Cannabinoids Protect Astrocytes from Ceramide-induced Apoptosis through the Phosphatidylinositol 3-Kinase/Protein Kinase B Pathway*†

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Cannabinoids, the active components of marijuana and their endogenous counterparts, exert many of their actions on the central nervous system by binding to the CB1 cannabinoid receptor. Different studies have shown that cannabinoids can protect neural cells from different insults. However, those studies have been performed in neurons, whereas no attention has been focused on glial cells. Here we used the pro-apoptotic lipid ceramide to induce apoptosis in astrocytes, and we studied the protective effect exerted by cannabinoids. Results show the following: (i) cannabinoids rescue primary astrocytes from C2- ceramide-induced apoptosis in a dose- and time-dependent manner; (ii) triggering of this anti-apoptotic signal depends on the phosphatidylinositol 3-kinase/protein kinase B pathway; (iii) ERK and its downstream target p90 ribosomal S6 kinase might be also involved in the protective effect of cannabinoids; and (iv) cannabinoids protect astrocytes from the cytotoxic effects of focal C2-ceramide administration in vivo. In summary, results show that cannabinoids protect astrocytes from ceramide-induced apoptosis via stimulation of the phosphatidylinositol 3-kinase/protein kinase B pathway. These findings constitute the first evidence for an “astroprotective” role of cannabinoids.

The effects exerted by marijuana and their derivatives through Δ9-tetrahydrocannabinol (THC)1 and other cannabinoid constituents have been known for many centuries. However, the molecular basis of these actions were not understood until the discovery of an endogenous cannabinoid system comprising two plasma membrane Gαs-coupled cannabinoid receptors (CB1 (1) and CB2 (2)) and a family of endogenous ligands for those receptors (3, 4). Cannabinoid receptors mediate cannabinoid effects by coupling to different signaling pathways. Both the CB1 and the CB2 receptor signal inhibition of adenylyl cyclase (5) and stimulation of extracellular signal-regulated kinase (ERK) (6), whereas the CB1 receptor is also coupled to modulation of Ca2+ and K+ channels (7), stimulation of the stress-activated p38 and c-Jun N-terminal kinases (8), stimulation of the focal adhesion kinase (9), hydrolysis of sphingomyelin (10), and stimulation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) (11).

The study of the potential therapeutic applications of cannabinoids has become one of the most exciting areas in the field. Ongoing research is determining whether cannabinoid ligands may be effective agents in the treatment of pain, glaucoma, and the wasting and emesis associated with acquired immunodeficiency syndrome and cancer chemotherapy (7, 12). In addition, cannabinoids are being investigated as potential antitumoral drugs (13–15) and therapeutic agents for neurological and neurodegenerative disorders (16, 17). Neuroprotection by cannabinoids has related to the CB1-mediated inhibition of voltage-sensitive Ca2+ channels to reduce Ca2+ influx, glutamate release and excitotoxicity (12, 18), and to the ability of cannabinoids to act as antioxidants (19, 20). However, nothing is known about the possible protective effect of cannabinoids on the major cell population of the central nervous system, namely the astrocytes, despite the pivotal role played by these cells in brain homeostasis. In addition, although the CB1 receptor is coupled to PI3K/PKB (11) and ERK activation (6), and both signaling routes are essential for neural cell survival (21), their possible involvement in the protection of neural cells by cannabinoids is as yet unknown.

Ceramide, a sphingosine-based lipid, regulates a variety of cellular processes including differentiation, proliferation, and apoptosis (22). Interestingly, the pro-apoptotic effect of ceramide may be due, at least partially, to its ability to inhibit PKB (23, 24). In addition, it has been shown that accumulation of ceramide in astrocytes leads to apoptosis (25). Here we employed a cell-permeable analog of ceramide to induce apoptosis in astrocytes, and we studied (i) the protective role of cannabinoids and (ii) the involvement of PI3K/PKB and ERK pathways in such effect.

**EXPERIMENTAL PROCEDURES**

Materials—The following materials were kindly donated: HU-210 by Dr. R. Mechoulam (Hebrew University, Jerusalem, Israel); SR 141716 by Sanofi Synthelabo (Montpellier, France); antibodies against total PKB and RSK and the specific PKB/RSK peptide substrate (cross-tide) by Dr. D. Alessi (University of Dundee, Dundee, UK); and wild-type and dominant-negative PKB adenoviral vectors by Dr. W. Ogawa (Kobe University, Kobe, Japan). DNA fragmentation and TUNEL staining kits and biotin-16-dUTP were from Roche Molecular Biochemicals; deoxyribonuclease/transfection was from Invitrogen; streptavidin Alexa Fluor 488 was from Molecular Probes (Leiden, The Netherlands); wortmannin, LY 294002, PD 098059, Ro 318220, and C2-ceramide were from Alexis Biochemicals (San Diego, CA); anti-HA antibody was from Roche Molecular Biochemicals; anti-phospho-ERK antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-PKB Thr-380 and phospho-PKB Ser-473 were from Cell Signaling Technology (Beverly,
MA); anti-glial fibrillary acidic protein (GFAP) polyclonal antibody was from DAKO (Glostrup, Denmark); ABC complex was from Pierce; and WIN 55,212-2 and THC were from Sigma.

Astrocyte Isolation and Culture—Cortical astrocytes were prepared from 24- to 48-h Wistar rats as described previously (25). Briefly, cerebral hemispheres were dissected in PBS supplemented with 0.03% glucose, treated with trypsin (5 mg/ml, 30 min at 37 °C), and after stopping the reaction by addition of 10% serum-containing medium, incubated with DNase I (10 μg/ml, 5 min at 37 °C). Subsequently cells were mechanically dissociated, centrifuged, and seeded (3 × 10^4 cells/cm^2) on plastic plates previously coated with 5 μg/ml poly-l-ornithine and cultured in a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1, v/v) supplemented with 0.5% (v/v) glucose, 5 mg/ml streptomycin, 5 units/ml penicillin, and 10% fetal calf serum. After 10–12 days, cells were trypsinized and reseded until they reached confluence. Finally, cells were trypsinized, seeded at a density of 3 × 10^5 cells/cm^2, and 24 h before the experiment transferred to a chemically defined serum-free medium consisting of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1, v/v).

Apoptosis and Cell Viability—Cell viability was determined by trypan blue exclusion. Oligonucleosomal DNA fragmentation, a characteristic biochemical feature of apoptotic cell death, was measured using a nucleosomal DNA enzyme-linked immunosorbent assay, which quantitatively records histone-associated DNA fragments, according to manufacturer’s instructions. TUNEL staining was performed as described previously (26).

PKB and RSK Kinase Assays—PKB and RSK activities were determined as described (11). Briefly, PKB or RSK was immunoprecipitated from cell lysates with 2 μg of anti-PKBα or anti-RSK antibodies bound to protein G-Sepharose, and kinase activity was determined as the incorporation of [γ-32P]ATP into a specific peptide substrate (GRPRSSPFAQG).

PKB and ERK Phosphorylation—Western blot analyses were performed with antibodies that recognize ERK phosphorylated on Thr-202/Tyr-204, PKB-phosphorylated on Thr-308, and PKB-phosphorylated on Tyr-204, PKB-phosphorylated on Thr-308, and PKB-phosphorylated on Ser-473.

Adenovirus Infections—Adenoviral vectors encoding HA-tagged dominant-negative and wild-type PKB were amplified as described (27). Astrocytes were transferred to serum-free medium, infected for 3 h with the corresponding adenoviral vector at the multiplicity of infection indicated in the figures, washed with PBS, and transferred to a 10% fetal calf serum medium for 12 h to recover from the infection. Before performing the experiments, infected cells were incubated for 24 h in serum-free medium. Pilot experiments using adenoviruses encoding the green fluorescent protein showed that >95% were infected in our experimental conditions. Expression of HA-tagged wild-type and dominant-negative forms of PKB was confirmed in the infected astrocytes by Western blot analysis with anti-HA antibody.

In Vivo Ceramide Administration—Male Wistar rats (320–350 g) were anaesthetized with equitesin (3.5 ml/kg) and injected stereotactically with C2-ceramide (10 μg/ml in Me2SO) at two sites in the hippocampus. In preliminary experiments the volume and number of sites of C2-ceramide injection were established. Twenty μg were injected into the dorsal dentate gyrus and another 20 μg into the dorsal hippocampus (anteroposterior, bregma −3.8 mm; lateral −3.0 mm, and ventral to the surface of the brain −3.4 and −2.6 mm, respectively). C2-ceramide or vehicle were slowly injected (1 μl/min). The needle was left in place for 2 min before retraction to the more dorsal coordinate, and after injection at the second site left in place for a further 5 min before final retraction. WIN 55,212-2 (2.5 mg/kg, intraperitoneal in 1 ml/kg of 10% Me2SO in saline) was administered 10 min before anesthetic injection and 30 min before focal injection. All procedures were conducted according to the guidelines of the European Community (EC) and were approved by the ethical committee of the Centro Superior de Investigaciones Científicas (CSIC).

Immunohistochemistry—Two days post-injection animals were decapitated, the brains removed, and 4 mm coronal slabs around the injected area cut, fixed by immersion in 4% paraformaldeyde in 0.1 M phosphate buffer for 3 days, and cryoprotected with 15% sucrose for 24 h and then with 30% sucrose for a further 24 h at 4 °C. Finally, brain slabs were flash-frozen in hexane (−70 °C) and stored at −20 °C until sectioning. 45 μm in a cryostat. TUNEL staining of mounted tissue sections was performed according to the manufacturer’s instructions. GFAP immunostaining was performed on free-floating sections. Sections were washed 3 times in PBS, treated with 3% H2O2 for 15 min to block endogeneous peroxidase, and rinsed 3 times in PBS. After incubation with 10% normal goat serum (NGS) in PBS containing 0.3% Triton X-100 for 30 min, sections were incubated with anti-GFAP polyclonal antibody (1:1000 dilution, DAKO). After washing with PBS, sections were incubated with biotinylated anti-goat antibody (1:1000, DAKO) followed by ABC complex and Vector red (1:1000) (Vector Laboratories, Burlingame, CA) for 30 min. Thereafter, sections were counterstained with DAPI (1:1000) for 5 min. Images were captured on a Zeiss LSM 510 confocal microscope (Zeiss, Germany). Immunohistochemical detection of caspase-3 and -9 was performed using a nucleosomal DNA enzyme-linked immunosorbent assay, which quantitatively records histone-associated DNA fragments, according to manufacturer’s instructions. TUNEL staining was performed as described previously (26).

Fig. 1. Cannabinoids rescue primary astrocytes from ceramide-induced death. Astrocytes were incubated in serum-free medium for 24 h and treated with 15 μM C2-ceramide or vehicle (Control) for 90 min. Then, the medium was changed, and vehicle (−) or the corresponding cannabinoid was added. The protective effect of each cannabinoid was determined at the indicated times as the percentage of viable cells with respect to the controls. A, cell viability was determined 18 h after the addition of vehicle or the indicated cannabinoid (1 μM THC, 25 nM HU-210 (HU), or 25 nM WIN 55,212-2 (WIN)). B, cell viability was determined 18 h after the addition of vehicle or the indicated doses of WIN 55,212-2. C, cell viability was determined at the indicated times after addition of vehicle or 25 nM WIN 55,212-2 (WIN). Results correspond to six different experiments. * significantly different (p < 0.01) from the controls.
Cannabinoids Prevent Astrocytes from Apoptosis

It is well established that stimulation of the PI3K activation—PKB pathway leads to activation of the anti-apoptotic kinase PKB (29). As shown in Fig. 4A, incubation of astrocytes with HU-210 stimulated and incubation with ceramide inhibited PKB activity. Interestingly, incubation with HU-210 also prevented ceramide-induced inhibition of PKB activity (Fig 4A). Because activation of PKB depends on its phosphorylation on residues Thr-308 and Ser-473 (29), we monitored the phosphorylation status of PKB in astrocytes by using specific antibodies raised against the phosphorylated forms of the kinase. Fig. 4B shows that changes in PKB phosphorylation paralleled changes in enzyme activity. Thus, incubation of astrocytes with HU-210 increased and incubation with ceramide decreased PKB phosphorylation on Thr-308 and Ser-473. In addition, after ceramide challenge, incubation with cannabinoids led PKB phosphorylation to the control level.

To confirm the involvement of PKB in the anti-apoptotic effect of cannabinoids, we expressed dominant-negative or wild-type forms of PKB (27) in astrocytes. Because primary cells are transfected with very low efficiency, we used adenoviral vectors to ensure that 95% of the cells express the exogenous proteins. As shown in Fig. 4C, expression of a dominant-negative form of PKB abrogated the protective effect of cannabinoids. In addition, infection with the wild-type form of PKB led to a dose-dependent blockade of the apoptotic effect of ceramide (Fig. 4D), suggesting that the pro-apoptotic effect of this lipid may be mediated, at least partially, by PKB inhibition.

PI3K-dependent Stimulation of the ERK Pathway May Be Involved in the Anti-apoptotic Effect of Cannabinoids—As data in Fig. 3 indicated that the protective effect of cannabinoids on
Astrocytes could also involve the ERK pathway. We determined the extent of ERK activation in the cells by using an antibody raised against the phosphorylated (active) form of this kinase. As shown in Fig. 5A, incubation with HU-210 increased the phosphorylation extent of ERK in the presence and in the absence of ceramide, whereas incubation with ceramide only slightly stimulated ERK. Incubation with SR 141716 or wortmannin partially prevented ERK activation after challenge to ceramide plus HU-210. We also determined the activity of the ERK downstream kinase RSK. As shown in Fig. 5B, incubation with cannabinoids or ceramide alone induced a 60–80% stimulation of RSK, and treatment with both compounds led to an additive stimulation. The latter effect was prevented by both wortmannin and SR 141716. By contrast, ceramide stimulation of RSK was not affected by incubation with wortmannin or SR141716.

Cannabinoids Protect Brain Astrocytes from Focal Injection of Ceramide—We next examined the role of cannabinoids in protecting astrocytes in vivo. As shown in Fig. 6A, treatment with WIN 55,212-2 prevented the toxic effects of focal administration of C2-ceramide in astrocytes. Thus, whereas administration of ceramide induced an area absolutely devoid of GFAP immunoreactivity coinciding with the site of injection (the ventral dentate gyrus), rats treated with WIN 55,212-2 showed a homogeneous GFAP staining throughout the whole hippocampus and did not present an injured area in the zone of injection. GFAP staining remained increased compared with normal rats or to the contralateral non-injected hemisphere of the brain in both cannabinoid- and vehicle-treated rats. In addition, as shown in Fig. 6B there was a high number of TUNEL-positive nuclei in ceramide-injected hippocampus that was significantly reduced by cannabinoid administration (number of TUNEL-positive nuclei/mm2):
DISCUSSION

During the last few years, a number of reports have indicated that cannabinoids protect nervous cells from different insults (reviewed in Refs. 12 and 17). In line with those observations, data presented here show that cannabinoids, via activation of the CB1 receptor, protect astrocytes from ceramide-induced apoptosis in vitro and in vivo. Astrocytes have been traditionally considered as secondary players in the central nervous system scenario, and therefore all the previous studies on the protective role of cannabinoids on neural cells have involved neurons (see Refs. 18 and 30–34, for example). However, it is currently well established that astrocytes, the most abundant cells of the mammalian brain, are involved in numerous functions such as supply of nutrients to neurons (35), establishment of synapses (36), and generation of neurons (37). In addition, in the context of the present study astrocytes are known to take up (38) and produce (39) endocannabinoids. Thus, most likely the complex mechanisms underlying defense against brain injury (and in particular the mechanisms mediated by cannabinoids) also involve protection of astrocytes.

Several observations presented in this report indicate that cannabinoids protect primary astrocytes from ceramide-induced apoptosis via CB1 receptor-mediated stimulation of the PI3K/PKB pathway. (i) Blockade of the CB1 receptor or inhibition of PI3K abolishes the protective effect of cannabinoids. (ii) Cannabinoid treatment leads to reactivation of PKB in parallel to prevention of apoptosis. (iii) Overexpression of a dominant-negative form of PKB abrogates the protective effect of cannabinoids. Thus, most likely the complex mechanisms underlying defense against brain injury (and in particular the mechanisms mediated by cannabinoids) also involve protection of astrocytes.

994 ± 236 after C2-ceramide treatment, 624 ± 193 after WIN 55,212-2 plus C2-ceramide treatment, p < 0.01. No TUNEL-positive nuclei were observed in vehicle-injected controls.
may be at least partially due to dephosphorylation and inactivation of PKB by a ceramide-activated phosphatase (23, 24). Our results suggest that cannabinoids (via activation of the PI3K pathway) and ceramide (via phosphatase activation) may compete for the modulation of PKB activity in astrocytes. Supporting this notion, overexpression of ceramide-sensitive wild-type PKB abrogated the apoptotic effect of ceramide. Because activation of PKB triggers the phosphorylation of different targets involved in preventing apoptosis, including Bad, forkhead transcription factors, IκB kinase, and caspase 9 (29), ceramide inhibition of PKB could lead to suppression of the survival signal, whereas cannabinoid-dependent reactivation of the pathway would restore it.

Expression of a dominant-negative form of PKB abolishes the protective effect of cannabinoids but does not induce apoptosis by itself, indicating that the apoptotic effect of ceramide and therefore the generation of a survival signal may also depend on the modulation of additional pathways. Thus, several data suggest that the ERK pathway may participate together with PKB activation in the anti-apoptotic effect of cannabinoids as follows: (i) inhibition of the ERK pathway also prevents the protective effect of cannabinoids, and (ii) astrocyte challenge with cannabinoids leads to activation of both ERK and RSK. One of the mechanisms whereby ERK prevents apoptosis in neural cells involves activation of its downstream kinase RSK as this kinase phosphorylates Bad and the transcription factor CAM-response element-binding protein (21). Thus RSK may act synergistically with PKB to prevent apoptosis (40). In our model, triggering of the survival signal is accompanied by a consistent activation of ERK and RSK. Nevertheless, incubation with ceramide leads to apoptosis and activation of ERK and RSK, although to a lower extent than with cannabinoid co-treatment. Interestingly, blockade of PI3K prevents the effect of cannabinoids on ERK and RSK but not ceramide-induced activation of these kinases. These data are in line with recent results of our group showing that stimulation of ERK by cannabinoids depends on PI3K and suggest that the latter may be involved in the pro-survival effect of cannabinoids also via activation of the ERK/RSK pathway. It is worth noting that RSK activation also depends on phosphorylation by 3-phosphoinositide-dependent kinase 1 on its N-terminal domain (41). Although that phosphorylation site has been suggested to be constitutive (41), it cannot be ruled out that under certain circumstances PI3K activation could lead to 3-phosphoinositide-dependent kinase 1-dependent phosphorylation and activation of RSK (42).

In short, data presented here indicate that cannabinoids protect primary astrocytes from ceramide-induced apoptosis via activation of the PI3K/PKB pathway. Our data also suggest that cannabinoids are involved in protecting astrocytes in vivo. Although the mechanisms of ceramide generation in astrocytes in vivo are still unknown, it is possible that exposure to proinflammatory cytokines (43) or to saturated fatty acids (25) may increase ceramide production in astrocytes during situations of brain injury. It is curious that, unlike this protective effect on astrocytes, cannabinoids induce apoptosis of glioma cells (13, 14, 26). This difference between transformed (glioma) and non-transformed cells (astrocytes) could be due to their ability to synthesize ceramide in response to cannabinoids. Thus, cannabinoids induce apoptosis on glioma cells via stimulation of ceramide synthesis de novo (26), whereas challenge to cannabinoids does not induce ceramide synthesis de novo in astrocytes. Taken together, these data suggest that cannabinoid receptors are coupled to different pathways and therefore lead to different responses in glioma cells and astrocytes. Accordingly, cannabinoids are being tested as potential antitumor drugs in the treatment of malignant gliomas and, given the crucial role of astrocytes in brain homeostasis and neuroprotection, our results raise the suggestive although still speculative idea of their usage as therapeutic agents for the management of neurodegenerative disorders.

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Cannabinoids Protect Astrocytes From Apoptosis

36533