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5-Lipoxygenase and anandamide hydrolase (FAAH) mediate the antitumor activity of cannabidiol, a non-psychoactive cannabinoid

P. Massi,* M. Valenti,† A. Vaccani,† V. Gasperi,‡ G. Perletti,† E. Marras,† F. Fezza,§,¶ M. Maccarrone‡,¶ and D. Parolaro†

*Department of Pharmacology, Chemotherapy and Toxicology, University of Milan, Milan, Italy

†Department of Structural and Functional Biology, Pharmacology Section, Center of Neuroscience, University of Insubria, Busto Arsizio, Italy

‡Department of Biomedical Sciences, University of Teramo, Teramo, Italy

§Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, Italy

¶European Center for Brain Research (CERC)/IRCCS S. Lucia Foundation, Rome, Italy

Abstract

It has been recently reported that cannabidiol (CBD), a non-psychoactive cannabinoid, is able to kill glioma cells, both *in vivo* and *in vitro*, independently of cannabinoid receptor stimulation. However, the underlying biochemical mechanisms were not clarified. In the present study, we performed biochemical analysis of the effect of CBD both *in vivo*, by using glioma tumor tissues excised from nude mice, and *in vitro*, by using U87 glioma cells. *In vivo* exposure of tumor tissues to CBD significantly decreased the activity and content of 5-lipoxygenase (LOX, by ~ 40%), and of its end product leukotriene B_4 (~ 25%). In contrast cyclooxygenase (COX)-2 activity and content, and the amount of its end product prostaglandin E_2, were not affected by CBD. In addition, *in vivo* treatment with CBD markedly stimulated (~ 175%) the activity of fatty acid amide hydrolase (FAAH), the main anandamide-degrading enzyme, while decreasing anandamide content (~ 30%) and binding to CB_1 cannabinoid receptors (~ 25%). *In vitro* pre-treatment of U87 glioma cells with MK-886, a specific 5-LOX inhibitor, significantly enhanced the antimitotic effect of CBD, whereas the pre-treatment with indomethacin (pan-COX inhibitor) or celecoxib (COX-2 inhibitor), did not alter CBD effect. The study of the endocannabinoid system revealed that CBD was able to induce a concentration-dependent increase of FAAH activity in U87 cells. Moreover, a significantly reduced growth rate was observed in FAAH-over-expressing U87 cells, compared to wild-type controls. In conclusion, the present investigation indicates that CBD exerts its antitumoral effects through modulation of the LOX pathway and of the endocannabinoid system, suggesting a possible interaction of these routes in the control of tumor growth.

Keywords: cannabidiol, cyclooxygenase, endocannabinoid system, glioma, lipoxygenase.

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Address correspondence and reprint requests to Daniela Parolaro, Department of Structural and Functional Biology, Pharmacology Section, Center of Neuroscience, University of Insubria, Via A. da Giussano 10, Busto Arsizio (VA), Italy. E-mail: daniela.parolaro@uninsubria.it or Mauro Maccarrone, Department of Biomedical Sciences, University of Teramo, Piazza A. Moro 45, 64100 Teramo, Italy.
E-mail: mmaccarrone@unite.it

Abbreviations used: AA, arachidonic acid; AEA, anandamide; CBD, cannabidiol; CBR, cannabinoid receptor; CLX, celecoxib; COX, cyclooxygenase; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; GAR-AP, goat anti-rabbit; LOX, lipoxygenase; LTB_4, Leukotriene B4; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; NAPE-PLD, *N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D; NArPE, *N*-arachidonoylphosphatidylethanolamine; PBS, phosphate-buffered saline; PGE_2, Prostaglandin E2; ROS, reactive oxygen species; RP, reversed phase; RTX, resiniferatoxin; SV40, simian virus 40; TRPV1, transient receptor potential vanilloid type-1.

Investigations carried out during the past few years have shown that administration of cannabinoids exerts a growth inhibitory action on various cancer cell types and, more importantly, slows down the growth of different models of tumor xenografts in rats and mice (Kogan 2005; Bifulco *et al.* 2006). These observations have attracted great interest on the clinical exploitation of cannabinoid-based therapies. However, despite the considerable progress that has been made towards the understanding of the molecular mechanisms that underlie the antitumoral actions of cannabinoids, at present there is no universal mechanism whereby cannabinoids may affect cell viability and proliferation, and no clear structure-activity relationships have been established for the anticancer effect of cannabinoids (Kogan 2005). In some cases, (endo)cannabinoids act through cannabinoid receptors (CBR) and/or vanilloid receptors (transient receptor potential vanilloid type-1, TRPV1), while in other studies they have been reported to inhibit tumor cell growth through mechanisms unrelated to receptor stimulation (Ruiz *et al.* 1999; Jacobsson *et al.* 2001; Massi *et al.* 2004; De Petrocellis and Di Marzo 2005; Bifulco *et al.* 2006; Maccarrone 2006). The mechanisms of the regulation of cell fate by cannabinoids differ between cell lines and depend on the concentration of cannabinoids. It has been reported that in tumor cells cannabinoids act preferentially through the ceramide pathway and apoptotic death, but other mechanisms including cell cycle arrest, oxidative stress damage and increase in calcium concentration have been documented (De Petrocellis and Di Marzo 2005; Bifulco *et al.* 2006).

Cannabinoids can also act by targeting angiogenesis and cell migration (Blazquez *et al.* 2003; Vaccani *et al.* 2005; Fernandez-Ruiz *et al.* 2006), and they can interfere with the action of some growth factors, thus blocking mitogenic factor-dependent cancers. Furthermore, intriguing investigations propose that drugs modulating the endogenous cannabinoid system (ECS) might be used in cancer therapy, providing protection from the growth and spread of cancer (De Petrocellis and Di Marzo 2005; Jonsson *et al.* 2006). The ECS includes amides and esters of polyunsaturated fatty acids, like *N*-arachidonoylethanolamine (anandamide, AEA), the enzymes that synthesize (*N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D, NAPE-PLD) or degrade AEA (fatty acid amide hydrolase, FAAH), and the AEA-binding CBR and TRPV1 receptors (Bari *et al.* 2006). In addition, the ECS includes AEA congeners like 2-arachidonoylglycerol, for which specific biosynthetic (Bisogno *et al.* 2003) and hydrolytic (Dinh *et al.* 2002) enzymes have been recently discovered. However, 2-arachidonoylglycerol does not seem to play a major role in controlling the cell choice between survival and death (Guzman 2003; Maccarrone 2006).

Recently, we were able to demonstrate that cannabidiol (CBD), a non-psychotropic cannabinoid, is capable of killing glioma cells, both *in vivo* and *in vitro*, independently of CBR and TRPV1 stimulation. Instead, CBD induced apoptosis and caspase activation with a mechanism involving an early increase in reactive oxygen species (ROS) and glutathione depletion (Massi *et al.* 2004, 2006).

Substantial evidence has been collected supporting a fundamental role for lipoxygenase (LOX)- and cyclooxygenase (COX)-catalyzed arachidonic acid (AA) metabolism in cancer development (Furstenberger *et al.* 2006). In particular, 5-LOX (Maccarrone *et al.* 2000a; Manev *et al.* 2000) and COX-2 (Funk 2001) are the isozymes most involved in the control of cell growth and death within the CNS. Remarkably, 5-LOX (van der Stelt *et al.* 2002) and COX-2 (Kozak *et al.* 2004) can also oxygenate endocannabinoids, thus modulating their ability to recognize their targets within the ECS (van der Stelt *et al.* 2002). In the present study we sought to investigate the possibility that 5-LOX and COX-2, as well as the ECS, could be modulated by CBD, in order to limit tumor growth. To this end, we performed biochemical analysis of 5-LOX, COX-2 and ECS both *in vivo*, by using tumor tissues excised from nude mice bearing subcutaneous human glioma tumor, and *in vitro*, by using U87 human glioma cell cultures. These studies were further extended to purified isozymes, in order to ascertain any direct effect of CBD on LOX and COX activity. We show that CBD exerts its antitumoral effect through the modulation of the LOX pathway, and subsequently of the ECS. Our results suggest that the non-psychoactive CBD might be exploited in the future as an effective antitumor drug, alone or in combination with 5-LOX inhibitors.

Materials and methods

Materials

Cannabidiol was a generous gift from GW Pharmaceuticals (Salisbury, UK). Chemicals were of the purest analytical grade. Anandamide (*N*-arachidonoylethanolamine, AEA), CP55,940 (5-(1,10-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl)-cyclohexyl] phenol), arachidonic acid, bovine serum albumin, indomethacin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), MK-886, URB597 and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Resiniferatoxin (RTX) and celecoxib (CLX) were from Alexis Corporation (Lausen, Switzerland). *N*-Arachidonoyl-phosphatidylethanolamine (NArPE) was synthesized from AA and phosphatidylethanolamine as reported (Fezza *et al.* 2005). [^{3}H]AEA (205 Ci/mmol), [^{3}H]CP55.940 (126 Ci/mmol), and [^{3}H]RTX (43 Ci/mmol) were from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA). [^{3}H]NArPE, (200 Ci/mmol) was from ARC (St. Louis, MO, USA). Anti-FAAH polyclonal antibodies were prepared by Primm S.r.l. (Milan, Italy) as reported (Maccarrone *et al.* 1998). Anti-5-LOX polyclonal antibodies were from Chemicon International (Temecula, CA, USA), and anti-COX-2 polyclonal antibodies were from Cayman Chemical Co. (Ann Arbor, MI, USA). Goat anti-rabbit (GAR-AP) antibodies conjugated to alkaline phosphatase were from Bio-Rad (Hercules, CA, USA).

Enzymes

5-Lipoxygenase (arachidonate:oxygen oxidoreductase; E.C. 1.13.11.34; 5-LOX) was purified to electrophoretic homogeneity from ungerminated barley (*Hordeum vulgare*), as reported previously (van Aarle *et al.* 1991). 15-Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12; 15-LOX) was purified to electrophoretic homogeneity from soybean (*Glycine max*) seeds as reported (Finazzi-Agrò *et al.* 1973). Cyclooxygenase-1 (prostaglandin H synthase 1; EC 1.14.99.1; COX-1), isolated from ram seminal vesicles, and human recombinant cyclooxygenase-2 (prostaglandin H synthase 2; EC 1.14.99.1; COX-2) were purchased from Sigma. Purified FAAH used as a positive control in western blot analysis was a kind gift from Dr. Benjamin F. Cravatt (The Scripps Research Institute, La Jolla, CA, USA).

Nude mouse xenograft model of human glioma

Athymic female CD-1 nude (*nu/nu*) mice (Charles River, Milan, Italy), 8 week-old, were used as previously described (Massi *et al.* 2004). Briefly, the animals were injected subcutaneously on the left flank with 3×10^6 U87 human glioma cells in 0.1 mL of PBS. Seven days after inoculation, when the tumor had reached an average volume of about 70 mm^3, mice were randomly divided in two groups. The mice were then treated peritumorally with CBD (dissolved in 0.1 mL sterile PBS supplemented with 5 mg/mL defatted and dyalized bovine serum albumin) or its vehicle, at a dose of 0.5 mg/mouse (once a day, 5 days/week). On the last day of the 3 week *in vivo* experiments (day 23) when CBD-treated mice had significantly smaller tumors than control mice (Massi *et al.* 2004), tumors were excised from both groups and used for biochemical assays.

This protocol was conducted in accordance with the Italian regulation for the welfare of animals in experimental neoplasia (Permission no. 94/2000A) and met the European Community directives regulating animal research.

Cell culture

U87 human glioma cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells were cultured in 75-cm^2 flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mmol/L L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 10% heat-inactivated fetal bovine serum. Cells were seeded in serum-free medium, consisting of DMEM supplemented with 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite, in multiwell plates or Petri dishes according to the experimental protocol. After 24 h incubation, the medium was removed and new culture medium, containing the compounds to be tested, was added.

Receptor binding

For CBR studies, membrane fractions were prepared from tissues, as reported (Maccarrone *et al.* 2005), and were stored at −80°C for no longer than 1 week. The membrane fractions were used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP55.940 (500 pmol/L), as described previously (Maccarrone *et al.* 2005). To assess the presence of vanilloid receptors (TRPV1), also binding of [^{3}H]RTX (500 pmol/L) was evaluated by rapid filtration assays and performed as described previously (Ross *et al.* 2001). In all experiments, unspecific binding was determined in the presence of cold agonists (1 μmol/L CP55.940 or 1 μmol/L RTX), as reported (Ross *et al.* 2001; Maccarrone *et al.* 2005). Receptor binding was expressed as fmol [^{3}H]ligand bound/mg protein.

Fatty acid amide hydrolase activity and expression

The hydrolysis of [^{3}H]AEA by fatty acid amide hydrolase (*N*-arachidonoylethanolamine amidohydrolase, E.C. 3.5.1.4; FAAH) was assayed in cell or tissue homogenates. These (10 μg/test) were incubated at pH 9.0 with 10 μmol/L [^{3}H]AEA as substrate, and the release of [^{3}H]AA was evaluated through reversed phase (RP)-HPLC, as reported (Maccarrone *et al.* 1998). FAAH expression was determined in homogenates by ELISA (10 μg/test) or western blot (50 μg/lane), using anti-FAAH polyclonal antibodies (1 : 250) and GAR-AP (diluted 1 : 2000) as first and second antibody (Maccarrone *et al.* 1998). Purified FAAH (2 μg/lane) was used as a positive control in western blot analysis.

Western blot analysis

Cells were scraped and collected by centrifugation, lysed with 20 mmol/L HEPES, pH 7.2, 150 mmol/L NaCl, 1% Triton X-100 in the presence of a protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy), at 4°C for 30 min. Extracted proteins (40 μg/well) were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by electroblotting onto nitrocellulose membrane. The membrane was then overnight – probed with polyclonal anti-FAAH antibody (Chemicon, Milan, Italy, 1 : 200 in 3% non-fat dry milk in Tris-Buffered Saline), followed by specific IgG antibody conjugated to horseradish peroxidase (Santacruz, Milan, Italy). Detection of proteins was then performed by ECL system. The protein content was determined with bicinchonic acid protein assay using bovine serum albumin as a standard.

NAPE-PLD activity

The synthesis of AEA through the activity of *N*-acylphosphatidylethanolamines (NAPE)-hydrolyzing phospholipase D (E.C. 3.1.4.4; NAPE-PLD) was assayed in tissue homogenates (100 μg/test), by measuring the release of [^{3}H]AEA from [^{3}H]NArPE (100 μmol/L) as described (Fezza *et al.* 2005).

Endogenous levels of anandamide

For the evaluation of the endogenous levels of AEA, tumor samples were homogenized with an UltraTurrax T25 in 50 mmol/L Tris-HCl, 1 mmol/L EDTA pH 7.4 and 1 mmol/L phenylmethanesulfonyl fluoride buffer, at a 1 : 10 (wt/vol) homogenization ratio. Lipids were then extracted, the organic phase was dried under nitrogen and dry pellet was derivatized as reported (Maccarrone *et al.* 2005). Briefly, 25 μL of 10 mmol/L DBD COCl (4-(*N*-chloroformyl-mytlmethyl-*N*-methyl)amino-7-*N,N*-dimethyl-aminosulphonyl-2,1,3-benzoxadiazole, from Tokyo Kasei Kogyo Co., Tokyo, Japan) was added to 500 μL anhydrous dichloromethane. The mixture was then heated at 60°C for 1 h, dried by centrifugal concentrator and reconstituted in 50 μL acetonitrile. RP-HPLC with fluorimetric detection was carried out using a S-200 fluorescence detector (Perkin-Elmer). The separation was performed with a mobile phase of acetonitrile in water (700 mL/L) at a flow rate of 1.0 mL/min. The concentration of AEA was quantified by comparison with known amounts of standard, as reported (Maccarrone *et al.* 2005).

Lipoxygenase and cyclooxygenase activity

The activity of 5-LOX in tumor samples was measured by RP-HPLC, incubating tissue homogenates for 10 min at 37°C in the presence of 1 mmol/L ATP, 2 mmol/L $CaCl_2$ and 40 μmol/L AA (Maccarrone *et al.* 2002). The activity of pure 5-LOX and pure 15-LOX was assayed spectrophotometrically in 100 mmol/L sodium phosphate (pH 6.8) or borate (pH 9.0) buffer, respectively, at 25°C, by recording the formation of conjugated hydroperoxides from linoleic acid at 234 nm (Maccarrone *et al.* 2001). Tissue 5-LOX activity was expressed as pmol 5-hydroperoxyeicosatetraenoic acid formed per min per mg protein, whereas the activity of the pure enzymes was expressed as nmol/L hydroperoxide formed per min.

The activity of tissue COX, and that of purified COX-1 and COX-2 were measured polarographically, at 30°C, in 0.1 mol/L potassium phosphate, pH 7.2, 1 mmol/L phenol, 1 μmol/L hemin and 75 μmol/L AA (Kulmacz and Wang 1995; Maccarrone *et al.* 2000a). COX activity was expressed as nmol O_2 consumed per min per mg protein (tissue COX), or as nmol O_2 consumed per min (purified COXs).

Lipoxygenase and cyclooxygenase expression

ELISA tests of tissue extracts were performed as previously reported (Maccarrone *et al.* 2000a). Anti-5-LOX or anti-COX-2 polyclonal antibodies (diluted 1 : 200 and 1 : 400, respectively) were used as first antibody, and GAR-AP (diluted 1 : 2000) was used as second antibody. Color development of the alkaline phosphatase reaction was followed at 405 nm, using *p*-nitrophenylphosphate as substrate (Maccarrone *et al.* 2000a).

LTB_4 and PGE_2 level

Leukotriene B4 (LTB_4) and Prostaglandin E2 (PGE_2) metabolites were extracted from tissue homogenates on octadecyl-SPE (solid phase extraction) columns (Baker, Deventer, The Netherlands) and were analyzed by RP-HPLC on a C18 3 × 3 CR column (SGE, Austin, TX, USA) (Jakobsson *et al.* 1992). RP-HPLC was performed on a Perkin Elmer 1022 LC Plus liquid chromatograph (Norwalk, CT, USA) at a flow rate of 1.2 mL/min, using methanol (700 mL/L) and trifluoroacetic acid (0.7 mL/L) in water as mobile phase (Maccarrone *et al.* 2000a). Chromatograms were recorded at 270 nm, assessing peak identity by comparison with authentic standards (Cayman Chemical Co.).

MTT test

To determine the effects of CBD on cell viability, the MTT colorimetric assay was carried out as previously reported (Massi *et al.* 2004). Briefly, U87 glioma cells were seeded in a 96 flat bottom multiwell at a density of 8000 cells/well. After 24 h, cells were treated with CBD and/or inhibitors at the indicated concentrations. At the end of the incubation with the drugs, MTT (0.5 mg/mL final concentration) was added to each well and the incubation was continued for further 4 h. The insoluble formazan crystals were solubilized by the addition of 100 μL of 100% dimethyl sulfoxide. Plates were read at 570 nm, using an automatic microtiter plate reader.

Fatty acid amide hydrolase over-expression in U87 cells

The transfer vector pWPT/FAAH/GFP was generated as previously described (Osti *et al.* 2006). Briefly, an Eco RI-Hind III fragment containing the simian virus 40 (SV40) early promoter was subcloned in the corresponding sites of pBluescriptII SK(−) (Stratagene, La Jolla, CA, USA). A 2472 bp EcoRI fragment containing the cDNA of FAAH was inserted into the EcoRI site of pBluescriptII SK(−), upstream of the SV40 early promoter. A BamHI-BssHII fragment containing the FAAH cDNA and the SV40 early promoter was finally isolated from pBluescriptII SK(−) and ligated into the BamHI-MluI sites of the pWPT/GFP vector. Thus, in the resulting construct (pWPT/FAAH/GFP) the FAAH and GFP cDNAs were placed under the transcriptional control of the elongation factor-1α-short and SV40-early promoters.

For *in vitro* FAAH-over-expression experiments, 30% confluent U87 cells were infected for 4 h with 10 M.O.I. lentiviral vectors; the particle-containing medium was replaced with fresh medium and cells were incubated at 37°C for 48 h. Cells were then harvested and processed for western blot analysis (for control) and FAAH activity measurements.

Statistical analysis

Results are given as mean ± SEM. The significance of differences was evaluated by Student *t*-test or one-way ANOVA, followed by Tukey's test performed by Prism (GraphPad Software for Science, Inc., San Diego, CA, USA).

Results

In vivo experiments on human glioma tumors

Recently, we demonstrated the *in vivo* efficacy of CBD in reducing tumor growth, by using athymic nude mice inoculated subcutaneously with U87 glioma cells (Massi *et al.* 2004). The rationale of the present study was based on the evidence that, as no clear biochemical target is demonstrated for the marked antiproliferative effect of CBD, then this cannabinoid might interfere with important routes for the control of tumor cell growth, like the LOX and COX pathways, and the endocannabinoid system.

To demonstrate that CBD could act through the modulation of LOX, COX or ECS, we subjected to biochemical analysis tumor samples that had been excised on the last day from nude mice treated *in vivo* with repeated administration of CBD (or its vehicle) for 23 days (Massi *et al.* 2004). Thus, we first checked for 5-LOX and COX activity, for 5-LOX and COX-2 levels, and then for the level of 5-LOX and COX terminal products. *In vivo* exposure to CBD significantly decreased (by ∼ 35%) 5-LOX activity in tumor tissues, and this reduction was paralleled by a similar (∼ 40%) decrease of 5-LOX content (Fig. 1a). Instead, cellular COX activity was not significantly affected by *in vivo* treatment with CBD (Fig. 1a). As COX-1 is a constitutive isozyme whereas the expression of COX-2 is inducible (Funk 2001), we also checked the effect of CBD on the level of COX-2 protein, and found that there was none (Fig. 1a).

The inhibition of 5-LOX was paralleled by the decrease (∼ 25%) of its LTB_4 product in CBD-exposed tumor tissues

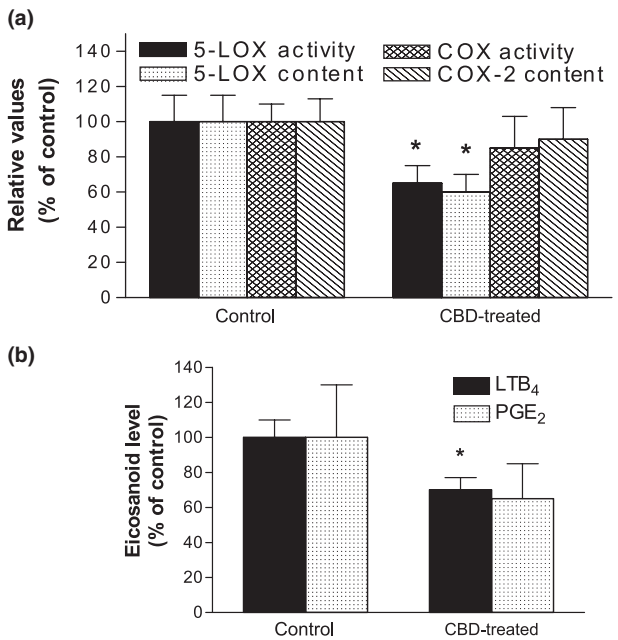

Fig. 1 (a) Modulation of 5-lipoxygenase (LOX) and cyclooxygenase (COX) activity, and 5-LOX and COX-2 levels in tumor samples excised from nude mice treated *in vivo* with cannabidiol (CBD). 100% = 300 ± 30 pmol/min per mg protein (5-LOX activity), 0.230 ± 0.035 A_{405} units (5-LOX content), 70 ± 10 nmol O_2/min per mg protein (COX activity), and 0.340 ± 0.040 A_{405} units (COX-2 content). (b) Modulation of LTB_4 and PGE_2 in tumor samples excised from nude mice treated *in vivo* with CBD. 100% = 25 ± 3 pmol per mg protein (LTB_4), or 70 ± 8 nmol per mg protein (PGE_2). In both panels, *denotes $p < 0.05$ versus control.

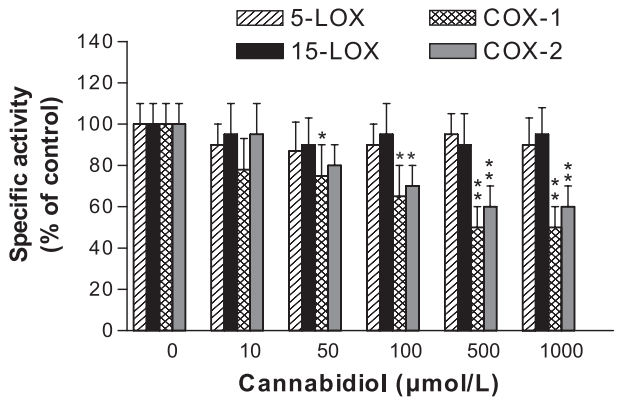

Fig. 2 Modulation of the activity of purified lipoxygenases (LOXs) and cyclooxygenases (COXs) by *in vitro* exposure to cannabidiol. 100% = 2.0 ± 0.3 nmol/L/min (5-LOX), 4.0 ± 0.2 nmol/L/min (15-LOX), 35 ± 4 nmol O_2/min (COX-1), and 15 ± 3 nmol O_2/min (COX-2). *Denotes $p < 0.05$ and **denotes $p < 0.01$ versus control.

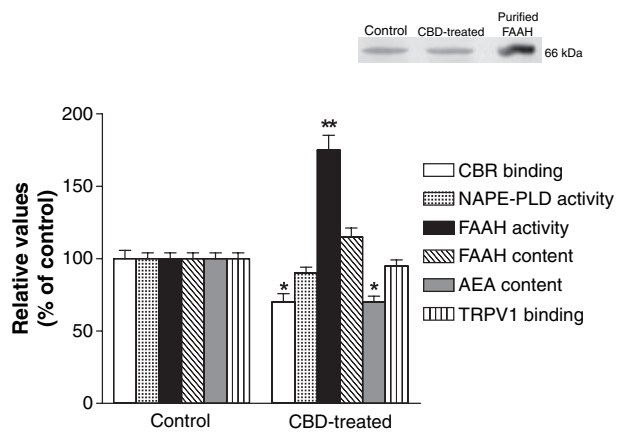

Fig. 3 Modulation of the endocannabinoid system in tumor samples after *in vivo* treatment with cannabidiol (CBD, 0.5 mg/mouse) for 23 days. Results are expressed as percentage of controls [100% = 100 ± 10 fmol per mg protein, for cannabinoid receptors (CBR) binding; 4.50 ± 0.80 pmol/min per mg protein, for *N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D (NAPE-PLD) activity; 196 ± 20 pmol/min per mg protein, for fatty acid amide hydrolase (FAAH) activity; 0.420 ± 0.050 A_{405} units, for FAAH content; 7.43 ± 1.05 pmol per mg protein, for anandamide (AEA) content; 65 ± 7 fmol per mg protein, for transient receptor potential vanilloid type-1 (TRPV1) binding]. Inset. Western blot analysis of FAAH in control and CBD-treated tumor samples (50 μg/lane). *Denotes $p < 0.05$ and **denotes $p < 0.01$ versus corresponding controls.

(Fig. 1b). Instead, the level of the COX product PGE_2 was not significantly affected (Fig. 1b).

To further ascertain whether CBD could affect directly LOX and COX activity, we treated *in vitro* purified enzymes with CBD. This substance did not affect the activity of purified 5-LOX, neither did it affect the activity of the other major mammalian isozyme 15-LOX (Brash 1999), in the 10–1000 μmol/L concentration range (Fig. 2). On the other hand, the activity of COX-1 and COX-2 isozymes was significantly inhibited by CBD, but only at the very high concentration of 50 μmol/L, or at even higher doses (Fig. 2).

We next sought to characterize in glioma tumor tissues the ECS, and measured CBR binding, NAPE-PLD activity, FAAH activity and content, endogenous tone of AEA, and TRPV1 binding. We found a significant decrease (∼ 25%) of CBR binding in CBD-treated tumors compared to controls, whereas the other AEA-binding TRPV1 receptor was not affected (Fig. 3). In addition, *in vivo* treatment of nude mice with CBD markedly enhanced the activity of the AEA-degrading enzyme FAAH, up to ∼ 175% of the controls; however, FAAH protein expression was not affected by CBD, as ascertained by ELISA test (Fig. 3) and western blot analysis (Fig. 3, inset). Also a ∼ 30% decrease in AEA levels was observed in CBD-treated tissues, whereas the activity of the AEA-synthase NAPE-PLD was not affected (Fig. 3). Taken together, these data suggest that *in vivo* exposure to CBD reduced the level of AEA and of AEA-binding CBRs in glioma tumors. They also demonstrate that the reduction of the endogenous tone of AEA was entirely because of enhanced degradation by FAAH.

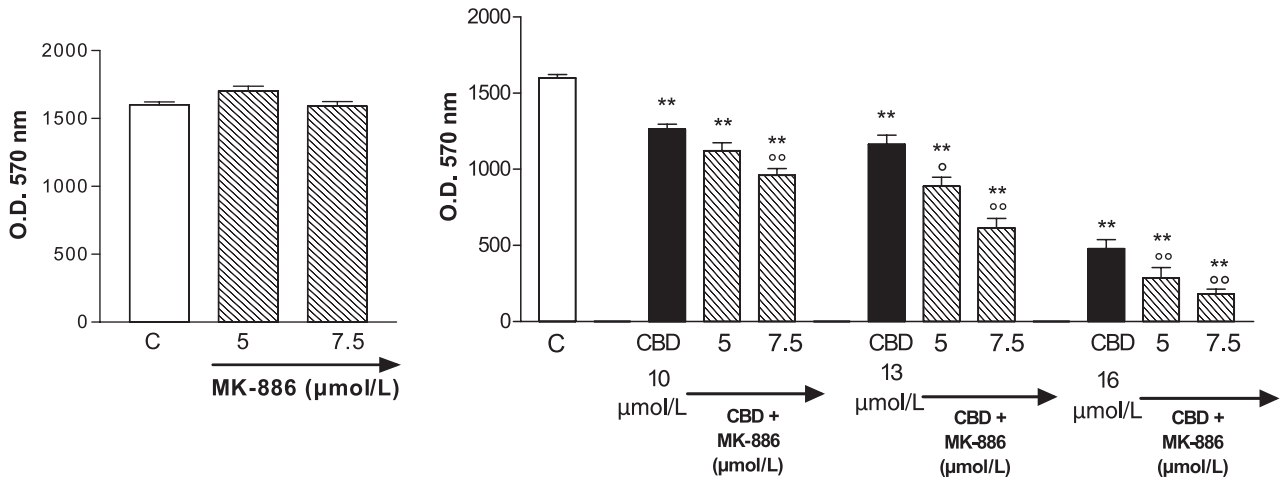

Fig. 4 Effect of pre-treatment of U87 cells with the 5-lipoxygenase inhibitor MK-886 on the antiproliferative effect induced *in vitro* by cannabidiol (CBD). **Denotes $p < 0.01$ versus control (C), °denotes $p < 0.05$ versus CBD, and °°denotes $p < 0.01$ versus CBD.

In vitro experiments on human glioma U87 cells

Based on the results obtained in the *in vivo* model, we further characterized *in vitro* the role of LOX and COX on the antiproliferative effect of CBD. *In vitro* pre-treatment of U87 cells with MK-886, a specific 5-LOX inhibitor that was used at concentrations not affecting *per se* cell viability, enhanced the antimitotic effect of CBD in a concentration-dependent manner (Fig. 4). In contrast, pre-treatment of U87 cells with either the COX-2 selective inhibitor CLX (Fig. 5) or the pan-COX inhibitor indomethacin (Fig. 6), did not alter the antimitotic activity of CBD, whatever the inhibitors concentration used.

Next, we sought to evaluate the role of FAAH, that was markedly increased *in vivo* (Fig. 3), and checked for the modulation of FAAH activity in U87 cells after 24 h of exposure to CBD *in vitro*. In keeping with the *in vivo* data, CBD induced a dose-dependent increase of FAAH activity, up to 2–3-fold over the untreated controls (Fig. 7) without affecting protein levels (Fig. 7, inset). As already reported in *in vivo* experiments, also a ~ 70% decrease in AEA levels was observed in U87 cells exposed *in vitro* to CBD (Table 1).

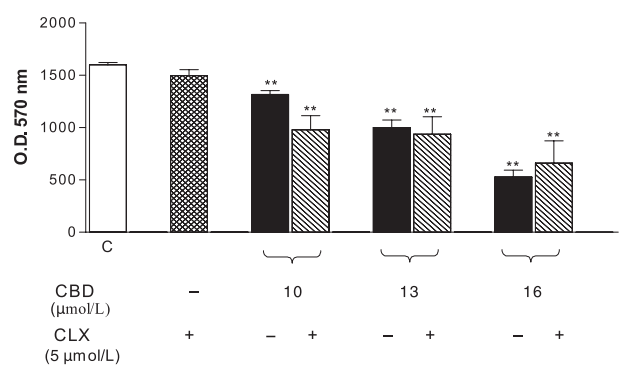

Fig. 5 Effect of pre-treatment of U87 cells with the cyclooxygenase-2 inhibitor celecoxib (CLX) on the antiproliferative effect induced *in vitro* by cannabidiol (CBD). **Denotes $p < 0.01$ versus control (C).

To further confirm the involvement of FAAH enzyme in the antimitotic effect of CBD, additional pharmacological studies were performed. The inhibition of the basal activity of FAAH by URB597 completely prevented the antiproliferative action of CBD on U87 cells (Fig. 8).

In an attempt to better understand the role of FAAH *per se* in tumor growth, we reproduced *in vitro* high intracellular levels of FAAH, by enhancing *faah* gene expression through lentivirus-mediated *in vivo* gene-transfer (Osti *et al.* 2006). Lentivirus-transduced U87 cells (U87 FAAH) over-expressed FAAH by ~ 40-fold compared to U87 wild type and mock-transduced (U87 WPT) cells, as evaluated by semi-quantitative analysis of western blots (Fig. 9a). U87 FAAH cells also showed an ~ 50-fold increased enzyme activity compared to the U87 WT cells (Table 1). Interestingly, U87 FAAH cells showed a significantly reduced growth rate compared to U87 WT and U87 WPT cells, based on both MTT test and Trypan blue dye exclusion (Fig. 9b,c). The observed impairment in U87 FAAH transfected cells growth appeared correlated to the level of FAAH activity and inversely correlated to AEA level (Table 1).

Discussion

In this investigation we report unprecedented evidence that in glioma tumor tissues excised from nude mice exposed *in vivo* to CBD, there was a significant decrease in the activity and content of 5-LOX, and consistently of its terminal product LTB_4, and a very high and sustained increase of FAAH activity. Also a significant reduction of the endogenous level of the FAAH substrate AEA and of the AEA-binding CBRs was observed. On the other hand, CBD treatment did not

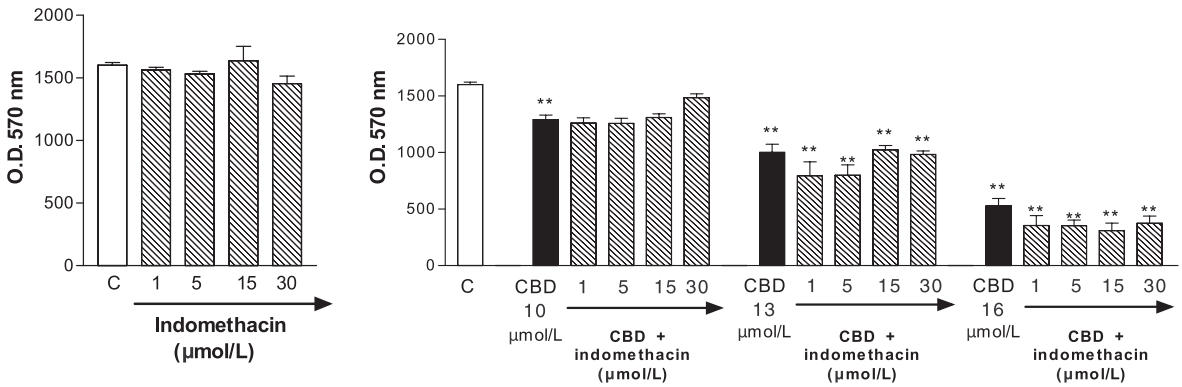

Fig. 6 Effect of pre-treatment of U87 cells with the pan-cyclooxygenase inhibitor indomethacin on the antiproliferative effect induced *in vitro* by cannabidiol (CBD). **Denotes $p < 0.01$ versus control (C).

Table 1 Anandamide (AEA) levels and fatty acid amide hydrolase (FAAH) activity in U87 cells treated with 16 μmol/L cannabidiol (CBD) for 24 h, or transfected with pWPT/FAAH/GFP (FAAH) lentiviral vectors

Sample	AEA level[a]	FAAH activity[b]
Control U87 cells	15.5 ± 1.3	0.23 ± 0.02
U87 cells + 16 μmol/L CBD	4.2 ± 1.0*	3.20 ± 0.80*
U87 FAAH-transduced cells	8.7 ± 2.1*	11.52 ± 1.80*

[a]Expressed as pmol per mg protein. [b]Expressed as pmol/min per mg protein. *$p < 0.01$ versus control ($n = 3$).

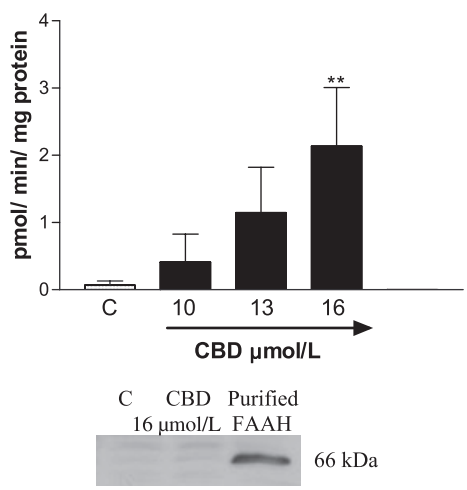

Fig. 7 Effect of *in vitro* cannabidiol (CBD) on fatty acid amide hydrolase (FAAH) activity in U87 cells, incubated for 24 h with the compound. Inset. Western blot analysis of FAAH in control (C) and CBD-treated U87 cells. **Denotes $p < 0.01$ versus control (C).

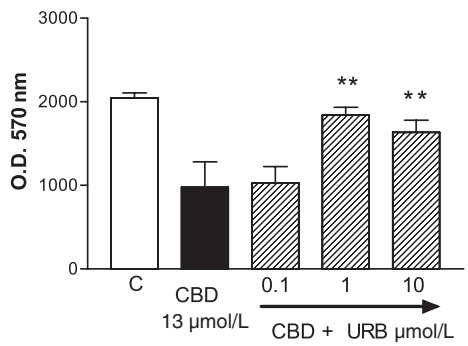

Fig. 8 Effect of pre-treatment of U87 cells with the fatty acid amide hydrolase selective inhibitor URB597 (URB) on the antiproliferative effect induced *in vitro* by cannabidiol (CBD). **Denotes $p < 0.01$ versus CBD-treated cells.

modify COX-2 activity and content, nor the COX end product PGE_2, nor the other elements of the ECS that bind (TRPV1) or synthesize (NAPE-PLD) AEA. These data suggest that 5-LOX, rather than COX-2, and FAAH may be the main downstream mediators of CBD signaling responsible for the marked antiproliferative effect of this compound on glioma tumor growth.

Up to date, the interaction between (endo)cannabinoids and LOX or COX pathways has been scarcely investigated. For instance, a role for COX in cannabinoid antiproliferative effect has been recently reported (Patsos *et al.* 2005; Eichele *et al.* 2006). The present study is the first to demonstrate that CBD may induce tumor and cell growth inhibition through, at least in part, the modulation of the LOX pathway. So far, it is not definitely clear how CBD can affect 5-LOX activity and content, however the data on purified LOX isozymes clearly rule out any direct effect of CBD on enzyme activity. Therefore, it can be proposed that indirect pathways evoked *in vivo* by CBD may be responsible for the modulation of the expression of the *5-lox* gene, and as a consequence of the 5-LOX activity and protein content. In line with this, CBD has been shown to act as a potent modulator of intracellular calcium homeostasis (Drysdale *et al.* 2006; Ligresti *et al.* 2006), as well as of ROS production (Massi *et al.* 2006;

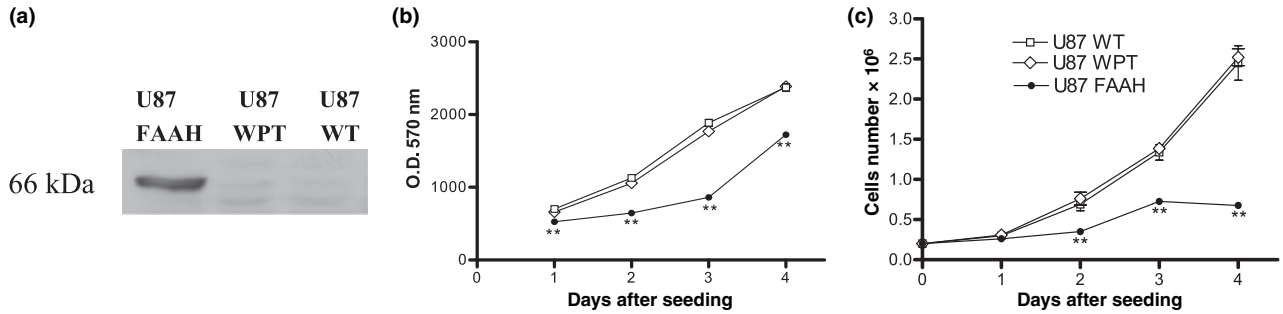

Fig. 9 (a) Western blot analysis of fatty acid amide hydrolase (FAAH) in U87 cells over-expressing FAAH (U87 FAAH), mock-transduced (U87 WPT) cells, and U87 wild-type (U87 WT) cells. (b,c) Effect of FAAH over-expression on U87 glioma cells viability. (b) MTT test (c) Trypan blue test. (□) Wild-type U87 cells; (◇) U87 cells transfected with pWPT/GFP lentiviral vector; (●) U87 cells transfected with pWPT/FAAH/GFP lentiviral vector. **Denotes $p < 0.01$ versus U87 WT and versus U87 WPT.

McKallip *et al.* 2006). Both calcium and ROS are known to control the LOX pathway (Brash 1999).

In the last few years, it has become evident that LOX isozymes may have fundamental and converging functions, not only in inflammation but also in cell proliferation, neoangiogenesis and degenerative diseases (Furstenberger *et al.* 2006), with a clear therapeutic potential (Werz and Steinhilber 2006). In line with this, it has been reported that 5-LOX is over-expressed in high grade astrocytomas (Nathoo *et al.* 2006), and that there is a relevant role for the LOX pathway, but not for the COX pathway, in promoting cell proliferation in U-373 MG human astrocytoma cells (Kim *et al.* 1998; Nathoo *et al.* 2006). Our results further corroborate existing data, and point to 5-LOX as a potential executioner of the antiproliferative effect of CBD.

In addition, our *in vitro* experiments provided evidence that the antiproliferative action of CBD can be enhanced by suboptimal concentrations of the 5-LOX inhibitor MK-886. Such a synergism further supports the evidence that modulation of 5-LOX may play a role in controlling glioma cell proliferation. A similar synergism was already documented by Klegeris *et al.* (2003), who investigated the anti-inflammatory properties of the selective CB2R agonist JWH-015 in human monocytic THP-1 cells, in the presence of the LOX inhibitor REV 5901. Conversely in U87 cells, much alike the excised tumors, COX activity did not seem to play any prominent role, because neither the pan-COX inhibitor indomethacin nor the COX-2 selective inhibitor CLX affected the activity of CBD on cell growth. It should be also stressed that the ability of CBD to inhibit *in vivo* 5-LOX activity and expression, and the synergism between CBD and 5-LOX inhibitors observed *in vitro*, may be of clinical relevance. In fact, an over-expression and/or activation of 5-LOX have been related to tumor development, as well as to neurodegenerative disorders (Manev *et al.* 2000; Montine and Morrow 2005). Thus, the pharmacological modulation of 5-LOX by the non-toxic and non-psychoactive CBD could become a suitable and safe tool to manage *in vivo* glioma growth, either alone or in combinatory therapies.

Another major finding of this study is the modulation of the ECS following repeated administration of CBD *in vivo*. We found in tumor tissues a very high increase of FAAH activity not paralleled by increased FAAH content, and consistently a significant reduction in AEA level and CBR binding, without alterations in AEA-synthesizing NAPE-PLD activity. A similar increase in FAAH activity was also observed in U87 cells exposed to CBD *in vitro*. As 5-LOX products can inhibit FAAH in the low micromolar range (van der Stelt *et al.* 2002), it is possible that the observed up-regulation of FAAH activity may be a consequence of the down-regulation of 5-LOX (and hence of its FAAH-inhibiting products) in CBD-treated tumor tissues. In support of this hypothesis, it has been shown in human mastocytes that inhibition of 5-LOX can disclose the activity of a cryptic FAAH, undetectable in untreated cells (Maccarrone *et al.* 2000b). On the other hand, the endocannabinoid system is often viewed as a defense mechanism of the body against cancer cell proliferation. AEA has been reported to have proapoptotic effect on cell cultures and the inhibition of its degradation has been suggested as a useful tool to limit either *in vivo* (Bifulco *et al.* 2004) or *in vitro* (Jonsson *et al.* 2003) the growth of thyroid transformed and C6 glioma cells, respectively. However, in our experimental model the view of AEA as a proapoptotic stimulus does not appear to hold true, as the antiproliferative effect of CBD *in vivo* correlated with a $\sim$ 30% decrease in AEA level. In this context, it should be recalled that opposite actions of (endo)cannabinoids have been often reported (Fride *et al.* 1995; Sulcova *et al.* 1998; Kogan 2005), depending on their concentrations and on the cells or tissues used. Thus it is possible that, because of the complexity of the *in vivo* glioma model used in this study, the higher level of AEA in untreated compared to CBD-treated tumors can act as a natural growth factor, rather than a proapoptotic agent. As a matter of fact, a role for AEA as neurotrophin has been

proposed (Derkinderen *et al.* 2003), as well as a role as growth factor for hematopoietic cells (Derocq *et al.* 1998). Therefore, in untreated tumor tissues the lower activity and expression of FAAH, and the subsequently higher level of AEA, might represent a growth stimulus contributing to cancer development. In keeping with this hypothesis, (endo)cannabinoids have been shown to behave as pro-cancer agents (Gardner *et al.* 2003; Hart *et al.* 2004; Kogan 2005). Petersen *et al.* (2005) reported that glioblastoma showed high levels of AEA. As exogenous AEA has been described to possess *in vitro* antiproliferative effects (Jacobsson *et al.* 2001), the authors concluded that endocannabinoids can behave as endogenous lipid mediators of tumor cell anti-proliferation, although the hypothesis was not supported by pharmacological studies. The present results showing an inverse correlation between intrinsic AEA levels and tumor growth, suggest a new possible physiological/pathological role of endocannabinoids as procancer agents. As a matter of fact, the reported evidence (Petersen *et al.* 2005) that in meningioma, a benign form of tumor, the level of AEA are low, seems to further support this hypothesis.

Overall, it is tempting to suggest that the endogenous tone of AEA can be finely regulated by CBD through 5-LOX inhibition and hence FAAH activation, ultimately leading to antitumoral properties. Also over-expression of FAAH in U87 cells seems to favor this hypothesis.

In summary, this is the first report demonstrating that CBD induces tumor growth inhibition through a specific modulation of the procarcinogenic LOX pathway and, consequently, of the endocannabinoid system, thus suggesting a novel model through which (endo)cannabinoids can control tumor growth. These data also support the hypothesis that a concomitant use of CBD and 5-LOX inhibitors may be exploited for cancer therapy, in order to treat more effectively tumor growth.

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