

Effects of Anandamide on Polyamine Levels and Cell Growth in Human Colon Cancer Cells

MICHELE LINSALATA^{1*}, MARIA NOTARNICOLA^{1*}, VALERIA TUTINO¹,
MAURIZIO BIFULCO², ANTONIETTA SANTORO², CHIARA LAEZZA^{3,4},
CATERINA MESSA¹, ANTONELLA ORLANDO¹ and MARIA GABRIELLA CARUSO¹

¹Laboratory of Biochemistry, National Institute for Digestive Diseases S. de Bellis, Castellana Grotte, Bari, Italy;

²Department of Pharmaceutical Sciences, University of Salerno, Italy;

³Institute of Endocrinology and Experimental Oncology-CNR, Napoli, Italy;

⁴Department of Biology and Cellular Pathology, University Federico II, Napoli, Italy

Abstract. *Background:* Anandamide (AEA) is an endogenous agonist for cannabinoid receptor CB1-R and seems to be involved in the control of cancer growth. Polyamines are compounds that play an important role in cell proliferation and differentiation. Our aim was to investigate the effect of AEA on the polyamine levels (putrescine, spermidine and spermine) and cell growth of three human colon cancer cell lines, positive for CB1-R. *Materials and Methods:* After AEA treatment of DLD-1, HT-29 and SW620 cells, polyamine analysis was performed by high-performance liquid chromatography (HPLC) and cell growth was measured by 3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. CB1 gene expression was determined using reverse transcription and polymerase chain reaction (RT-PCR). *Results:* AEA significantly reduced polyamine levels and cell proliferation dose-dependently when the tested cell lines were exposed for 24 h and 48 h. This inhibitory effect was mediated by CB1-R, since SR 1411716A, a selective CB-1 receptor antagonist, was able to entirely antagonize the effect of AEA. CB1-R mRNA levels were enhanced after AEA treatment in DLD-1 cells, whereas no induction was found in HT-29 and SW620 cells. *Conclusion:* It appears that mechanisms by which AEA may affect growth of colon cancer cells involve a decrease in cell proliferation rate by reducing the polyamine levels.

Cannabinoids are a class of hydrophobic substances found in *Cannabis sativa*. Mammalian tissues contain at least two types of cannabinoid receptors (CB1-R and CB2-R) (1, 2). Both are G_{i/o}-protein-coupled transmembrane receptors, and the subsequent signalling pathways negatively regulate adenylyl cyclase and activate mitogen-activated protein kinase (3, 4). Endogenous agonists for these receptors (endocannabinoids) have also been discovered, the most important being arachidonoyl ethanolamide (AEA), 2-arachidonoyl glycerol and 2-arachidonoyl glyceryl ether (5).

AEA is an arachidonic acid derivative that may exert its effects through the two CB-Rs or *via* a CB-R-independent pathway; however, it binds to CB2-R with less affinity than to CB1-R (6). AEA has been detected in different regions of the central nervous system and also in peripheral tissues such as the spleen, heart and skin (7). Moreover, the presence of AEA has also been reported in human colon tissue and its levels are greatly increased when normal mucosa is transformed to adenomatous or neoplastic tissue (8).

Endocannabinoids regulate multiple physiological and pathological conditions *e.g.* food intake, immunomodulation, chronic pain, inflammation and carcinogenesis. A growing body of evidence suggests that AEA has antitumoral effects by decreasing the viability, adhesion and migration of cancer cells, as well as modulating angiogenesis and metastasis (9). Data also suggest that AEA inhibits the proliferation of a wide range of cancer cells, including colorectal carcinoma cells through CB-1-like receptors (10-11). Nevertheless, little is known about the signalling mechanisms through which endocannabinoids modulate neoplastic cell growth. An antiproliferative effect of AEA has been observed which was not due to toxicity or to apoptosis of cells but was accompanied by a reduction of cells in the S-phase of the cell cycle (10). Moreover, its action seems to depend on the degree of differentiation and malignancy of the cells in question (8).

*These Authors contributed equally to this work.

Correspondence to: Maria Gabriella Caruso, MD, Laboratory of Biochemistry, National Institute for Digestive Diseases, Via Turi 27, 70013 Castellana Grotte, Bari, Italy. Tel: +39 0804994669, Fax: +39 0804994313, e-mail: gabriella.caruso@ircsdebellis.it

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The cellular polyamines spermidine and spermine, as well as their precursor putrescine, are ubiquitous short-chain aliphatic amines that play an important role in cell proliferation and differentiation (12). It has been observed that polyamines are required at different phases of cell cycle progression and an inhibition of G₁- to S-phase is due to polyamine depletion (13). Abnormal hyperproliferative cells exhibit very high requirements for polyamines to sustain cell growth by means of elevated DNA, RNA and protein synthesis (14). Intracellular polyamine content homeostasis is lost in dysregulation of cell proliferation, leading to cancer development. Moreover, the mucosal polyamine levels are known to be elevated in tumor cells compared to normal ones and they have been suggested as specific markers for neoplastic proliferation (15).

On this basis, we designed this study to investigate the effects of increasing concentrations of AEA on polyamine levels and cell proliferation of three human colon cancer cell lines, positive for CB1-R, with different degrees of differentiation. We also explored the issue of whether AEA exerts its effects according to a CB1-R-dependent process in these cell lines.

Materials and Methods

Cell culture conditions. Human colon cell lines DLD-1, HT-29 and SW620 were obtained from the ICLC (IST, Genoa, Italy). DLD-1, HT-29 and SW620 were routinely grown in RPMI-1640, McCoy's 5A and Dulbecco modified Eagle medium (DMEM), respectively, supplemented with 10% foetal bovine serum (FBS), 1% non essential amino acids, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, in monolayer cultures, and incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. At confluence, the grown cells were harvested by means of trypsinization and serially subcultured at a 1:4 split ratio. All cell culture components were purchased from Sigma-Aldrich (Milan, Italy).

AEA treatment. DLD-1, HT-29 and SW620 cells were seeded at a density of 2×10⁵ cells/5 ml in their corresponding phenol red-free growth media containing 10% FBS in 60 mm tissue culture dishes (Corning Costar Co., Milan, Italy). After 24 h to allow for attachment, the medium was removed and replaced by fresh culture medium containing AEA at increasing concentrations (0.1 µM, 1 µM, 5 µM, 10 µM, 20 µM and 50 µM) dissolved in absolute ethanol. AEA was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). The cells were allowed to grow for a further 24 and 48 hours and then trypsinized. The cell pellet obtained after low-speed centrifugation was used for subsequent analyses. Each experiment included an untreated control and a control containing solvent alone at the same concentration used when adding AEA. The solvent reached a concentration not exceeding 0.3% in all experiments.

Triplicate cultures were set up for each AEA concentration and for controls, and each experiment was repeated 4 times. Cell viability, determined using the trypan blue exclusion test, always exceeded 90%.

Table I. Sequences of amplification primers used.

Gene	Primer
<i>CB1-R</i>	
Sense	5'-GGAGAACATCCAGTGTGGGG-3'
Antisense	5'-CATTGGGGCTGTCTTTACGG-3'
<i>β-Actin</i>	
Sense	5'-AAAGACCTGTACGCCAACACAGTGTCTGTGG-3'
Antisense	5'-CGTCATACTCCTGCTTGCT GATCCACATCTGC-3'

Polyamine analysis. For the evaluation of the polyamine levels after AEA treatment, each cell culture pellet was homogenized in 700 µl of 0.9% sodium chloride mixed with 5 µl (174 nmol/ml) of internal standard (1,10-diaminodecane).

To precipitate the proteins, 50 µl of 3 M perchloric acid were added to the homogenate. After 30 min of incubation on ice, the homogenate was centrifuged for 15 min at 7000 ×g. The supernatant was filtered (Millex-HV13 pore size 0.45 µm; Millipore, Bedford, MA, USA) and lyophilized. The residue was dissolved in 250 µl of HCl (0.1 N). Aliquots (100 µl) were reacted with dansyl chloride, and the dansyl-polyamine derivatives were determined by high-performance liquid chromatography as previously described (16). Polyamine levels were expressed as concentrations (nmol/mg of protein).

Assessment of cell proliferation. After DLD-1, HT-29 and SW-620 cells had been cultured for 24 and 48 h with different concentrations of AEA (0.1 µM, 1 µM, 5 µM, 10 µM, 20 µM and 50 µM), the proliferative response was estimated by colorimetric MTT test (Sigma-Aldrich). To determine cell growth by the colorimetric test, MTT stock solution (5 mg/ml in medium) was added to each dish at a volume of one-tenth the original culture volume and incubated for 2 h at 37°C in humidified CO₂. At the end of the incubation period, the medium was removed, and the blue formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol). MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometry at an optical density of 570 nm.

CB1-R mRNA analysis. Each cell line, cultured with 0.1 µM, 5 µM and 20 µM of AEA for 24 hours, was washed twice in phosphate-buffered saline (PBS) and then trypsinized and centrifuged at low speed. The cell pellets were resuspended in 0.3 ml of pure distilled water and used for RNA extraction.

Total cell RNA was extracted using Tri-Reagent (Molecular Research. Centre Inc. Cincinnati, OH, USA), following the manufacturer's instruction. Approximately 2 µg of total cell RNA, extracted from both control and treated cells, were used for cDNA synthesis. Reverse transcription (RT) was performed in 20 µl of final volume at 41°C for 60 min, using 30 pmol of antisense primer (Table I) for analyses of the *CB1-R* gene. The *β-actin* gene was utilized as an internal control.

Real-time PCRs were performed in 25 µl of final volume containing 2 µl of cDNA, master mix with SYBR Green (iQ SYBR Green Supermix; Bio-Rad, Milan, Italy) and sense and antisense primers for the *CB1-R* and *β-actin* genes (Table I).

Table II. Polyamine content (nmol/mg protein) of DLD-1 cells after exposure to different concentrations of AEA for 24 and 48 h (panels A and B, respectively). All data represent the mean \pm SE of four consecutive experiments. The effect of AEA, in the presence of 141716A (SR), after 24 and 48 h of treatment is also shown. The *p*-value was determined by one-way analysis with Dunnett's post test.

A				
	Putrescine	Spermidine	Spermine	Total polyamines
Control	0.38 \pm 0.01	5.45 \pm 0.15	6.80 \pm 0.09	12.77 \pm 0.17
0.1 μ M	0.34 \pm 0.01	5.37 \pm 0.12	6.32 \pm 0.13	12.04 \pm 0.22
1 μ M	0.19 \pm 0.01*	4.57 \pm 0.10*	5.07 \pm 0.09*	9.84 \pm 0.19*
5 μ M	0.20 \pm 0.01*	3.52 \pm 0.08*	4.92 \pm 0.14*	8.65 \pm 0.23*
10 μ M	0.18 \pm 0.01*	3.12 \pm 0.06*	3.67 \pm 0.14*	6.98 \pm 0.19*
20 μ M	0.16 \pm 0.01*	1.67 \pm 0.08*	3.23 \pm 0.09*	5.04 \pm 0.14*
50 μ M	0.15 \pm 0.01*	1.42 \pm 0.07*	3.01 \pm 0.10*	4.63 \pm 0.10*
10 μ M AEA + 0.01 μ M SR	0.37 \pm 0.01	5.30 \pm 0.16	6.30 \pm 0.14	12.08 \pm 0.11
B				
	Putrescine	Spermidine	Spermine	Total polyamines
Control	0.33 \pm 0.01	5.87 \pm 0.14	7.17 \pm 0.16	13.36 \pm 0.30
0.1 μ M	0.30 \pm 0.01	5.51 \pm 0.16	6.82 \pm 0.13	12.76 \pm 0.39
1 μ M	0.21 \pm 0.05*	4.47 \pm 0.13*	5.35 \pm 0.18*	10.04 \pm 0.18*
5 μ M	0.15 \pm 0.01*	3.40 \pm 0.12*	4.90 \pm 0.09*	8.45 \pm 0.17*
10 μ M	0.12 \pm 0.01*	2.60 \pm 0.10*	4.20 \pm 0.10*	6.91 \pm 0.07*
20 μ M	0.11 \pm 0.01*	2.17 \pm 0.08*	3.77 \pm 0.08*	6.05 \pm 0.08*
50 μ M	0.10 \pm 0.01*	1.77 \pm 0.11*	3.12 \pm 0.11*	5.00 \pm 0.22*
10 μ M AEA + 0.01 μ M SR	0.31 \pm 0.01	5.52 \pm 0.11	6.80 \pm 0.09	12.48 \pm 0.16

**p*<0.05 versus control.

Real-time PCR was carried out in an iCycler Thermal Cycler System apparatus (Bio-Rad) using the following parameters: one cycle at 95°C for 1 min and 30 s, followed by 45 cycles at 94°C for 10 s, 55°C for 10 s and 72°C for 30 s and a further melting curve step at 55-95°C with a heating rate of 0.5°C per cycle for 80 cycles. The PCR products were quantified by external calibration curves, obtained with serial dilutions of known copy number of molecules (10²-10⁷ molecules). All expression data were normalized against the expression of the housekeeping gene β -actin, used as internal control. The specificity of the PCR product of each tested gene was confirmed by gel electrophoresis.

Statistical analysis. Data were statistically evaluated by ANOVA followed by the Dunnett's multiple comparison test. Differences were considered significant at a 5% probability level. Correlations between the polyamine content and increasing AEA concentrations were analyzed by Pearson's correlation coefficient.

Results

Effects of AEA on polyamine levels. The administration of increasing concentration of AEA (from 0.1 μ M to 50 μ M) led to a decrease of the single and total polyamine contents

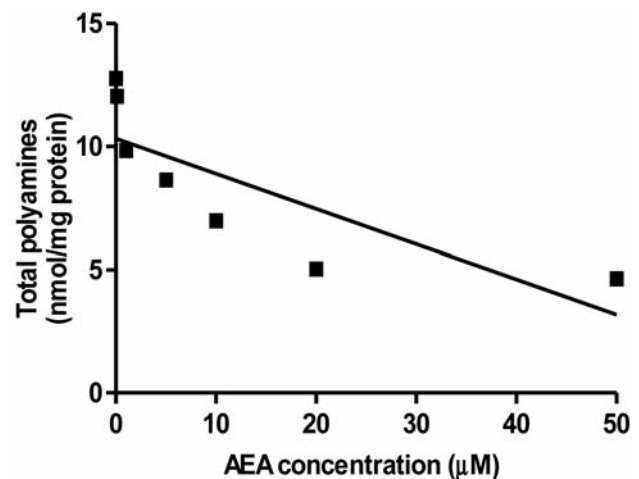


Figure 1. The significant inverse relationship between AEA concentration and the total polyamine content in DLD-1 cells after 24 h of treatment ($r=-0.81$ $p=0.024$; Pearson's correlation coefficient).

in all the cell lines studied. Table II shows the polyamine profile in the DLD-1 cell line after AEA treatment. Both after 24 h (panel A) and 48 h (panel B) of treatment, the decrease was significant ($p<0.05$, Dunnett's post test) starting at a concentration of 1 μ M for single and total polyamines as compared to control cells, and was maintained up to 50 μ M for all parameters considered. Exposure of DLD-1 cells to the selective antagonist of CB1-R, SR 141716A, for 24 and 48 h, reversed the inhibitory effect of AEA on the polyamine content in this cell line. Moreover, in DLD-1 cells, the total polyamine content was inversely correlated to AEA concentrations both after 24 h and 48 h of treatment ($r=-0.81$, $p=0.024$ and $r=-0.80$, $p=0.038$ respectively; Pearson's correlation coefficient). Figure 1 shows the significant and inverse relationship between AEA concentration and the total polyamine content in DLD-1 cells after 24 h of treatment.

Analysis of the polyamine profile in the HT-29 and SW620 cell lines after AEA treatment showed results similar to those detected in DLD-1 cells (data not shown.). Both after 24 h and 48 h of treatment, the inhibitory effect of AEA on single and total polyamine content was significant, starting at a concentration of 1 μ M, and was maintained up to 50 μ M. Moreover, the addition of SR 141716A reversed the inhibitory effect of AEA on the polyamine content for HT-29 and SW620 cells too.

Finally, for HT-29 cells, significant inverse correlation was found between the total polyamine and AEA concentrations after 24 h and 48 h of treatment ($r=-0.79$ $p=0.034$ and $r=-0.80$ $p=0.029$ respectively; Pearson's correlation coefficient). The total polyamine content was also inversely correlated to AEA concentration in SW620 cells (after 24 h

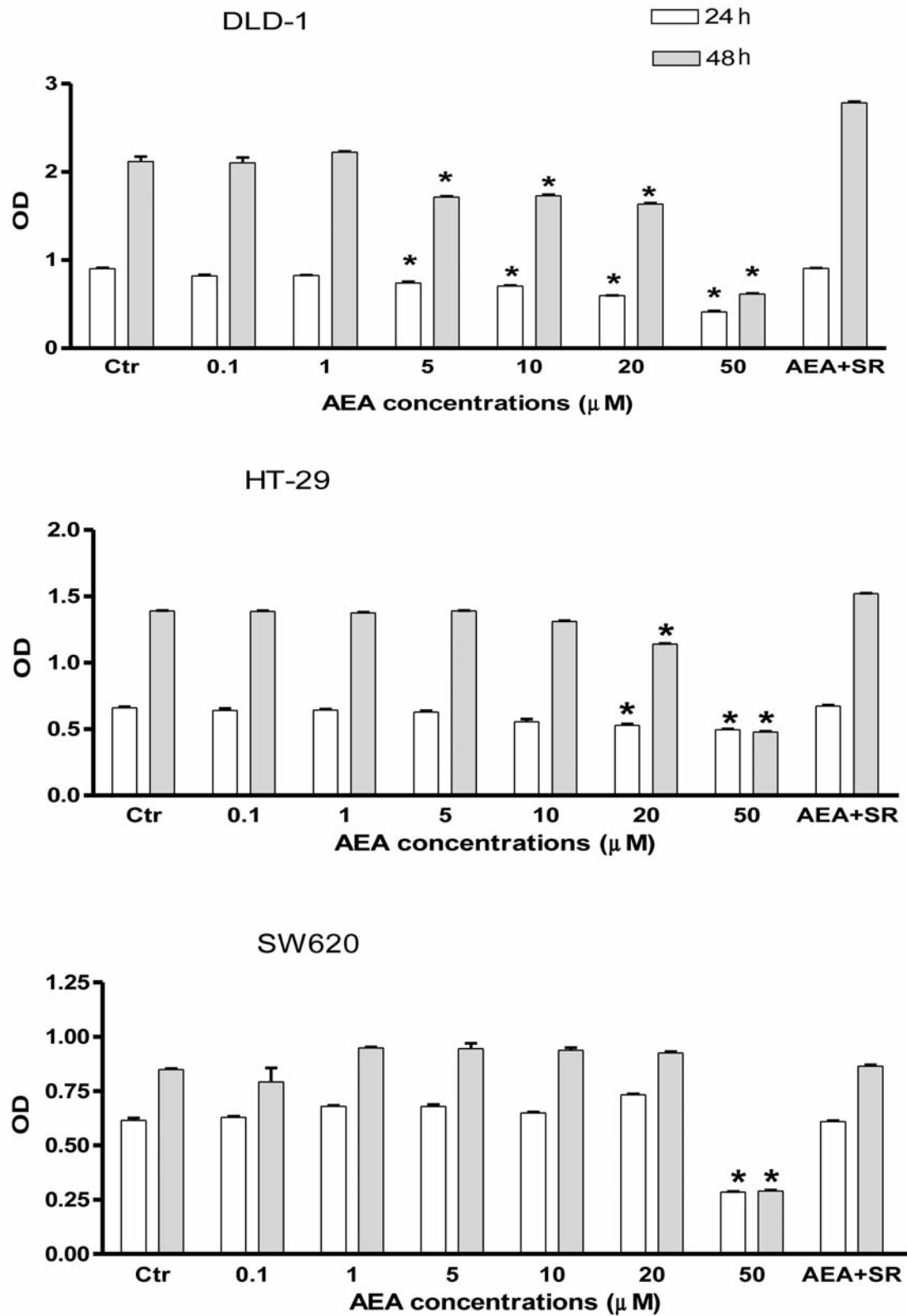


Figure 2. Effect of increasing concentration of AEA on the conversion of MTT tetrazolium salt in DLD-1, HT-29 and SW620 cells, after 24 and 48 h of treatment. The effect of AEA in the presence of SR 141716A (SR), after 24 and 48 h of treatment is also shown. All data represent the results of 4 different experiments (mean value \pm SE). The p-value was determined by one-way analysis with Dunnett's post test. *p<0.05 versus control.

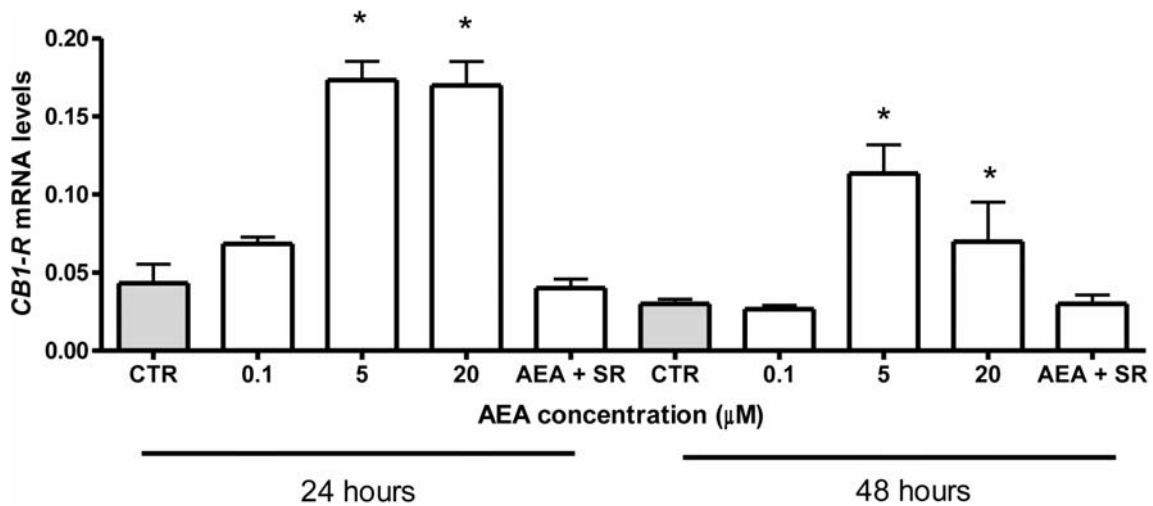


Figure 3. Effects of 0.1, 5 and 20 μM of AEA on CB1-R mRNA levels of DLD-1 cells cultured for 24 and 48 h. The effect of 10 μM of AEA in the presence of 0.01 μM of SR 141716A (SR) after 24 and 48 h of treatment is also shown. All data represent the results of 4 different experiments (mean value \pm SE). The P-value was determined by one-way analysis with Dunnett's post test. * $p < 0.05$ versus control.

of treatment: $r = -0.83$, $p = 0.020$; after 48 h of treatment: $r = -0.82$, $p = 0.022$; Pearson's correlation coefficient).

Effects of AEA on cell proliferation. AEA dose-dependently inhibited the proliferation of all tested cell lines exposed for 24 and 48 h (Figure 2). In the DLD-1 cell line, concentrations of AEA from 5 μM to 50 μM caused a significant reduction in the conversion of MTT tetrazolium salt as compared with untreated control cells, after both 24 and 48 hours of treatment ($p < 0.01$). When cell proliferation was measured in the HT-29 and SW620 cell lines, the effect of AEA was significantly more evident at higher concentrations (20 and 50 μM , respectively) after both 24 and 48 h of cell exposure. The inhibitory effect of AEA on the proliferation of the three tested cell lines was abrogated by co-incubation of cells with 10 μM AEA and 0.01 μM SR 141716A.

Effects of AEA on CB1-R gene expression. Exposure of DLD-1 cells to increasing concentrations of AEA, for 24 and 48 h, caused a significant induction of CB1-R mRNA as compared to control untreated cells (Figure 3). The induction was statistically significant starting from 5 μM of AEA ($p < 0.05$, Dunnett's post test) and was reversed by using SR 141716A.

No induction of CB1-R mRNA levels by AEA was detectable in the HT-29 and SW-620 cell lines after 24 and 48 h of AEA exposure (data not shown).

Discussion

There is growing evidence that in addition to recognized uses of endocannabinoids in the clinic as appetite stimulants and antiemetics, they may have therapeutic potential as

neuroprotective (17, 18) or anticancer agents (19, 20). *In vitro* experiments have provided numerous data about the anticarcinogenic properties of endocannabinoids, with particular reference to AEA. As a matter of fact, antiproliferative and antiangiogenic activities of AEA have been proven in a wide range of cell lines, including colon cancer cells (21, 22). However, the precise mechanisms through which endocannabinoids can influence neoplastic cell growth are not completely known.

Data from the present study clearly demonstrate that the concentration of AEA for 24 h – 48 h of treatment influences both polyamine levels and the proliferation rate of three human colon cancer cell lines of different grades of differentiation in a dose-dependent manner.

Particularly, by employing concentrations between 1 μM and 50 μM , DLD-1 cells showed a significant decrease in single and total polyamine levels. The same behaviour was observed in the HT-29 and SW620 lines, although these are known to be less-differentiated cancer cells as compared to DLD-1. The ability of AEA to modify the polyamine profile in colon cancer cell lines is also underlined by the significant inverse relationship found between the AEA concentration and the total polyamine content in all three tested cell lines. Therefore, the antiproliferative action of AEA on colon cancer cells may be related to its ability to reduce the polyamine levels.

To our knowledge, this is the first study investigating the behaviour of polyamine levels after AEA administration, however a similar polyamine response has also been observed in the same and other cell lines treated with drugs or natural molecules that inhibit polyamine biosynthesis (23–25). Polyamines are able to stabilize chromatin and nuclear

enzymes due to their ability to form complexes with organic polyanions, such as groups of proteins and DNA. It was postulated that stabilization of the chromatin structure by polyamines may be a mechanism by which these molecules affect nuclear processes including cell division and apoptosis (13). Moreover, it is known that polyamines influence the expression of various genes involved in cell proliferation, tumor invasion and metastasis (26).

The inhibitory effect exerted by AEA on colon cancer cell proliferation was further supported by our data obtained with the MTT test, although a significant inhibition for this marker of proliferation required higher AEA concentrations than those utilized to reduce the cellular polyamine content in DLD-1 cells. Moreover, higher doses of AEA were necessary in order to elicit a significant reduction in MTT in the HT-29 and SW620 lines.

Small amounts of AEA may be sufficient to produce inhibition of the biosynthesis of polyamines, which are necessary for cells to initiate their proliferative processes independently from their grade of differentiation. As a matter of fact, it has been observed that polyamine synthesis represents an early event during the G₁ phase of the cell cycle (27) and an inhibition of the G₁- to S-phase is due to polyamine depletion (10). The MTT assay estimates cell growth ability on the whole and depends both on the number of viable cells present and on the mitochondrial activity per cell (28). Therefore, we can presume that one of the mechanisms by which AEA may affect growth of colon cancer cells involves a decrease in cell proliferation rate by reducing a lessening the polyamine content that consequently induces the arrest of cell cycle progression.

Our findings also show that the inhibitory effect of AEA on cell proliferation rate and polyamine levels was mediated by CB₁-R in all the tested cells, since SR 1411716A, a selective CB₁-R antagonist, was able to entirely antagonize the effect of AEA. This finding suggests that AEA might function as an endogenous inhibitor of colon cancer cell growth by binding to CB₁-R as previously found for breast and prostatic cancer (29).

CB₁-R mRNA levels were in fact enhanced after AEA treatment in DLD-1 cells; however, no induction was found in HT-29 cells and the lymph node metastatic cell line SW620, suggesting that changes in endocannabinoid signaling occurring in colon cancer cells may depend on their degree of differentiation. The absence of *CB₁-R* induction by AEA in SW620 might also be due to higher basal levels of *CB₁-R* mRNA present in this cell line than in DLD-1 cells, as previously reported (11).

In conclusion, our results suggest a functional role for AEA in activating biochemical pathways leading to an inhibition of cell proliferation *via* a CB₁-R-mediated process in human colon cancer cell lines. The marked decrease in the single and total polyamine content observed after AEA administration

particularly suggests that polyamine metabolism could be considered a target for the antiproliferative and antineoplastic properties of endocannabinoids. This further supports the notion that drugs directed at regulating the endocannabinoid system may prove to be valuable tools in the fight against various types of cancer.

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