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ORIGINAL ARTICLE

Cannabinoid-induced cell death in endometrial cancer cells: involvement of TRPV1 receptors in apoptosis

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Abstract

Among a variety of phytocannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most promising therapeutic compounds. Besides the well-known palliative effects in cancer patients, cannabinoids have been shown to inhibit in vitro growth of tumor cells. Likewise, the major endocannabinoids (eCBs), anandamide (AEA) and 2-arachidonoylglycerol (2-AG), induce tumor cell death. The purpose of the present study was to characterize cannabinoid elements and evaluate the effect of cannabinoids in endometrial cancer cell viability. The presence of cannabinoid receptors, transient receptor potential vanilloid 1 (TRPV1), and endocannabinoid-metabolizing enzymes were determined by qRT-PCR and Western blot. We also examined the effects and the underlying mechanisms induced by eCBs and phytocannabinoids in endometrial cancer cell viability. Besides TRPV1, both EC cell lines express all the constituents of the endocannabinoid system. We observed that at concentrations higher than 5 μM, eCBs and CBD induced a significant reduction in cell viability in both Ishikawa and Hec50co cells, whereas THC did not cause any effect. In Ishikawa cells, contrary to Hec50co, treatment with AEA and CBD resulted in an increase in the levels of activated caspase −3/−7, in cleaved PARP, and in reactive oxygen species generation, confirming that the reduction in cell viability observed in the MTT assay was caused by the activation of the apoptotic pathway. Finally, these effects were dependent on TRPV1 activation and intracellular calcium levels. These data indicate that cannabinoids modulate endometrial cancer cell death. Selective targeting of TPRV1 by AEA, CBD, or other stable analogues may be an attractive research area for the treatment of estrogen-dependent endometrial carcinoma. Our data further support the evaluation of CBD and CBD-rich extracts for the potential treatment of endometrial cancer, particularly, that has become non-responsive to common therapies.

Keywords Endocannabinoids · Phytocannabinoids · Endometrial cancer · Apoptosis

Abbreviations

AEA	Anandamide
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CPZ	Capsazepine
CPS	Capsaicin
2-AG	2-Arachidonoylglycerol
DMSO	Dimethylsulfoxide
E2	Estradiol
EC	Endometrial cancer
eCBs	Endocannabinoids
ER	Estrogen receptor
FAAH	Fatty acid amide hydrolase
iRTX	5'-Iodoresiniferatoxin
MAGL	Monoacylglycerol lipase
MTT	3-(4–5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAPE	*N*-Arachidonoyl phosphatidylethanolamine
PI	Phosphatidylinositol
PR	Progesterone receptor

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Introduction

The endocannabinoid system is defined as the signaling system composed of G-protein-coupled cannabinoid receptors (CB1, CB2), their endogenous ligands, called endocannabinoids

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(eCBs), and the enzymes that produce and degrade eCBs [14]. Anandamide (AEA) was the first endogenous ligand for cannabinoid receptors discovered, which together with 2-arachidonoylglycerol (2-AG) are the best known eCBs.

AEA and 2-AG are synthesized from *N*-arachidonoyl phosphatidylethanolamine (NAPE) and phosphatidylinositol (PI) lipid precursors, respectively. The release of AEA from NAPEs occurs via a specific phospholipase D enzyme (NAPE-PLD), whereas 2-AG is synthesized from PI by diacylglycerol lipase (DAGL) enzyme. The eCBs have a very short half-life due to the enzymatic hydrolysis mediated primarily by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) for AEA and 2-AG, respectively.

Although, the activity of eCBs was primarily focused in the central nervous system, these compounds also revealed important physiological activities in peripheral tissues, including energy metabolism, as well as cardiovascular, respiratory, and reproductive functions [23]. In addition, they present important properties in the control of cell fate by modulating the decision of cell survival/death [7]. Indeed, cannabinoid receptors were found to be differentially expressed between healthy and malignant cells [6]. Although its relevance is still in debate, it highlights the endocannabinoid system as a potential target in the control of cancer progression. Moreover, eCBs demonstrated in vitro anti-tumorigenic activity in breast, prostate, and bone cancer cells [13, 17].

Endometrial cancer (EC) is the most common gynecological cancer in developed countries, with more than 320,000 new cases diagnosed annually worldwide [4, 32]. The EC cases are increasing and are mainly attributed to a variety of environmental and lifestyle factors, like increased average life-span, obesity, and post-menopausal hormone replacement therapy [2]. However, it is estimated that 5–10% are due to genetic causes [8].

There are distinct EC categorized into type 1 and type 2. The former is estrogen-dependent and constitutes 80–90% of newly diagnosed EC, presenting a better prognosis, whereas the latter is not estrogen-dependent and requires a more aggressive treatment [8].

Due to the difficulties of studying EC in vivo, the Ishikawa cell line was developed in 1980 from a 39-year-old type 1 endometrial adenocarcinoma patient, while Hec50co, a cell line of type 2 EC, was primarily established from a metastatic lesion from an advanced case [26]. Ishikawa cells express both estrogen (ER) and progesterone receptors (PR). However, the ability of these cells to grow in estrogen-free media demonstrates that they are not strictly dependent on estrogen for survival [3, 20]. The Hec50co cells express trace levels of steroid hormone receptors [12, 18].

Besides the use of cannabinoid derivatives in palliative treatments, these compounds have also shown the ability to inhibit tumor cell growth. Among a variety of cannabinoids, Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most promising therapeutic compounds. This fact increased the interest in studying the potential role of phytocannabinoids and the modulation of the endocannabinoid system in cancer treatment [5, 15]. Therefore, our aim was to investigate the effects of eCBs, AEA, and 2-AG and of the phytocannabinoids THC and CBD in endometrial cancer by using Ishikawa and Hec50co cells as models.

Results

Expression of the endocannabinoid system elements in endometrial cancer cells

To investigate the expression of endocannabinoid system elements in the endometrial cancer cell lines, Ishikawa and Hec50co, and in a non-tumoral cell line, HFF-1, qRT-PCR and Western blot analysis for CB1, CB2, NAPE-PLD, FAAH, DAGL, and MAGL were performed (Fig. 1a–c). In addition, as AEA and CBD can also exert their actions through the transient receptor potential vanilloid 1 (TRPV1), the expression of this cation channel was also investigated (Fig. 1). The Western blot and qRT-PCR analysis revealed that both endometrial cancer and HFF-1 cells expressed all the endocannabinoid system machinery and TRPV1 (Fig. 1a–c).

Effect of endocannabinoids in endometrial cancer cells

To assess the cell viability response of the Ishikawa, Hec50co, and HFF-1 cell lines to eCBs, CBD, and THC treatment, MTT and LDH release assays were employed (Fig. 2). Treatment of Ishikawa cells with both eCBs and CBD decreased cell viability in a concentration-dependent manner (Fig. 2a). The THC did not induce significant loss of cell viability for the tested concentrations (0.01–25 μM), even after 72 h of treatment (data not shown). On the other hand, Hec50co cells treated with 1 μM of eCBs or 25 μM of CBD demonstrated a significant reduction in cell viability after 48 h of treatment (Fig. 2a). As for Ishikawa cells, THC did not affect Hec50co cell viability even for concentrations of 25 μM (Fig. 2a). In order to investigate the cytotoxicity of cannabinoids, their effects were evaluated in HFF-1. None of the tested compounds induced a decrease in HFF-1 cell viability (Fig. 2a). So, the effects in cell viability in either type 1 or type 2 endometrial cancer cells were different, particularly for 2-AG at 48 h, which presented a more pronounced effect on Hec50co compared to Ishikawa cells (Fig. 2a).

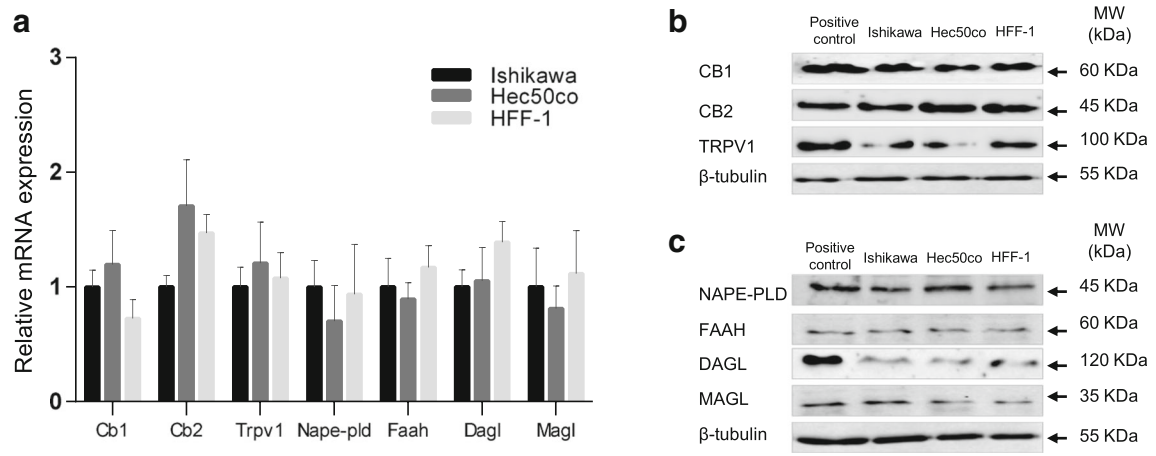

Fig. 1 Expression of the endocannabinoid system members and TRPV1 in Ishikawa and Hec50co endometrial cancer cells and in non-tumoral cell line, HFF-1. **a** The qRT-PCR revealed that all the studied elements are expressed. **b** Representative Western blots showing expression of cannabinoid receptors (CB1, CB2) and TRPV1 in HFF-1, Ishikawa and Hec50co cells. Brain homogenates were used as positive control for CB1 and TRPV1, whereas spleen was used for CB2. **c** Representative Western blots for the endocannabinoid metabolic enzymes NAPE-PLD, FAAH, DAGL, and MAGL. Brain homogenates or spleen were used as positive contro

In Ishikawa cells, only concentrations of 10 μM, or higher, of eCBs and CBD resulted in a significant release of LDH to the culture medium after 48 h (Fig. 2b). However, in Hec50co cells, concentrations of 1 μM, or higher, significantly increased LDH release (Fig. 2b), suggesting that these compounds have a cytotoxic effect. The CBD also induced LDH release, but only for the highest concentrations, whereas THC did not affect LDH release in either Ishikawa or Hec50co cell lines (Fig. 2b). None of the tested compounds induced LDH release in HFF-1 cells (Fig. 2b).

Effect of endocannabinoids in endometrial cancer cell morphology

The impact of eCBs in endometrial cancer cell morphology was investigated by Giemsa and Höechst staining (Fig. 3). In Ishikawa cells, eCBs and CBD caused chromatin condensation and nuclear fragmentation, as revealed by Giemsa staining, all of which were characteristic morphological alterations associated with apoptosis (Fig. 3). For the Hec50co cells, at 5 μM, these compounds also induced an evident decrease in

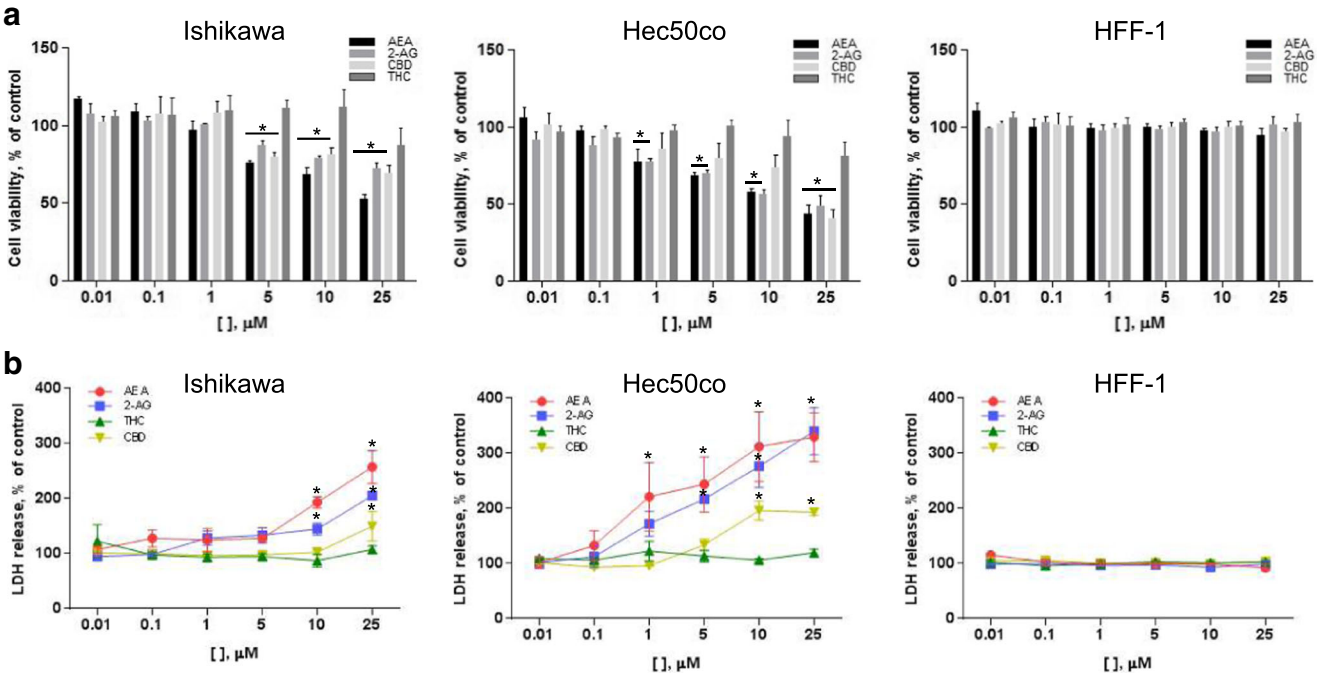

Fig. 2 Effect of endocannabinoids (AEA; 2-AG) and phytocannabinoids (THC; CBD) on the viability of Ishikawa, Hec50co, and HFF-1 cells after 48 h of treatment. Cell viability evaluated by MTT assay (**a**) and LDH release (**b**) in response to eCBs and phytocannabinoids (*$P < 0.05$ compared to respective untreated control; one-way ANOVA with Tukey's ad hoc post-test)

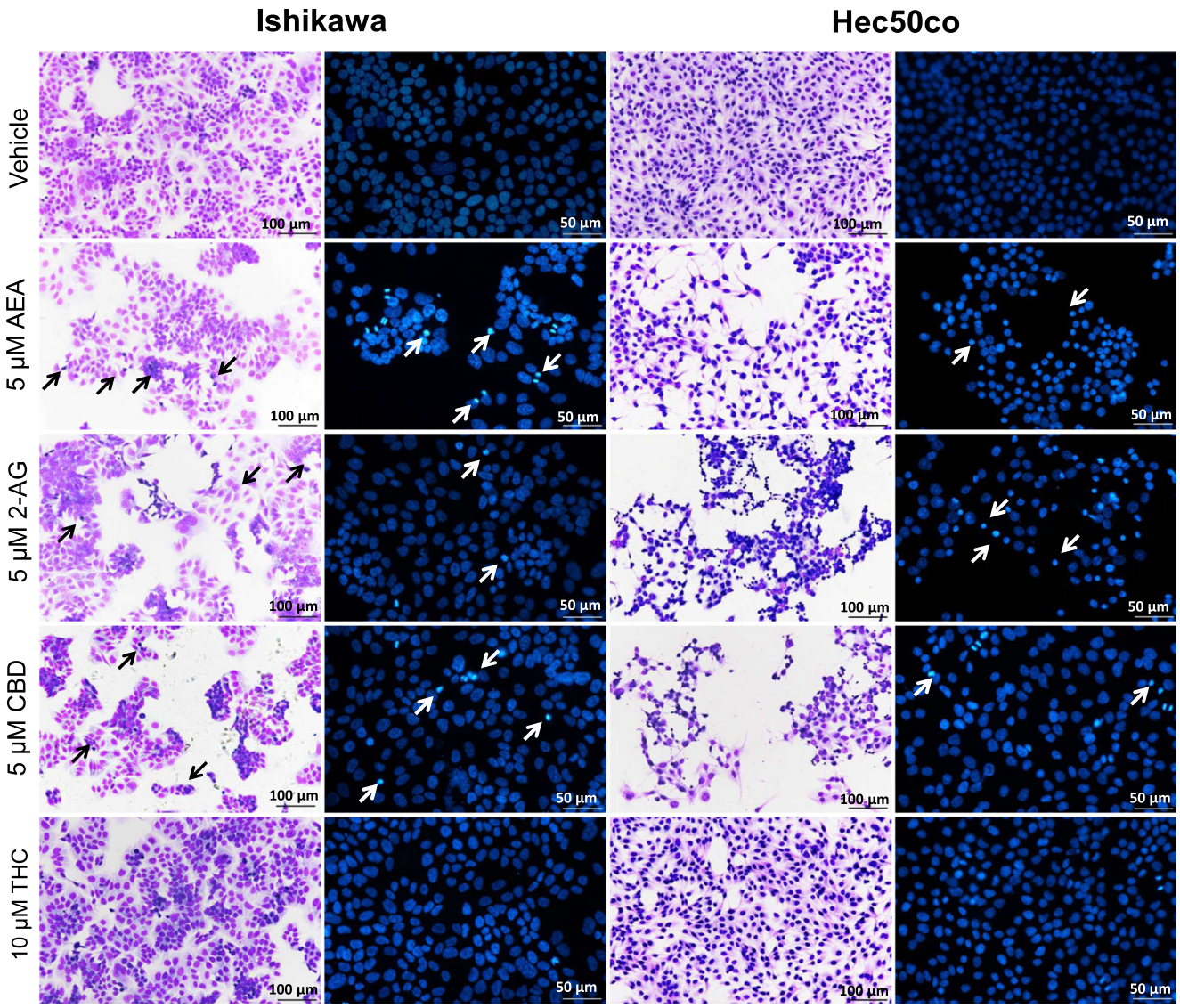

Fig. 3 Evaluation of endocannabinoids (AEA; 2-AG) and phytocannabinoids (THC; CBD) on the morphology of Ishikawa and Hec50co cells after 48 h of treatment. Ishikawa and Hec50co cells treated with AEA (5 μM), 2-AG (5 μM), CBD (5 μM), and THC (10 μM) and stained with Giemsa and Höechst. Both eCBs and CBD induced a decrease in cell number with morphological alterations, particularly evident in Giemsa staining and on Hec50co cells. In Höechst staining, AEA and CBD induced the appearance of condensed chromatin, particularly evident on Ishikawa cells, whereas the effects of 2-AG were not so pronounced. Control cells were treated with vehicle only

cell number accompanied by a dramatic change in cell morphology with distended cytoplasm (Fig. 3). In Höechst staining, eCB- and CBD-treated cells displayed chromatin condensation and pyknotic nucleus (Fig. 3). The THC treatment did not affect cell density and cell morphology (Fig. 3).

Mechanisms of cell death triggered by eCBs in endometrial cancer cells

As suggested by Höechst staining and to confirm the programmed cell death induced by these compounds, the activities of the effector caspases −3/−7 were evaluated. Contrary to 2-AG, AEA and CBD in Ishikawa cells caused an increase of 39 and 23% in caspase-3/7 activities, respectively (Fig. 4a). Interestingly, although Höechst staining showed chromatin condensation in Hec50co cells, any increase in caspase −3/−7 activities was detected for all the cannabinoids (Fig. 4a).

A biochemical event that accompanies apoptosis in many cell types is the proteolytic cleavage of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair. In order to clarify the observed chromatin condensation and increase in caspase −3/−7 activities, it was investigated the appearance of cleaved PARP. While in Ishikawa cells it was observed an increase in cleaved PARP in response to AEA, 2-AG, or CBD, no differences were detected in Hec50co cells (Fig. 4b). Curiously, an increase in the transcription factor

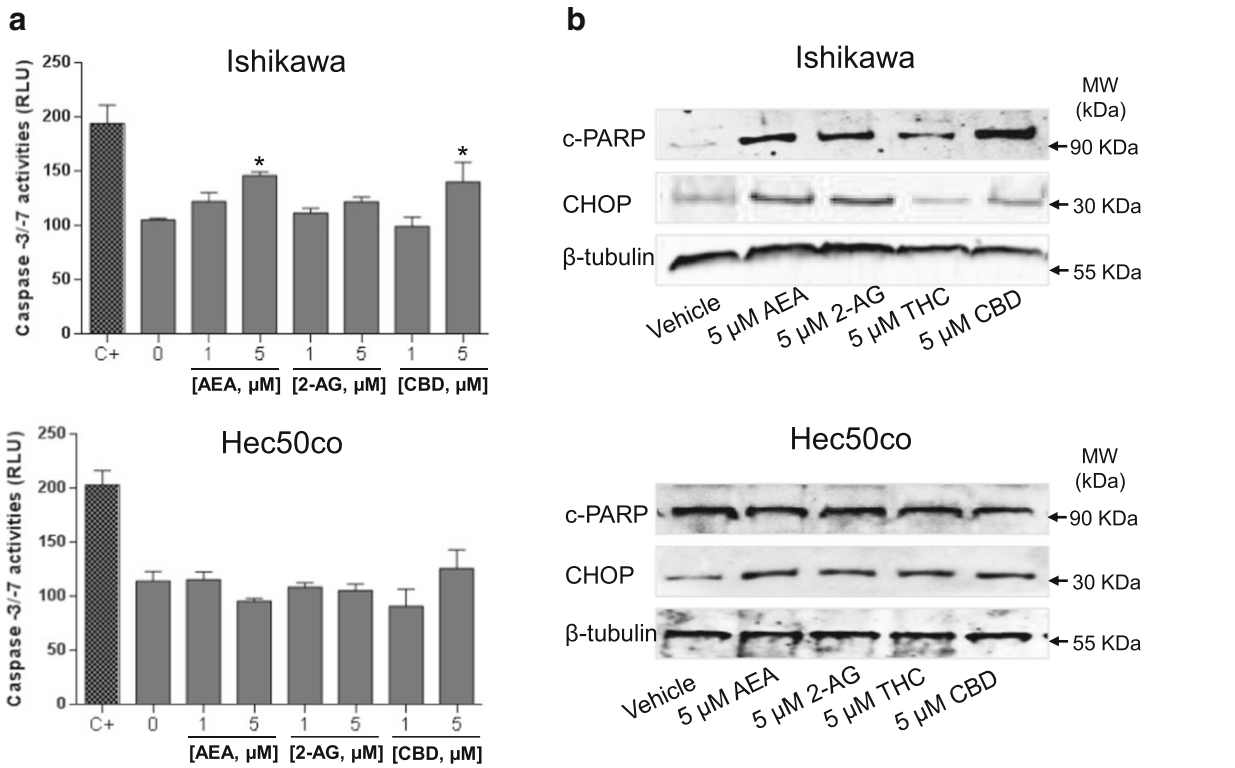

Fig. 4 Evaluation of apoptotic markers in Ishikawa and Hec50co cells treated with eCBs and CBD after 48 h of treatment. **a** AEA and CBD, at 5 μM, induced a significant increase of caspase −3/−7 activities in Ishikawa cells. Caspase −3/−7 activities on Hec50co cells were not influenced by the treatments. **b** Representative Western blots showing the effects on the expression of cleaved PARP (c-PARP) and CHOP. (*$P < 0.05$ compared to respective untreated control; one-way ANOVA with Tukey's ad hoc post-test)

C/EBP homologous protein (CHOP) was obtained in both cell lines treated with both eCBs and CBD (Fig. 4b).

Involvement of receptors on cell viability loss

To study the role of CB1, CB2, and TRPV1 receptors in the eCB-mediated suppression of cell viability, the effects of the antagonists for CB1 (AM281), CB2 (AM630), and TRPV1 (iRTX) by MTT assay were evaluated (Fig. 5). Cells were pre-incubated with each antagonist for 30 min (which had no effect on the cell viability) and then treated with eCBs or CBD. The antagonists for CB1 or CB2 did not reverse the reduction in the viability loss caused by AEA, 2-AG, or CBD (Fig. 5a, b). However, under our conditions, pre-treatment with the TRPV1 antagonists induced a significant protective effect for AEA and CBD on Ishikawa, but not on Hec50co cells (Fig. 5a, b). Moreover, the observed increase in caspase −3/−7 activities induced by either AEA or CBD in Ishikawa cells was also partially reversed by the TRPV1 receptor antagonist (Fig. 5c).

Furthermore, in the Ishikawa cells, AEA and CBD caused a loss of 22 and 24% of mitochondrial membrane potential, respectively (Fig. 6a), and an increase in the generation of ROS/RNS (Fig. 6b). These AEA-induced effects were attenuated by the pre-treatment of cells with the TRPV1 antagonists (Fig. 6a, b). Curiously, ROS generation induced by CBD was not reverted by any of the antagonist. 2-AG did not affect the mitochondrial potential in these cells, though it induced an increase in ROS generation, which was attenuated by the CB2 antagonist (Fig. 6b). In the Hec50co-treated cells, the mitochondrial membrane potential remained unchanged (Fig. 6c), and it was also observed an increase in ROS/RNS production that was independent of cannabinoid or vanilloid receptor activation (Fig. 6d).

Apoptosis is mediated by increased intracellular calcium levels

The TRPV1 receptor is a cation channel with preference for calcium ions, and its activation mediates an increase in intracellular calcium. In order to confirm whether the TRPV1 activation contributes to AEA and CBD promotion of apoptosis, we determined intracellular Ca^{2+} levels in Ishikawa cells. Anandamide, CBD, and CPS (5 μM) induced a rapid twofold increase of intracellular Ca^{2+} levels, after 20 s of exposure (Fig. 5d). These effects were reversed by iRTX, suggesting that this Ca^{2+} influx occurs through TRPV1 (Fig. 5d).

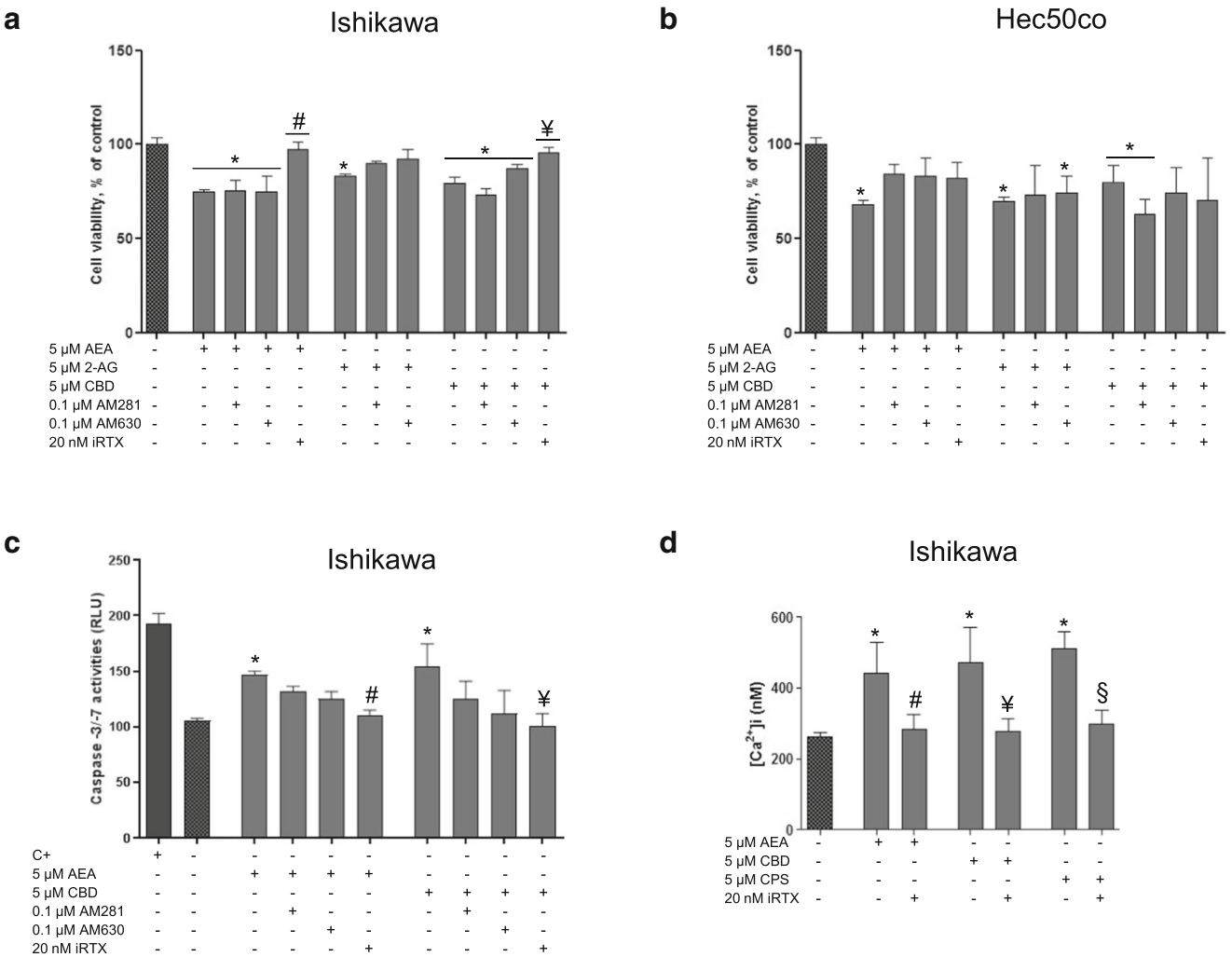

Fig. 5 Involvement of cannabinoid receptors and TRPV1 in cannabinoid-induced decrease in cell viability and in the intracellular calcium levels ([Ca^{2+}]i). **a** Evaluation of cell viability of Ishikawa cells in response to either eCBs or CBD after 48 h in the presence of specific antagonists for CB1 (AM281), CB2 (AM630), and TRPV1 (iRTX). **b** Evaluation of cell viability of Hec50co cells in response to cannabinoids after 48 h and in the presence of the specific antagonists. **c** The increased caspase −3/−7 activities induced by 5 μM of AEA or CBD after 36 h were attenuated by the pre-treatment with the TRPV1 antagonist (iRTX). Staurosporine was used as positive control for caspase −3/−7 evaluations. **d** Both AEA (5 μM), CBD (5 μM), and capsaicin (CPS; 5 μM) induced a rapid increase of [Ca^{2+}]. The TRPV1 antagonist, iRTX, was able to block the increase in intracellular calcium levels ($^{*}P < 0.05$ compared to respective untreated control; $^{\#}P < 0.05$ vs AEA 5 μM; $^{¥}P < 0.05$ vs CBD 5 μM; $^{§}P < 0.05$ vs CPS 5 μM)

Discussion

Endometrial cancer is still the most frequently diagnosed malignancy of the female genital tract, particularly in developed countries. The significance of sex steroid hormones in the biology of the uterus has been well established. Basically, estrogen stimulates, whereas progesterone inhibits endometrial growth. Thus, any disturbance in hormone exposure can lead to neoplastic changes, including hyperplasia and adenocarcinoma. The evidences that eCBs may modulate cell survival/death led us to investigate the involvement of eCBs, THC, and CBD in Ishikawa and Hec50co cell lines, two models of the relevant types of endometrial cancers.

Earlier reports have documented the expression of all the endocannabinoid system elements in a healthy endometrium [31]. However, despite the work of Maurizio Guida, who showed overexpression of CB2 receptor in human endometrial carcinoma biopsies [16], no studies have characterized the endocannabinoid system or evaluated the role of either eCBs or phytocannabinoids in endometrial cancer cells. Moreover, although TRPV1 receptor was already identified in endometrium, no indications were proposed for its function and impact on endometrial biology [25]. Here, we present evidences that the non-tumoral human foreskin fibroblasts cell line (HFF-1) and both endometrial cancer cell lines Ishikawa and Hec50co express all the elements of endocannabinoid system, as

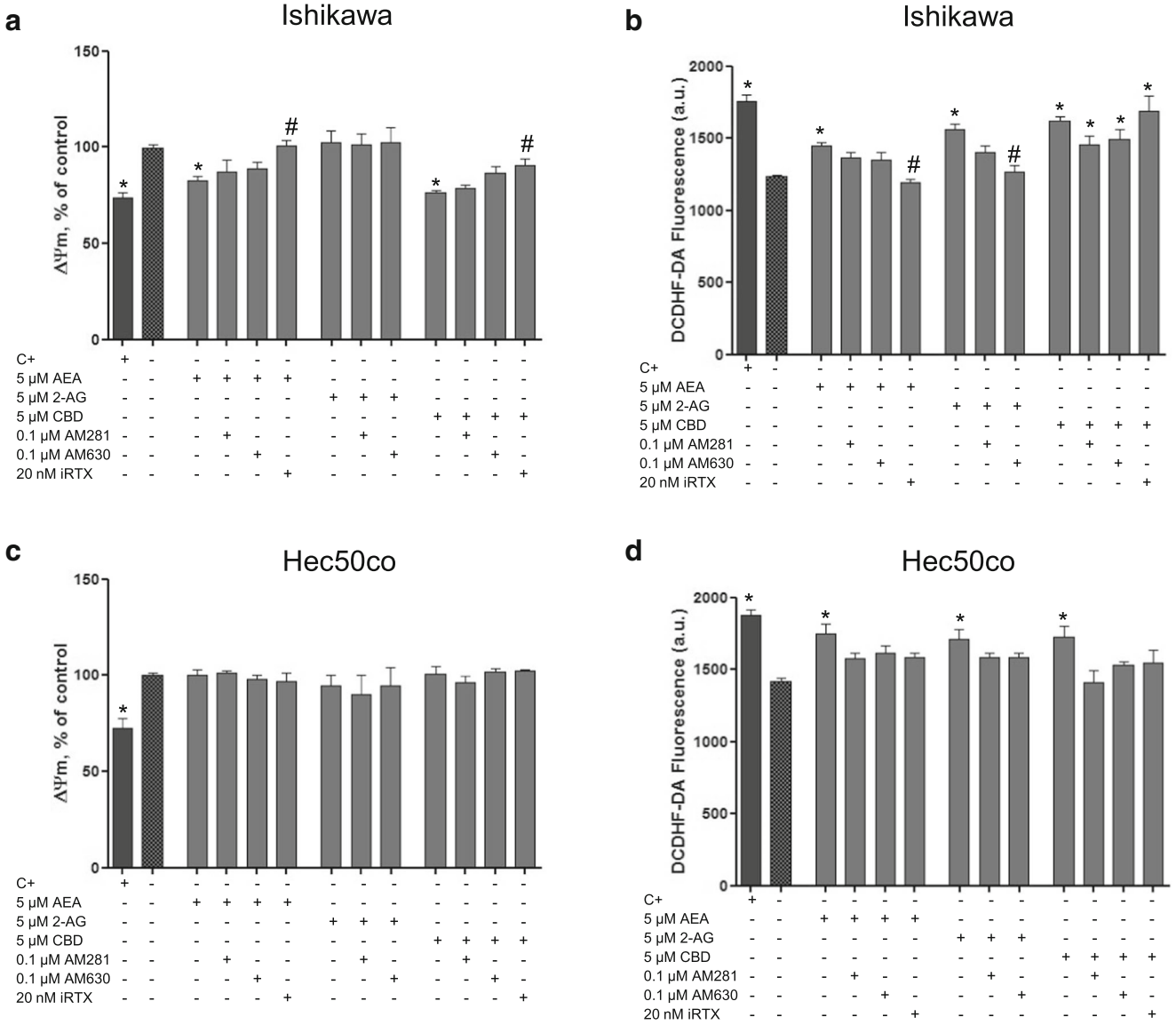

Fig. 6 Effects of eCBs and CBD in mitochondrial membrane potential and reactive oxygen/nitrogen species generation in endometrial cancer cell lines. **a** In Ishikawa cells, after 36 h of treatment, AEA (5 μM) induced a loss of Δψm, an effect reversed by the TRPV1 antagonist capsazepine (CPZ). Similar effects were observed with CBD, whereas 2-AG has no effects on Δψm. **b** After 48 h of treatment, both eCBs and CBD increased the levels of ROS/RNS in Ishikawa cells. Contrary to CBD, the effect induced by AEA was reversed by the TRPV1 antagonist, whereas the effects induced by 2-AG were attenuated by the pre-treatment with the CB2 antagonist. **c** In Hec50co, neither eCBs nor CBD were able to induce a loss of Δψm. **d** In Hec50co, both eCBs and CBD induced an increase in the levels of ROS/RNS, though any antagonist was able to attenuate these effects (*$P < 0.05$ compared to respective untreated control; $^{\#}P < 0.05$ compared to AEA 5 μM; one-way ANOVA with Tukey's ad hoc post-test)

well as the AEA-binding receptor TRPV1, which highlights new potential targets on these cells.

Treatment with eCBs and phytocannabinoids did not induce alterations in HFF-1 cell viability. On the other hand, a significant reduction in cell viability was observed with eCBs and CBD in both EC cell types, while, with THC treatment (0.01–25 μM), no changes were observed. At concentrations of 5 μM, both eCBs and CBD, in Ishikawa cells, induced chromatin condensation, though only AEA and CBD were able to induce a decrease in mitochondrial membrane potential and an increase in caspase −3/−7 activities. At this concentration, an increase in PARP cleavage was also observed. Although the pre-treatment with CB1 and CB2 antagonists attenuated cell viability loss and caspase activation, only the pre-treatment with TRPV1 antagonist was effective in the reversion of the observed effects. In addition, both eCBs and CBD induced an increase in ROS/RNS generation and CHOP expression, suggesting the involvement of endoplasmic reticulum (ER) stress. The TRPV1 antagonist was able to attenuate the effects induced by AEA in these cells, while the CB2

antagonist also reversed the ROS/RNS generation induced by 2-AG. AEA already demonstrated anti-proliferative effects via induction of ER stress and oxidative stress in hepatic stellate cells and non-melanoma skin cancer cells, though these effects occurred via receptor-independent signaling pathways [22, 30].

Interestingly, although the eCBs were more effective in reducing cell viability in Hec50co cells, they did not cause any increase in the apoptotic markers, suggesting that these cells are more susceptible to eCB-induced cytotoxic effects. However, there was a significant release of LDH and ROS/RNS generation, which was receptor-independent.

TRPV1 receptor is a non-selective cation channel with preference for calcium ions. According to our results, AEA and CBD may modulate the intracellular levels of calcium in the endometrial cancer cell line Ishikawa. In addition, since calcium is important for cell apoptosis [27] and our results indicate that TRPV1 activation interferes with endometrial cancer cell death, AEA and/or CBD signaling through TRPV1 activation may be relevant for the regulation of type 1 endometrial cancer cell progression.

AEA was reported to act as a full agonist at both rat and human TRPV1 receptor and, thus, suggested as a potent "endovanilloid" [28]. Also, CBD was shown to dose-dependently activate and rapidly desensitize TRPV1, producing a TRPV1-mediated intracellular calcium elevation, in vitro, in neurons [19]. Accordingly, with our data, previous observations referred a pro-apoptotic action of AEA in different cervical carcinoma cell lines via aberrantly expressed TRPV1 receptor, whereas AEA binding to the classical cannabinoid receptors (e.g., CB1 and CB2) mediated a protective effect [9]. In human glioma cells, AEA also induced apoptosis through TRPV1 receptor, suggesting a role for AEA via this receptor in the modulation of cancer cell growth [10]. Cannabidiol also induced apoptosis of human breast cancer cells via activation of cannabinoid CB2 and TRPV1 receptors and elevation of intracellular calcium and ROS/RNS generation [21]. Additionally, in human breast cancer cells, CBD caused ER stress and, subsequently, inhibition of AKT and mTOR signaling as shown by decreased levels of phosphorylated mTOR, 4EBP1, and cyclin D1, highlighting an intricate interplay between apoptosis and autophagy in CBD-induced breast cancer cell apoptosis [29]. The increase in CHOP expression induced by AEA and CBD also suggests a coordinated cross-talk between apoptosis and ER stress in endometrial cancer cells.

Although the anti-tumor properties of phytocannabinoids have been suggested for years, more recently, the endocannabinoid system has become an attractive novel target for pharmacological intervention in the fight against many cancers. Altogether, these results suggest that cannabinoids differentially modulate EC cell viability. Whereas in type 1, estrogen-dependent EC, the most potent endocannabinoid, AEA, and the major non-psychoactive constituent of cannabis, CBD, induce apoptosis through TRPV1 activation, the treatment of type 2 with cannabinoids resulted in a drastic cytotoxic effect, which was independent of cannabinoid or TRPV1 receptors. Interestingly, it was also shown that CBD is a more promising compound than THC for these types of tumors. Thus, the selective targeting of TPRV1 by AEA, CBD, or other stable analogues may be an attractive research area for the treatment of estrogen-dependent endometrial carcinoma. The biochemical regulation of TRPV1 is, however, complex, and our data suggest that TRPV1 should be studied accordingly to tumor cell type and disease state. Also, the role of cannabinoids in endometrial cancer and their involvement in the interplay between apoptosis and ER stress require further attention.

Materials and methods

Reagents

All chemicals were from Sigma–Aldrich Co. (St. Louis, MO, USA), except the following: AEA, 2-AG, CBD, capsaicin, AM630, and 5'-Iodoresiniferatoxin (5-iRTX) (Tocris Bioscience, Bristol, UK); AM281 (Santa Cruz Biotechnology, Dallas, TX, USA); Fluo-3 AM (Molecular Probes, Eugene, OR, USA); and THC (Lipomed, Arlesheim, Switzerland).

Cell culture

We used the human endometrial cancer cell lines Ishikawa and Hec50co (Hec50 cell subline), kindly provided by Dr. Kim K. Leslie (The University of New Mexico Health Sciences Center, Albuquerque, USA), and the human foreskin fibroblasts cell line (HFF-1, ATCC, Manassas, VA, USA). The HFF-1 cells were routinely maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM), whereas Ishikawa and Hec50co cells were maintained in DMEM/F12 medium (Gibco Invitrogen Co., Carlsbad, CA, USA). Cell culture medium was supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine (Gibco Invitrogen Co., Carlsbad, CA, USA), and 1% antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Gibco Invitrogen Co., Carlsbad, CA, USA), and cells cultured at 37 °C with 95% air/5% CO_2 in humidified atmosphere.

Cell viability/cytotoxicity

Cells were seeded in a 96-well plate at a density of 2×10^3 cells per well. For all experiments, the medium was replaced

with DMEM/F12 containing 2% charcoal-stripped FBS, 2 mM glutamine, and 1% antibiotic–antimycotic solution 24 h before adding the compounds. Varying concentrations of AEA, 2-AG, CBD, and THC (0.01–25 μM) were added, and cells were incubated at 37 °C in 5% CO_2 for 24, 48, or 72 h. Cell viability was ascertained by MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Lactase dehydrogenase (LDH) activity was determined accordingly to kit instructions (Non-Radioactive Cytotoxicity Assay, Promega). For both LDH and MTT assays, cell viability was quantified by calculating the absorbance as a percentage of the absorbance of the relevant control.

Cell morphological analysis

Morphological changes induced by eCBs were analyzed by Giemsa and Höechst 33342 staining. Briefly, Ishikawa and Hec50co cells were seeded onto glass coverslips in a 24-well plate, at a density of 2×10^4 per well. Cells were incubated for 24 h to adhere in DMEM/F12 with 2% charcoal-stripped FBS. Varying concentrations of tested compounds were added, and cells were incubated for the required time at 37 °C in 5% CO_2. Then, cells were fixed with 4% paraformaldehyde, washed and stained with Giemsa for 30 min, analyzed under a light microscope or exposed to 0.5 μg/ml Höechst 33342 (in PBS) for 20 min, and observed under a fluorescence microscope equipped with an excitation filter with maximum transmission at 400 nm (Eclipse E400, Nikon, Japan).

qRT-PCR analysis

Cells were collected in TRIzol reagent, and total RNA was extracted according to the manufacturer's instructions and quantified in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). RNA quality was assessed using a bioanalyzer (Experion RNA, Bio-Rad Laboratories, USA) and reverse transcribed into cDNA with the iScript™ Select cDNA Synthesis (Bio-Rad Laboratories, USA). For quantitative PCR, cDNA was amplified with KAPA SYBR® FAST qPCR Master Mix 2×

Table 1 Primer sequences for control and target genes. Detailed description of all genes tested, primer pairs' sequences and Q-PCR conditions

Symbol	Accession number	Description	Primers (5′-3′)	Amplification conditions[1)]	Tm (°C)	Reference
CB1	NM_001160259.1	*Homo sapiens* cannabinoid receptor 1	S: CTTCCCACAGAAAT TCCC AS: TACCTTCCCATCCT CAGA	95 °C, 03 s 62 °C, 30 s 72 °C, 90 s	87	[1]
CB2	NM_001841.2	*Homo sapiens* cannabinoid receptor 2	S: CGTGGCTGTGCTCT ATCTGA AS: ATCTCGGGGCTTCT TCTTTT	95 °C, 03 s 62 °C, 30 s 72 °C, 90 s	87	[1]
TRPV1	NC_000017.11	*Homo sapiens* transient receptor potential cation channel subfamily V member 1	S: CAAGAACATCTGGA AGCTGC AS: CTTCTCCCCGGAAG CGGCAGG	95 °C, 03 s 62 °C, 30 s 72 °C, 90 s	86.5	[24]
FAAH	NM_001441.2	*Homo sapiens* fatty acid amide hydrolase	S: GGCCGTCAGCTACA CTATGC AS: ATCAGTCGCTCCAC CTCCC	95 °C, 03 s 59 °C, 30 s 72 °C, 90 s	87	[1]
NAPE-PLD	NM_001122838.1	*Homo sapiens* N-acyl phosphatidylethanolamine phospholipase D	S: AGATGGCTGATAAT GAGAA AS: TTCTCCTCCCACCA GTC	95 °C, 03 s 58 °C, 30 s 72 °C, 90 s	83.5	[1]
DAGL-α	NM_006133.2	*Homo sapiens* diacylglycerol lipase, alpha	S: TGCTCTTCGGCCTG GTCTAT AS: CGCATGCTCAGCCA GATGAT	95 °C, 03 s 61 °C, 30 s 72 °C, 90 s	86	[11]
MAGL	BC000551.2	*Homo sapiens* monoglyceride lipase	S: CAAGGCCCTCATCT TTGTGT AS: ACGTGGAAGTCAGA CACTAC	95 °C, 03 s 57 °C, 30 s 72 °C, 90 s	85.5	[11]
β-actin	NM_001101.3	*Homo sapiens* actin, beta	S: AACTCCATCATGAA GTGTGACG AS: GATCCACATCTGCT GGAAGG	95 °C, 03 s 60 °C, 30 s 72 °C, 90 s	85.5	[11]
GAPDH	NC_000012.12	*Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase	S: AGAACATCATCCCT GCCTC AS: GCCAAATTCGTTGT CATACC	95 °C, 03 s 55 °C, 30 s 72 °C, 90 s	78	[1]

[1)] Cycling conditions were as follows: an initial step at 95 °C 3 min for enzyme activation in all cases, followed by up to 40 cycles of denaturation, annealing, and primer extension as described

Kit (Kapa Biosystems, Woburn, MA, USA), according to kit instructions within a MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA).

The PCR conditions and primer sequences are described in Table 1 [1, 11, 24]. The specificity of the amplified PCR product was evaluated by the melting curve analysis. The expression levels were corrected for the levels of β-actin using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Protein samples (50 μg) were separated by electrophoresis through 10 or 12% SDS-polyacrylamide (PAGE) gels and transferred onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). After blocking for 1 h at room temperature (5% dry milk in PBS with Triton™ X-100 0. 1%), membranes were incubated with specific polyclonal antibodies in blocking solution, overnight at 4 °C, as described in Table 2. Membranes were then incubated with secondary horseradish-peroxidase conjugated antibody for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Super Signal West Pico; Pierce, Rockford, USA); immunoreactive bands were visualized by ChemiDocTM Touch Imaging System (BioRad, Laboratories Melville, NY, USA). Blots were then stripped and re-probed with monoclonal anti-β-tubulin (1:500) (Santa Cruz Biotechnology, Dallas, TX, USA), as a loading control, and signal intensities were quantified by densitometry. Brain or spleen homogenates were used as positive controls.

Determination of caspase-3/7 activities

Cells were seeded in 96-well white plates and treated with AEA, 2-AG, or CBD for 36 h in the presence or absence of antagonists for CB1, CB2, or TRPV1 receptors. At the end of incubation period, Caspase-Glo® −3/−7 reagent (Promega Corporation, Madison, WI, USA) was added and the assay performed according to the manufacturer's protocol. Luminescence was assessed in a 96-well Microplate Luminometer (BioTek Instruments, VT, USA).

Evaluation of mitochondrial membrane potential and intracellular reactive oxygen and nitrogen species

For the assessment of mitochondrial membrane potential (Δψm) and reactive oxygen and nitrogen species (ROS/RNS) production, cells were seeded in 96-well black plates and treated with AEA, 2-AG, or CBD for 48 h. For Δψm studies, cells were washed and incubated with DiOC6 100 nM, for 20 min, at 37 °C, in the dark. For the evaluation of ROS/RNS production, cells were washed and incubated with the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA), for 1 h, at room temperature. For both assays, the resulting fluorescence was measured in a Microplate Fluorimeter (BioTek

Table 2 Detailed description of all protein tested, antibodies, and conditions used for Western blot (WB) analyses

Primary antibody	Host species	Source[a]	WB working dilution	WB secondary antibody (dilution)	Cell function
CB1	Rabbit	Santa Cruz Biotechnology cat. # sc-20754	1:200	Santa Cruz Biotechnology cat. # sc-2357 (1:2000)	Cannabinoid receptor 1
CB2	Rabbit	Santa Cruz Biotechnology cat. # sc-25494	1:100	Santa Cruz Biotechnology cat. # sc-2357 (1:1000)	Cannabinoid receptor 2
TRPV1	Goat	Santa Cruz Biotechnology cat. # sc-12500	1:100	Santa Cruz Biotechnology cat. # sc-2354 (1:1000)	Transient receptor potential vanilloid 1
FAAH	Goat	Santa Cruz Biotechnology cat. # sc-26427	1:200	Santa Cruz Biotechnology cat. # sc-2354 (1:1000)	AEA-hydrolyzing enzyme
NAPE-PLD	Rabbit	Cayman Chemical cat. # 10306	1:100	Santa Cruz Biotechnology cat. # sc-2357 (1:1000)	AEA-synthesizing enzyme
DAGL-α	Rabbit	Santa Cruz Biotechnology cat. # sc-133307	1:200	Santa Cruz Biotechnology cat. # sc-2357 (1:1000)	2-AG-synthesizing enzyme
MAGL	Rabbit	Santa Cruz Biotechnology cat. # sc-134789	1:100	Santa Cruz Biotechnology cat. # sc-2357 (1:1000)	2-AG-hydrolyzing enzyme
PARP-1	Mouse	Santa Cruz Biotechnology cat. # sc-53643	1:1000	Santa Cruz Biotechnology cat. # sc-2005 (1: 5000)	Involved in DNA repair
CHOP	Rabbit	Santa Cruz Biotechnology cat. # sc-575	1:200	Santa Cruz Biotechnology cat. # sc-2357 (1: 5000)	Transcription factor
β-Tubulin	Rabbit	Santa Cruz Biotechnology cat. # sc-9104	1:500	Santa Cruz Biotechnology cat. # sc-2357 (1:2500)	Cytoskeletal protein

CB1, cannabinoid receptor 1; *CB2*, cannabinoid receptor 2; *TRPV1*, transient receptor potential vanilloid receptor; *FAAH*, fatty acid amide hydrolase; *NAPE-PLD*, N-acyl phosphatidylethanolamine phospholipase D; *DAGL-α*, diacylglycerol lipase alpha; *MAGL*, monoacylglycerol lipase; *PARP-1*, poly(ADP-ribose) polymerase-1

[a] Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA); Cayman Chemical (Cayman Europe, Tallinn, Estonia)

Instruments, VT, USA) (excitation 485 ± 10 nm; emission 530 ± 12.5 nm). The positive controls for $\Delta\psi m$ or ROS/RNS production were the mitochondrial depolarizing carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 mM) or H_2O_2 (200 mM), respectively. The results were expressed in relative values, in comparison to the control ± SEM.

Measurement of intracellular calcium levels

Ishikawa cells plated in 96-well black plates were incubated with 5 μM of the calcium probe Fluo-3/AM diluted in FBS-free culture media, with shaking at room temperature for 60 min. in the dark. Then, cells were washed and incubated without the probe for 30 min at 37 °C and used for experiments. After the addition of TRPV1 antagonist, 5'-Iodoresiniferatoxin (iRTX), cells were treated with AEA, CBD, or the TRPV1 agonist, capsaicin (CPS). The fluorescence intensity was immediately registered using a dual wavelength spectrophotometer (BioTek Instruments, VT, USA) (excitation at 485 nm, emission at 525 nm), at 37 °C, during 2 min and 30 s. Intracellular calcium levels where calculated according to the formula $[Ca^{2+}]i = Kd\ [F - Fmin]/[Fmax - F]$, where the Kd corresponds to the dissociation constant of the dye for Ca^{2+} (400 nM) and the fluorescence intensity of Fluo-3 under Ca^{2+}-free and Ca^{2+} saturation conditions is abbreviated as Fmin and Fmax, respectively. To corroborate that $[Ca^{2+}]i$ alterations resulted from Ca^{2+} influx from the extracellular environment, ethylene glycol tetraacetic acid (EGTA; 1 mM) was added to the cells prior to the incubation with AEA and CPS (data not shown).

Statistical analysis

The results are the mean of at least three independent experiments carried out in triplicate. Data are expressed as the mean ± SEM, and differences were considered to be statistically significant at $P < 0.05$. Data were graphed and statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA). Then, Tukey's multiple comparison test was used to make pairwise comparisons of individual means.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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