Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity and of Ras farnesylation mediate antitumor effects of anandamide in human breast cancer cells

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Abstract

The endocannabinoid system regulates cell proliferation in human breast cancer cells. Recently, we described that a metabolically stable anandamide analog, 2-methyl-2'-F-anandamide, by activation of CB1 receptors significantly inhibited cell proliferation of human breast cancer cell lines. In this study, we observed that the activation of the CB1 receptor, in two human mammary carcinoma cell lines, MDA-MB-231 and MCF7, caused the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity due to a reduction of HMG-CoA reductase transcript levels. The decrease of HMG-CoA reductase activity induced the inhibition of the prenylation of proteins, in particular of the farnesylation of *Ras* oncogenic protein involved in cell proliferation of these cell lines. We suggest that the inhibitory effect of anandamide analog on tumor cell proliferation could be related to the inhibition of Ras farnesylation.

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Introduction

The endocannabinoid system inhibits cell proliferation in various cancer cells (Bifulco & Di Marzo 2002, Bifulco *et al.* 2006, 2008). We have previously described that the activation of the CB1 receptor induced the arrest of cell growth of mammary carcinoma cells by blocking the cell cycle in G₁/S phase (De Petrocellis *et al.* 1998, Melck *et al.* 1999, Grimaldi *et al.* 2006, Laezza *et al.* 2006a,b). Moreover, we demonstrated that the activation of this receptor inhibited cell migration of a highly invasive human breast cancer cell line MDA-MB-231 by a RhoA-ROCK-dependent signaling pathway due to the inhibition of RhoA activity (Laezza *et al.* 2008). An important event for the biological activity of RhoA protein is a post-translational modification by an isoprenoid compound geranylgeranyl diphosphate

(GGPP). Isoprenoids are generated by the mevalonate (MVA) pathway where the endoplasmic reticulum enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase catalyzes the synthesis of MVA, precursor of donor isoprenoids, as farnesyl diphosphate (FPP) and GGPP that are required for the biological activity of a class of biologically relevant proteins, which include monomeric GTPase-proteins like Rho and Ras family proteins (Laezza *et al.* 2006a,b). The rate-limiting enzyme for the synthesis of MVA is HMG-CoA reductase, a critical regulator of cell proliferation in normal as well as in tumor cells (Bifulco *et al.* 1995). At the transcriptional level, HMG-CoA reductase is negatively controlled by its end-product (cholesterol) since the enzyme is expressed at a relatively high rate when cells are MVA starved (Bifulco *et al.* 1995). Moreover, we have

reported that in FRTL-5 rat thyroid cells, HMG-CoA reductase is transcriptionally induced by TSH, via cAMP (Bifulco *et al.* 1995). TSH is the physiologic mitogen in FRTL-5 cells and when TSH starved, these cells become quiescent. TSH challenge increased HMG-CoA reductase gene expression and HMG-CoA reductase activity which preceded the TSH-increased [³H]thymidine incorporation into DNA and cell doubling. We reported that TSH by increasing cAMP production induced HMG-CoA reductase gene transcription through the cAMP-responsive element (CRE) that is present in the promoter of the enzyme (Nakanish *et al.* 1988, Bifulco *et al.* 1995, Ngo *et al.* 2002). CRE sites were originally identified as *cis*-acting elements that confer transcriptional regulation in response to elevated cAMP levels (Sands & Palmer 2008). Cannabinoids are classically documented to signal through the inhibition of adenylyl cyclase (AC). The cannabinoid CB1 and CB2 receptors are preferentially coupled to inhibitory *G*_{ai/o} proteins to inhibit AC activity by reducing intracellular cAMP levels (Bosier *et al.* 2009). Moreover, it has been described that the activation of cannabinoid receptors led to the regulation of several DNA-binding proteins, including activator protein 1 (AP-1) and the transcription factor CRE-binding protein (CREB protein; Bosier *et al.* 2008). In this study, we observed that the activation of CB1 receptor by 2-methyl-2'-F-anandamide (Met-F-AEA) inhibited the HMG-CoA reductase activity due to the decrease of HMG-CoA reductase transcript levels. This event reduced the pattern of prenylated proteins involved in the proliferation of breast cancer cells.

Materials and methods

Materials

Met-F-AEA was purchased from Calbiochem (San Diego, CA, USA). The selective CB1 antagonist, SR141716, was kindly provided by Sanofi-Aventis (Montpellier, France). 3-Hydroxy-3-methyl-3-[¹⁴C]glutaryl-CoA was supplied by Amersham; HMG-CoA sodium salt and the mevalonic acid lactone were supplied by Sigma. Glucose-6-phosphate dehydrogenase was from Calbiochem; glucose-6-phosphate disodium salt and NADP disodium salt were from Boehringer. Bisindolmaleimide was from Alexis Corporation (San Diego, CA, USA). Anti-phospho-CREB (S133), anti-CREB were from Cell Signaling (Boston, MA, USA). Anti-Pan-Ras was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and cocktail of protease inhibitors was from Sigma.

Cell culture

MDA-MB-231, an invasive human breast carcinoma cell line, was grown in RPMI 1640 medium (Gibco BRL Life Technologies) supplemented with 10% inactivated fetal bovine serum (FBS) and 2 mM L-glutamine (Bifulco & Di Marzo 2002, Grimaldi *et al.* 2006). MCF7, a noninvasive human breast cancer cell line, was grown in DMEM supplemented with 10% inactivated FBS and 2 mM L-glutamine (De Petrocellis *et al.* 1998). Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere.

Proliferation assay

The effects of Met-F-AEA on MDA-MB-231 and MCF7 proliferation were evaluated *in vitro*, by [³H]thymidine incorporation. The 96-well plates were seeded with 5×10⁴ cells/ml, and the cells were immediately treated with the drugs, incubated for 24 h at 37 °C (5% CO₂), then pulsed with 0.5 µCi/well of [³H]thymidine, and harvested 12 h later. Radioactivity was measured in a scintillation counter (Wallac, Turku, Finland) (Grimaldi *et al.* 2006).

Subcellular fractionation

The supernatant was centrifuged at 100 000 *g* for 60 min at 4 °C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (wt/vol) Triton X-100. The homogenate was kept at 4 °C for 60 min with occasional stirring and then centrifuged at 100 000 *g* for 60 min at 4 °C. The resulting supernatant was used as the membrane fraction (Laezza *et al.* 2008).

HMG-CoA reductase activity

The enzyme activity was performed, following the method described by Brown *et al.* (1979) with slight modifications. Cells were preincubated 24 h before the HMG-CoA reductase assay with medium without serum and were treated with several drugs for 24 and 48 h. Then, the cells were washed twice with 5 ml of 0.15 M cold NaCl and were harvested by scraping. The cell suspensions were homogenized and centrifuged (900 *g*) at 4 °C for 15 min. The supernatant was then centrifuged in a 75 Ti Beckman rotor for 30 min at 55 400 r.p.m. (200 000 *g*). The supernatant was discarded, and the pellet, containing the microsome fraction, was dissolved in 100 µl of 20 mM imidazole, pH 7.4, 5 mM dithiothreitol (DTT), and it was stored at −80 °C until the HMG-CoA reductase assay. The HMG-CoA reductase activity was evaluated starting from 20 µl microsome samples containing 20–50 µg protein. The samples were preincubated for 30 min at

37 °C with 30 µl of 20 mM imidazole, 5 mM DTT, 83 mM MgCl₂, and 10 units/ml of *Escherichia coli* alkaline phosphatase. Then, 50 µl of 0.2 M potassium phosphate (pH 7.4), 40 mM glucose-6-phosphate, 20 mM disodium EDTA, 10 mM DTT, 5 mM NADP, and 50 µg/ml glucose-phosphate dehydrogenase were added to the preincubation solution. The reaction was started by adding 25 µl of 5 mM [¹⁴C]HMG-CoA (specific activity 8000–9000 c.p.m./nmol); after 30 min of incubation at 37 °C, the [¹⁴C]MVA was converted to lactone and was isolated by thin layer chromatography (Mondola *et al.* 2002).

Reverse transcriptase-PCR

Total RNA was isolated from liver (100–150 mg) using TRIzol. Single strand cDNA was synthesized from 2 µg of total RNA, using Moloney murine leukemia viruses reverse transcriptase. The cDNAs for HMGR and β₂ microglobulin (β₂m) (housekeeping genes) were PCR amplified using the following specific primers: HMGR (sense) 5'-TAC CAT GTC AGG GGT ACG TC-3' and (antisense) 5'-CCA GTC CTA ATG AAA CCT TAG AAG T-3'; β₂MIC (sense) 5'-CCT GGA TTG CTA TGT GTC TGG GTT TCA TCC-3' and (antisense) 5'-GGA GCA ACC TGC TCA GAT ACA TCA AAC ATG-3' respectively. PCR amplification was carried out as follows: HMGR, 1 × (95 °C, 1 min), 32 × (93 °C, 30 s; 61 °C, 45 s and 69 °C, 35 s), and 1 × (69 °C, 5 min); and β₂MIC, 1 × (95 °C, 50 s), 28 × (93 °C, 30 s; 60 °C, 1 min and 69 °C, 1 min), and 1 × (69 °C, 5 min). Amplified products were resolved by 2% agarose gel electrophoresis and were stained with ethidium bromide.

Western blotting analysis

After 24 h of incubation, cells were washed twice with PBS and resuspended in lysis buffer (HEPES 50 mM, NaCl 150 mM, EDTA 50 mM, NaF 100 mM, Na orthovanadate 2 mM, glycerol, Na₄P₂O₇ 10 mM, and 10% triton, pH 7.5), and they were passed through a 23-gauge needle 10 times before centrifugation at 12 000 *g* at 4 °C. Aliquots of the cellular lysates (40 µg of protein) were boiled for 5 min, electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with antibodies. The blots were blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk for 1 h at room temperature. The filters were then probed overnight with primary specific antibodies. Immunodetection of specific proteins was carried out with HRP-conjugated donkey anti-mouse or anti-rabbit IgG (Bio-Rad), using the enhanced chemiluminescence system (Amersham).

Incorporation of [³H]-MVA into cellular proteins

Cells were incubated with drugs for 24 h. During the last 7 h of incubation, 10 µM lovastatin and 30 µCi/ml [³H]-MVA were added to medium. Density of cell culture ranged between 1.5 and 2.0 × 10⁶ cells/ml in 100 mm Petri dishes. Cells were then washed three times with ice-cold PBS, scraped from the dish and lysed in hypotonic buffer (10 mM Tris-HCl, pH 7.2 plus inhibitor of protease), disrupted by sonication, and centrifuged at 3000 r.p.m. (1000 *g*) for 10 min at 4 °C. Equal amounts of each protein extract (100 µg) were analyzed by 12% SDS-PAGE as described (Laezza *et al.* 2006a).

Immunoprecipitation and SDS-PAGE

[³H]-MVA-labeled cells were washed three times with PBS and were lysed in RIPA buffer (20 mM Tris/150 mM NaCl/1 mM EDTA/0.5% (vol/vol) nonidet P-40/0.5% (wt/vol) Na deoxycholate/0.1% (vol/vol) Trasylol/0.2 mM phenylmethylsulfonyl fluoride, pH 7.4). After 10 min on ice, the lysates were centrifuged at 12 000 *g* for 10 min, and the supernatants were immunoprecipitated with 5 µg of preimmune rat serum or anti-pan-p21ras mAb (Santa Cruz Biotechnology) followed by incubation with protein A-Sepharose. Immunoprecipitates were washed three times with RIPA buffer and once with 100 mM Tris-Cl (pH 6.8), and then they were dissolved in Laemmli loading buffer with 1 mM DTT before electrophoresis in a 12.5% SDS-PAGE. Gels then were permeated with Amplify fluorographic enhancer (Amersham), and they were dried and autoradiographed at –80 °C.

Densitometric and statistical analyses

The intensities of bands obtained from western blots and RT-PCR were estimated with Alpha ImagerTm2200 (Alpha Innotech Corporation, Santa Clara, CA, USA). All the measurements were made in triplicate, and all values are represented as mean ± s.d. The significance of difference between the treatments and/or with the controls was obtained with Student's *t*-test. A *P* value <0.05 was considered statistically significant.

Results

Met-F-AEA inhibited the HMG-CoA reductase activity

Recently, we described that Met-F-AEA, a stable analog of anandamide, inhibited the cell proliferation of MDA-MB-231 and MCF7 breast cancer cell lines

Figure 1 (A) Cell number of human breast cancer cells MDA-MB-231 and MCF7 by trypan blue dye exclusion assay. Cells (5×10^4 /well) were cultured in triplicate for 24 h with $10 \mu\text{M}$ of Met-F-AEA. (B) Cells were cultured in triplicate with several drugs, and after 18-h incubation, [^3H]thymidine incorporation ($0.5 \mu\text{Ci}/\text{well}$) was measured. Co-addition of mevalonate at $700 \mu\text{M}$, forskolin at $0.5 \mu\text{M}$, and SR141716 at $0.1 \mu\text{M}$ recovered the inhibition of cell proliferation. Data represent results from at least three separate experiments each performed in triplicate. The graph reports the mean $\pm$ s.d. values of three independent experiments. Inhibition of proliferation was statistically significant. Met-F-AEA data were compared to the controls, whereas all other treatments were compared to Met-F-AEA-treated cells. (with Student's *t*-test ** $P < 0.0001$, * $P < 0.001$, Met-F-AEA versus control).

in a dose-dependent manner. We observed that the maximal inhibition occurred at $10 \mu\text{M}$ of Met-F-AEA after 24 h. The arrest of cell growth was reverted by the CB1 receptor antagonist SR141716 at $0.1 \mu\text{M}$, suggesting that this effect was mediated by the CB1 receptor (De Petrocellis *et al.* 1998, Melck *et al.* 1999, Grimaldi *et al.* 2006, Laezza *et al.* 2006a,b). Moreover, we observed that the activation of CB1 receptor by the stable analog Met-F-AEA inhibited the activity of Ras and RhoA proteins (Bifulco *et al.* 2001, Laezza *et al.* 2008), two GTP-binding proteins whose activity are dependent on post-translational modification by an isoprenoid compound as FPP and GGPP. These isoprenoids are generated by the MVA pathway, where the endoplasmic reticulum enzyme HMG-CoA reductase catalyzes the synthesis of MVA, precursor of donor isoprenoids (Laezza *et al.* 2006a,b). Based on this background, we hypothesized that the activation of CB1 receptor could affect the activity of HMG-CoA reductase. First, we have studied if the arrest of cell

growth by Met-F-AEA at $10 \mu\text{M}$ was reverted by the concomitant addition of MVA at $700 \mu\text{M}$, and of forskolin at $0.5 \mu\text{M}$, an activator of AC (Bosier *et al.* 2009), at Met-F-AEA-treated cells. As shown in Fig. 1A and B, the co-addition of MVA and forskolin at treated cells recovered the cell proliferation. Because the synthesis of MVA is catalyzed by HMG-CoA reductase (Goldstein & Brown 1990), we evaluated whether the activation of CB1 receptor affected the activity of the enzyme by performing an assay of HMG-CoA reductase activity in cells treated with Met-F-AEA at $10 \mu\text{M}$ for 24 h. As shown in Fig. 2, the CB1 activation by Met-F-AEA decreased the HMG-CoA reductase activity in MDA-MB-231 and MCF7 cells after 24 h of treatment reaching the 45% of reduction of activity after 48 h of treatment. This effect was reverted both by co-treatment with forskolin at $0.5 \mu\text{M}$ and SR141716 at $0.1 \mu\text{M}$, antagonist of CB1 receptor. In order to ascertain if the reduction of HMG-CoA activity was dependent on the mRNA expression of this gene, we analyzed the mRNA levels using quantitative RT-PCR. Treatment with Met-F-AEA caused a decline in mRNA levels of both cell lines in comparison with control, and the effect was reverted when the cells were co-treated with forskolin at $0.5 \mu\text{M}$ or SR141716 at $0.1 \mu\text{M}$ (Fig. 3A and B). To assess if the effect of Met-F-AEA on HMG-CoA reductase activity was due to the expression levels of HMG-CoA reductase protein, we performed a western blot analysis of microsomal protein isolated from both cell lines treated with Met-F-AEA. We observed that the Met-F-AEA

Figure 2 Effect of Met-F-AEA on HMG-CoA reductase activity of MDA-MB-231 and MCF7 cell lines. *In vitro* HMG-CoA reductase activity was evaluated in microsomes, after incubation with Met-F-AEA $10 \mu\text{M}$ after 24 and 48 h. Co-addition of forskolin at $0.5 \mu\text{M}$ or SR141716 at $0.1 \mu\text{M}$ after 24 h recovered the HMG-CoA reductase activity. The value is expressed as percent of control sample assumed as 100%. The data were expressed as mean $\pm$ s.d. The picture presented is representative of three different experiments. ** $P < 0.001$ and * $P < 0.01$ as compared to control.

Figure 3 Effect of Met-F-AEA on mRNA expression of HMG-CoA reductase of MDA-MB-231 and MCF7 cell lines. (A) Ratio of HMG-CoA reductase and $\beta 2m$ densitometric analysis. Cells were grown in cell culture dishes for 24 h and were then exposed to several drugs for 24 h followed by total RNA isolation and quantitative RT-PCR. (B) Representative u.v. exposure of HMG-CoA reductase cDNA on 1.5% agarose gel. Data shown in A are means $\pm$ S.E.M. of three samples. Each bar represents the mean $\pm$ S.D. of three different experiments. ** $P < 0.05$ versus control.

treatment evoked a significant decrease in protein levels in treated cells in comparison with control cells, and they were recovered in cells co-treated with forskolin and SR141716 (Fig. 4A).

Met-F-AEA inhibited the activity of transcription factor CREB

In the previous section, we observed that forskolin at $0.5 \mu M$ recovered cell proliferation activity, mRNA and protein levels of HMG-CoA reductase of Met-F-AEA-treated cells. Forskolin, an activator of AC, causes an increase of cAMP levels which is translated into pleiotropic intracellular effects by a panel of cAMP-binding effector proteins, which include cAMP-dependent protein kinase A (PKA) and members of the CREB protein family. The CREB protein, primarily regulated via cAMP/PKA-dependent phosphorylation, has been recognized as an important transcription factor involved in the control of HMG-CoA reductase gene transcription; moreover, we described that TSH, enhancing cAMP production, induced an increase of HMG-CoA reductase gene expression by activation of CREB protein (Bifulco *et al.* 1995). As recent reports described that activation

of cannabinoid receptors leads to the regulation of CREB protein (Herring *et al.* 1998), we investigated whether the CB1 activation by Met-F-AEA affected the activity, using an anti-phospho-CREB (Ser 133), and the expression of CREB protein by western immunoblotting analysis. As shown in Fig. 4B in the lysates of Met-F-AEA-treated cells, we revealed a significant decrease in the levels of phosphorylated CREB after 1 h in comparison with untreated cells, while co-treatment of cells with forskolin or SR141716 increased the levels of phosphorylated CREB in comparison with Met-F-AEA-treated cells. The levels of CREB protein did not change in the treated cells in comparison with control cells.

Met-F-AEA inhibited the protein prenylation

As the activation of CB1 receptor inhibited the HMG-CoA reductase activity by reducing the synthesis of downstream products as FPP and GGPP required for the prenylation of proteins, we analyzed the pattern of prenylated proteins in Met-F-AEA-treated cells. We metabolically labeled these cells with [3H]-MVA a labeled precursor of both cholesterol and several isoprenoid intermediates. To allow the incorporation of [3H]-MVA into prenylated proteins, endogenous MVA synthesis was blocked by the addition of $10 \mu M$ lovastatin, a specific inhibitor

Figure 4 (A) Western blot and densitometric analysis of HMG-CoA reductase protein in MDA-MB-231 and MCF7 cells treated with Met-F-AEA at $10 \mu M$, forskolin at $0.5 \mu M$, and SR141716 at $0.1 \mu M$. (B) Western blot and densitometric analysis of phospho-CREB protein levels in MDA-MB-231 cells. The cells were incubated with Met-F-AEA for the times indicated. Co-addition of forskolin at $0.5 \mu M$ or SR141716 at $0.1 \mu M$ for 1 h recovered the phosphorylation levels of CREB protein. The picture presented is representative of three different experiments. ** $P < 0.001$, as compared to control; * $P < 0.01$.

Figure 5 Incorporation of (RS)-[5-³H]-mevalonolactone into protein in MDA-MB-231 (A) and MCF7 (B) cells. Cells were treated with Met-F-AEA at 10 μ M and Met-F-AEA plus SR141716 at 0.1 μ M for 24 h. Whole cell extracts were analyzed by SDS-PAGE and fluorography; 100 μ g of whole cell protein were loaded onto each lane. These data are representative of three independent experiments. The autoradiogram was exposed for 20 days.

of HMG-CoA reductase (Laezza *et al.* 2006a,b). Under these conditions, MDA-MB-231 (Fig. 5A) and MCF7 (Fig. 5B) cells incorporated [³H]-MVA very efficiently. Radiolabeled proteins in cellular lysates are separated by electrophoresis showing a variety of proteins with a molecular weight ranging between 14 and 90 kDa (Laezza *et al.* 2006a,b). Cells treated with

Met-F-AEA at 10 μ M for 24 h showed a decrease into the incorporation of the [³H]-MVA into several cellular proteins. Cells exhibited a substantial decrease in the intensity of 46 and 68 kDa, and 20–30 kDa protein bands (Fig. 5A and B), these latter include Ras and Rho proteins. The incorporation of [³H]-MVA in prenylated proteins was reverted when cells were co-treated with SR141716 at 0.1 μ M. The change in prenylation protein was not induced by a decrease of protein synthesis (data not shown).

Met-F-AEA inhibited ras farnesylation

As the translocation of Ras protein from the cytoplasm to the plasma membrane, an essential step for its biological activity, is dependent on farnesylation (Konstantinopoulos *et al.* 2007), we investigated if CB1 activation by Met-F-AEA affected Ras farnesylation by a specific immunoprecipitation with an anti-pan-Ras mAb from [³H]-MVA-radiolabeled cellular lysates treated with Met-F-AEA at 10 μ M for 24 h in comparison with untreated cellular lysates. As shown in Fig. 6A, Met-F-AEA lowered the prenylation level of Ras protein. In order to determine if Met-F-AEA treatment affected Ras membrane localization, we incubated MDA-MB-231 and MCF7 cells with Met-F-AEA for 24 h, and subsequently we determined Ras protein levels in cytosolic and membrane fractions by immunoblotting assay. Figure 6B shows that in control cells cultures, Ras is predominantly associated with the cell membrane fraction, while in cell cultures treated with Met-F-AEA, Ras is predominantly found in the cytosolic fraction, and the amount of Ras protein associated with the cell membrane is greatly reduced. Co-treatment with MVA at 700 μ M or with SR141716 at 0.1 μ M reverted this effect (Fig. 6B).

Discussion

In this study, we described a new role of the CB1 receptor in the regulation of the MVA pathway through the modulation of HMG-CoA reductase activity. The data presented in this report clearly reveal that the significant decrease in HMG-CoA reductase activity correlates well with the changes in the mRNA levels of this enzyme suggesting that the CB1 activation decreases the HMG-CoA reductase activity through its transcriptional regulation. The CB1 receptor is a member of the superfamily of G-protein-coupled receptors. The major mediators of CB1 receptor signaling are the Gi/o proteins of the G-protein family, which inhibit the adenylyl cyclase in most

Figure 6 (A) Immunoprecipitation of ³H-MVA-labeled Ras protein obtained from radiolabeled MDA-MB-231 and MCF7 cellular lysates with anti-pan-p21ras mAb. The immunoprecipitates were subjected to SDS-PAGE. Autoradiogram was exposed for 20 days. (B) Met-F-AEA induced translocation of Ras protein from the cell membrane to cytosol. Western blots indicate the particulate fractions (cell membrane) and soluble fractions (cytosol) of MDA-MB-231 and MCF7 cells treated with Met-F-AEA at 10 μ M, Met-F-AEA plus mevalonate at 700 μ M, Met-F-AEA plus forskolin and untreated cells. Equal amounts of protein were loaded in each lane. Immunoblot analyses were performed using specific anti-pan-Ras and anti-actin antibodies as loading control. The experiments were performed at least three different times, and the results were always comparable. The figure shows a representative blot of three independent experiments. The intensities of the bands were expressed as arbitrary units and were calculated as mean ratio $\pm$ s.d. of control protein (actin). The diagram shows quantification of the intensity of bands, calibrated to the intensity of the actin bands, expressed as means $\pm$ s.e.m. (* P < 0.05 for Met-F-AEA versus control).

tissues and cells, and regulate ion channels, including calcium and potassium (Demuth & Molleman 2006). Inhibition of AC activity was the first characterized agonist stimulated CB1 receptor signal transduction pathway (Howlett & Fleming 1984). Several reports indicate that activation of cannabinoid receptors leads to the regulation of several DNA-binding proteins, including AP-1 (Porcella *et al.* 1998) and CREB protein (Herring *et al.* 1998). Recently, it has been reported that HU 210 and CP 55940, generally used as reference cannabinoid agonists, operate reciprocal influences on the expression of tyrosine hydroxylase (TH) through distinct regulations at the transcriptional level of the *cis*-enhancer elements AP-1 and CRE present in the TH gene promoter, suggesting that both CRE and AP-1 activities are regulated through activation of Gi/o-type G-proteins coupled to the CB1 receptor (Bosier *et al.* 2008, 2009). In the present study, we observed that the activation of the CB1 receptor by Met-F-AEA inhibited the phosphorylation of CREB protein essential for the activation of this transcription factor, and this effect was reverted in cells co-treated with forskolin that induced CREB phosphorylation through an increase in cAMP levels. In the past, we described that in the promoter region of HMG-CoA reductase, there exists a CRE site that is an important co-activator for transcriptional regulation of HMG-CoA reductase gene expression; moreover, we observed that TSH, a physiologic mitogen of thyroid cells, enhanced the production of cAMP, and induced an increase of HMG-CoA reductase gene expression by activation of CREB protein (Bifulco *et al.* 1995, Perillo *et al.* 1995). These results support the hypothesis that the CB1 activation through the regulation of the *cis*-enhancer element CRE present in the HMG-CoA reductase gene promoter could inhibit the transcription of this gene as a consequence of the HMG-CoA reductase protein levels determining a decrease of its activity. Moreover, we observed that the inhibition of cell proliferation upon CB1 activation is reversed by the addition of MVA. MVA is synthesized from HMG-CoA reductase, the rate-limiting enzyme of the MVA pathway. This pathway performs several key functions within cells, leading to the production of sterols, such as cholesterol essential to membrane formation, and isoprenoids. The isoprenoids are essential for switching on the function of GTPase proteins by post-translational lipid modifications – a process known as ‘prenylation’. These modifications include the covalent attachment of a nonsterol isoprenoid (either FPP or GGPP) to the cysteine residue of the CAAX motif by farnesylation and geranylgeranylation respectively (McTaggart 2006).

In this study, we focused on the inhibition of prenylation protein and in particular of Ras farnesylation because the Ras proteins (H-Ras, N-Ras, K-Ras4A, and K-Ras4B) are generally considered key molecular targets in the signal transduction pathways leading to cell proliferation, differentiation, or death. Ras is also involved in uncontrolled growth. Indeed, an increased expression of normal or mutated ras has been observed in 30–40% of human cancers, including colon cancer, pancreatic, breast carcinoma, and anaplastic and follicular thyroid carcinoma (Laezza et al. 1998). The Ras farnesylation is essential to target this protein to the membrane where it performs its biological activity. In a previous study, we observed that anandamide may be efficacious for the inhibition of K-ras-induced epithelial cancer cell growth *in vivo* through the activation of CB1 receptors, inhibition of p21ras activity and blockade of the cell cycle (Laezza et al. 1998). Here we have observed that the inhibition of p21ras activity is due to the inhibition of its farnesylation, which allows the translocation of this protein from cytosol to cell membrane. These data strongly suggest that the inhibition of the farnesylation of Ras protein is critical for its biological activity and consequently for cancer cell proliferation. So, the crucial point of the antitumor action of Met-F-AEA could be related to the inhibition of Ras farnesylation, causing the disruption of Ras localization to the membrane, required to modulate its interaction with upstream and downstream signaling components, thereby attenuating the ability of Ras protein to promote cell proliferation. Finally, we observed that CB1 receptor agonists emulate other inhibitors of prenylation protein, as lovastatin and/or bisphosphonates (Graaf et al. 2004) in both cancer cell lines (data not shown). The finding of possible antitumor effect for these substances might have a tremendous potential for therapeutic intervention in preventing the progression of breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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