Archives of Medical Research 48 (2017) 46–54

ORIGINAL ARTICLE

Polyunsaturated Fatty Acids Differentially Modulate Cell Proliferation and Endocannabinoid System in Two Human Cancer Lines

Repossi Gastón,[a,b,c] Pasqualini María Eugenia,[a] Undurti N. Das,[d,e] and Aldo R. Eynard[a,c]

[a]*Biología Celular, Histología y Embriología, Facultad de Ciencias Médicas, Universidad Nacional Córdoba, Argentina*
[b]*Cátedra de Histología y Embriología, Universidad Nacional de La Rioja, La Rioja, Argentina*
[c]*INICSA, CONICET-UNC, Córdoba, Argentina*
[d]*Department of Medicine and BioScience Research Centre, GVP Hospital, Gayatri Vidya Parishad College of Engineering, Visakhapatnam, India*
[e]*UND Life Sciences, Federal Way, Washington, USA*

Received for publication March 4, 2016; accepted January 16, 2017 (ARCMED-D-16-00137).

Background and Aims. Evidence suggests that quantity and quality of dietary polyunsaturated fatty acids (PUFAs) play a role in the development of cancer. However, the mechanisms involved in this interaction(s) are not clear. Endocannabinoids are lipid metabolites known to have growth modulatory actions. We studied the effect of supplementation with PUFAs ω-6 and ω-3 (essential fatty acids, EFAs), saturated and monounsaturated fatty acids (non-EFAs) on the growth of tumor cells and modifications in their endocannabinoid content.

Methods. Cell cultures of human glioblastoma (T98G) and breast cancer (MCF7) were supplemented with 50 or 100 mmol EFAs and non-EFAs for 72 h. Cell proliferation was then determined by MTT, anandamide (AEA) levels by HPLC, total fatty acids profiles by GLC, CB1 receptor expression by WB and FAAH activity by spectrophotometric method.

Results. Fatty acids profile reflected the incorporation of the lipids supplemented in each assay. Arachidonic acid (EFA ω-6) supplementation increased AEA levels and inhibited the growth of T98G, whereas palmitic acid (non-EFA) enhanced their proliferation. In breast cancer (MCF7) cells, eicosapentaenoic acid (EFA ω-3) reduced and oleic acid (non-EFA) enhanced their proliferation. CB1 expression was higher in T98G and no differences were observed in FAAH activity.

Conclusions. The growth of tumor cells can be differentially modulated by fatty acids and, at least in part, can be attributed to their ability to act on the components of the endocannabinoid system.

Key Words: Cell proliferation, Endocannabinoid system, Anandamide, Human glioblastoma cells, Human breast cancer cells, Essential fatty acids.

Introduction

Clinical and experimental trials indicated some correlation between quantity and quality of dietary fat intake and the incidence and growth of cancer (1,2). Epidemiologic studies reported a positive correlation with saturated dietary fat intake with an increase in cancer risk for breast, colon, bladder and prostate (1–5). On the other hand, increased consumption of ω-3 fatty acids (FA) was found to be related to a low incidence of cancer (1) and the same was observed for high dietary intake of essential fatty acids (EFA) (3,4). Data from studies performed in animal tumor models and experimental treatments of humans show that ω-6 PUFAs can inhibit cell growth and/or promote cell death in central nervous system (CNS) tumors (5–8). The inhibitory effects of PUFAs may be linked to their ability to serve as precursors to several bioactive lipids that have anti-tumor activities. Some of these bioactive lipids derived

Address reprint requests to: Undurti N. Das, UND Life Sciences, 2020 S 360th St., #K-202, Federal Way, WA 98003, USA; Phone: 216-231-5548; FAX: +543514334020; E-mail: Undurti@hotmail.com.

0188-4409/$ - see front matter.
http://dx.doi.org/10.1016/j.arcmed.2017.01.009

mainly from ω-6 PUFAs are eicosanoids and endocannabinoids (EC) (9).

The relative proportions and quality of PUFAs in cell membranes as well as cell type are the primary factors that determine the type and quantity of eicosanoids and EC that will be formed in a given situation. Intriguingly, the hydrolytic release of ω-3 and ω-6 PUFAs from phospholipids appears to occur indiscriminately (6). Since the main PUFA substrate for EC in cell membrane is AA (20:4 ω6), most of the ligands are endogenous AA-derived lipids. These ECs are released in the brain and many other tissues and have been implicated in a wide array of physiological and pathological processes including cancer (10−15). The two major endogenous cannabinoid ligands are arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG).

The biological effects of EC are due to their binding and activation of the cannabinoid receptors (CBR), called CB1 and CB2, a family of transmembrane G-protein-coupled receptors, densely distributed throughout the autonomic and central nervous system (CNS), immune system and other human tissue (11). Binding of CB1 or CB2 receptors to endogenous ligands can cause excitation or inhibition of certain cellular activities, depending on the enzyme cascades linked to the receptors (12,13). It is likely that EC arising from other PUFAs (other than AA) or even monounsaturated FAs may have different activities, although this has not yet been fully explored.

EC are not stored as in the case of conventional water-soluble neurotransmitters, but are rather rapidly synthesized from PUFAs of the cell membrane (9). Once extracellularly released by a putative EC transporter, they are quickly degraded by fatty acid amide hydrolase (FAAH), which cleaves AEA and 2-AG into AA and ethanolamide and into AA and glycerol, respectively (16).Thus, EC are akin to eicosanoids and are very short-lived substances, a property very useful for regulatory mechanisms in almost all body tissues (9,12).

In vitro and *in vivo* stimulation of CBR by EC ligand influence intracellular events that play a significant role in the proliferation and apoptosis of a wide variety of cancer cells, thereby leading to antitumor effects (10,17−19). Studies in mammals (rat, mouse and pigs) showed that dietary PUFAs influence tissue and cellular concentration of EC (20−23). These data indicate that dietary PUFAs can alter the levels and types of EC to a significant extent mainly due to the availability and release of the precursor AA or other PUFAs from the membrane phospholipids (12). Evidence shows that high availability of different kinds of FA could have pro- or anti-tumor effects and these effects could at least be partially mediated by EC.

Human tumor cell lines with different origins have been reported that express CBR (19) as glioblastoma T98G, an astrocytic-derived line and human breast cancer MCF7, an epidermal ductal strain. The level of expression of receptors, agonists and related enzymes in these different cell lines may lead to diverse physiological responses after activation of the ECS.

Our previous epidemiological and experimental studies revealed the modulatory effects of FA, particularly PUFAs, on cancer process (2,3,24−27). In an extension of these studies, we evaluated supplementation of EFAs (precursors of EC metabolites) and non-EFAs to two human tumors cell lines with different origins, physiology, and metabolic responses. Data obtained confirm that the kind of FA used differentially affects cell growth and viability *in vitro*. This effect could be due, at least in part, to ECS activation in T98G cells, whereas in the MCF7 line the observed response was independent of endocannabinoids.

Materials and Experimental Procedures

The cell lines were purchased from American Type Culture Collections (ATCC, Rockville, MD). Free and methyl esters of fatty acids were obtained from Nu Check (USA). Cell culture medium was obtained from Gibco (USA). Decanoyl p-nitroaniline, URB597, Rimonabant (SR141716), MTT kit and Anandamide standards were purchased from Cayman Chemicals (USA). Antibodies and Western blot molecular weight standards were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO). Solvents and other chemicals of analytical grade were obtained from Anedra (Argentina) and Sigma-Aldrich.

Cell Culture

Human glioblastoma T98G (ATCC CRL-1690) and human breast cancer MCF7 (ATCCHTB-22) were cultured in 75 cm^2 flasks or 96-well plates (for MTT essays) in DMEM medium with 10% fetal bovine serum (FBS), 100 IU/mL penicillin G and 40 μg/mL gentamycin sulfate and incubated at 37°C in a 5% CO_2 atmosphere. Twenty four hours after seeding, cells were supplemented with AA, EPA, oleic acid (OA) and palmitic acid (PA). All FA were dissolved in ethanol at 50 or 100 mmol concentration (final ethanol concentration was $<0.1\%$). The FAAH specific inhibitor URB597 (5 mmol) and CB1 agonist Rimobanant® (50 mmol) were added in DMSO (final concentration $<$ 0.05%). Controls were treated with ethanol and DMSO at the same concentration as experimental treatments. Cells were cultured for 72 h with and without fatty acids and were harvested for various analyses (26). FA concentrations and culture time used were selected after exploratory dose-time assays (data not shown).

Fatty Acid Determination

The fatty acid profile of the cells under different experimental conditions was determined. Cell lipids were extracted

using Folch's method and then methylated by sodium methoxide (28). The separation, identification and quantification of FA methyl esters (FAMEs) were all performed using a capillary column (BPX 70 30 m long, 0.25 mm ID, 0.25 μm film, Phenomenex, Torrance, CA) in a gas chromatograph (Clarus 500 Perkin Elmer, Waltham, MA). The FAMEs were identified using commercial standards.

Cell Proliferation (CP)

Cells were seeded in 96-well plates (2×10^4 cells per well) for 24 h and were supplemented with DMEM containing different FAs as per the design of the experiments for 72 h. Cell proliferation was evaluated by MTT method at 570 nm. For the later experiments, only those FA that modified CP were selected.

Fatty Acid Amide Hydrolase Activity (FAAH)

Specific activity of FAAH was determined by spectrophotometric assay (410 nm) using as substrate decanoyl p-nitroaniline (29). Protein content was measured with Bradford protein assay and enzymatic activity was adjusted by total protein concentration.

Western Blot Analysis to Detect CB1 Receptor

The expression of the CB1 receptor was determined only for controls and those treatments that showed CP modulatory capacity in each cell line. Cell lysates were electrophoretically separated in SDS-PAGE with 3% stacking gel and 10% resolving gel. Samples were electrophoresed at 200 V for 1 h and blotted onto nitrocellulose at 300 mA for 1.5 h using a Trans Blot Mini Protean II apparatus (BioRad, Hercules, CA). After blocking with TBS-5% bovine serum albumin, the nitrocellulose was incubated overnight at 4°C with 1:500 dilution of anti-CB1 antibody (rabbit polyclonal) (Santa Cruz Biotechnology). After three washes with TBS, nitrocellulose was incubated for 1 h with 1:800 dilutions of peroxidase-conjugated goat antibodies against rabbit immunoglobulins and revealed with H_2O_2/DAB (Sigma-Aldrich Co.). The molecular weight was calculated using a calibration curve obtained with molecular weight standards. Negative controls were obtained without adding primary antibodies. Actin protein was used as positive control. CB1 expression was evaluated with ImageJ v.1.48 software to measure the area units marked by Western blot.

Endocannabinoid Ligands

The metabolites were extracted using a STRATA C-18 cartridge (1 mL) (Phenomenex) of supernatant obtained from the cell lysate and reserved for the determination of EC that was detected by reverse-phase high-performance liquid chromatography (HPLC). Analyses were conducted with a C18 Phenosphere-Next column (5 μm; 4.6 × 250 mm,

Table 1. Profile of total fatty acids of T98G cells cultured under different FA supplementation

	T98G cell treatments				
		EFA		Non-EFA	
Fatty acid	**Control**	**AA (ω6)**	**EPA (ω3)**	**OA**	**PA**
14:0	2.11 ± 0.51	1.57 ± 0.36	1.95 ± 0.38	-	1.06 ± 0.27[a]
16:0	20.95 ± 3.53	22.01 ± 4.02	20.81 ± 5.64	20.28 ± 4.66	28.84 ± 3.40[a]
18:0	21.43 ± 3.95	21.24 ± 3.68	23.08 ± 3.49	22.33 ± 2.74	23.18 ± 2.75
20:0	-	0.53 ± 0.15	-	-	0.12 ± 0.02
Total saturated	44.49 ± 6.90	45.35 ± 5.92	45.84 ± 4.87	42.61 ± 4.83	53.20 ± 5.72[a]
16:1	7.07 ± 1.65	4.62 ± 0.98	5.69 ± 1.18	6.33 ± 1.10	12.64 ± 2.36[a]
18:1	19.25 ± 2.25	16.34 ± 3.19	15.97 ± 2.08	32.01 ± 5.69[a]	19.18 ± 2.72
24:1	1.72 ± 0.54	0.96 ± 0.23[a]	1.15 ± 0.31	2.00 ± 0.28	1.69 ± 0.35
Total monounsaturated	28.04 ± 3.68	21.92 ± 4.05	22.81 ± 3.93	40.34 ± 6.56[a]	33.51 ± 4.17
18:2 ω6	7.61 ± 1.18	8.02 ± 0.91	6.76 ± 0.87	5.90 ± 0.61	4.51 ± 0.64
18:3 ω6	3.04 ± 0.63	4.01 ± 0.56	3.24 ± 0.47	1.69 ± 0.33[a]	2.01 ± 0.44[a]
20:4 ω6	3.87 ± 0.43	8.73 ± 1.10[a]	1.75 ± 0.34	1.8 ± 0.24	1.55 ± 0.20
Total PUFA ω6	14.52 ± 2.65	20.76 ± 3.31[a]	11.75 ± 2.27	9.39 ± 1.46[a]	8.07 ± 2.05[a]
18:3 ω3	8.86 ± 1.14	7.23 ± 0.93	8.39 ± 1.27	4.98 ± 0.87[a]	3.11 ± 0.69[a]
20:3 ω3	0.11 ± 0.03	0.64 ± 0.18	0.20 ± 0.04[a]	0.17 ± 0.03	-
20:5 ω3	0.80 ± 0.22	2.34 ± 0.56[a]	6.87 ± 1.25[a]	0.92 ± 0.19	1.02 ± 0.27
22:5 ω3	0.23 ± 0.07	0.64 ± 0.15	0.08 ± 0.02	-	-
22:6 ω3	2.85 ± 0.43	2.12 ± 0.46[a]	4.06 ± 0.47[a]	1.59 ± 0.72[a]	1.09 ± 0.38[a]
Total PUFA ω3	12.85 ± 2.69	10.21 ± 2.10	19.52 ± 1.78[a]	7.66 ± 1.73[a]	5.22 ± 1.63[a]
Ratio Saturated/PUFAs	1.63	1.02	1.46	2.50	4.00
Ratio PUFAs ω6/ω3	1.36	2.30	0.61	1.23	1.55

Values are mean ± SEM of three independent experiments run in duplicate. Determined by GC and expressed as % of area.
[a]Indicates significant difference from control ($p < 0.05$), ANOVA with Dunnett post-hoc test.

Phenomenex) equipped with a Beckman System Gold Programmable Module Model 126. Metabolic separation was achieved using a time program. A linear gradient from solvent A: methanol:water:acetic acid, 50:50:0.02 (v/v), pH 6 to solvent B: methanol, was over 20 min. UV Programmable Detector Beckman System Gold Model 166 linked with a computer for data processing (24). UV analysis of absorbance of the AEA was at 204 nm.

Statistical Analysis

Data obtained from experiments were analyzed by ANOVA with Dunnett post-hoc tests for the comparison of means and Student t test in Western blot analysis; $p < 0.05$ was chosen to define significant differences. Statistical tests were performed using the InfoStat 2013 software.

Results

Determination of Profiles of FA in Tumoral Cell Lines

The FA profile of analyzed cells reflected the incorporation of the FA added to the culture medium or its derivatives, and the different treatments showed quantitative and qualitative modifications of these profiles in both cell lines. The total percentages of FAs grouped as saturated, monounsaturated and PUFAs of the ω6 and ω3 families were determined. Using these data, the ratios saturated/PUFAs and ω6/ω3 were calculated, in which the difference in the cell profiles under different treatments can be clearly seen (Tables 1 and 2). Differences were observed in basal profiles (control treatments) between cell lines, presence of 20:4 ω3 and 20:2 ω6. Higher levels of monounsaturated and lower PUFAs n3 were detected in MCF7 cells compared to T98G.

Furthermore, in regard to incorporation of FA added to the culture medium, in those T98G cells grown with PA, an increase in monounsaturated percentages, decrease in total PUFAs, presence of 20:0, and 20:3 and 22:5 of the ω3 family absence were observed compared to control (Table 1). Analyzing the calculated ratios, it was observed that all analyzed treatments altered the total FA profile in T98G cells (Table 1).

In MCF7cells, in addition to the aforementioned incorporation of FAs added to the cell culture, 14:0 absence in non-EFA treated cells was observed. Meanwhile, 22:5 ω3 was detected in EFA-treated cells only. Cells treated with EFAs and OA did not show detectable levels of 20:0 and in those treated with AA, 20:3 ω3 and 20:4 ω3 FA were not found (Table 2). As observed for the T98G line, the ratios showed that all analyzed treatments produced modifications in the FA profiles of MCF7 cells (Table 2).

Table 2. Profile of total FA of MCF7cells cultured under different FA supplementation

	MCF7 cells treatments				
		EFA		Non-EFA	
Fatty acid	**Control**	**AA (ω6)**	**EPA (ω3)**	**OA**	**PA**
14:0	0.53 ± 0.11	0.67 ± 0.16	0.03 ± 0.01[a]	-	-
16:0	31.15 ± 4.26	32.73 ± 3.87	31.09 ± 4.34	27.65 ± 3.72	40.07 ± 4.35[a]
18:0	13.85 ± 1.78	14.26 ± 2.15	11.12 ± 3.11	12.12 ± 2.23	13.21 ± 1.72
20:0	0.63 ± 0.20	-	-	-	0.76 ± 0.16
Total saturated	46.16 ± 4.91	47.66 ± 5.02	42.24 ± 5.25	39.77 ± 4.05	54.04 ± 6.10
16:1	5.87 ± 1.17	2.51 ± 0.4[6a]	6.02 ± 1.25	6.95 ± 1.23	9.43 ± 1.79[a]
18:1	30.88 ± 4.46	27.62 ± 3.01	25.50 ± 2.75	44.68 ± 5.24[a]	30.69 ± 3.73
Total monounsaturated	36.75 ± 4.72	30.13 ± 4.16	31.52 ± 3.98	51.63 ± 6.73[a]	40.12 ± 5.68
18:2 ω6	3.40 ± 0.87	3.72 ± 0.61	2.57 ± 0.25	0.92 ± 0.36[a]	1.08 ± 0.20[a]
18:3 ω6	4.52 ± 0.84	4.07 ± 0.56	2.57 ± 0.46[a]	2.28 ± 0.27[a]	0.65 ± 0.16[a]
20:2 ω6	1.29 ± 0.15	1.06 ± 0.18	0.68 ± 0.21[a]	1.17 ± 0.19	0.09 ± 0.04[a]
20:4 ω6	4.70 ± 0.73	10.53 ± 1.92[a]	5.33 ± 0.56	1.96 ± 0.30[a]	1.16 ± 0.42[a]
Total PUFA ω6	13.91 ± 2.69	19.38 ± 3.47[a]	13.15 ± 2.16	6.33 ± 1.87[a]	2.98 ± 0.72[a]
18:3 ω3	0.47 ± 0.10	0.40 ± 0.14	1.53 ± 0.26[a]	1.17 ± 0.30[a]	0.50 ± 0.08
20:3 ω3	1.04 ± 0.21	-	0.95 ± 0.35[a]	0.23 ± 0.11[a]	0.41 ± 0.17[a]
20:4 ω3	0.24 ± 0.06	-	0.82 ± 0.33[a]	0.18 ± 0.05	0.23 ± 0.08
20:5 ω3	1.43 ± 0.50	0.79 ± 0.13[a]	7.46 ± 1.65[a]	1.44 ± 0.32	0.88 ± 0.31
22:5 ω3	-	0.94 ± 0.15	0.73 ± 0.22	-	-
22:6 ω3	0,27 ± 0.19	1.10 ± 0.25[a]	3.40 ± 1.45[a]	0.25 ± 0.16	0.78 ± 0.35
Total PUFA ω3	3.45 ± 0.52	3.23 ± 0.44	14.89 ± 3.23[a]	3.27 ± 0.57	2.80 ± 0.48
Ratio Saturated/PUFAs	2.66	2.11	1.51	4.14	9.35
Ratio PUFAs ω6/ω3	4.03	6.00	0.88	1.94	1.06

Values are mean ± SEM of three independent experiments run in duplicate. Determined by GC and expressed as % of area.
[a]Indicates significant difference with control ($p < 0.05$). ANOVA with Dunnett post-hoc test.

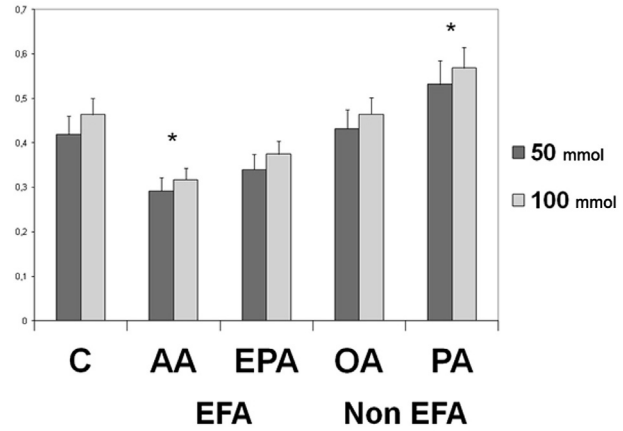

Figure 1. Cell proliferation of T98G cells determined by MTT method. Values are mean of three independent experiments run in triplicate. EFA, essential fatty acids added. Non-EFA, non-essential fatty acids added. C, control. DMEM, no FA added. AA: DMEM+AA (50 or 100 mmol). EPA, DMEM+ EPA (50 or 100 mmol). OA, DMEM+OA (50 or 100 mmol). PA: DMEM+PA (50 or 100 mmol). *Indicates significant differences at both concentrations compared to C (p <0.05), ANOVA with Dunnett post-hoc test.

Cell Proliferation (CP)

The addition of EFAs decreased and non-EFAs increased CP in both cell lines (Figures 1 and 2). In T98G cells AA addition arrested proliferation, whereas PA significantly enhanced cell proliferation at both concentrations used (50 mmol and 100 mmol). EPA reduces (statistically non-significant) and OA did not affect the CP (Figure 1). MCF7 cells showed that EPA at 50 mmol and 100 mmol decreased and OA addition increased CP to a significant degree. AA shows a trend, which does not become significant, to increase CP. No significant differences were observed with the PA addition (Figure 2).

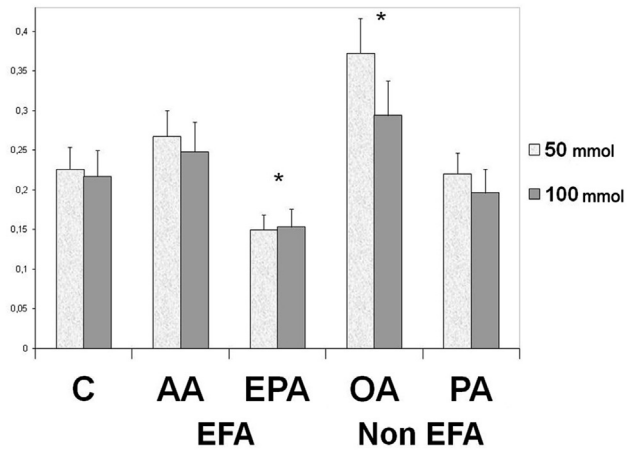

Figure 2. Cell proliferation of MCF7 cells determined by MTT method. Values are mean of three independent experiments run in triplicate. Conditions as in Figure 1. *Indicates significant differences at both concentrations compared to C (p <0.05). ANOVA with Dunnett post-hoc test.

FAAH Specific Activity

No changes in the activity of FAAH were recorded in response to treatments performed. All cells cultured with FA addition showed greater FAAH specific activity than its controls, but this difference did not reach statistical significance in our experiments (Figure 3).

CB1 Receptor

T98G cells cultured with AA and PA were analyzed. Meanwhile, in MCF7 cells CB1 receptor expression was determined in EPA and OA treatments. Levels of CB1 expression did not show any significant difference among treatments within each cell line. However, the measured expression of CB1 was significantly lower (~56 %) in the MCF7 compared to T98G cells. This fact could indicate different basal expression of this receptor in the studied cell lines (Figure 4).

AEA Levels

In T98G cells treated with AA alone or AA plus Rimobanant® (CB1 blockage) or AA plus URB597 (FAAH inhibitor) the levels of AEA were increased (2–6 fold) compared to their respective controls. Cells cultured only with addition of Rimobanant® (C+Rimobanant®) decrease 49.5% its level of AEA, whereas those treated only with URB597 (C+URB597) concentrations 73-fold higher than in untreated cells (C) were detected. It is remarkable that in the cells added with AA and the URB597 inhibitor the detected levels of AEA were, on average, 246 times higher than baseline. In contrast, treatment with PA plus URB597 decreased 84% of AEA level compared to control (Table 3).

In MCF7 cells, only significant increases of AEA were observed in those treated with AA plus URB597 compared with control and those treated only with URB597 (C+URB597) compared with baseline (C) level (Table 3).

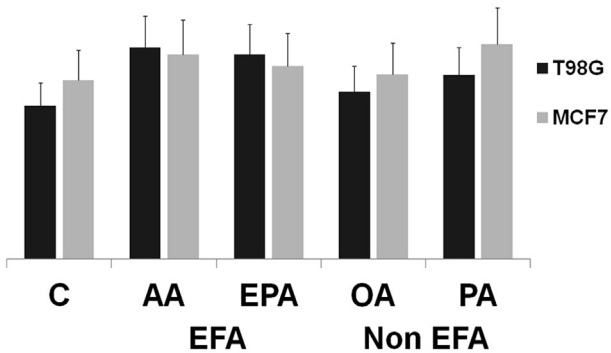

Figure 3. Specific activity of FAAH. Activity of EC degradative enzyme from cell homogenates of cultures with medium supplemented with different FA. Values are mean of three independent experiments run by triplicate, expressed as µmol of product/µg of protein. Conditions as shown in Figure 1. No significant differences were found at p <0.05. ANOVA with Dunnett post-hoc tests.

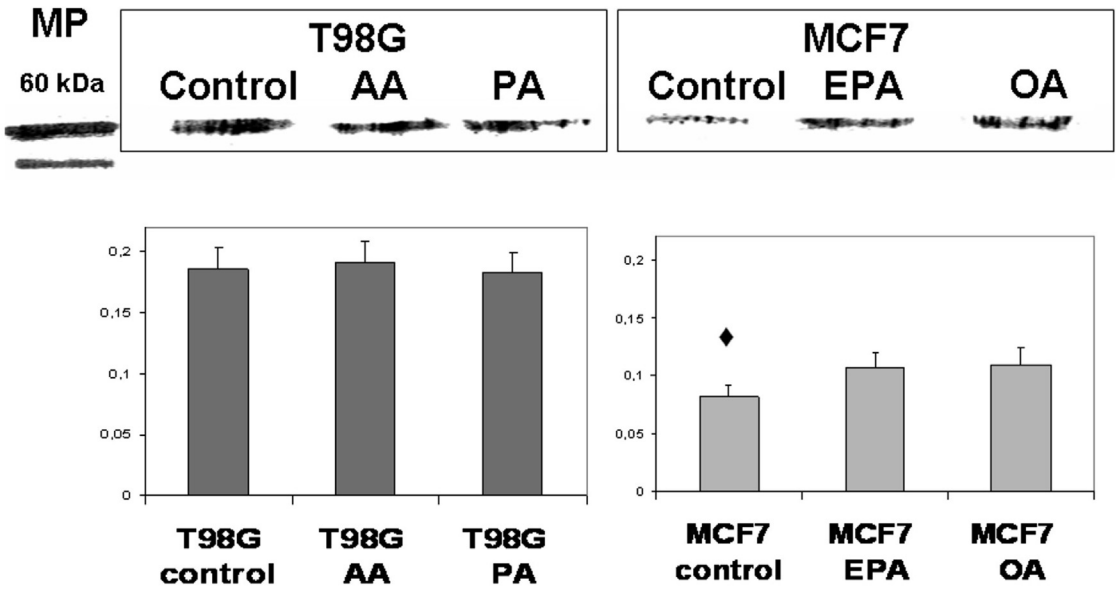

Figure 4. Western blot to detect CB1 protein expression. CB1 was detected in homogenates from both human tumor cell lines tested. Values are mean of two independent experiments run in duplicate and are expressed as area units by software ''Scion Image for Windows'' that measured the marked area. Conditions as in Figure 1. ◆ Indicates significant difference with T98G control ($p < 0.05$), Student t test.

Discussion

Effect of FAs on cancer cell proliferation depends of the type of FA supplemented and the strain/type of cell lines and/or tumor variety. Depending on the FAs that are being tested, they may either enhance or inhibit tumor growth. In general, it is reported that high-fat diets, especially saturated FA, have a cancer-promoting action (1,3,4). Epidemiological and experimental studies revealed that optimum amounts of ω-6 and ω-3 PUFAs could prevent or inhibit tumor growth. It is also known that an imbalance between ω-6/ω-3 FA, with lower ω-3 levels, may have proliferative effects on cancer (5,9,27,30,31). There appear to be multiple mechanisms by which FAs would exert their effects on tumor growth. Some of which may include generation of various eicosanoids and endocannabinoids, formation of increased amounts of lipoperoxides, modification of mitochondria function, perturbation of the plasma membrane properties (3,6,9,27,32), interaction with nuclear receptors of the PPAR-type (33,34).

A growing body of evidence supports the argument that cannabinoids (endogenous and exogenous) have the ability to regulate development, growth and migration, at least on some types, of tumor cells (9−12,18,19,35,36). It is likely that EC would act in conjunction with other cell signaling pathways in bringing about their actions (5,36,37).

Table 3. Levels of AEA detect by HPLC from cell cultures

T98G cell		MCF7 cells	
Treatment	**AEA level**	**Treatment**	**ANAE level**
	1.03 ± 0.28	C	1.07 ± 0.39
EFA (AA)	5.64 ± 1.48[a]	EFA (AA)	1.63 ± 0.66
		EFA (EPA)	0.56 ± 0.31
Non-EFA (PA)	0.71 ± 0.17[a]	Non-EFA (OA)	0.49 ± 0.26
C+Rimobanant®	0.51 ± 0.12	C+Rimobanant®	0.61 ± 0.33
		EFA (AA)+Rimobanant®	0.48 ± 0.05
EFA (AA)+ Rimobanant®	1.15 ± 0.36[a]	EFA (EPA)+Rimobanant®	0.24 ± 0.15
Non-EFA (PA)+Rimobanant®	0.73 ± 0.40	Non-EFA (OA)+ Rimobanant®	0.65 ± 0.20
C+URB597	73.74 ± 14.89	C+URB597	6.15 ± 0.94[a]
EFA (AA)+URB597	253.86 ± 36.6[3a]	EFA (AA)+URB597	8.36 ± 0.6[2a]
		EFA (EPA)+URB597	5.51 ± 0.86
Non-EFA (PA)+URB597	11.51 ± 2.39[a]	Non-EFA (OA)+URB597	5.45 ± 1.10

Values are mean (±SEM) of three independent experiments run in duplicate and expressed as area units.
Conditions as Figure 1. Rimonabant® (SR141716) and URB597 were added to 50 mmol and 5 mmol concentrations, respectively.
[a]Indicates significant differences from control ($p < 0.05$).

Data obtained in this study confirm that there could feature a differential regulation of CP by the exogenous kind of FA added, as previously reported (6,24,25,27,31,38) and suggest that observed changes in the rate of CP may involve activation of ECS. T98G and MCF7 showed differential sensitivity to the FA supplemented and this was reflected in cell growth rate and production of EC released as well (Figures 1 and 2 and Table 3).

The enhanced proliferation of tested tumor cells with non-EFAs such as PA and OA may be due to competition among substrates sharing the same metabolic pathways with the EFAs resulting in pro-tumor action and/or blocking synthesis EFA-derived metabolites such as lipoperoxides (because EFAs can undergo significant peroxidation compared to saturated and monounsaturated fatty acids), which have pro-apoptotic or anti-proliferative actions on cancer cells (3–5,39,40).

AA-enriched T98G cultures showed significantly increased levels of AEA, high expression of CB1 receptor and diminution of CP. This is in agreement with the higher concentrations of AEA and 2-AG and higher density of CBR found in cells belonging to the nervous tissue (41) and this density of CB1 receptors in T98G cells could explain the sensitivity to EC. This cancer cell line derived from CNS produced an amount of EC metabolites that normally undergo rapid degradation by the FAAH system. Inhibition of this enzyme with URB597 allows high concentrations of AEA to be reached (Table 3). Our results lend further support to previous reports that experimental animals fed diets rich in AA showed an increase of AEA in CNS (20–23). Indeed, the pathways that regulate CP depend on the enzyme cascades linked to the CBR in each cell population (12). The addition of Rimobanant® caused a decrease in the levels detected of AEA in both cell lines (Table 3), probably due to negative feedback regulation mechanisms.

On the other hand, MCF7 cells displayed a lack of response to AA supplementation, but did not show a significant increment in AEA levels and their CP remained unchanged. Whereas cells treated with ω-3 EFA showed a decrease in their growth rate, it in agreement with reports that EPA and ALA inhibited proliferation in MCF7 cells (24,39,42). The results of the present study showed that the effect of ω-3 EFA (EPA) on CP decrement in this cell line would follow a different pathway, without intervention of the ECS (9). It has been reported that EPA can directly inhibit tumor growth (*in vitro* and *in vivo*) by activation of PPAR-γ receptors (42) and/or by synthesis of eicosanoids derived from ω-3 PUFAs. The action of these metabolites on mitochondria and gene expression may regulate the apoptotic activity in MCF7 cells and other cancer cell lines and tumors (6,42,43). There is a large amount of evidence supporting the anti-proliferative, anti-inflammatory and antitumor effects of ω-3 PUFAs (1–3,5–9,38,43).

However, some types of tumors such as glioblastomas are relatively refractory to ω-3 and sensitive to ω-6 PUFAs treatments, in agreement with what was observed in our assays (43). Both *in vitro* and *in vivo* studies revealed that ω-6 PUFAs, especially gamma-linolenic acid (GLA), had a modulatory influence on the growth and proliferation of glioma-derived cell lines (5–7,40) and successful therapeutic trials were reported in patients with grade IV gliomas using GLA (44–46). Recently, investigators from the Department of Neurosurgery of Wuhan University (China) reported the effectiveness of AEA to increase apoptosis, suppress migration, adhesion and invasion capabilities of glioma cells *in vitro*. Furthermore, AEA inhibited tumor growth *in vivo* (47). In our experiments, we demonstrated that the *in vitro* addition of exogenous ω-6 PUFAs to glioma cells activates high AEA synthesis and decreases cell proliferation.

Similar mechanisms with ω-6 PUFAs conversion to EC ligands, CBR activation and triggering of apoptotic pathways could partially explain the efficacy of experimental trials with GLA in patients with advanced gliomas (5,7,39–41).

The results of the present study suggest that the growth of tumor cells can be differentially influenced by FAs. Data obtained in our assays would indicate that in T98G and MCF7 cell lines, the tested EFAs inhibit growth, whereas non-EFAs can stimulate cell proliferation. Our results demonstrated that ω-6 PUFAs (AA) differentially increase the levels of synthesis of AEA. This fact, plus high expression of CBR in glioma cells, could be linked to diminution of cell proliferation. Very high concentrations of AEA were measured in T98G cells following treatment with an inhibitor of the FAAH enzyme and addition of AA.

The effects reported by therapeutic utilization of ω-6 PUFAs on gliomas in animals and humans can be related to its modulatory action on ECs (5,38–41).

Based on the results of the present study, it is suggested that more assays are needed to explore the use of ω-6 PUFAs and specific inhibitors of FAAH in order to develop newer therapeutic strategies in the management of cancer, especially cannabinoid-sensitive tumors such as CNS tumors.

Acknowledgments

This work was supported by the funds provided by CONICET PIP 112-200801-03014, SECYT-UNC 05/H297, SECYT-UNLAR 063-2011 (Argentina). We are grateful to Dr. Fulvio Magni for providing CBR antibodies. We are indebted to Mrs. Ester Sticcotti for English technical revision. U.N. Das received a Ramalingaswami Fellowship of the Department of Biotechnology, India during the period of this study.

References

1. World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective (monograph on the internet). Washington, DC:

AICR; 2007. (cited March 15, 2013). Available from: http://eprints.ucl.ac.uk/4841/1/4841.pdf.
2. Eynard AR. Potential of essential fatty acids as natural therapeutic products for human tumors. Nutrition 2003;4:386−388.
3. Eynard AR. Role of dietary polyunsaturated fatty acids (PUFA) on tumorigenesis. Cancer J 1996;9:142−144.
4. Eynard AR. Is the risk of urinary tract tumorigenesis enhanced by a marginal chronic essential fatty acid deficiency (EFAD). Nutrition 1998;14:211−216.
5. Sandrone SS, Repossi G, Candolfi M, et al. Polyunsaturated fatty acids (PUFAs) and gliomas: a critical review of experimental, clinical and epidemiological data. Nutrition 2014;30:1104−1109.
6. Das UN, Begin ME, Ells G, et al. Polyunsaturated fatty acids augment free radical generation in tumor cells in vitro. Biochem Biophys Res Commun 1987;1:15−24.
7. Faragó N, Fehér LZ, Kitajka K, et al. MicroRNA profile of polyunsaturated fatty acid treated glioma cells reveal apoptosis-specific expression changes. Lipids Health Dis 2011;10:173.
8. Puskás LG, Fehér LZ, Vizler C, et al. Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells. Lipids Health Dis 2010;9:56.
9. Pasqualini ME, Berra MA, Yurawecz MP, et al. Dietary manipulation of precursor polyunsaturated fatty acids modulates eicosanoid and endocannabinoid synthesis: A potential tool to control tumor development. Curr Nutr Food Sci 2008;4:161−175.
10. Guindon J, Hohmann AG. The endocannabinoid system and cancer: therapeutic implication. Br J Pharmacol 2011;163:1447−1463.
11. Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 2004;3:771−784.
12. Repossi G, Dain A, Eynard AR. Dietary manipulations of polyunsaturated fatty acids (PUFA) substrates of endocannabinoids: implications in human health and diseases. Curr Nutr Food Sci 2009;5:112−125.
13. Dain A, Repossi G, Das UN, et al. Role of polyunsaturated fatty acids, the precursors of endocannabinoids, in human obesity and diabetes development. Front Biosci 2010;E2:1432−1447.
14. Pertwee RG, Ross RA. Cannabinoid receptors and their ligands. Prostaglandins Leukot Essent Fatty Acids 2002;66:101−121.
15. Muccioli GG, Nephi S. Microglia produce and hydrolyze palmitoylethanolamide. Neuropharmacology 2008;54:16−22.
16. Deutsch DG, Ueda N, Yamamoto S. The fatty acid amide hydrolase (FAAH). Prostaglandins Leukot Essent Fatty Acids 2002;66:201−210.
17. Laezza C, Pisanti S, Crescenzi E, et al. Anandamide inhibits Cdk2 and activates Chk1 leading to cell cycle arrest in human breast cancer cells. FEBS Lett 2006;580:6076−6082.
18. Bifulco M, Di Marzo V. The endocannabinoid system as a target for the development of new drugs for cancer therapy. Recenti Prog Med 2003;94:194−198.
19. Oesch S, Gertsch J. Cannabinoid receptor ligands as potential anticancer agents, high hopes for news therapies? J Pharm Pharmacol 2009;61:839−853.
20. Berger A, Crozier G, Bisogno T, et al. Anandamide and diet: inclusion of dietary arachidonate and docosahexaenoate leads to increased brain levels of the corresponding N-acylethanolamines in piglets. Proc Natl Acad Sci U S A 2001;98:6402−6406.
21. Watanabe S, Doshi M, Hamazaki T. n-3 Polyunsaturated fatty acid (PUFA) deficiency elevates and n-3 PUFA enrichment reduces brain 2-arachidonoylglycerol level in mice. Prostaglandins Leukot Essent Fatty Acids 2003;69:51−59.
22. Matias I, Carta G, Murru E, et al. Effect of polyunsaturated fatty acids on endocannabinoid and N-acyl-ethanolamine levels in mouse adipocytes. Biochim Biophys Acta 2008;1781:52−60.
23. Artmann A, Petersen G, Hellgren LI, et al. Influence of dietary fatty acids on endocannabinoid and N-acylethanolamine levels in rat brain, liver and small intestine. Biochim Biophys Acta 2008;1781:200−212.
24. Comba A, Maestri MD, Berra MA, et al. Effect of w-3 and w-9 fatty acids rich oil on lipoxygenases and cyclooxygenases enzymes and on the growth of a mammary adenocarcinoma model. Lipids Health Dis 2010;9:112−118.
25. Cremonezzi DC, Díaz MP, Valentich MA, et al. Neoplastic and preneoplastic lesions induced by melamine in rat urothelium are modulated by dietary polyunsaturated fatty acids. Food Chem Toxicol 2004;42:1999−2007.
26. Soria EA, Eynard AR, Quiroga PL, et al. Differential effects of quercetin and silymarin on arsenite-induced cytotoxicity in two human breast adenocarcinoma cell lines. Life Sci 2007;81:1397−1402.
27. Pasqualini ME, Berra MA, Calderón RO, et al. Dietary lipids modulate eicosanoid release and apoptosis of cells of a murine lung alveolar carcinoma. Prostaglandins Leukot Essent Fatty Acids 2005;72:235−240.
28. Grasso EJ, Bongiovanni GA, Pérez RD, et al. Precancerous changes in urothelial endocytic vesicle leakage, fatty acid composition, and As and associated element concentrations after arsenic exposure. Toxicology 2011;284:26−33.
29. Patricelli MP, Cravatt BF. Characterization and manipulation of the acyl chain selectivity of fatty acid amide hydrolase. Biochemistry 2001;40:6107−6115.
30. Liu M, Zhou L, Zhang B, et al. Elevation of n-3/n-6 PUFAs ratio suppresses mTORC1 and prevents colorectal carcinogenesis associated with APC mutation. Oncotarget 2016;7:76944−76954.
31. Finocchiaro C, Segre O, Fadda M, et al. Effect of n-3 fatty acids on patients with advanced lung cancer: a double-blind, placebo-controlled study. Br J Nutr 2011;25:1−7.
32. Eynard AR, Monis B. Concanavalin A sites in urothelium and milk fat globules of essential fatty acid (EFA) deficient rats. J Submicrosc Cytol 1983;15:375−382.
33. Avis I, Martinez A, Tauler J, et al. Inhibitors of the arachidonic acid pathway and peroxisome proliferator-activated receptor ligands have superadditive effects on lung cancer growth inhibition. Cancer Res 2005;65:4181−4190.
34. Sampath H, Ntambi J. Polyunsaturated fatty acid regulation of gene expression. Nutr Rev 2004;62:333−339.
35. Pisanti S, Bifulco M. Endocannabinoid system modulation in cancer biology and therapy. Pharmacol Res 2009;60:107−116.
36. Velasco G, Hernández-Tiedra S, Dávila D, et al. The use of cannabinoids as anticancer agents. Prog Neuropsychopharmacol Biol Psychiatry 2016;64:259−266.
37. Parolaro D, Massi P. Cannabinoids as potential new therapy for the treatment of gliomas. Expert Rev Neurother 2008;8:37−49.
38. Hopkins MM, Zhang Z, Liu Z, et al. Eicosopentaneoic acid and other free fatty acid receptor agonists inhibit lysophosphatidic acid- and epidermal growth factor-induced proliferation of human breast cancer cells. J Clin Med 2016;26:E16.
39. Yamashita Y, Kumabe T, Cho YY, et al. Fatty acid induced glioma cell growth is mediated by the acyl-CoA synthetase 5 gene located on chromosome 10q25.1-q25.2, a region frequently deleted in malignant gliomas. Oncogene 2000;19:5919−5925.
40. Antal O, Hackler L, Shen J, et al. Combination of unsaturated fatty acids and ionizing radiation on human glioma cells: cellular, biochemical and gene expression analysis. Lipids Health Dis 2014;13:142.
41. Zurolo E, Iyer AM, Spliet WG, et al. CB1 and CB2 cannabinoid receptor expression during development and in epileptogenic developmental pathologies. Neuroscience 2010;170:28−41.
42. Sauer LA, Dauchy RT, Blask DE, et al. Eicosapentaenoic acid suppresses cell proliferation in MCF-7 human breast cancer xenografts in nude rats via a pertussis toxin−sensitive signal transduction pathway. J Nutr 2005;135:2124−2129.

43. Manda K, Kriesen S, Hildebrandt G, et al. Omega-3 fatty acid supplementation in cancer therapy: does eicosapentanoic acid influence the radiosensitivity of tumor cells? Strahlenther Onkol 2011;187: 127–134.
44. Das UN. From bench to the clinic: gamma-linolenic acid therapy of human gliomas. Prostaglandins Leukot Essent Fatty Acids 2004;70: 539–552.
45. Sánchez C, De Ceballos ML, Gómez del Pulgar T, et al. Inhibition of glioma growth in vivo by selective activation of the CB2 cannabinoid receptor. Cancer Res 2001;61:5784–5789.
46. Das UN. Gamma-linolenic acid therapy of human glioma–a review of in vitro, in vivo, and clinical studies. Med Sci Monit 2007;13:119–131.
47. Ma C, Wu TT, Jiang PC, et al. Anti-carcinogenic activity of anandamide on human glioma in vitro and in vivo. Mol Med Rep 2016;13:1558–1562.