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Research Article

The CB_2 cannabinoid receptor signals apoptosis via ceramide-dependent activation of the mitochondrial intrinsic pathway

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ABSTRACT

Δ^9-Tetrahydrocannabinol and other cannabinoids exert pro-apoptotic actions in tumor cells via the CB_2 cannabinoid receptor. However, the molecular mechanism involved in this effect has remained elusive. Here we used the human leukemia cell line Jurkat—that expresses CB_2 as the unique CB receptor—to investigate this mechanism. Our results show that incubation with the selective CB_2 antagonist SR144528 abrogated the pro-apoptotic effect of Δ^9-tetrahydrocannabinol. Cannabinoid treatment led to a CB_2 receptor-dependent stimulation of ceramide biosynthesis and inhibition of this pathway prevented Δ^9-tetrahydrocannabinol-induced mitochondrial hypopolarization and cytochrome c release, indicating that ceramide acts at a pre-mitochondrial level. Inhibition of ceramide synthesis de novo also prevented caspase activation and apoptosis. Caspase 8 activation—an event typically related with the extrinsic apoptotic pathway—was also evident in this model. However, activation of this protease was post-mitochondrial since (i) a pan-caspase inhibitor as well as a selective caspase 8 inhibitor were unable to prevent Δ^9-tetrahydrocannabinol-induced loss of mitochondrial-membrane transmembrane potential, and (ii) cannabinoid-induced caspase 8 activation was not observed in Bcl-x_L over-expressing cells. In summary, results presented here show that CB_2 receptor activation signals apoptosis via a ceramide-dependent stimulation of the mitochondrial intrinsic pathway.

Introduction

The hemp plant *Cannabis sativa* produces approximately 60 unique compounds known as cannabinoids, of which Δ^9-tetrahydrocannabinol (THC) is the most important owing to its high potency and abundance in cannabis [1]. THC exerts a wide variety of biological effects by mimicking endogenous substances—the endocannabinoids anandamide [2] and 2-arachidonoylglycerol [3]—that bind to and activate specific cannabinoid receptors. So far, two cannabinoid-specific $G_{i/o}$ protein-coupled receptors, CB_1 [4] and CB_2 [5], have been cloned and characterized from mammalian tissues [6]. The CB_1 receptor is particularly abundant in discrete areas of the brain, but is also expressed in peripheral nerve terminals and

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various extra-neural sites. In contrast, the CB_2 receptor was initially described to be present in the immune system [5], although recently it has been shown that expression of this receptor also occurs in cells from other origins [7–9].

Extensive molecular and pharmacological studies have demonstrated that cannabinoids inhibit adenylyl cyclase through CB_1 and CB_2 receptors. The CB_1 receptor also modulates ion channels, inducing, for example, inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of G protein-activated inwardly rectifying K^+ channels [6]. Besides these well-established signaling events, cannabinoid receptors also modulate several pathways that are more directly involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase [10], c-Jun N-terminal kinase and p38 mitogen-activated protein kinase [11], phosphatidylinositol 3-kinase/Akt [12] and focal adhesion kinase [13]. In addition, cannabinoids have been shown to stimulate the generation of the bioactive lipid second messenger ceramide [14] via two different pathways: sphingomyelin hydrolysis [15] and ceramide synthesis de novo [7,16,17].

One of the most exciting areas of research in the cannabinoid field is the study of the potential application of cannabinoids as therapeutic agents [18,19]. Among these possible applications, cannabinoids are being investigated as potential antitumoral drugs [20]. Thus, cannabinoid administration has been shown to curb the growth of several models of tumor xenografts in rats and mice [20]. These antitumoral actions of cannabinoids rely, at least in part, on the ability of these compounds to inhibit tumor angiogenesis [21–23] and metastasis [23]. In addition, cannabinoid treatment—via induction of apoptosis or cell cycle arrest—affects directly the viability of a wide spectrum of tumor cells in culture [20]. Both CB_1 [7,9,24,25] and CB_2 receptors [9,26,27] have been shown to mediate the antiproliferative actions of THC and related cannabinoids in tumor cells [20]. In particular, in gliomas [27], skin carcinomas [9] and lymphomas [26], the CB_2 receptor plays a major role in the pro-apoptotic effect of cannabinoids. Several observations have shown that ceramide accumulation participates in cannabinoid-induced apoptosis of glioma cells [7,16], a mechanism that was shown to rely on the activation of the CB_2 receptor [27]. However, the downstream targets of ceramide have not been identified yet and the precise molecular mechanism involved in CB_2 receptor-mediated apoptosis is not completely understood. In the present study, we used a human leukemia cell line to investigate the role of de novo-synthesized ceramide in the mechanism of CB_2 receptor-induced apoptosis.

Materials and methods

Reagents

Δ^9-Tetrahydrocannabinol and SR144528 were kind gifts from Dr. Javier Fernández-Ruiz (Complutense University, Madrid) and Sanofi-Synthelabo (Montpellier, France), respectively. Anti-Fas (IgM) monoclonal antibody (clone CH11) was from Upstate Biotechnology (Lake Placid, NY). Z-IETD-FMK (caspase 8 inhibitor), Z-DQMD-FMK (caspase 3 inhibitor), Z-VAD-FMK (pan-caspase inhibitor) and rotenone were from Sigma Chemical Co (St. Louis, MO). Myriocin (ISP-1) was from Biomol (Plymouth Meeting, PA). The fluorescent probes Hoechst 33258, Hoechst 33342 and tetramethylrhodamine methyl ester (TMRM) as well as annexin V-PE and annexin V-FITC were from Molecular Probes (Leiden, The Netherlands). Anti-CB_1 and anti-CB_2 polyclonal antibodies were from Affinity Bioreagents (Golden, CO), polyclonal anti-α-tubulin was from Sigma Chemical Co, anti-caspase 3 and anti-PARP polyclonal antibodies were from Cell Signalling (Beverly, MA), anti-cytochrome *c* monoclonal antibody and anti-Bcl-x polyclonal antibody were from BD Pharmingen (San Diego, CA). Horseradish peroxide-conjugated secondary antibodies and radiochemicals were from Amersham Biosciences (Piscataway, NJ). G418 was from Gibco (Carlsbad, CL).

Cell culture and viability

Exponentially growing Jurkat cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 5 units/ml penicillin and 5 mg/ml streptomycin. Jurkat cells stably transfected with Bcl-x_L expression vector were maintained in the same medium supplemented with 1 mg/ml G418. Cells were transferred to a serum-free medium 30 min before performing the different treatments and were maintained in this medium for the rest of the experiment. Cell viability was determined by Trypan blue exclusion.

Drugs and treatments

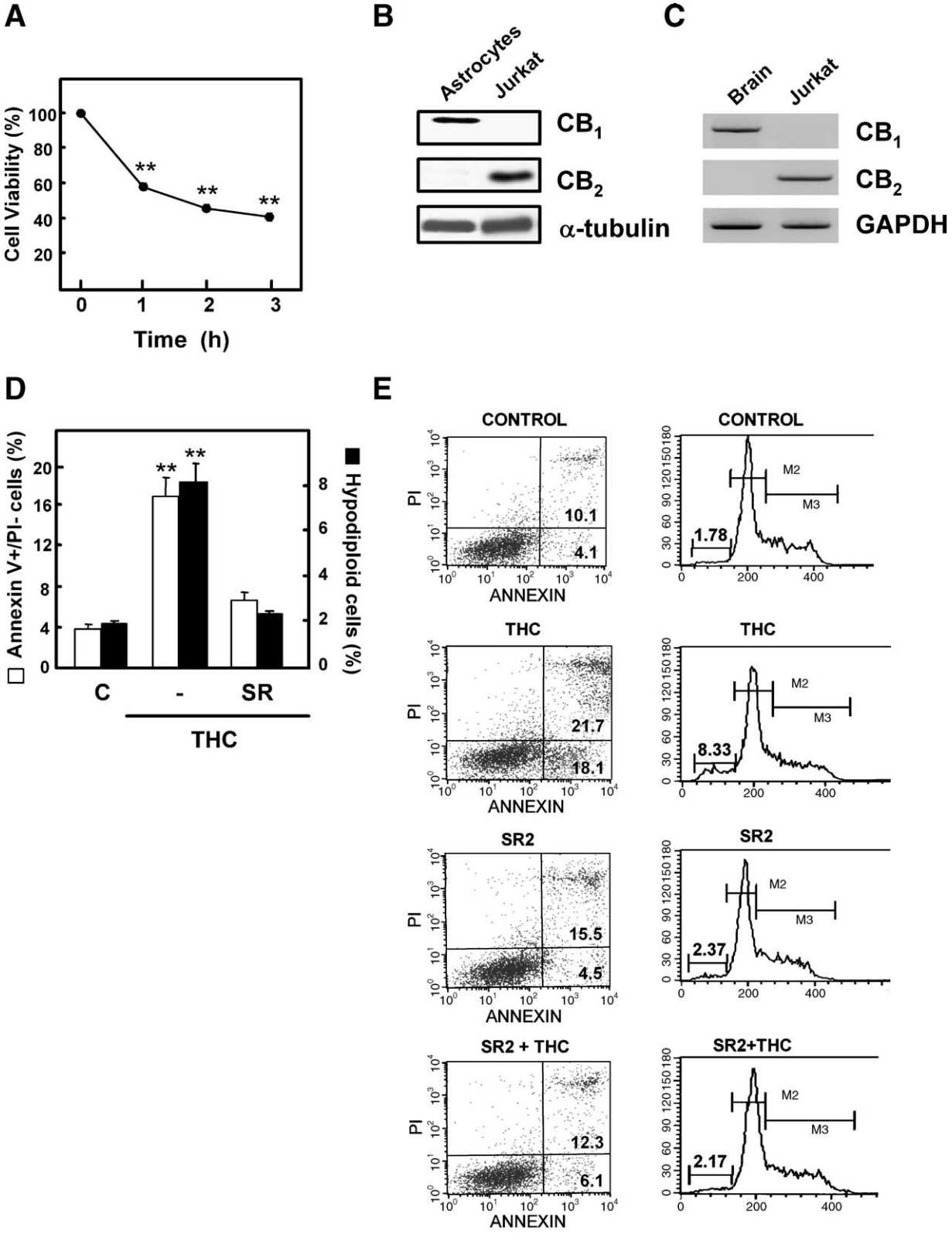
Cells were pre-incubated with vehicle or the corresponding inhibitor for 20 min before adding THC. The standard doses of THC, SR144528 and ISP-1 used in these experiments were 1.5,

Fig. 1 – THC induces apoptosis via CB_2 receptor activation. (A) Cells were treated with 1.5 μM THC for the indicated times and cell viability was determined by Trypan blue exclusion. Results correspond to 10 different experiments and are expressed as the percentage of viable cells relative to vehicle-treated cells. (B and C) Whole-cell extract and total RNA were obtained from Jurkat cells. CB_1, CB_2 and α-tubulin protein expression was analyzed by Western blot (B) and CB_1, CB_2 and GAPDH mRNA levels were determined by RT-PCR (C). Extracts from rat astrocytes and human brain cortex were used as positive controls for CB_1 protein and mRNA expression, respectively. A representative experiment of 4 is shown. (D and E) Jurkat cells were pre-incubated with vehicle (–) or 2 μM SR144528 (SR) for 20 min, further treated with vehicle (C) or 1.5 μM THC for 1 h and apoptosis was analyzed by flow cytometry. Results correspond to 4 different experiments and are expressed as the percentage of annexin V-positive/PI-negative cells (white bars) or of hypodiploid cells (black bars). Dot plots (E, left column) or DNA content profiles (E, right column) of representative experiments are shown. Values in the lower right corner and the upper right corner of the dot plots correspond to the percentage of annexin V-positive/PI-negative cells and annexin V-positive/PI-positive cells, respectively, in that experiment. Values in the lower left corner of the DNA content profiles correspond to the percentage of hypodiploids cells in that experiment. **Significantly different ($P < 0.01$) from vehicle-treated cells.

2.0 and 1.0 μM, respectively. Except if otherwise indicated in the corresponding figure legend, mitochondrial membrane potential, annexin V/PI, DNA fragmentation, caspase activity and cell viability experiments were performed 15 min, 1 h, 1 h, 1 h and 2 h, respectively, after THC addition.

Analysis of nuclear DNA content by flow cytometry

Cells were collected by centrifugation at 1500 × *g* for 5 min, washed once and incubated in PBS containing 1% (w/v) BSA, 30% ethanol and 5 μg/ml Hoechst 33342 (a fluorescent cell-



permeable dye that binds to DNA and allows quantification of the cellular DNA content) for 30 min at room temperature. Fluorescence intensity was analyzed using a LSR flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells were recorded in each analysis.

Analysis of apoptosis by phosphatidylserine exposure and propidium iodide uptake

Cells (approximately 0.55×10^6 cells per assay) were collected by centrifugation at 1500 × *g* for 5 min, washed once and incubated for 30 min at room temperature in 50 μl of binding buffer (10 mM HEPES, pH 7.4, 2.5 mM $CaCl_2$, 140 mM NaCl) supplemented with 3 μl annexin V-FITC. After incubation for 1 additional minute with the same medium containing 4.5 μg/ml propidium iodide, fluorescence intensity was analyzed using a FACSCalibur flow cytometer. Ten thousand cells were recorded in each analysis.

Analysis of mitochondrial transmembrane potential

Cells (approximately 0.55×10^6 cells per assay) were collected by centrifugation at 1500 × *g* for 5 min, washed once and incubated for 30 min at room temperature with binding buffer (see above) plus 0.25 μM TMRM (A cell-permeable dye that becomes fluorescent once it is inside the mitochondria. Since mitochondrial membrane potential is the driving force of TMRM pumping into this organelle, TMRM fluorescence allows quantification of mitochondrial membrane potential) and 3 μl annexin V-FITC. After incubation for an additional minute with 1 μM Hoechst 33258 (a fluorescent dye that binds to DNA and allows discrimination of living cells since it only enters membrane-disrupted dead cells), fluorescence intensity was analyzed using a LSR flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells were recorded in each analysis.

Sample preparation

Whole-cell extracts

Whole cell extracts were prepared as follows. Cells were lysed in 50 mM Tris–HCl, pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol supplemented with 0.5 μM microcystin-LR, 17.5 μg/ml PMSF, 0.1 μM DTT and a protease inhibitor cocktail (Roche, Basel, Switzerland), sonicated for 10 s and frozen.

Membrane and cytosol fractions

Membrane and cytosol fractions were isolated as described in [28]. Briefly, cells were permeabilized in 250 mM sucrose, 25 mM Tris–HCl pH 6.8, 1 mM EDTA, 0.05% (w/v) digitonin, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin.

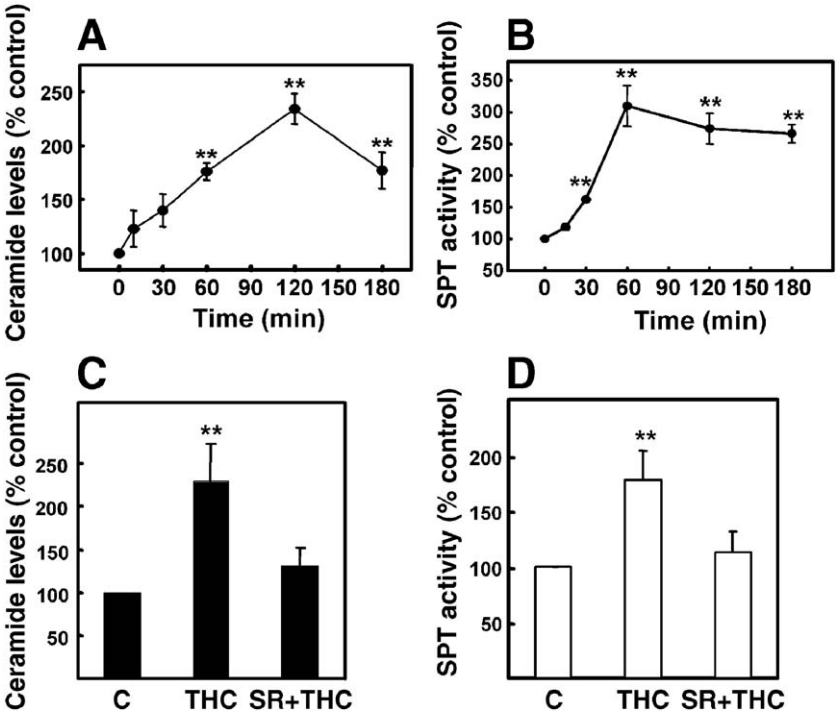


Fig. 2 – THC treatment stimulates ceramide synthesis de novo. (A and B) Cells were treated with vehicle or 1.5 μM THC for the indicated times and ceramide levels (A) and SPT activity (B) were determined. Results correspond to 3 different experiments and are expressed as the percentage of ceramide content (A) or SPT activity (B) relative to vehicle-treated cells. (C and D) Cells were pre-incubated with vehicle or 2 μM SR144528 (SR) for 20 min, further treated with vehicle (C) or 1.5 μM THC for 2 (C) or 1 (D) additional hour and ceramide levels (C) or SPT activity (D) were determined. Results correspond to 3 different experiments and are expressed as the percentage of ceramide content (C) or SPT activity (D) relative to vehicle-treated cells. **Significantly different ($P < 0.01$) from vehicle-treated cells.

Samples were then centrifuged at 13,000×*g* for 3 min at 4°C and supernatants (cytosol-enriched fraction) and pellets (mitochondria-enriched fraction) were collected, denatured with Laemmli loading buffer and frozen.

Western blot analysis

Samples (15–45 μg/condition) were subjected to SDS/PAGE in 10% or 15% polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked for 1 h with TBST [50 mM Tris–HCl, pH 7.8, 100 mM NaCl and, 0.1% (v/v) Tween 20] supplemented with 5% (w/v) fat-free dried milk, and incubated for 12–16 h at 4°C with the selected primary antibody (1:1000 in TBST). After washing, membranes were incubated for 1 h at room temperature with the corresponding horseradish peroxide-conjugated secondary antibody (1:5000 in TBST), and subjected to chemiluminescence detection.

Reverse transcription-PCR analysis

RNA was isolated using the RNeasy Protect kit (Qiagen, Hilden, Germany) including a DNase digestion step using the RNase-free DNase kit (Qiagen). cDNA was subsequently obtained using the first strand cDNA synthesis kit (Roche, Basel, Switzerland). The following sense and antisense primers were used respectively to amplify human CB_1 [CGTGGGCAGCCTGTTCCTCA and CATGCGGGCTTGGTCTGG (408 bp product)], human CB_2 [CGCCGGAAGCCCTCATACC and CCTCATTCGGGCCATTCCTG (522 bp product)], and GADPH [GGGAAGCTCACTGGCATGGCCTTCC and CATGTGGGCCATGAGGTCCACCAC (322 bp product)]. PCR reactions were performed using the following parameters: 95°C for 15 s, 58°C for 15 s and 72°C for 30 s (35 cycles) followed by a final extension step of 72°C for 5 min. Finally, PCR products were separated on 1.5% agarose gels.

Analysis of caspase 3/7 and caspase 8 activities

Caspase 3/7 (DVEDase activity) and caspase 8 (LETDase activity) were determined according to manufacturer instructions using a luminogenic substrate (Caspase Glo, Promega, Madison, WI). Luminiscence was determined in a Microplate Fluorescence Reader FLUOstar Optima (BMG Labtech, Offernburg, Germany).

Analysis of serine palmitoyltransferase activity

Serine palmitoyltransferase (SPT) activity was determined as previously described [16] in digitonin-permeabilized Jurkat cells. Briefly, reactions were started by the addition of an assay mixture containing 2.1 μg/ml digitonin, 100 mM HEPES, pH 8.3, 200 mM sucrose, 2.5 mM EDTA, 5 mM dithioerythritol, 50 μM pyridoxal phosphate, 1 mg/ml BSA, 0.3 mM palmitoyl-CoA and 0.25 mM L-[U-^{14}C]serine (3 μCi/assay). After 30 min, reactions were stopped with 0.5 M NH_4OH and [^{14}C]ketosphinganine product was extracted with choloroform/methanol/1%NaCl.

Ceramide levels

Ceramide levels were determined as previously described [16]. Briefly, after incubation of the cells in the different conditions, lipids were extracted, saponified and incubated with *E. coli* diacylglycerol kinase in the presence of [γ-^{32}P]ATP. Finally, ceramide 1-phosphate was resolved by thin-layer chromatography.

Directed mutagenesis of LCB1

The "quick change" direct mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce a Trp in position 133 of LCB1—a mutation that has been shown to dominantly inactivate SPT [29]—in the template plasmid pSV-LCB1 [30]. Mutagenic primers were C133W-F (GTATGGTGTGGGTACCTATGGACCTCGAGGATTTTATGG) and C133W-R (CCATAAAATCCTCGAGGTCCATAGGTACCCACACCATAC). The mutated plasmid pSV-LCB1-C133W was sequenced to confirm that no further mutations were introduced during this procedure.

Transient transfections and analysis of apoptosis by phosphatidyserine exposure

Jurkat cells were transiently transfected with the corresponding plasmids using Lipofectamine 2000 (Life technologies, Gibco, BRL) according to manufacturer recommendations. Phosphatidylserine exposure was analyzed 48 h after transfection using annexin-PE and propidium iodide as described above. Fluorescence intensity of GFP-positive cells was determined using a FACSCalibur flow cytometer. Ten thousand cells were recorded in each analysis.

Statistics

Results shown represent means ± SD. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student–Neuman–Keuls test.

Results

THC induces apoptosis via CB_2 receptor activation

In order to investigate the molecular mechanisms involved in apoptosis via the CB_2 receptor, we used the human leukemia cell line Jurkat. Firstly, we confirmed that THC treatment of Jurkat cells reduces cell viability (Fig. 1A) and induces apoptosis—as determined by the annexin V/propidium iodide test and DNA fragmentation (Figs. 1D and E). We also examined the presence of CB receptors in our model. Only the CB_2 receptor protein (Fig. 1B) and mRNA (Fig. 1C) were shown to be expressed. These results were further confirmed by using quantitative real-time PCR (CB_2 amplification was evident after 22 cycles whereas no amplification of CB_1 receptor mRNA was detected after 45 cycles). We also confirmed that the CB_2 receptor mediates the pro-apoptotic effect of THC as incubation with the selective CB_2 antagonist SR144528 (2.0 μM) prevented THC (1.5 μM; 1 h)-induced apoptosis of Jurkat cells (Figs. 1D and E).

De novo-synthesized ceramide is involved in THC-induced apoptosis

Accumulation of de novo-synthesized ceramide has been implicated in the pro-apoptotic action of cannabinoids in

glioma [7,16,27]. Therefore, we investigated whether ceramide participates in THC-induced apoptosis in our model. Cell incubation with THC led to a CB_2 receptor-dependent increase in ceramide levels (Figs. 2A and C). This was concomitant to a stimulation of serine palmitoyltransferase (SPT), the enzyme that catalyzes the first step of sphingolipid synthesis de novo (Figs. 2B and D). In addition, incubation with the selective SPT inhibitor ISP-1 prevented THC-induced

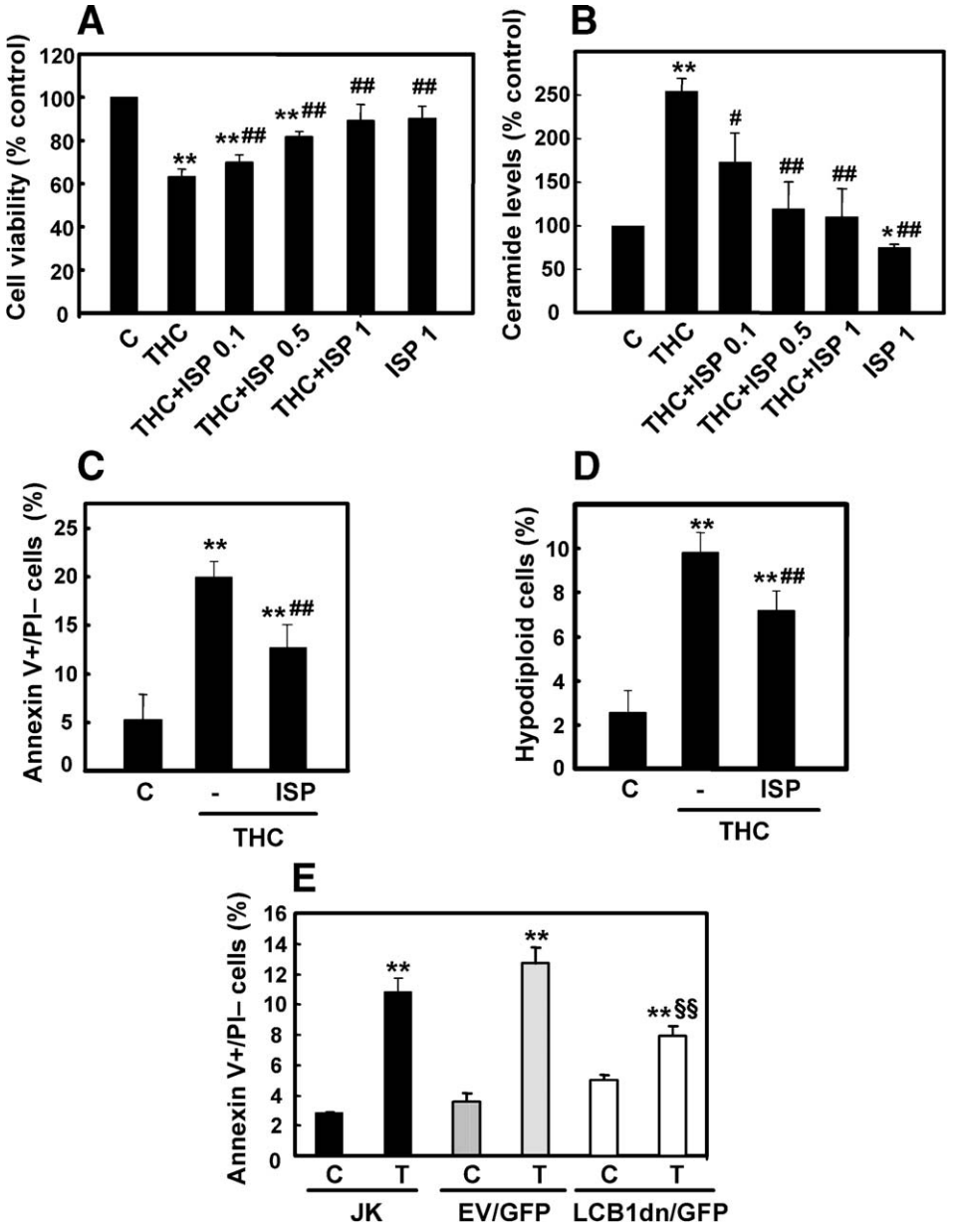


Fig. 3 – Ceramide synthesis de novo is involved in THC-induced apoptosis. (A and B) Cells were pre-incubated with vehicle or different doses of ISP-1 (ISP; 0.1, 0.5 or 1.0 μM) for 20 min, further treated with vehicle (C) or 1.5 μM THC for 2 h, and cell viability (A) and ceramide levels (B) were determined. Results correspond to 6 different experiments and are expressed as the percentage of viable cells (A) or ceramide level (B) relative to vehicle-treated cells. (C and D) Cells were pre-incubated with vehicle (–) or 1 μM ISP-1 for 20 min, treated with vehicle (C) or 1.5 μM THC for 1 h and phosphatidylserine exposure (C) and nuclear DNA content (D) were analyzed by flow cytometry. Results correspond to 9 different experiments and are expressed as the percentage of annexin V-positive/PI-negative cells (C) and hypodiploid cells (D). (E) Jurkat cells were transiently co-transfected with the plasmid pSV-LCB1-C133W (LCB1dn) or the empty vector pSV (EV) and with a GFP-carrying vector (GFP). Twenty-four hours after transfection, cells were treated with vehicle (C) or 1.5 μM THC for 1 h and phosphatidylserine exposure was analyzed by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of annexin V-positive/GFP-positive/PI-negative cells. Significantly different ($^{**}P < 0.01$; $^{*}P < 0.05$) from vehicle-treated cells. Significantly different ($^{\#\#}P < 0.01$, $^{\#}P < 0.05$) from cells treated only with THC. §§Significantly different ($P < 0.01$) from THC-treated EV/GFP cells.

cell death (Fig. 3A) and ceramide accumulation (Fig. 3B). In line with this observation, pharmacological blockade of ceramide synthesis de novo with ISP-1 (Figs. 3C and D) or transient transfection of Jurkat cells with a SPT dominant negative form [29] (Fig. 3E) significantly attenuated THC-induced apoptosis.

THC-induced accumulation of de novo-synthesized ceramide mediates loss of mitochondrial membrane potential, cytochrome c release and caspase activation

Mitochondria undergo two major changes during early apoptosis: (i) the inner-mitochondrial membrane transmembrane-potential ($\Delta\psi_m$) is decreased, and (ii) the outer-mitochondrial membrane becomes permeable to proteins, resulting in the release of soluble intermembrane proteins [31,32]. Since it has been recently reported that THC leads to apoptosis of Jurkat cells via the mitochondrial intrinsic pathway [33], we examined whether ceramide is involved in the triggering of these events. As shown in Fig. 4A, an increase in the number of cells with decreased $\Delta\psi_m$ was observed in THC-treated cells and pharmacological inhibition of ceramide synthesis de novo significantly prevented this effect (Fig. 4B). Incubation with ISP-1 also reduced THC-induced cytochrome c release from mitochondria (Fig. 4C).

Cleavage of specific substrates by caspases is one of the key events involved in the triggering and execution of apoptosis. We tested therefore whether activation of these proteases is involved in THC-induced apoptosis of Jurkat cells. Incubation with the pan-caspase inhibitor (Z-VAD-FMK) as well as with the selective caspase 3 inhibitor (Z-DQMD-FMK) or the selective caspase 8 inhibitor (Z-IETD-FMK) prevented THC-induced apoptosis (Fig. 5A). Moreover, challenge with THC led to the cleavage of the caspase 3 selective substrate PARP (Fig. 5B), and activation of caspase 3/7 (Fig. 5C) and caspase 8 (Fig. 5D). Pharmacological inhibition of ceramide biosynthesis attenuated these effects (Figs. 5B–D).

THC induces activation of caspase 8 at a post-mitochondrial level

Since caspase 8 activation has been shown to be involved in the extrinsic apoptosis pathway [32], we next investigated whether activation of this protease mediates the mitochondrial changes observed in our model. Incubation with a pan-caspase inhibitor prevented apoptosis (1 h; Figs. 5A and 6B) but did not abolish the $\Delta\psi_m$ loss observed 15 min after the exposure to THC (Figs. 6A and B). Likewise, incubation with the selective inhibitors of caspase 3 and caspase 8 did not prevent $\Delta\psi_m$ loss (Fig. 6A), suggesting that changes in mitochondria were necessary for caspase activation to be observed.

To confirm the involvement of the mitochondrial intrinsic pathway in our model, we analyzed the effect of Bcl-x_L over-expression (Fig. 6C). As shown in Fig. 6D, challenge with THC (1 h) induced apoptosis in control cells but not in cells over-expressing Bcl-x_L. By contrast, treatment with an anti-Fas antibody (18 h) did induce apoptosis in both control and—although to a lower extent—Bcl-x_L over-expressing cells (Fig. 6E). In addition, Bcl-x_L over-expression prevented THC-

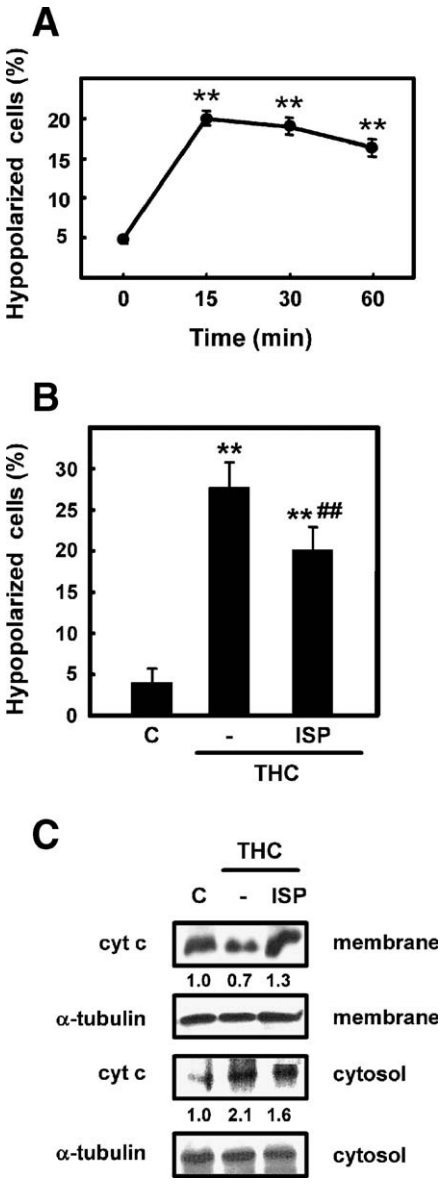


Fig. 4 – THC induces cytochrome *c* release and loss of mitochondrial transmembrane potential via de novo-synthesized ceramide. (A) Cells were treated with 1.5 μM THC for the indicated times and mitochondrial transmembrane potential changes ($\Delta\psi_m$) were analyzed by flow cytometry. Results correspond to 6 different experiments and are expressed as the percentage of cells with decreased $\Delta\psi_m$. (B and C) Cells were pre-incubated with vehicle (–) or 1 μM ISP-1 for 20 min, further treated with vehicle (C) or 1.5 μM THC for 15 min (B) or 30 min (C), and mitochondrial transmembrane potential (B) and cytochrome *c* and α-tubulin content in membrane and cytosol fractions (C) were determined. Results correspond to 8 different experiments and are expressed as the percentage of cells with decreased $\Delta\psi_m$ (B). A representative experiment of 3 (C) is shown. Optical density values of the bands are given in panel C. **Significantly different ($P < 0.01$) from vehicle-treated cells. ##Significantly different ($P < 0.01$) from THC-treated cells.

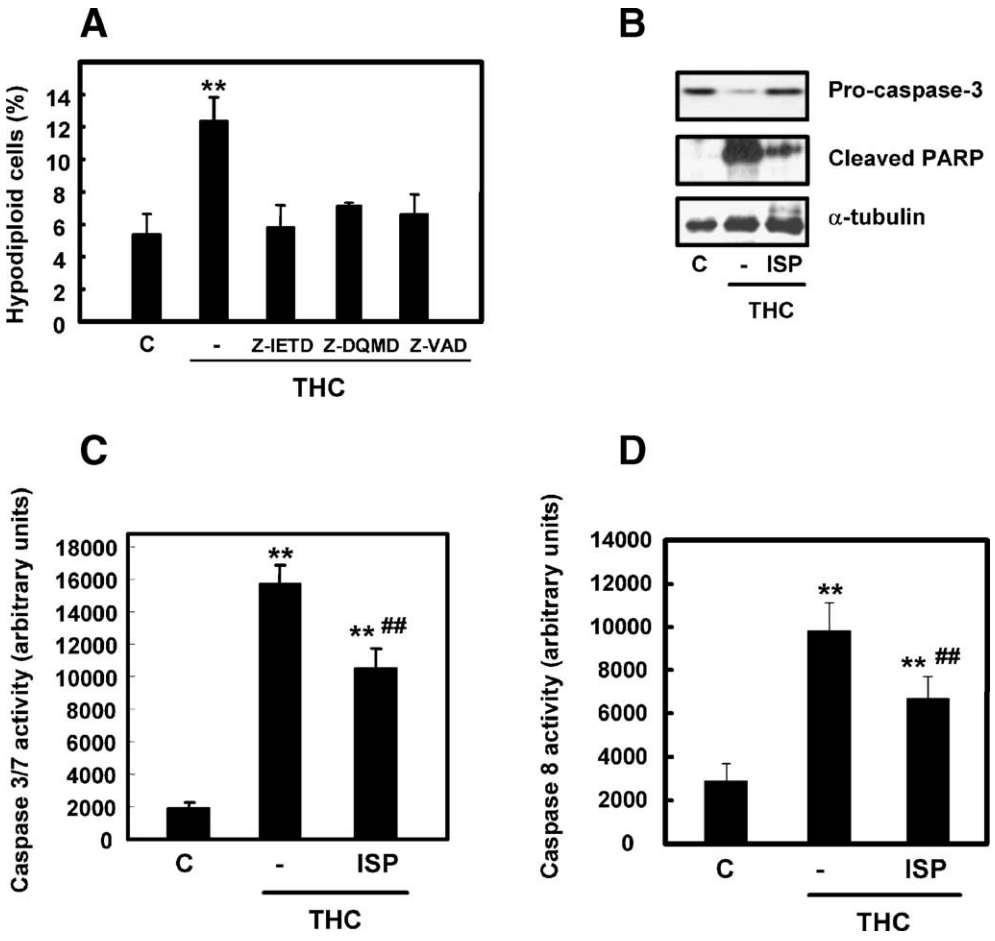


Fig. 5 – THC induces activation of caspases via de novo-synthesized ceramide. (A) Cells were pre-incubated with vehicle (–) or 50 μM Z-IETD-FMK (caspase 8 inhibitor), 50 μM Z-DQMD-FMK (caspase 3 inhibitor) or 10 μM Z-VAD-FMK (pan-caspase inhibitor) for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 1 h. Nuclear DNA content was analyzed by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of hypodiploid cells. (B, C and D) Cells were pre-incubated with vehicle (–) or 1 μM ISP-1 for 20 min, further treated with vehicle (C) or 1.5 μM THC for 1 h, and procaspase 3, PARP and α-tubulin content (B), DVEDase (caspase 3/7) and LETDase (caspase 8) activities (B and C) were determined. Results correspond to 4 (C and D) different experiments and are expressed in arbitrary units. A representative experiment of 3 (panel B) is shown. **Significantly different ($P < 0.01$) from vehicle-treated cells. ##Significantly different ($P < 0.01$) from THC-treated cells.

induced activation of caspase 8 (Fig. 6E), supporting again that the latter events depend on a previous mitochondrial alteration.

Discussion

Cannabinoids have been shown to induce apoptosis on a wide spectrum of transformed cells [20]. However, the molecular mechanism involved in this effect is not completely understood. Stimulation of the ceramide synthesis pathway has been previously implicated in the antitumoral effects of cannabinoids in glioma cells both in vitro [7,16] and in vivo [21]. Now we show that this process also operates in THC-induced apoptosis of leukemia cells, suggesting that it could be a common mechanism associated with the antitumoral effect of cannabinoids via the CB_2 receptor, and that it occurs via activation of the mitochondrial intrinsic pathway. The involvement of this receptor in tumor-cell apoptosis might be relevant for the design of potential cannabinoid-based antitumoral therapies as the psychoactive effects of cannabinoids, which severely limit the clinical application of these compounds, are mediated largely or entirely by CB_1 receptors within the brain [20].

A recent report has suggested that the mitochondrial intrinsic pro-apoptotic pathway is involved in cannabinoid-induced apoptosis of leukemia cells [33]. Results presented here now show that inhibition of ceramide synthesis de novo significantly prevents $\Delta\psi_m$ decrease and cytochrome c release—two early mitochondrial events that occur prior to caspase activation in our system as well as apoptosis, supporting that accumulation of de novo-synthesized ceramide (or ceramide-derived metabolites) is involved in the triggering of this pathway. In line with our observations, de novo-synthesized ceramide has been proposed to induce apoptosis by acting at a mitochondrial level [14,34].

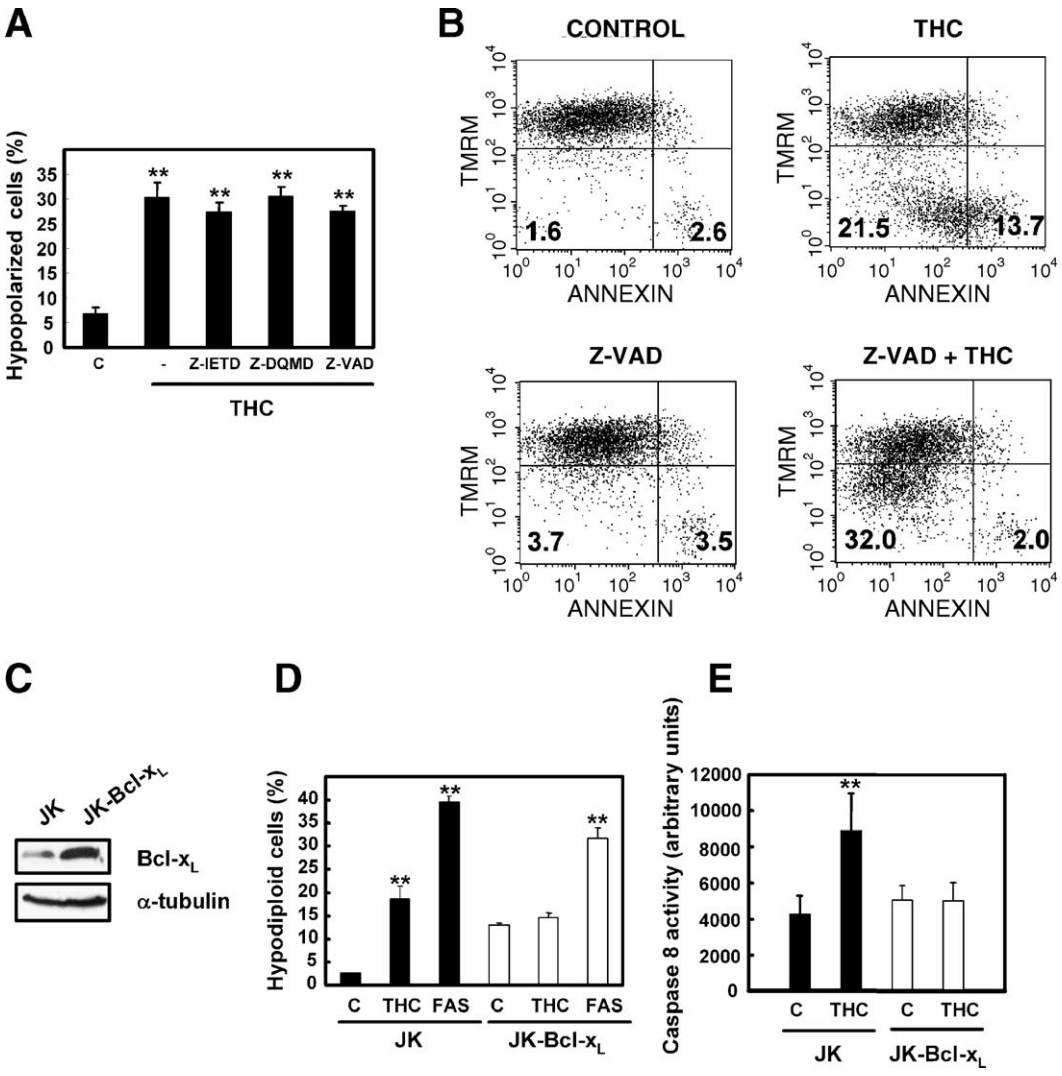


Fig. 6 – THC induces activation of caspase 8 at a post-mitochondrial level. (A) Cells were pre-incubated with vehicle (–) or 50 μM Z-IETD-FMK (caspase 8 inhibitor), 50 μM Z-DQMD-FMK (caspase 3 inhibitor) or 10 μM Z-VAD-FMK (pan-caspase inhibitor) for 20 min and further treated with vehicle (C) or 1.5 μM THC for 15 min. Mitochondrial transmembrane potential changes were analyzed by flow cytometry. Results correspond to 6 different experiments and are expressed as the percentage of cells with decreased $\Delta\psi_m$. (B) Cells were pre-incubated with vehicle or 10 μM Z-VAD-FMK for 20 min, further treated with vehicle or 1.5 μM THC for 15 min and mitochondrial transmembrane potential and phosphatidylserine exposure were analyzed by flow cytometry after incubation with TMRM (*y* axis) and annexin V (*x* axis). A representative experiment of 10 is shown. Values correspond to the percentage of low TMRM/annexin V-negative cells (lower left corner of each plot) or low TMRM/annexin V-positive cells (lower right corner of each plot). (C) Whole-cell extracts were obtained from control (JK) or Bcl-x_L over-expressing (JK-Bcl-x_L) Jurkat cells, and Bcl-x_L and α-tubulin content were analyzed by Western blot. A representative experiment of 3 is shown. (D) JK and JK-Bcl-x_L cells were treated with vehicle or 20 ng/ml anti-Fas mAb (FAS) for 18 h, or with vehicle or 1.5 μM THC for 1 h, and nuclear DNA content was analyzed by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of hypodiploid cells. (E) JK and JK-Bcl-x_L cells were treated with vehicle (C) or 1.5 μM THC for 1 h, and LETDase (caspase 8) activity was assayed. Results correspond to 3 experiments and are expressed in arbitrary units. **Significantly different ($P < 0.01$) from vehicle-treated cells.

Intriguingly, THC-induced $\Delta\psi_m$ decrease occurs before full ceramide accumulation and SPT activation ensues. A possible explanation for this observation is that early local accumulation of ceramide in the endoplasmic reticulum or in the mitochondria could be enough to trigger the observed pro-apoptotic response via the mitochondrial intrinsic pathway. Nevertheless, our data also suggest that in addition to these direct actions on mitochondria, ceramide may also be involved in the execution of apoptosis at a later stage.

We note that abrogation of ceramide synthesis de novo only partially prevents apoptosis in our model, which suggests that there should be additional pathways participating in the triggering of the apoptotic signal. We have observed that THC rapidly stimulates extracellular signal-regulated kinase, c-Jun

N-terminal kinase and p38 mitogen-activated protein kinase (p38 MAPK) in Jurkat cells [35]. Moreover, activation of p38 MAPK seems to play an important role in the execution of THC-induced apoptosis [35]. However, experiments of pharmacological inhibition support that none of these kinases is responsible for the ceramide-dependent limb of THC pro-apoptotic action. Moreover, p38 MAPK does not affect THC-induced $\Delta\psi_m$ decrease [35] (suggesting that this kinase acts at a post-mitochondrial level) nor is regulated by ceramide in this model (data not shown). These observations support that ceramide and p38 MAPK act at different levels to mediate CB_2-induced apoptosis.

Although CB_2 receptor activation leads to apoptosis of leukemia cells via the intrinsic mitochondrial pathway, we observed that challenge with THC also leads to caspase 8 activation, an event typically related with the extrinsic pathway in type II cells [32]. Nevertheless, we clearly show that caspase 8 is involved in THC-induced apoptosis at a post-mitochondrial level since incubation with a caspase 8-selective or a pan-caspase inhibitor did not prevent the loss of mitochondrial membrane potential triggered by THC. To further support this hypothesis, Fas ligand—the prototypic extrinsic pathway-triggering signal—exhibits a completely different pattern of apoptosis than THC. Moreover, Bcl-x_L over-expression completely abrogates THC- but not Fas ligand-induced apoptosis, supporting again that THC and Fas ligand signal apoptosis via different mechanisms, at least in Jurkat cells.

Caspase 8 activation during apoptosis induced by different stimuli has been described by other groups [36–38] as part of an apoptotic feedback loop in which cytochrome *c* and caspase 9 lead to the activation of caspase 3, which will subsequently cleave and activate caspase 8 as one of the execution events of apoptosis induction, downstream from caspase 3. If this were the case in our model, then z-IETD would not significantly change the efficiency of apoptosis induction by THC. Thus, the z-IETD-mediated protection from apoptosis could be due to a positive feedback loop on mitochondrial damage induction via tBid. However, inhibition of caspase 8 activity does not affect mitochondrial membrane potential, suggesting that caspase 8 acts at a later point and that other mechanisms could be involved in this amplification loop. Further research is therefore necessary to completely clarify the role of caspase 8 in THC-induced apoptosis.

In summary, results presented here show that THC induces apoptosis via CB_2 receptor activation, accumulation of de novo-synthesized ceramide and the mitochondrial intrinsic pathway. These findings significantly increase our understanding of how cannabinoids control tumor-cell death [20] and, to the best of our knowledge, show for the first time that signaling via a G-protein-coupled receptor leads to stimulation of ceramide synthesis de novo and apoptosis through the mitochondrial intrinsic pathway.

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