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Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa*, on β-amyloid-induced toxicity in PC12 cells

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Abstract

Alzheimer's disease is widely held to be associated with oxidative stress due, in part, to the membrane action of β-amyloid peptide aggregates. Here, we studied the effect of cannabidiol, a major non-psychoactive component of the marijuana plant (*Cannabis sativa*) on β-amyloid peptide-induced toxicity in cultured rat pheocromocytoma PC12 cells. Following exposure of cells to β-amyloid peptide (1 μg/mL), a marked reduction in cell survival was observed. This effect was associated with increased reactive oxygen species (ROS) production and lipid peroxidation, as well as caspase 3 (a key enzyme in the apoptosis cell-signalling cascade) appearance, DNA fragmentation and increased intracellular calcium. Treatment of the cells with cannabidiol (10^{-7}–10^{-4} M) prior to β-amyloid peptide exposure significantly elevated cell survival while it decreased ROS production, lipid peroxidation, caspase 3 levels, DNA fragmentation and intracellular calcium. Our results indicate that cannabidiol exerts a combination of neuroprotective, anti-oxidative and anti-apoptotic effects against β-amyloid peptide toxicity, and that inhibition of caspase 3 appearance from its inactive precursor, pro-caspase 3, by cannabidiol is involved in the signalling pathway for this neuroprotection.

Keywords: Alzheimer's disease, apoptosis, β-amyloid, cannabidiol, cannabinoid, neuroprotection.

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Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder (Koo *et al*. 1999). Regional neuronal degeneration, synaptic loss, presence of neurofibrillary tangles (NFTs) (Terry 1963) and senile plaques (Braak and Braak 1997) are specific hallmarks of this disease. While NFTs are the result of disposition of hyper-phosphorylated tau protein (Lee *et al*. 1991), senile plaques are complex extracellular lesions composed of a core of β-amyloid (Aβ) aggregates, surrounded by activated astrocytes, and dystrophic neuritis (Itagaki *et al*. 1989; Cotman *et al*. 1996).

Excessive accumulation of Aβ peptide has been proposed as a pivotal event in the pathogenesis of AD, although the precise mechanism by which Aβ induces neuronal death is still unknown (Hensley *et al*. 1994; Troy *et al*. 2001). Proposed mechanisms include production of oxygen free radicals (Behl *et al*. 1994), modification of cytosolic calcium homeostasis (Mattson 1992; Ueda *et al*. 1997), the Wnt pathway and activation of nuclear factor-kB (Green and Peers 2002; Caricasole *et al*. 2003). The cysteine proteases, known as caspases, are essential mediators of many of the pathways involved in executing the apoptotic programme following Aβ accumulation (Gervais *et al*. 1999). Caspase-mediated apoptosis can be modulated by several agents, including antioxidants (Behl *et al*. 1994), calcium channel blockers (Weiss *et al*. 1994) and growth factors (Mattson *et al*. 1993).

Cannabinoids are a group of C_{21} compounds occurring in the glandular hairs of *Cannabis sativa* (Indian hemp) and consequently in hashish and marijuana, the well known drug

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Abbreviations used: Aβ, β-amyloid; AD, Alzheimer's disease; $[Ca^{2+}]i$, changes in intracellular free calcium levels; CBD, cannabidiol; DCF, 2′,7′-dichlorofluorescein; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; H_2DCF-DA, dichlorofluorescin-diacetate; MDA, malonyl dialdehyde; MTT, 3(4,5-dimethylthiazol-2yl)2,5-diphenyl-2H-tetrazolium bromide; NFTs, neurofibrillary tangles; O.D., optical density; PBS, phosphate-buffered saline; RFU, relative fluorescence unit; ROS, reactive oxygen species; Δ^9-THC, Δ^9-tetrahydrocannabinol.

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of abuse (Samuelsson 1999; Nocerino *et al*. 2000). The most important representative cannabinoid is Δ^9-tetrahydrocannabinol (Δ^9-THC), which has psychoactive properties depending upon the interaction with the cannabinoid CB_1 receptors within the brain (Pertwee 1997). Good quality hashish contains 4–10% and marijuana 0.1–2.7% Δ^9-THC (Samuelsson 1999). Cannabidiol, the main component of the glandular hairs (up to 15%), is a non-psychoactive cannabinoid; it exerts a plethora of pharmacological effects, including anti-convulsive, sedative, hypnotic, anti-psychotic, anti-nausea and anti-inflammatory actions (Mechoulam *et al*. 2002). Cannabidiol is a potent antioxidant compound and it has been recently proposed to have a neuroprotective role during ischemic damage (Hampson *et al*. 1998, 2000).

The aim of the present study is to evaluate the possible neuroprotective effect of cannabidiol on β-amyloid-induced neurotoxicity. For this purpose we evaluated the effect of cannabidiol on cell viability, reactive oxygen species (ROS) formation and membrane lipoperoxidation, as well as neuronal apoptosis in cultured rat pheochromocytoma PC12 cells exposed to Aβ.

Materials and methods

Materials

All the materials for cell culture were purchased from Biowittaker (Caravaggio, BG, Italy). Fetal calf serum and horse serum were from Hyclone (Logan, UT, USA). Human β-amyloid peptide (fragment 1–42) and CP55,940 {(–)-*cis*-3-[2-hydroxy-4-(–, – dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol)} were from Tocris Cookson (Ballwin, UK). Antibody anti-caspase 3 was from Calbiochem (San Diego, CA, USA). Anti-mouse IgG was from Dako (Glostrup, Denmark). SR141716A [(*N*-piperidin-1-yl)-5-(4-chlorophenyl)-1–2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] was a gift from Dr Madaleine Mosse (SANOFI-Reserche). Cannabidiol, Vitamin E (DL-α-tocopherol) and all the other reagents used, unless otherwise stated, were from Sigma (Milan, Italy). Aβ fragment peptide was dissolved in pyrogen-free water, cannabidiol, vitamin E and SR141716A in dimethylsulfoxide (DMSO). The final DMSO concentration in the assay wells, irrespective of cannabinoid concentration, was always adjusted to 0.01%, a concentration that in preliminary experiments was found to have no effect on the response under study.

Cell culture

PC12 cells (American Tissue Culture Catalogue number CRL1721) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 15% horse serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% CO_2/95% air. Confluent cells were detached, counted and seeded into Petri dishes (10 cm diameter) at a density of 1×10^6 cells/mL and allowed to adhere for 24 h at 37°C. Thereafter, the medium was replaced with fresh medium and cells were treated with Aβ (1 μg/mL) in the presence or absence of cannabidiol (10^{-7}–10^{-4} M, given immediately before Aβ). In some experiments, the effect of cannabidiol (10^{-5} M) was evaluated in the presence of the CB_1 receptor antagonist SR141716A (10 min before cannabidiol). The concentration of Aβ was selected on the basis of our laboratory experience (i.e. a concentration which produced a submaximal effect on cell viability, lipid peroxidation and ROS formation) (data not shown).

MTT cell viability assay

Cell viability was determined using a 3(4,5-dimethylthiazol-2yl)2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay (Mosman 1983). Briefly, the cells were plated in 96-well culture plates at the density of 5×10^3 cells/well and allowed to adhere at 37°C for 2 h. Thereafter, the medium was replaced with fresh medium and the cells were incubated with Aβ peptide (1 μg/mL) in the presence or absence of cannabidiol (10^{-4}–10^{-7} M). After 24 h, 25 μL MTT (5 mg/mL in DMEM) were added and the cells incubated for an additional 3 h at 37°C. After this time, the cells were lysed and the dark blue crystals solubilized with 125 μL of a solution containing 50% (v/v) N,N, dimethylformamide, 20% (w/v) sodium dodecylsulfate, with an adjusted pH of 4.5. The optical density (O.D.) of each well was measured with a microplate spectrophotometer (Titertek Multiscan MCC/340; Labsystem) equipped with a 620 nm filter. The cell viability in response to the treatment with Aβ peptide in the presence or absence of cannabidiol was calculated as percentage of cell viability = (O.D. treated/O.D. control) × 100.

Measurement of reactive oxygen species (ROS)

The formation of ROS was evaluated by means of the probe 2′,7′-dichlorofluorescein (DCF) according to the method described by Bass *et al*. (1983) and Le Bel *et al*. (1992). Briefly, PC12 cells were seeded at a density of 5×10^3 cells/well into 96-well plates and allowed to grow for 48 h. 2′,7′-Dichlorofluorescein-diacetate (H_2DCF-DA, Sigma) was then added directly to the growth medium at a final concentration of 5 μM and the cells incubated for 1 h at 37°C. H_2DCF-DA is a non-fluorescent permeant molecule which diffuses passively into cells; the acetates are then cleaved by intracellular esterases to form H_2DCF which is thereby trapped within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidized to the highly fluorescent DCF. Therefore, cells were washed twice with phosphate-buffered saline (PBS), placed in fresh medium and treated with Aβ (1 μg/mL) with or without cannabidiol (10^{-7}–10^{-4} M) for 24 h. After treatment, cells were washed twice with PBS and the plates placed in a fluorescent microplate reader (LS 55 Luminescence Spectrometer; Perkin Elmer, Beaconsfield, Bucks, UK). Fluorescence was monitored using an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Results were expressed as Relative Fluorescence Units (RFU). In some experiments, the effect of the cannabinoid receptor agonist CP55,940 (10^{-8}–10^{-5} M) was also evaluated.

Lipid peroxidation assay

Malonyl dialdehyde (MDA), as the most abundant lipid peroxidation product from PC12 cells, was measured by the thiobarbituric acid colorimetric assay (Mihara and Uchiama 1978). Briefly, 24 h after treatment with Aβ (1 μg/mL) (in the presence or absence of cannabidiol 10^{-4}–10^{-7} M), the cells were washed three times with 1× PBS, then scraped in 1× PBS containing 0.5 mM EDTA and 1.13 mM butyl-hydroxytoluene. Cell lysis was performed by means of six cycles of freezing and thawing. To 450 μL of cellular lysate was

added 1 mL 10% (w/v) trichloroacetic acid. After centrifugation at 1000 *g* for 10 min, 1.3 mL 0.5% (w/v) thiobarbituric acid were added and the mixture was heated at 100°C for 20 min. After cooling, MDA formation was recorded (Absorbance 530 nm and Absorbance 550 nm) in a Perkin Elmer (Massachussets, MA, USA) spectrofluorimeter and the results are presented as ng MDA/1 $\times 10^6$ cells.

Preparation of cytosolic fractions

Extracts of PC12 cells stimulated for 24 h with Aβ (1 μg/mL) in the presence or absence of cannabidiol (10^{-7}–10^{-4} M) were prepared as previously described (Esposito *et al*. 2002). Briefly, harvested cells (1×10^6) were washed twice with ice-cold PBS and centrifuged at 180 *g* for 10 min at 4°C. The cell pellet was resuspended in 100 μL of ice-cold hypotonic lysis buffer [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1.5 μg/mL soybean trypsin inhibitor, pepstatin A 7 μg/mL, leupeptin 5 μg/mL, 0.1 mM benzamidine, 0.5 mM dithiothreitol (DTT)] and incubated in ice for 15 min. The cells were lysed by rapid passage through a syringe needle five or six times and the cytoplasmic fraction was then obtained by centrifugation at 13 000 *g* for 1 min.

Western blot analysis

Immunoblotting analysis of caspase 3 protein was performed on a cytosolic fraction of PC12 cells treated as described (Samali *et al*. 1999). Cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol 2-mercaptoethanol, 2 mg bromophenol/ml) in a ratio of 1 : 1, boiled for 5 min and centrifuged at 10 000 *g* for 10 min. Protein concentration was determined and equivalent amounts (50 μg) of each sample were separated under reducing conditions in 12% SDS–polyacrylamide minigel. The proteins were transferred onto nitrocellulose membrane according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked by incubation at 4°C overnight in high salt buffer (50 mM Trizma base, 500 mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin; they were then incubated for 1 h with anti-caspase 3 antibody (1 : 2000) (Chemicon, Temecula, CA, USA) for 1 h at room temperature, followed by incubation with horseradish peroxidase (HRP)-conjugate secondary antibody (Dako). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham, Cologno Monzese, Italy) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of caspase 3 and pro-caspase 3 on X-ray film were scanned and densitometrically analysed with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). Results are expressed as the ratio pro-caspase/caspase 3 (a low ratio is indicative of apoptosis).

DNA fragmentation analysis

To prepare cellular DNA, PC12 cells (4×10^6, incubated for 24 h with or without Aβ, alone, or in presence of cannabidiol 10^{-5} M and 10^{4} M) were detached from 10 cm ∅ culture dishes and the cell suspension was centrifuged at 100 *g* for 10 min (Gschwind and Huber 1995). The cell pellet was then washed twice with ice-cold PBS and cellular DNA was isolated using a DNA isolation kit (Roche, Basel, Switzerland) according the manufacturer's instructions. A 20 μL aliquot of each DNA sample was analysed on a 1.5% agarose gel containing ethidium bromide (1 μg/mL) in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA) and run for 90 min at 70 V. After electrophoresis, the DNA was visualized under UV light and photographed.

Measurement of intracellular calcium levels

Changes in intracellular free calcium levels ($[Ca^{2+}]_i$) in a suspension of PC12 cells were determined by the fura-2 method (Sandirasegarane *et al*. 1994; Zhou *et al*. 1996). Upon binding calcium, fura-2 shifts its excitation wavelength from 380 to 340 nm, and the ratio of the fluorescence caused by excitation at 340 nm to that at 380 nm ($R_{340/380}$) reflects changes in $[Ca^{2+}]_i$ (Zhou *et al*. 1996). PC12 cells were incubated with 5 μM fura-2/acetoxymethyl ester (fura-2/AM) in HBH (concentration in mM: NaCl 137, KCl 5.4, $MgCl_2$ 0.49, KH_2PO_4 0.44, Na_2HPO_4 0.64, $NaHCO_3$ 3, glucose 5.5, $CaCl_2$ 1.26 and HEPES 20, pH 7.4) in a 37°C incubator for 60 min, and washed twice before taking an aliquot for $[Ca^{2+}]_i$ measurement. Each aliquot contained 5×10^3 cells suspended in a total volume of 200 μL HBH. Basal $[Ca^{2+}]_i$ values were obtained after 2 min of equilibration and $[Ca^{2+}]_i$ measurements were performed 2, 5, 10 and 15 min after Aβ administration. As Aβ produced a maximal increase 5 min after its administration, the effect of cannabidiol (10^{-6}–10^{-4} M, administered 15 min before Aβ 1 μg/mL) or vitamin E (10^{-5} M, used as a reference compound) was assessed at this time-point. Measurements were performed with a spectrofluorometer with an LS55 luminescence spectrometer (Perkin Elmer).

Statistical analysis

Results were expressed as the mean ± SEM of *n* experiments. Statistical analysis was determined with ANOVA and multiple comparisons were performed by Bonferroni's test, with $p < 0.05$ considered significant.

Results

Cell viability

MTT assay was used to detect cell viability. Incubation of PC12 cells with Aβ (1 μg/mL) for 24 h caused 38.8 ± 2.2% of cell death (Fig. 1). Cannabidiol (10^{-7}–10^{-4} M), added to the cells immediately before treatment with Aβ, significantly reduced cell death. The effect of cannabidiol was significant for all the concentrations tested. The cannabinoid receptor agonist CP55,940 also reduced Aβ-induced cell death (cell death: Aβ 37.0 ± 3.8; CP55,940 10^{-8} M + Aβ 13.6 ± 7.9; CP55,940 10^{-7} M + Aβ 21.7 ± 9.0; CP55,940 10^{-6} M + Aβ 8.5 ± 4.2, $p < 0.05$, CP55,940 10^{-5} M + Aβ 6.6 ± 3.8, $p < 0.05$, $n = 3$–4).

Measurement of reactive oxygen species (ROS)

ROS accumulation, estimated using a converting reaction of the probe $DCFH_2$ to DCF, was significantly ($p < 0.001$) increased after incubation with Aβ (1 μg/mL), as indicated by the increase in RFU (Fig. 2). Incubation of PC12 with cannabidiol (10^{-7}–10^{-4} M) significantly inhibited Aβ-induced increase in ROS accumulation (Fig. 2). The effect of cannabidiol (10^{-5} M) was not significantly modified by the cannabinod CB1 receptor antagonist SR141716A (10^{-5} M) (RFU: control 53 ± 5; Aβ 146 ± 11; cannabidiol +

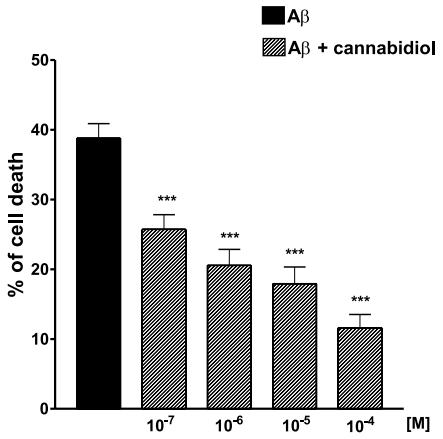


Fig. 1 Effect of cannabidiol (10^{-7}–10^{-4} M) on β-amyloid (Aβ, 1 μg/mL)-induced cell death. Cell death, evaluated by the reduction of the tetrazolium salt MTT, was assessed 24 h after incubation with Aβ. Cannabidiol was added immediately before Aβ. Results, expressed as the percentage of cell death, are the means ± SEM of five experiments in triplicate. Untreated cells were assumed to be vital (100% viability). ***$p < 0.001$ versus Aβ.

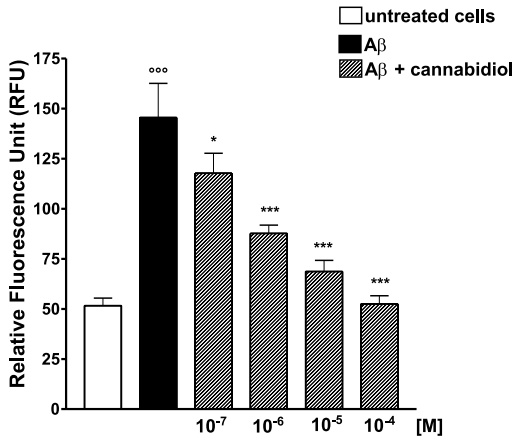


Fig. 2 Effect of cannabidiol (10^{-7}–10^{-4} M) on β-amyloid (Aβ, 1 μg/mL)-induced formation of reactive oxygen species (ROS). ROS formation, evaluated by the oxidation of 2′,7′-dichlorofluorescein (H_2DCF) to the fluorescent 2′,7′-dichlorofluorescein (DCF), was assessed 24 h after incubation with Aβ. Cannabidiol was added immediately before Aβ. Results are the means ± SEM of six experiments in triplicate. °°°$p < 0.001$ versus control (untreated cells); *$p < 0.05$ and ***$p < 0.001$ versus Aβ.

Aβ 61 ± 11; Aβ + cannabidiol + SR141716A 66 ± 6; $n = 5$). Given alone, SR141716A (10^{-5} M) did not modify ROS accumulation either in untreated cells or in cells incubated with Aβ (data not shown). Cannabidiol did not affect viability in untreated cells (data not shown).

Lipid peroxidation assay

The thiobarbituric acid colorimetric assay, which measures malondialdehyde (MDA) levels, was used to quantify lipid peroxidation. Exposure of PC12 cells to Aβ (1 μg/mL) caused a significant increase in MDA levels (indicating that lipid peroxidation occurs) (Fig. 3). Lipid peroxidation of Aβ-treated cells was concentration-dependently reduced by cannabidiol (10^{-7}–10^{-4} M). The effect of cannabidiol (10^{-5} M) was not significantly modified by the cannabinod CB_1 receptor antagonist SR141716A 10^{-5} M (MDA ng/10^6 cells: untreated cells 31 ± 3; Aβ 85 ± 4; cannabidiol + Aβ 45 ± 4; Aβ + cannabidiol + SR141716A 42 ± 3; $n = 5$). Given alone, SR141716A (10^{-5} M) did not change MDA levels either in untreated cells (MDA ng/10^6 cells: untreated cells 31 ± 3; SR141716A 32 ± 3; $n = 5$) or cells incubated with Aβ (MDA ng/10^6 cells: Aβ 85 ± 4; Aβ + SR141716A 81 ± 4; $n = 5$).

Apoptosis assay

We evaluated the appearance of the caspase 3 band in a cellular extract of PC12 cells by western blot analysis as a hallmark of apoptosis. In our time-course experiments, we showed that PC12 cells start to undergo to apoptosis 4 h after treatment with Aβ (1 μg/mL), but a marked apoptosis was evident at 6 h after treatment (Fig. 4a). Analysis of the pro-caspase/total caspase 3 ratio showed that Aβ significantly induced apoptosis (as indicated by the decreased ratio) 6 h after treatment, and this increase was significantly counteracted by cannabidiol (10^{-4} M) (Fig. 4b). These results suggest that apoptosis in Aβ-treated PC12 cells is mediated by caspase 3, and the protective action of cannabidiol may, at least in part, be attributed to inhibition of the caspase cascade.

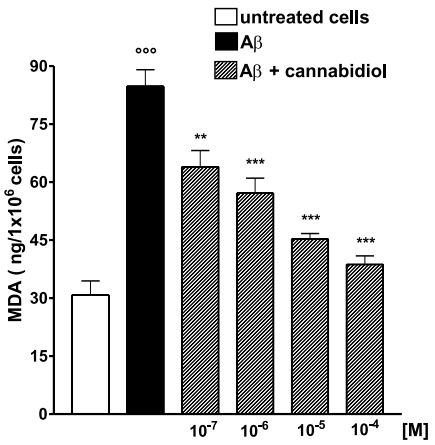


Fig. 3 Effect of cannabidiol (10^{-7}–10^{-4} M) on β-amyloid (Aβ, 1 μg/mL)-induced lipid peroxidation. Lipid peroxidation, evaluated by the thiobarbituric acid colorimetric assay [which measures malondialdehyde (MDA)], was assessed 24 h after incubation with Aβ. Cannabidiol was added immediately before Aβ. Results, expressed as concentration of MDA, are the means ± SEM of six experiments in triplicate. °°°$p < 0.001$ versus control (untreated cells); **$p < 0.01$ and ***$p < 0.001$ versus Aβ.

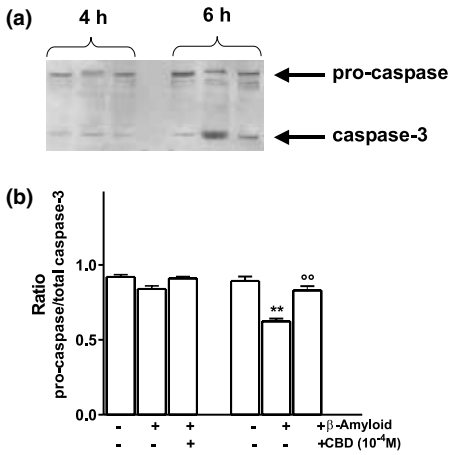


Fig. 4 Effect of cannabidiol (CBD, 10^{-4} M) on β-amyloid (1 μg/mL)-induced caspase 3 activation. (a) Representative western blot analysis of caspase 3 activation from its inactive precursor, pro-caspase 3, in PC12 cell homogenate treated with and without Aβ and in the presence of cannabidiol; arrows indicate cleaved (activated) caspase 3 at about 17 kDa and its precursor, pro-caspase, at about 43 kDa. (b) Ratio pro-caspase/total caspase 3 (a decreased ratio is indicative of apoptosis). $^{**}p < 0.01$ versus control cells (at 6 h), $^{\circ\circ}p < 0.01$ versus β-amyloid (at 6 h), $n = 3$ separate experiments.

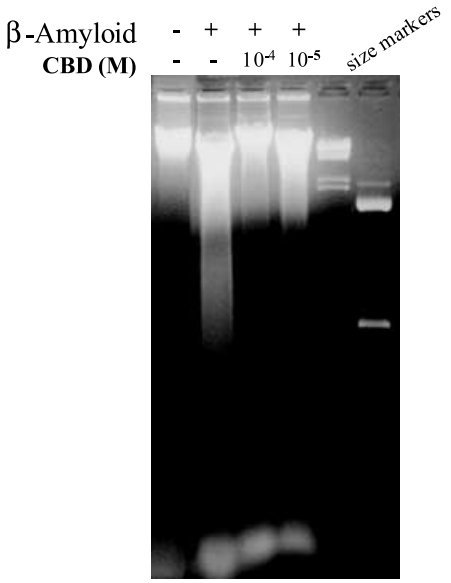


Fig. 5 DNA fragmentation in PC12 untreated cells and in PC12 cells treated with β-amyloid (1 μg/mL, alone or in the presence of cannabidiol 10^{-4} M and 10^{-5} M). Cellular DNA was extracted and visualized on agarose gel as described in the Materials and methods section. Each lane was loaded with the same amount of DNA. DNA size markers used were: (left) phage lambda DNA digested with Hind III restriction enzyme; (right) pBR322 DNA digested with Hinf I restriction enzyme.

DNA fragmentation

Experiments on DNA fragmentation are shown in Fig. 5. Untreated PC12 cells showed no detectable DNA fragmentation. By contrast, DNA extracted from PC12 cells after 24 h of exposure to Aβ was clearly fragmented. Pretreatment with cannabidiol (10^{-5} M and 10^{-4} M) reduced Aβ-induced DNA ladders, the effect being more evident for the 10^{-4} M concentration.

Intracellular calcium levels

Aβ (1 μg/mL) produced a significant increase in $[Ca^{2+}]_i$ 2–15 min after its administration, with a maximal effect after 5 min (data not shown). Therefore, the effects of cannabidiol were assessed at this time point. Aβ-induced changes in $[Ca^{2+}]_i$ were reduced by both cannabidiol ($R_{340/380}$: basal 0.90 ± 0.02; Aβ 1.20 ± 0.06; Aβ + cannabidiol 10^{-6} M 1.10 ± 0.07; Aβ + cannabidiol 10^{-5} M 1.08 ± 0.07; Aβ + cannabidiol 10^{-4} M 0.91 ± 0.07; $n = 3$–4, $p < 0.05$ for the 10^{-5} M and 10^{-4} M concentrations) and vitamin E 10^{-5} M ($R_{340/380}$: basal 0.90 ± 0.02; Aβ 1.20 ± 0.06; Aβ + vitamin E 0.93 ± 0.03; $n = 3$, $p < 0.05$).

Discussion

The accumulation of plaques containing Aβ is an invariant feature of AD pathology and there is abundant evidence suggesting that Aβ contributes to the aetiology of AD (Carlson 2003; Golde 2003). In the present paper we report that cannabidiol, a major non-psychotropic component of marijuana, prevents Aβ-induced neurotoxicity, ROS production and lipid peroxidation; it also inhibits caspase 3 appearance (from the inactive precursor pro-caspase) in PC12 cells.

The MTT assay has been widely used as an index of cell survival and proliferation. Previous investigators have shown that cannabidiol produces a modest or no reduction in C6 glioma cell viability after 6 days of incubation (Jacobsson *et al*. 2000). Here, we have shown that exposure of PC12 cells to Aβ produces a reduction in cell viability, and that cannabidiol protects, in a concentration-dependent manner, Aβ-induced neurotoxicity. Consistent with these results, cannabidiol has been shown to reduce glutamate-, N-methyl-D-aspartate- and kainate-induced toxicity in rat cortical neurones (Hampson *et al*. 1998).

The cellular events involving free radical oxidative stress may be one basic pathway leading to cell degeneration. Increased oxidative stress and disturbed defensive mechanisms occur in the brain of AD patients, which might result in a self-propagating cascade of neurodegenerative events (Markesbery 1997). ROS have been shown to be involved in cell damage and death induced by amyloid peptides (Mattson *et al*. 1993; Brera *et al*. 2000) and antioxidant therapy has led to an improvement in AD patients. Excessive ROS can lead to a number of detrimental effects, including lipid peroxidation. In our experiments, PC12 cells exhibited

increased ROS levels and lipid peroxidation following exposure to Aβ peptide. The ability of Aβ to increase both ROS and lipid peroxidation has been documented previously in a number of neuronal cell types, including PC12 cells (Xiao *et al*. 2002; Onoue *et al*. 2002; Guan and Nordberg 2003). However, we have shown for the first time that cannabidiol reduced, in a concentration-dependent manner, Aβ-induced ROS accumulation and lipid peroxidation. Since antioxidants are known to attenuate Aβ-induced oxidative injury (Cash *et al*. 2002), it is likely that the previously reported antioxidant properties of cannabidiol could contribute to its beneficial effect. Other studies have shown that cannabidiol antagonizes the oxidative stress induced by retinoid anhydroretinol in lymphoblastoid cells (Chen and Buck 2000) and prevents hydrogen peroxide-induced oxidative damage in neuronal cultures (Hampson *et al*. 1998).

In contrast to Δ^9-THC, cannabidiol is not a cannabinoid receptor agonist (Mechoulam *et al*. 2002); nevertheless, cannabidiol may enhance the endocannabinoid action at CB_1 receptors by its ability to block the uptake and the enzymatic degradation of the endocannabinoid anandamide (Bisogno *et al*. 2001). Furthermore, Petitet *et al*. (1998) have shown that cannabidiol, in the micromolar range, behaves as a CB_1 antagonist in the rat brain. The importance of CB_1 receptors in neuroprotection has been previously documented (Grundy *et al*. 2001; Van der Stelt *et al*. 2001; Marsicano *et al*. 2002). Milton (2002) showed that the endogenous cannabinoids anandamide and noladin ether have a protective effect on Aβ-induced toxicity through activation of CB_1 receptors in human teratocarcinoma cells. However, it is very unlikely that the protective (anti-oxidative) effect of cannabidiol observed here involves CB_1 receptors as the potent and selective CB_1 receptor antagonist, SR141716A, at a concentration previously shown to be fully effective (Esposito *et al*. 2001, 2002), did not show a protective effect (when administered alone), nor did it modify the protective action of cannabidiol. In agreement with our results, Molderings *et al*. (2002) showed (by using binding and PCR techniques) that rat pheochromocytoma PC12 cells are not endowed with CB_1 receptors. It is interesting to note that in these cells, we have shown that the cannabinoid agonist CP55,290 also has a protective effect on Aβ-induced toxicity, thus confirming the existence of a non-CB_1 mediated protective effect of cannabinoids.

Several experimental studies suggest an association between deposition of Aβ, oxidative stress and apoptosis associated with AD and ageing (Kokoszka *et al*. 2001; Marx 2001). The Aβ peptide has been shown to induce apoptosis in neurones, including PC12 cells, which may be responsible for neuronal death in AD (Martin *et al*. 2001; Troy *et al*. 2001). Apoptosis is associated with the activation of a family of aspartic acid-specific cysteine proteases, referred to as caspases (Nicholson and Thornberry 1997). There are at least 14 caspases identified in mammalian cells that are synthesized as inactive precursor molecules, pro-caspase, and are converted by proteolytic cleavage to the active heterodimer (Thornberry and Lazebnik 1998). The activation of pro-caspase 3 to caspase 3 is a central event in the execution phase of apoptosis and appears to serve as the convergence point of different apoptotic signalling pathways (Nicholson and Thornberry 1997). In the present study, we have shown that cannabidiol increased pro-caspase 3 levels and, in parallel, reduced caspase 3 levels in Aβ-treated PC12 cells, suggesting that the cannabinoid could exert a protective role at the execution phase of apoptosis. The inhibitory effect of cannabidiol on apoptosis was strengthened by the observation that this cannabinoid inhibited the fragmentation of DNA (a hallmark of apoptosis) induced by Aβ. The fragmentation of DNA after Aβ has been previously documented (Gschwind and Huber 1995).

The pharmacological manipulation of neuronal calcium homeostasis may be a useful therapeutic approach in neurodegenerative pathologies, including AD, and a rise in $[Ca^{2+}]_i$ has been suggested to be responsible for toxic effects of Aβ (Mattson 2002). Previous investigators have shown that Aβ-induced neurotoxic actions in PC12 cells are associated with an increase in $[Ca^{2+}]_i$ and that this effect is blocked by antioxidants such as vitamin E (Zhou *et al*. 1996). In addition, it has been suggested that the increase in $[Ca^{2+}]_i$ induced by Aβ in PC12 cells is not the result of an influx of calcium through voltage-dependent calcium channels, but rather it is the result of a free radical-mediated process. In the present study we have shown that cannabidiol counteracted the Aβ-induced increase in $[Ca^{2+}]_i$, which is in agreement with the antioxidant properties of this cannabinoid reported here and elsewhere (Hampson *et al*. 1998).

In summary, the results reported herein indicate, for the first time, that cannabidiol exerts a combination of neuroprotective, anti-oxidative and anti-apoptotic effects against Aβ insult. Inhibition of the activity of the apoptotic enzyme caspase 3 is likely to be involved in signalling pathways for the neurotrophic effect of cannabidiol. Given the low toxicity of cannabinol shown in humans (Cunha *et al*. 1980; Consroe *et al*. 1991), this non-psychoactive component of marijuana may play an important role in counteracting neuronal cell death occurring in Alzheimer's disease.

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