluorescence levels with Epics Elite ESP Flow-Cytometer (Beckman Coulter, Miami, FL, USA). The oxidized DCF fluorescence levels were always normalized to C6 cells autofluorescence.

Thiobarbituric acid assay

Lipid peroxidation was detected by the thiobarbituric acid assay as previously described [23]. The method measures the production of malondialdehyde or other related substances, i.e. TBARS. This method was a first global measure of peroxidation and was applied because of its sensitivity and simplicity [7].

After being exposed to fatty acids, the cells were scraped and washed with PBS. Then 0.375% 2-thiobarbituric acid and 15% trichloracetic acid were added. The tubes were placed in a water bath and kept at 95°C for 45 min. After cooling, the colour developed was read in a spectrophotometer (Beckman Coulter, Miami, FL, USA) at 535 nm. The concentration was calculated using an extinction coefficient of $1.56 \times 10^5\,M^{-1}\,cm^{-1}$ and expressed as nmol TBARS/mg protein. In addition, a calibration curve was established with malondialdehyde produced by hydrolysis of 1,1,3,3-tetramethoxypropane in 0.1 M HCl as standard.

Superoxide assay

Reduction of ferricytochrome *c* to ferrocytochrome *c*, with and without the addition of SOD (10 μg/ml), was used to measure the $O_2^{\bullet -}$ formation rate. Cells were washed three times with PBS containing DTPA 0.1 mM, and then 1 ml of DTPA containing 10 μM of cytochrome *c* was added. The reaction was monitored spectrophotometrically (Beckman Coulter) at 550 nm, using the extinction coefficient of $2.1 \times 10^4\,M^{-1}\,cm^{-1}$, which corresponds to ΔE between ferricytochrome *c* and ferrocytochrome *c* [24].

Glutathione determination

Reduced and oxidized glutathione levels (GSH and GSSG, respectively) were measured by high-performance liquid chromatography using fluorimetric detection, after derivatization with *o*-pthaldialdehyde [25]. Glutathione levels were quantified as nmol glutathione per mg protein and expressed as percentage with respect to control cells.

Enzymatic assay

Cells were harvested with a rubber policeman into the medium, and spun at 1200*g* in Eppendorf tubes for 10 min at 4°C. Cells were washed once with PBS and then resuspended in a solution containing 50 mM Na-phosphate and 0.5% (v/v) Triton X-100 at pH 7.5, and sonicated for 15-s bursts. Cells were then centrifugated at 15,000*g* × 10 min and the supernatant collected for enzymatic assays.

CAT activity was measured by monitoring the disappearance of hydrogen peroxide at 240 nm, following the procedure described by Aebi [26]. Catalase activity was expressed as μmol of H_2O_2 consumed min^{-1} mg $protein^{-1}$.

GPX activity was measured with a commercial kit (Oxis International Inc., Portland, USA). Absorbance at 340 nm was recorded in a spectrophotometer for 5 min. One unit of enzyme was defined as nmol of NADPH oxidized min^{-1} mg $protein^{-1}$.

G6PDH activity was determined, as already described [27] by measuring the rate of production of μmol of NADPH min^{-1} mg $protein^{-1}$.

Protein determination

Protein levels were measured by Bradford's method [28] with a commercial kit (BioRad Laboratories, Hercules, CA, USA), using a bovine serum albumin standard.

Statistical analysis

Results are expressed as mean ± SE. Group means were compared by one-factor ANOVA. Upon significant interactions, differences between group means were analyzed by Fisher's probable least-square difference (PLSD) test. Differences were considered statistically significant at $p < 0.05$.

Results

PUFA incorporation

To study the incorporation of PUFAs, the fatty acid pattern of glial cells was analysed at different times upon incubation with 100 μM fatty acids. Untreated C6 cells were rich in saturated and monounsaturated fatty acids (21% C16:0, 19% C18:0 and 31% C18:1) with low percentages of PUFA (6.6% ARA, 1.5% EPA and 5.2% DHA), which did not change significantly with incubation time. As shown in Figure 1, C6 glioma cells significantly incorporated PUFAs, reaching a peak at 48 h. PUFAs were elongated and desaturated; supplementation with ARA and EPA resulted in the accumulation of C22:4n − 6 and C22:5n − 3, respectively. As a consequence, these fatty acids became the major n − 6 and n − 3 fatty acids in cellular lipids. Glioma cells readily took up C22:6n − 3 from the medium and this was accompanied by a significant increase in C22:5n − 3 as compared with controls, indicating active chain shortening and retro-conversion of C22:6n − 3.

The exogenous PUFAs appear to have replaced the monounsaturated fatty acids, while the saturated fatty acid content remained largely unchanged (Figure 2**).** The unsaturation index significantly increased in C6 cells treated with ARA, EPA and DHA as compared with controls. Among the three groups, the only significant difference was observed at 24 h, the unsaturation index of EPA-treated cells was lower than that of ARA and DHA groups.

Effects of different PUFAs on cell viability

Cell viability was determined by measuring LDH release. LDH is a cytoplasmic enzyme that dying cells release in the culture medium. Figure 3 shows that the exposure to different long-chain fatty acids at a concentration of 100 μM did not significantly alter

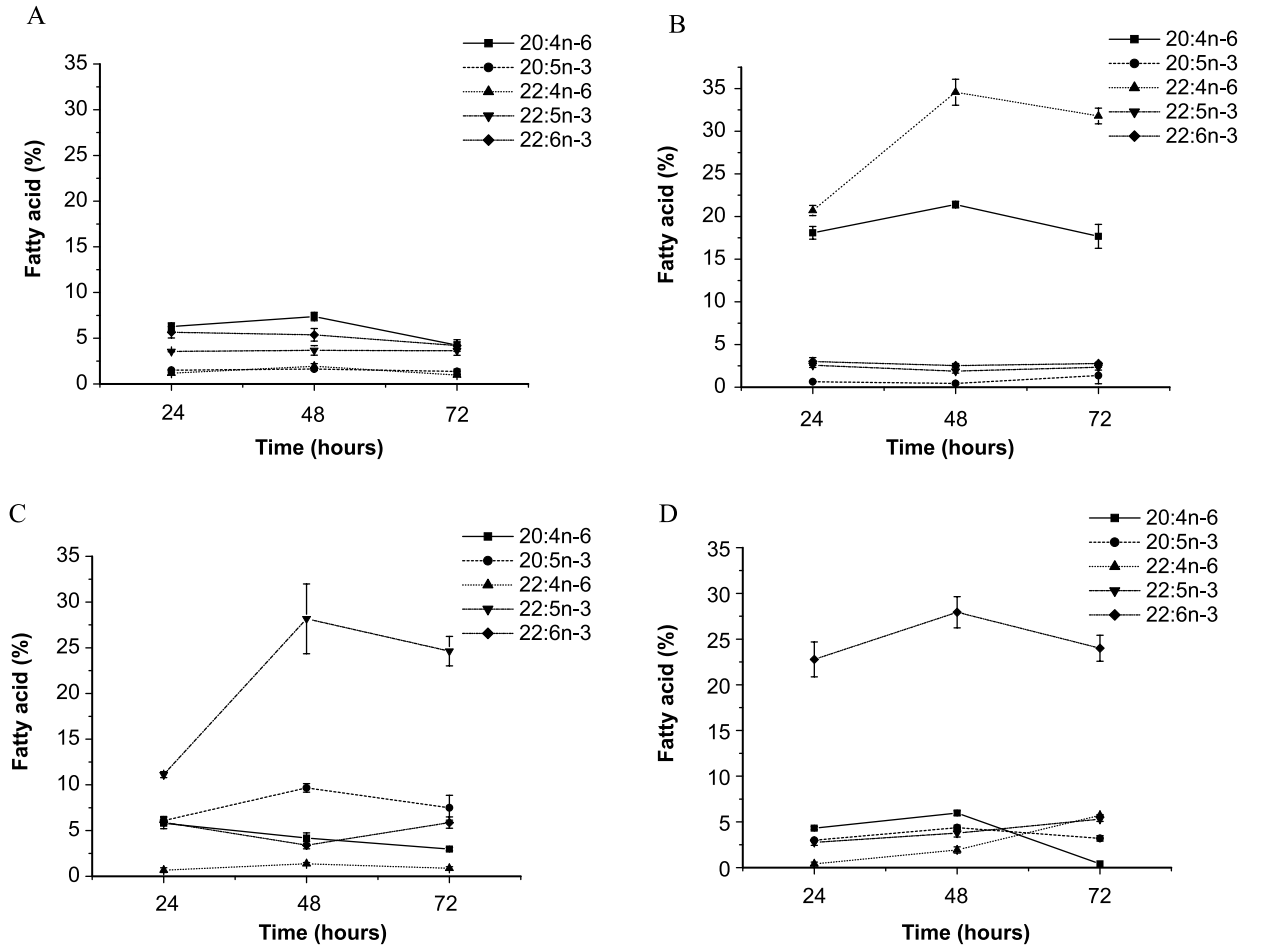

Figure 1. PUFAs pattern of C6 glioma cells without fatty acid exposure (control) (A) and with C20:4n − 6 (B), C20:5n − 3 (C) and C22:6n − 3 (D) supplementation at different time points.

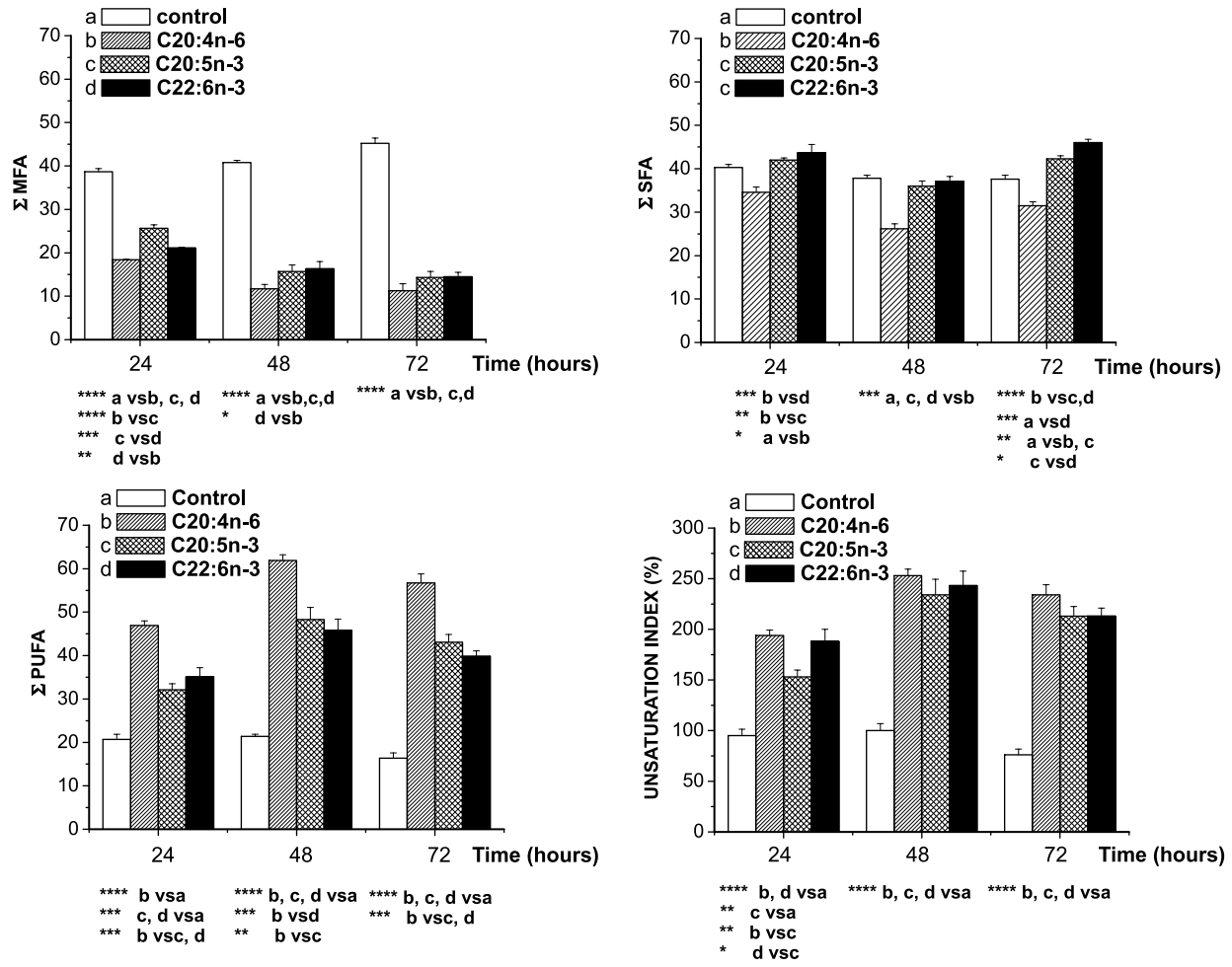

Figure 2. Fatty acid pattern (%) of C6 glioma cells, untreated (control) and supplementated with C20:4n − 6 (b), C20:5n − 3 (c) and C22:6n − 3 (d) at different time points. ΣSFA: saturated fatty acids; ΣMFA: monounsaturated fatty acids; ΣPUFA polyunsaturated fatty acids. The values represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (ΣSFA 24 h $p = 0.004$; ΣSFA 48 h $p = 0.0006$; ΣSFA 72 h $p < 0.0001$; ΣMFA 24, 48 and 72 h $p < 0.0001$; ΣPUFA 24, 48 and 72 h $p < 0.0001$; unsaturation index 24, 48 and 72 h $p < 0.0001$) followed by Fisher's PLSD test. ****$p < 0.0001$; ***$p < 0.001$; ** $p < 0.01$; *$p < 0.05$.

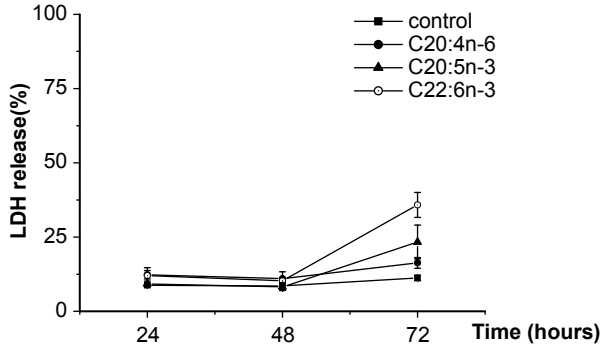

Figure 3. Cell viability of C6 glioma cells, untreated (control) and treated with PUFAs, was determined by LDH release. Cells were grown up to 72 h in the presence of 100 μM of either C20:4n − 6 (ARA) (b), or C20:5n − 3 (EPA) (c), or C22:6n − 3 (DHA) (d). The values represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (LDH 24 h $p = 0.481$; LDH 48 h $p = 0.4681$; LDH 72 h $p = 0.0042$) followed by Fisher's PLSD test. 72 h after PUFAs supplementation ***$p < 0.001$ DHA vs. Control; **$p < 0.01$ DHA vs. ARA; *$p < 0.05$ EPA vs. Control and DHA.

the extent of LDH leakage up to 48 h incubation as compared with the controls. Whereas, the cytotoxic effect was evident in C6 cells incubated with DHA for 72 h, the percentage of LDH leakage rose sharply. The increase in LDH leakage was significantly lower in C6 cells exposed to EPA. No cytotoxic effect was observed in cells treated with ARA for 72 h.

Effects of different PUFAs on cellular oxidative stress and antioxidant defence mechanisms

In order to demonstrate the PUFA-induced modification of the redox state, we determined the intracellular level of oxidant species by DCF fluorescence. The results shown in Figure 4 indicate that the presence of either ARA or EPA in the culture medium for 48 h causes only a slight increase in the oxidation of 2′,7′-dichlorodihydrofluorescein to 2′,7′-dichlorofluorescein as compared with untreated C6 cells in which the oxidant levels increase with time.

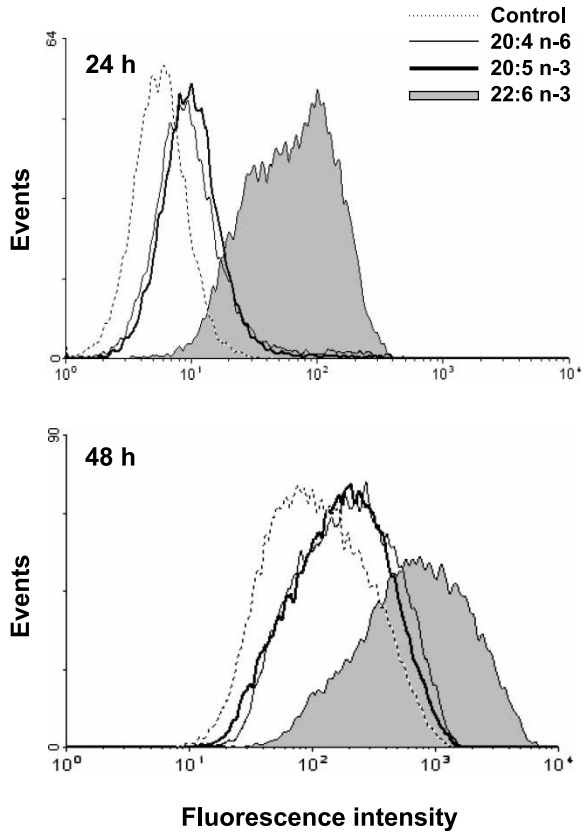

Figure 4. Intracellular oxidant species were determined by FACS analysis using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Control cells (dotted line) were compared with cells supplemented with C20:4 (ARA—black line), C20:5 (EPA—bold line) and C22:6 (DHA—grey histogram). The figure illustrates one of three separate experiments which gave similar results.

Conversely, the presence of DHA in the culture medium caused a significant production of oxidant species after only 24 h of incubation.

The possible involvement of different PUFAs in lipid peroxide production was investigated by measuring the intracellular TBARS content. C6 cells treated with different long-chain fatty acids showed a significant increase in TBARS levels compared with controls soon after 24 h incubation (Figure 5). The TBARS levels observed at 48 h were lower than those at 24 h because they increased two-fold in the controls. TBARS levels were significantly lower in the EPA- than in DHA-treated group, both in short and long-term incubation. The ARA group was no significantly different from EPA and DHA groups.

The supplementation of C6 cells with different PUFAs for 24 h increased superoxide release (Figure 6). The increment was proportional to the number of double bonds. In fact, superoxide release in C6 cells treated with ARA was significantly higher than in controls, but markedly lower than in cells incubated with EPA and DHA. The increase in the DHA group was somewhat higher, although not significant, than in the EPA group.

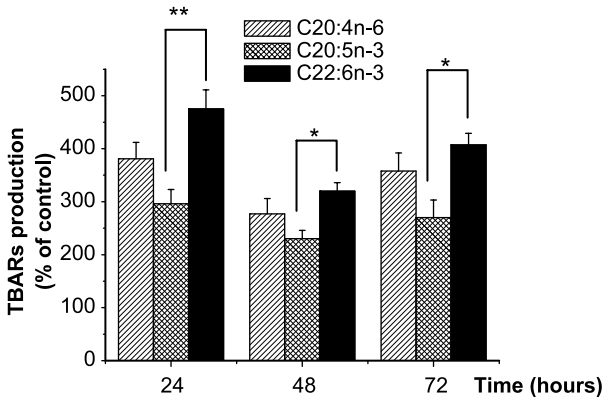

Figure 5. TBARS production in C6 glioma cells treated with 100 μM of either C20:4n − 6 (ARA), or C20:5n − 3 (EPA), or C22:6n − 3 (DHA). TBARS were measured as nmol/mg protein. The values, expressed as percentage of the levels detected in controls, represent the mean ± SE of three independent experiments. Statistical analysis was performed by one factor ANOVA (TBARS 24 h $p = 0.0127$; TBARS 48 h $p = 0.0466$; TBARS 72 h $p = 0.0455$) followed by Fisher's PLSD test. $^{**}p < 0.01$; $^{*}p < 0.05$.

Supplementation of the cell culture with either EPA or DHA for 24 h significantly reduced the levels of intracellular glutathione with respect to both control and ARA-treated cells (Figure 7). Oxidized glutathione remained undetectable under our conditions, indicating that GSH depletion was not coupled with increased levels of GSSG. After 24 h of PUFA supplementation, a significant concomitant increase in CAT activity and decrease in GPX activity was observed in DHA-treated cells as compared with the controls, while no major differences were observed for ARA and EPA-treated groups (Table I).

The inducible activity of G6PDH, the rate-limiting step in the pentose phosphate pathway, was examined in cells treated with different PUFAs for 24 h. As illustrated in Table I, a 55 and 62% increase was found in the specific activity of the EPA and DHA groups, respectively, as compared with the control and ARA groups. Again, the increments were proportional to the number of double bonds. The level of G6PDH activity was somewhat higher, although not significant, in the DHA group with respect to the EPA group and significantly higher in the ARA group with respect to controls.

Discussion

In the present study, C6 cells were used for their functional and morphological properties similar to glial cells- and because of their low PUFA content. This relative deficiency in PUFAs allowed us to manipulate their long-chain fatty acid content by exogenous supplementation in order to study the effect of individual PUFAs on oxidative balance.

PUFAs were efficiently incorporated. EPA and ARA were promptly metabolised as C22:5n − 3 and

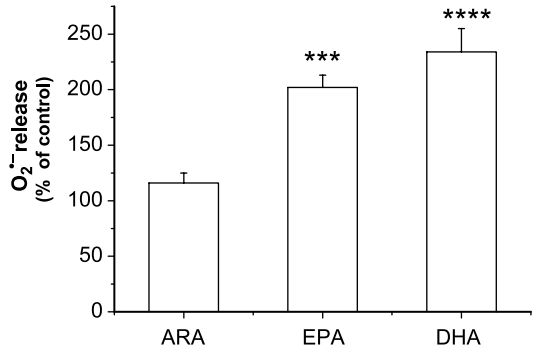

Figure 6. O_2^- release in C6 glioma cells incubated with C20:4n − 6 (ARA), or C20:5n − 3 (EPA), or C22:6n − 3 (DHA) for 24 h. The O_2^- release, quantified as nmol/mg protein, was expressed as percentage of levels detected in control cells. Data are the mean ± SE of three independent experiments, each performed in triplicate. Statistical analysis was performed by one factor ANOVA ($p < 0.0001$) followed by Fisher's PLSD test. ****$p < 0.0001$ DHA vs. ARA; ***$p < 0.001$ EPA vs. ARA.

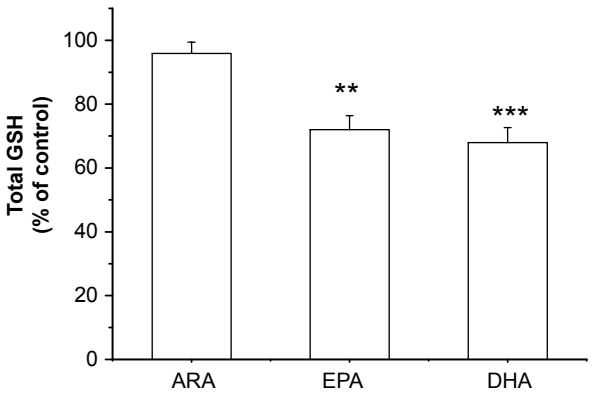

Figure 7. Glutathione content in C6 glioma cells exposed to C20:4n − 6 (ARA), or C20:5n − 3 (EPA), or C22:6n − 3 (DHA) for 24 h. Glutathione content was quantified as nmol of glutathione/mg protein and expressed as percentage of levels detected in control cells. Data are the mean ± SE of three independent experiments, each performed in triplicate. Statistical analysis was performed by one factor ANOVA ($p = 0.0008$) followed by Fisher's PLSD test. ***$p < 0.001$ DHA vs. ARA; **$p < 0.01$ EPA vs. ARA.

C22:4n − 6, respectively, whereas DHA remained mostly unchanged. The fact that DHA did not increase in EPA-treated cells can be attributed to an inhibitory effect of EPA on delta 6-desaturase [29,30], which has been postulated to be involved in the synthesis of C22:6n − 3 from C22:5n − 3[31].

The different fate of PUFAs in C6 cells could reflect differences in their roles and metabolism. It is well known that ARA and DHA are present in glioma cells at higher levels than EPA, which is preferentially utilized for energy purposes, e.g. β-oxidation. Our results agree with previous *in vivo* and *in vitro* studies. It has been reported that the administration of purified EPA to rats for 1 or 4 weeks led to an increased level of C22:5n − 3 in plasma and tissues [30–32], which was not accompanied by a concomitant increase in DHA levels. DPA enrichment was also obtained [33] in astrocyte cultures incubated with EPA.

The modification of the cellular fatty acid composition due to PUFA supplementation affected the unsaturation index that was higher in PUFA-treated C6 cells than in controls. Cells with a higher degree of membrane unsaturation are more susceptible to oxidative stress [34]. Nevertheless, in our study the oxidative stress seems not to be related to the degree of membrane unsaturation. In fact, DHA-enriched cells produced 4-fold more ROS than ARA- and EPA-enriched cells, even though the unsaturation index is similar in three groups. The specific amplification of ROS by DHA could suggest that DHA accumulated in a critical subcellular pool. Evidence [35] suggests that DHA increases ROS production by accumulating in cardiolipin (CL), where it alters electron transport efficiency. The inability of ARA and EPA to induce oxidative stress at levels similar to those produced by DHA was attributed to the fact that CL does not incorporate ARA or EPA. In the present study, the increase in ROS content in the DHA group after 24 h treatment could also be explained by the selective accumulation of DHA in the acyl chains of CL, which, in turn, may alter the mitochondrial potential.

ROS are a physiological product essential to many normal biological processes, but they can become hazardous to cells and tissues if their production is not strictly controlled. The GSH system is essential for the control of total cellular redox states and is the primary defence mechanism for the removal of peroxide from the brain [36,37], where it is considered to be distributed mainly in astrocytes [38]. The decrease in GSH content observed for the DHA group was accompanied by an increase in cellular antioxidant defences, as revealed by the significant increase in CAT activity. This may be attributed to an increase in

Table I. GPX, catalase and G6PDH activities in C6 glioma cells treated with different PUFAs for 24 h.

	Control	ARA	EPA	DHA	*p*-Value
GPX ($nmol^{-1}\,mg^{-1}\,min^{-1}$)	16.7 ± 0.9^{a}	15.5 ± 1.0	15.5 ± 0.6	13.6 ± 0.6^{b}	0.0833
Catalase ($\mu mol^{-1}\,mg^{-1}\,min$)	8.8 ± 1.1^{a}	9.6 ± 1.6^{a}	10.9 ± 1.5	14.1 ± 0.9^{b}	0.0861
G6PDH ($\mu mol\ NADPH^{-1}\,mg^{-1}min$)	13.6 ± 1.2^{c}	$18.7 \pm 2.1^{c,d}$	$30.5 \pm 2.3^{e,g}$	35.7 ± 2.6^{e}	<0.0001

Data are expressed as mean ± SE of three independent experiments performed in triplicate. Significance of difference between treatment and control groups was determined by one-factor ANOVA with post hoc testing using Fisher's PLSD test ($p < 0.05$). Values with different superscript are significantly different [a] vs. [b] $p < 0.05$; [d] vs. [g], $p < 0.01$; [c] vs. [e] $p < 0.0001$.

superoxide production in the mitochondria with the possible consequent increase in the formation of H_2O_2 during peroxisomal fatty acid oxidation. Surprisingly, a significant reduction of GPX activity was observed in C6 glioma cells treated with DHA. Previously, a reduction of GPX activity was also observed in bovine retinal endothelial cells incubated for 10 days with 10 μM of DHA, but not in bovine aortic endothelial cells [39]. The different effect of DHA on GPX activity in relation to cell type may suggest that this PUFA could activate different cellular pathways, directly or indirectly, depending on its availability and metabolism in the cells.

A perturbation of cellular oxidative state was also induced by EPA, since we observed an increase in $O_2^{\bullet -}$ release and a decrease in total glutathione. However, ROS production as measured by DCF was similar to that of controls. This result is not in discordant with the increased release of anion superoxide, for DCF is not supposed to detect $O_2^{\bullet -}$ while it does react with H_2O_2 and other oxidant species in the cell [40]. This hypothesis could be supported by the fact that only a slight, not significant increase in CAT activity was observed in EPA group. Furthermore, the inhibition of phospholipase A_2 by EPA [41,42] could be responsible for the low levels of TBARS and ROS production. Instead, ARA is a good substrate for phospholipase A_2 and, on the other hand, TBARS production is intimately involved in arachidonic acid metabolism [43]. Traditionally, DHA administration has been associated with COX inhibition [44], and this could explain the high production of oxidant products. Moreover, the most severe effect of DHA on cellular oxidative state could be partly due to its inhibitory effect on GPX.

Both EPA and DHA affected the oxidative state of C6 cells, but the mechanism(s) involved are probably different since the effects of the former were particularly evident only after long-term incubation and the oxidant species levels were lower in the EPA group than in the DHA group. The protection against cellular damage induced by oxidants could also be enhanced by increasing both the activity and the protein expression of glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the pentose phosphate pathway [45]. This pathway is an important source of NADPH, which plays a crucial role in the regulation of the intracellular redox state. NADPH is required for both the regeneration of GSH by GSSG through the action of glutathione reductase [46] and the formation of active catalase tetramers [47]. NADPH tightly binds to mammalian catalase preventing the H_2O_2-dependent conversion of the enzyme to an inactive state. Transient induction of G6PDH expression was observed after treatment with agents that either increase the intracellular concentration of $O_2^{\bullet -}$ or reduce GSH intracellular content [48].

Cell death could be observed only after 72 h of EPA or DHA incubation as suggested by the sharp increase in LDH release. Again, the effect of DHA is more pronounced than that of EPA. Further support for the deleterious effect of n − 3 PUFAs on cell survival was evident from the cell cycle analysis after staining with propidium iodide. The number of dead cells increased 5-, 8- and 10-fold in ARA, EPA- and DHA-supplemented cells, respectively (data not shown).

In summary, the present study demonstrates that n − 3 PUFAs alter the oxidative state of glial cells. The effects of DHA are more pronounced than those of EPA. It has been also shown that the oxidative state is independent of the unsaturation index, which is similar among PUFAs-treated groups. Preliminary results obtained with primary cell cultures indicated that EPA and DHA have a cytotoxic effect on rat oligodendrocytes similar to that observed in C6 cells (work in progress). The brain is a network of different cell types, each characterized by a specific fatty acid composition and a specific functional role. Several papers [3,49] have described the neuroprotective effect of DHA on neurons, where it is present at high levels. Recently, Wang et al. [50] demonstrated that DHA at 5–50 μg/ml successfully protected rat hippocampal cultures against glutamate-induced cytotoxicity. These neuronal cells could have developed antioxidative mechanism(s) to protect themselves by a high DHA content. Recently, it has been shown that in retina photoreceptors DHA modulates the levels of pro- and anti-apoptotic proteins of the Bcl-2 family, thus protecting photoreceptors from oxidative stress [51]. Conversely, glial cells, with their low DHA content, could be more susceptible to oxidative stress in conditions that alter their PUFA content, which results in an unbalanced n − 6/n − 3 ratio. This hypotheses is supported in our previous study [52], in which we observed adverse effects of n − 3 PUFAs on myelin deposition, but no effect on the cholinergic system of growing rats fed fish oil for 6 weeks.

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