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Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1

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Abstract

Neuroprotective effects have been described for many cannabinoids in several neurotoxicity models. However, the exact mechanisms have not been clearly understood yet. In the present study, antioxidant neuroprotective effects of cannabinoids and the involvement of the cannabinoid receptor 1 (CB1) were analysed in detail employing cell-free biochemical assays and cultured cells. As it was reported for oestrogens that the phenolic group is a lead structure for antioxidant neuroprotective effects, eight compounds were classified into three groups. Group A: phenolic compounds that do not bind to CB1. Group B: non-phenolic compounds that bind to CB1. Group C: phenolic compounds that bind to CB1. In the biochemical assays employed, a requirement of the phenolic lead structure for antioxidant activity was shown. The effects paralleled the protective potential of group A and C compounds against oxidative neuronal cell death using the mouse hippocampal HT22 cell line and rat primary cerebellar cell cultures. To elucidate the role of CB1 in neuroprotection, we established stably transfected HT22 cells containing CB1 and compared the protective potential of cannabinoids with that observed in the control transfected HT22 cell line. Furthermore, oxidative stress experiments were performed in cultured cerebellar granule cells, which were derived either from CB1 knock-out mice or from control wild-type littermates. The results strongly suggest that CB1 is not involved in the cellular antioxidant neuroprotective effects of cannabinoids.

Keywords: CB1 knock-out, cell lines, oxidative stress, primary neuronal cultures.

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Neuroprotective effects have been proposed for natural, synthetic and endogenous cannabinoids in several *in vitro* and *in vivo* neurotoxicity models (e.g. Hampson *et al*. 1998; Nagayama *et al*. 1999; Sinor *et al*. 2000). Although most of the actions of cannabinoids in the central nervous system appear to be exerted by the 'brain type' cannabinoid receptor 1, CB1 (Matsuda *et al*. 1990; Ledent *et al*. 1999; Zimmer *et al*. 1999), some cannabinoids such as the non-CB1-binding components of *Cannabis sativa*, cannabinol and cannabidiol, were also shown to protect cells from oxidative stress (Hampson *et al*. 1998; Chen and Buck 2000). These observations would indicate a completely CB1-independent mechanism of neuroprotection of cannabinoids. Indeed, for many natural and synthetic phenolic compounds, a protective activity against oxidative stress, independent of any specific receptor-mediated action, has recently been proposed, e.g. for oestrogens (Moosmann and Behl 1999). However, CB1 has been implicated in some neuroprotective mechanisms. WIN 55,212-2, a potent CB1 agonist belonging to the family of aminoalkylindoles, exerted potent neuroprotection in *in vivo* rat models of global and focal ischaemia (Nagayama *et al*. 1999), which was blocked by the pre-administration of the specific CB1 antagonist SR 141716A, suggesting an involvement of CB1 in this particular paradigm.

In the present study, the antioxidant neuroprotective effects of cannabinoid compounds and the involvement of CB1 in

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Abbreviations used: AIBN, 2,2′-azo-bis-(2-methylpropionamidine); AM 404, all-Z Eicosa-5,8,11,14-tetraenoic acid (4-hydroxy-phenyl)-amide; CB1, cannabinoid receptor 1; CBN, CB1-null mutant mouse line; CP 55,940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol; Δ^9-THC, Δ^9-tetrahydrocannabinol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FSK, forskolin; HU 210, (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; IBMX, 3-isobutyl-1-methylxanthine; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-naphtalenylmethanone.

these effects were analysed in detail, employing cell-free biochemical assays and cell culture models of oxidative stress and neurodegeneration. First, several cannabinoid compounds were classified on the basis of their chemical structure and according to their ability to bind to CB1, and a structure–activity relationship analysis was performed in different cell-free biochemical assays to test their chemical antioxidant potential. Their neuroprotective effects were then tested in cellular models of oxidative stress, i.e. in neuronal cell lines and in rat primary cerebellar granule cells. Subsequently, in order to analyse the involvement of CB1 in the neuroprotective effects of various cannabinoid compounds, two genetic approaches were adopted. First, cannabinoids belonging to different subgroups (classed on their ability to activate CB1 and their chemical antioxidant properties) were tested in oxidative cell death assays, using cell lines stably transfected with CB1 cDNA and control-transfected cells lacking CB1. Second, a potent CB1 agonist (CP 55,940) was tested as neuroprotective agent in primary cerebellar granule cell cultures derived either from CB1 knock-out mice or from wild-type littermate controls. We found that CB1 is not directly involved in the mechanism(s) by which antioxidant cannabinoids protect cells from oxidative stress *in vitro*.

Materials and methods

Chemicals

Cannabinoids (Δ^9-THC, cannabinol and cannabidiol) were purchased from Sigma (Deisenhofen, Germany), CP 55,940, WIN 55,212-2, methanandamide, HU 210 and AM 404 were purchased from Tocris (Cologne, Germany) and SR 141716A was from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program. These compounds were prepared as 10 mM stock solutions in 100% ethanol (Δ^9-THC, cannabinol, cannabidiol and methanandamide) or in 100% dimethylsulfoxide (DMSO; CP 55,940, WIN 55,212-2, HU 210, AM 404 and SR 141716A). IBMX and forskolin (FSK), were purchased from Sigma and were prepared as 200 mM and 10 mM stock solutions in DMSO, respectively.

Animals

Sprague–Dawley rats and mice with a deficiency in the CB1 gene were used for the study. The generation of the CB1-deficient mouse line CBN/CBN, lacking the entire CB1 open reading frame, will be described elsewhere (G. Marsicano *et al.* in preparation). For genotyping of the CBN allele, PCR was performed with DNA derived from tails of 1–2-day-old-pups. Wild-type (WT) and homozygous CBN/CBN mice (CB1 KO) were used for the experiments. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

Biochemical oxidation assays

Brain lipid oxidation assays

For the preparation of native brain lipids, dissected cerebral cortex of adult Sprague–Dawley rats was homogenized in 3 volumes of degassed lipid buffer (20 mM Tris–HCl, 1 mM $MgCl_2$, 5 mM KCl, pH 7.4) with a Kontes glass homogenizer (all preparative steps were performed at 4°C). After centrifugation (3000 *g*, 5 min), the pellet was solubilized by sonication in 3 volumes of lipid buffer supplemented with 0.5 M NaCl, incubated for 10 min and centrifuged (100 000 *g*, 20 min). This step was repeated and followed by three washing steps using 3 volumes of degassed water instead of lipid buffer. Finally, the pellet was resuspended in water at a concentration of 5 mg/mL protein, snap-frozen in liquid nitrogen and stored at −80°C.

For the oxidation assay, the rat brain membrane preparation was diluted with phosphate-buffered saline (PBS) to a concentration of 0.6 mg/mL protein and sonicated. Cannabinoids to be tested were added to the 1-mL aliquots at various concentrations and the oxidative chain reaction was started by adding 50 μM ascorbate and incubation at 37°C. Six hours later, single photon counting was done for 1 min in a Beckman scintillation counter set in the visible light range. Data were corrected for the baseline photocurrent and normalized to control values.

Low-density lipoprotein oxidation assays

The oxidation of human blood plasma low-density lipoprotein (LDL) was essentially performed as described (Moosmann and Behl 1999). In brief, fresh human LDL (0.1 mg/mL protein, diluted in PBS supplemented with 0.5 mM $MgCl_2$) was oxidized catalytically by the addition of 10 μM $CuSO_4$ at 37°C with or without the concomitant addition of the cannabinoids. The reaction products of LDL decomposition (conjugated dienes) were measured photometrically as an increase in UV absorption at 234 nm. Results were expressed as percentage (mean ± SEM) of the increase in absorption after 1 h of the cannabinoid-treated samples versus the vehicle-treated samples; the absorption of the blank samples (without oxidant) at 234 nm did not change during the assay time; the same applied to all the measured samples at a wavelength of 600 nm, indicating a stable LDL sample solution.

Peroxyl radical scavenging assays

To establish the direct radical scavenging properties of the cannabinoids in aqueous medium, they were challenged by a hydrophilic azo-initiator of free peroxyl radicals, 2,2′-azo-bis-(2-methylpropionamidine), AIBN. The carbon-centred radicals initially formed by the thermal decomposition of this compound react very fast with dissolved aqueous oxygen to form peroxyl radicals which in turn attack nucleophilic sites on biomolecules. As a biological indicator of the free peroxyl radical load, the phycobilinosomal fluorescent protein *Porphyrium cruentum* B-phycoerythrin was employed, and the temporal decrease in protein fluorescence was measured as a consequence of the free radical-induced structural decomposition of the protein. 10 nM B-phycoerythrin in PBS were oxidized employing 500 μM AIBN at 37°C; the temporal decrease in intrinsic protein fluorescence was quantified by a flash fluorimetry setting (excitation window 340 ± 50 nm, emission window 572 ± 6 nm, counting delay 25 μs, counting window 30 μs, number of repeats per sample 1000). Under the employed conditions, no significant photobleaching occurred, and the control protein kept stable for several hours. Results were calculated by comparing the decrease in fluorescence of the cannabinoid-treated samples versus the not treated samples (AIBN only) at three measuring times, after

190, 200 and 210 min. Data are presented as the grand mean ± SEM of all individual measurements (quadruplicate determinations at each measuring time).

Cell cultures

Cell lines

The neuronal cell lines PC12 and HT22 (kind gift of Dr Dave Schubert, Salk Institute, San Diego, CA, USA) were used for oxidative stress experiments. Cells were cultured in complete DMEM at 37°C, 5% CO_2 in humidified atmosphere. Complete DMEM consists of Dulbecco's modified Eagle's medium (Life Technologies Gibco, Karlsruhe, Germany), supplemented with 15% (PC12) or 10% (HT22) fetal calf serum (FCS, Life Technologies Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (100× penicillin/streptomycin solution, Life Technologies Gibco). Cells were kept on 10-cm cell culture dishes. One day prior to the experiment, cells were plated onto 96-well plates at a density of 10^4 cells/well (PC12 cell line), 10^3 cells/well (HT22 cell line), each well containing 100 μL of complete DMEM.

Primary cerebellar granule cells

Primary cerebellar granule cells were obtained from newborn Sprague–Dawley rats (3 days old) and from newborn mice (3–6 days old). The procedures for isolation and culture of primary cerebellar granule cells were identical for rats and mice. Newborn animals were decapitated, and cerebella were isolated and put into ice-cold, sterile DMEM containing 100 U/mL penicillin/streptomycin. Cerebella were dissected free of meninges and put into a 50-mL Falcon tube containing 10 mL of cold DMEM. Then, 10 mL of 0.1% Trypsin/1 mM EDTA were added to the tube. Cerebella were incubated with gentle shaking for 10–20 min at 37°C and then pipetted up and down first with a 5-mL plastic pipette and then with a Pasteur glass pipette. Tissues were centrifuged at 500 *g* for 4 min and then resuspended in 10 mL complete DMEM medium. After a new centrifugation and resuspension in 1 mL of complete DMEM, 100 μl of cells were plated onto poly-L-lysine-treated (Sigma) 96-wells plates, at a density of about 10^5 cells/well. After 2 days of incubation, the cytostatic drug cytosine arabinofuranoside (Sigma) was added to each well at a final concentration of 10 μM, in order to block the growth of fibroblasts and glial cells. After 10–15 days of incubation, the cultures appeared to contain > 90% neurons and were used in oxidative stress experiments.

Establishment of CB1-expressing cell lines

The mouse CB1 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, the Netherlands), which was then used for the stable transfection of CB1 into HT22 cells. Cells were electroporated with 1 μg/μL of linearized plasmid and selected with 3.5 mg/mL geneticin (G418, Life Technologies Gibco), following standard procedures.

Northern blot analysis

RNA extraction from transfected clonal cells was performed using the PeqGold RNApure kit (PeqLab, Erlangen, Germany), following the manufacturer's instructions. RNA samples were electrophoresed in a 1% agarose/6% formaldehyde gel under standard conditions (Sambrook *et al*. 1989). After electrophoresis, capillary blotting was performed in 10 × saline–sodium citrate buffer (SSC), as described in Sambrook *et al*. (1989) to transfer RNA onto Hybond NX membranes (Amersham/Pharmacia, Freiburg, Germany). Hybridization was performed using a mouse CB1-specific probe.

cAMP accumulation assay

One day before the experiment, cell clones expressing CB1 mRNA were plated onto 48-well plates in 500 μL of complete DMEM at the density of 2×10^5 cells/mL. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Then, 0.5 mM IBMX (Sigma, Deisenhofen, Germany) was added 5 min before the initiation of the reaction to prevent the degradation of accumulated cAMP. Cells were pre-incubated with WIN 55,212-2 alone or in combination with the CB1 antagonist SR 141716A for 1 h. Then, 5 μM forskolin (FSK, Sigma) were added. The reaction was terminated 1 h later by aspiration of the medium and addition of 500 μL ice-cold 6% trichloroacetic acid followed by an incubation overnight at 4°C. CB1 receptor ligands were dissolved in DMSO. DMSO alone served as a vehicle control and had no effect on cAMP accumulation (data not shown). To remove the trichloroacetic acid, the extracts were treated twice with 3 mL diethylether, dried overnight in a lyophilisator and reconstituted in DMEM. Intracellular cAMP levels were measured with a competitive protein binding assay following the manufacturer's recommendations (non-acetylated procedure; NEN Life Science Products, Inc., Boston, MA, USA). Data obtained in the cAMP accumulation assays were expressed as percentage of FSK-stimulated cAMP accumulation. Samples were measured in duplicate in two independent experiments. Data are given as mean percentages with standard error of mean (SEM).

Oxidative stress toxicity assays

The day before the experiment, cell lines were plated onto 96-well plates as described above. Cannabinoids were prepared as predilutions in ethanol or DMSO and added to the wells. All wells contained a final concentration of 1% ethanol or 1% DMSO. Experiments were performed in triplicate or quadruplicate and were repeated at least twice for each cell clone or primary cell culture. After adding the drug or vehicle, cells were incubated overnight, and then different concentrations of H_2O_2 were added to obtain final concentrations ranging from 60 to 250 μM. After overnight incubation, 10 μL of 5 mg/mL of dimethylthiazolyl-diphenyl-tetrazolium bromide in H_2O (MTT, Sigma) were added to each well, and the plates were incubated at 37°C for 4 h. Then, 100 μL of cell lysis solution [45% dimethylformamide, 10% sodium dodecyl sulfate (SDS), pH 4.2] were added to each well. Lysis was done overnight at room temperature, and then the plates were read with a Dynatec microplate reader (Dynatec, El Paso, TX, USA) set at 570 nm. Decreased cell survival was indicated by a decreased MTT reduction, and thus, by a decreased absorption at 570 nm. Data were calculated as relative protection and are given as averages of the triplicate or quadruplicate experiments with SEM.

Results

Cannabinoids as antioxidant neuroprotective agents

Eight compounds were tested in biochemical oxidation experiments: four 'classical cannabinoids' (Δ^9-THC,

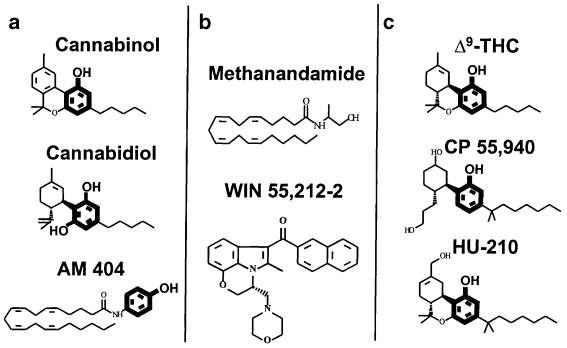

Fig. 1 Classification of cannabinoids on the basis of CB1 binding and the presence of phenolic moieties (in bold). (a) Phenolic cannabinoids with no or very low affinity for CB1. (b) Non-phenolic compounds with high affinity for CB1. (c) Phenolic cannabinoids with high affinity for CB1.

cannabinol and cannabidiol as *Cannabis sativa* derivatives, and the synthetic HU 210); one synthetic 'non-classical cannabinoid' (CP 55,940); one aminoalkylindole (WIN 55,212-2); one metabolically stable synthetic analogue of anandamide (methanandamide); one inhibitor of 'anandamide transporter' (AM 404). These compounds can be classified into three groups, based on whether or not they have a phenolic structure and whether or not they bind to cannabinoid receptors: phenolic compounds that do not bind to CB1 (Fig. 1a); non-phenolic compounds that bind to CB1 (Fig. 1b); and phenolic compounds that bind to CB1 (Fig. 1c).

The antioxidant properties of these compounds were evaluated in various biochemical assays (Fig. 2a–c). Compounds containing a phenolic group, such as cannabinol, cannabidiol and AM 404 (not binding to CB1, Fig. 2a1), and Δ^9-THC, CP 55,940 and HU 210 (binding to CB1, Fig. 2a3) were shown to be potent antioxidants in the brain lipid oxidation assay. However, cannabinoids not containing a phenolic ring, such as methanandamide and WIN 55,212-2 did not show any antioxidant activity in the same assay (Fig. 2a2). Similar results were obtained in the blood plasma LDL oxidation assay (Fig. 2b), with one exception: cannabinol was not protective. This discrepancy could be due to intrinsic molecular characteristics of cannabinol, which contains a biphenyl moiety that confers rigidity to the molecule and may prevent the penetration into the LDL particles. Figure 2(c) shows that all compounds were unable to protect globular proteins from oxidative destruction in aqueous medium, as it was expected from the lipophilic character of the compounds.

Chemical antioxidant properties often reflect the protective potential of the tested compounds in oxidative toxicity experiments in HT22 cells, as shown for oestrogenic drugs (Moosmann and Behl 1999). Therefore, this notion was tested here for cannabinoids. Indeed, the same compounds that showed biochemical antioxidant properties (Fig. 2a1 and a3) also exhibited potent cytoprotection from H_2O_2-induced oxidative cell death in HT22 cells (Fig. 2d1 and d3), whereas the non-phenolic compounds were not able to protect the cells from oxidative cell death (Fig. 2d2). Similar results were obtained in oxidative stress assays conducted on PC12 cells (data not shown). Thus, phenolic cannabinoids are antioxidant, cytoprotective drugs.

Cannabinoids are neuroprotective in rat cerebellar granule cells

As HT22 and PC12 cells do not express CB1 (see below, Fig. 4 and data not shown), it is not possible to analyse the participation of CB1 to the neuroprotective activity of cannabinoids by experiments conducted using these cell lines. Therefore, as a source of CB1-expressing primary neurons, rat cerebellar granule cell cultures, which are known to contain CB1 protein (e.g. Hillard *et al*. 1999), were assessed and tested in similar oxidative stress paradigms. Three CB1 agonists were chosen for these tests, the non-phenolic compound methanandamide, and the two phenolic compounds CP 55,940 and HU 210. Figure 3(a) shows that CP 55,940 and HU 210 possess similar neuroprotective potentials in cerebellar neurons as in HT22 cells (Fig. 2d3). These results indicate that phenolic cannabinoids are able to effectively protect primary granule cells against oxidative nerve cell death. In contrast, methanandamide did not have any neuroprotective effect on granular neurones at any of the concentrations tested (Fig. 3a).

Cannabinoid-mediated neuroprotection in a neuronal cell line expressing CB1

Antioxidant CB1 agonists protect cells that express CB1 (primary cerebellar cultures) or that lack CB1 (neuronal cell lines), suggesting a purely chemical antioxidant mechanism of action. However, to rule out definitely the participation of the receptor to the cannabinoid-induced neuroprotection, it is necessary to test the protection potential of the drugs in identical cellular model systems that differ only in the expression of CB1 and to compare the pharmacological effects in its presence or absence.

Therefore, HT22 cells were stably transfected with an expression vector coding for the mouse CB1, and G418-resistant clones were analysed by northern blot. Hybridization signals were detected at approximately 6.0 kb for cortex RNA (used as positive control), as previously described (Matsuda 1997) and at approximately 1.6 kb for many G418-resistant CB1-transfected clones (Fig. 4a). cAMP accumulation assays revealed that some of the clones expressing CB1 mRNA also expressed a functional receptor, as the presence of the potent CB1 agonist WIN 55,212-2 was able to decrease the forskolin (FSK)-induced cAMP accumulation in CB1-expressing cells (HT22 CB1), but not in cells

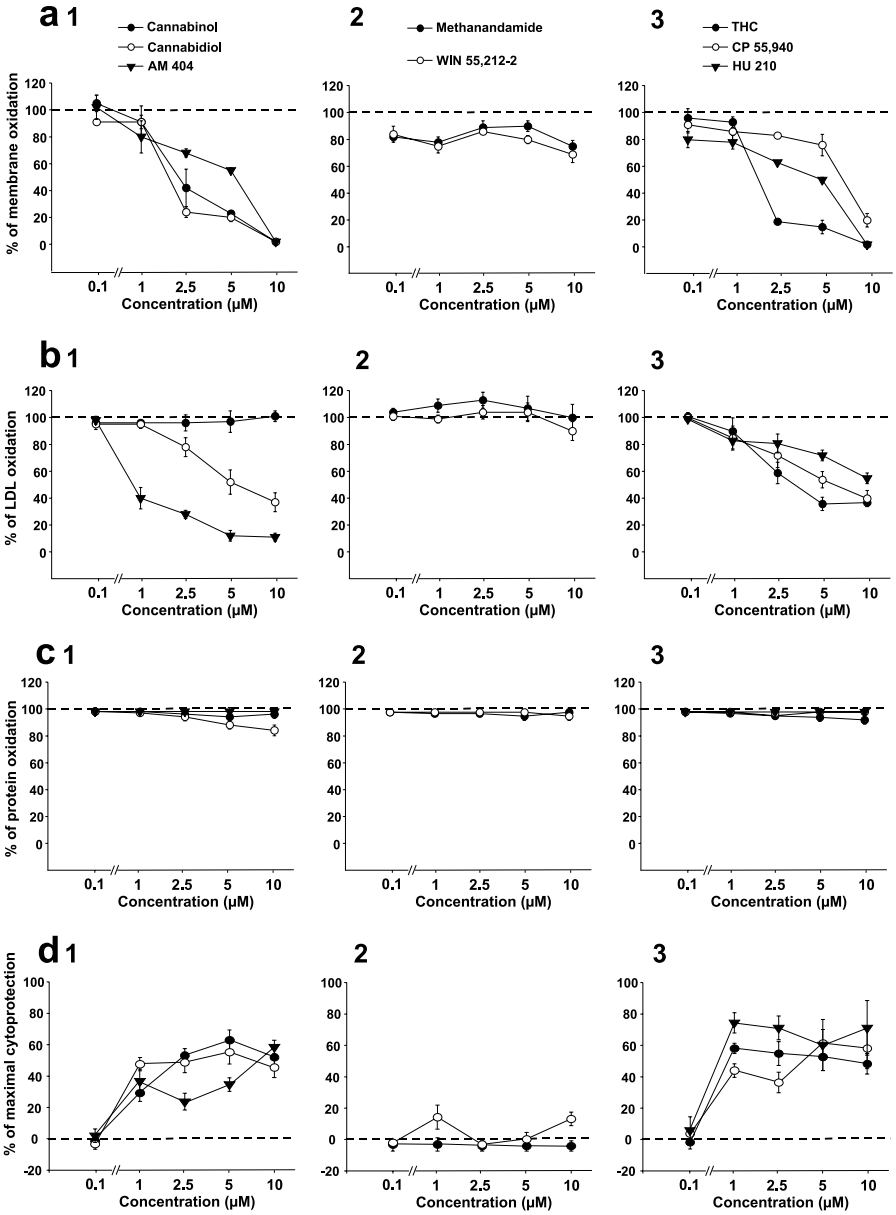

Fig. 2 Antioxidant properties of cannabinoids and protection against oxidative stress in HT22 cells. (a) Percentages of oxidation of rat brain lipids as induced by 50 μM ascorbate. (b) Percentages of oxidation of human blood plasma LDL as induced by 10 μM copper sulfate. (c) Percentage of oxidative destruction of globular proteins by peroxyl radicals deliberated from an azo compound (AIBN). (d) Percentages of protection against oxidative stress in HT22 cells, induced by 120 μM of H_2O_2. In (a–c), 100% indicates maximal oxidation in the absence of any cannabinoid. In (d), 0% and 100% indicate the value in the absence of cannabinoids (only H_2O_2) and the value for untreated cells (i.e. without cannabinoids and without H_2O_2), respectively. Data are indicated as means ± SEM.

transfected with an empty vector (HT22 WT) or in parental cells (Fig. 4b and data not shown). The extent of reduction of FSK-induced cAMP accumulation was approximately 30%, consistent with reported values in other heterologous CB1 expression systems (Song and Bonner 1996). After confirming that the clonal cells expressed a functional CB1, HT22 CB1 and HT22 WT were used for oxidative stress assays in the presence of cannabinoids. The four CB1 agonists to be tested were chosen in light of their previously determined effects in the antioxidant assays and in the oxidative stress assays in parental HT22 cells and primary cerebellar granule cell cultures (Figs 2d and 3). Δ^9-THC and CP 55,940 were used as prototypes of the phenolic antioxidant group, while methanandamide and WIN 55,212-2 represented non-phenolic and non-antioxidant compounds. As shown in Fig. 5(a and b), the two phenolic

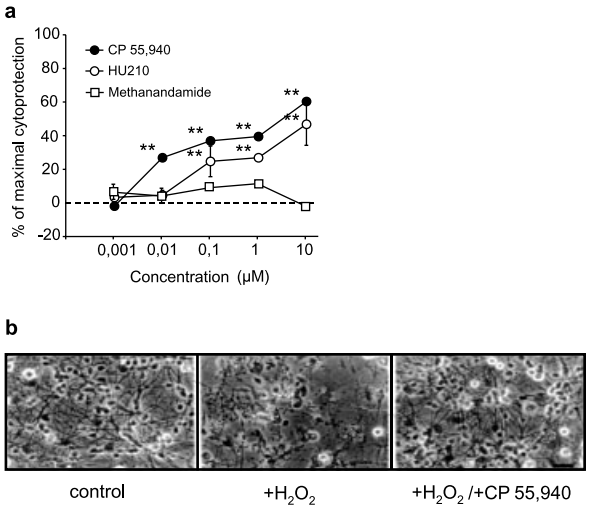

Fig. 3 Cannabinoid-mediated neuroprotection in oxidative stress assays on cultured primary rat cerebellar granule cells. (a) Neuroprotective effects of CP 55,940 and HU 210 (phenolic antioxidant CB1 agonists) and methanandamide (non-phenolic CB1 agonist). (b) Phase-contrast micrographs showing examples of CP 55,940-mediated neuroprotection in cultured primary rat cerebellar granule cells. In (a), results are presented as in Fig. 2(d). $^{**}p < 0.01$, as compared to control (Student's *t*-test; $n = 3$).

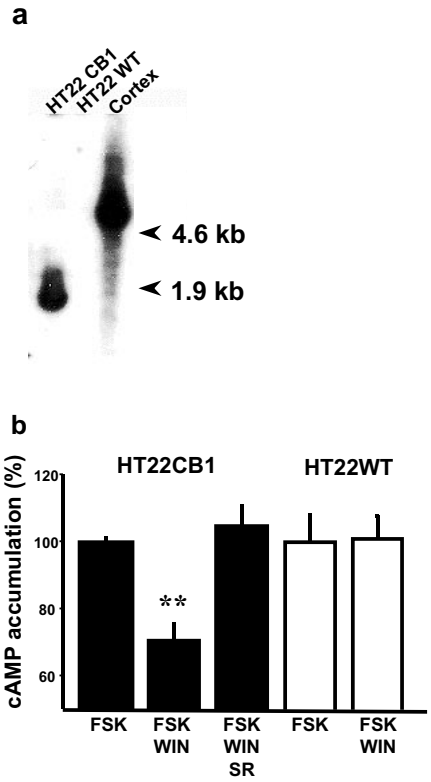

Fig. 4 Heterologous expression of mouse CB1 in HT22 cells. (a) Northern blot analysis of total RNA from HT22 cell clones after stable transfection with an expression vector containing the mouse CB1 cDNA (HT22 CB1) and an empty control vector (HT22 WT). Ribosomal RNA was used as molecular weight marker (28S, approximately 4.6 kb; 18 S, approximately 1.9 kb). The CB1 open reading frame was used as hybridization probe. Mouse cortex RNA, containing high levels of CB1 mRNA (about 6.0 kb; Matsuda 1997), was used as positive control. The positive signal in clone HT22 CB1 corresponds to a band of about 1.6–1.7 kb, consistent with the expected size. (b) cAMP accumulation assays using the same clonal cells as in (a). Results are presented as percentages of the control stimulus induced by 5 μM forskolin (FSK). WIN is the CB1 agonist WIN 55,212-2 (1 μM), SR is the CB1 antagonist SR 141716A (1 μM). (■) Clone HT22 CB1; (□) clone HT22 WT. Data are means ± SEM $^{**}p < 0.01$, as compared to FSK control (Student's *t*-test, $n = 4$).

compounds Δ^9-THC and CP 55,940 were able to protect HT22 WT cells up to values of approximately 70% and 60%, respectively. However, no differences were observed between the HT22 WT and the CB1-expressing cells HT22 CB1. The dose–response curves were basically identical, thus indicating that the presence of CB1 was altering neither the efficacy nor the potency of the drugs. Once again, methanandamide (Fig. 5c) showed no ability to protect cells from oxidative stress, neither in the absence nor in the presence of CB1. Also WIN 55,212-2 (Fig. 5d) did not show any significant protective effect even at concentrations as high as 10 μM. These observations indicate that CB1 is not required for the protective activity of cannabinoids in *in vitro* oxidative stress toxicity paradigms in neuronal cell lines.

Cannabinoid-mediated neuroprotection in primary cerebellar granule cells from CB1-deficient mice

Neuronal cell lines, such as HT22, are of clonal origin and represent good models for neurotoxicity studies. Nevertheless, they share only a limited amount of characteristics with primary neurones. Therefore, we wondered whether the presence of CB1 in freshly prepared primary neurones could influence the neuroprotective actions of CB1 agonists. In order to address this notion, primary cerebellar granule cell cultures were assessed from WT and CB1 knock-out mice (CB1-KO). CP 55,940 was used as phenolic antioxidant CB1 agonist, and its effects were compared between WT and CB1-KO primary cerebellar cultures. Figure 6 shows that CP 55,940 was able to protect both WT and CB1-KO neurons. However, no differences were observed between WT and CB1-KO cells. These results clearly show that CB1 is not necessary for the neuroprotective activity of a potent CB1 agonist in oxidative stress assays using H_2O_2.

Discussion

In this study, the neuroprotective properties of cannabinoids were analysed in *in vitro* oxidative stress assays. Oxidative stress represents one of the major events that occur during neurodegeneration in many neurological diseases such as

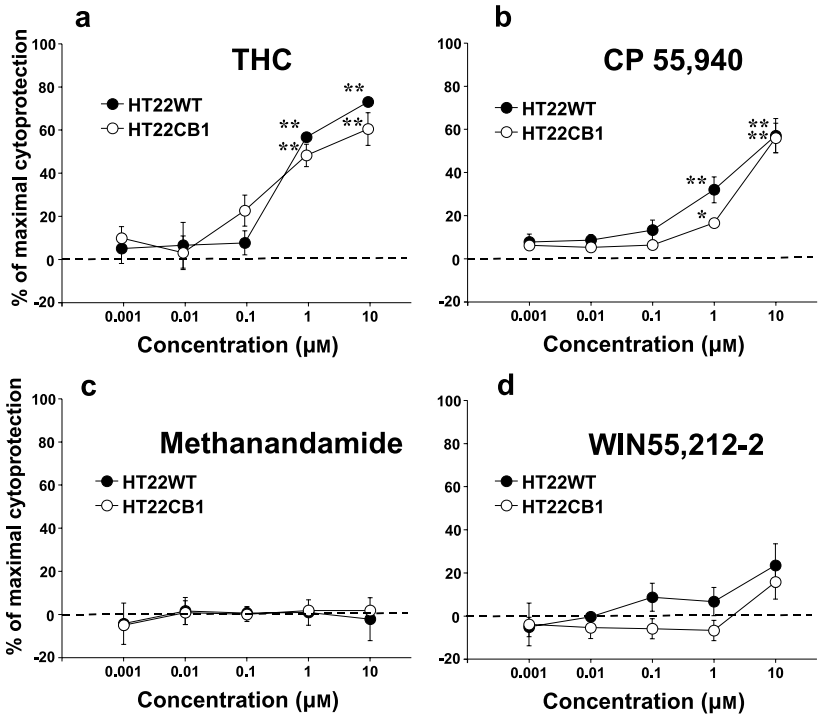

Fig. 5 Cannabinoid-mediated neuroprotection in oxidative stress assays on HT22 cells expressing CB1 (HT22 CB1) and not expressing CB1 (HT22 WT). Results are presented as in Fig. 2(d). Data are means ± SEM $^{*}p < 0.05$; $^{**}p < 0.01$, as compared to control (Student's *t*-test, $n = 4$). No significant differences were observed between the two genotypes.

Alzheimer's disease (Simonian and Coyle 1996; Behl 1999a; Markesbery and Carney 1999). Oxidative neurodegeneration can be mimicked *in vitro* through the toxicity of H_2O_2 which is a mediator of various disease-related neurotoxins (Behl *et al*. 1994). Therefore, drugs that are able to inhibit these oxidative processes are promising candidates for the treatment of such diseases. Chemical antioxidants, e.g. vitamin E (Behl 1999b), are examples for these neuroprotective drugs. Many cannabinoids have structural features typical for phenolic antioxidants and, on the other hand, through CB1 activation, they are able to inhibit the excitability of the cells, by increasing K^+ and decreasing Ca^{2+} permeability (for review see Pertwee 1997). Therefore, they could potentially exert neuroprotective activities through different mechanisms and, thus, possess very interesting therapeutic potential for the treatment of several neurodegenerative diseases.

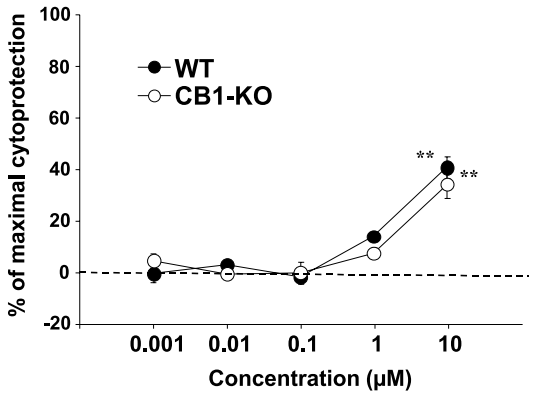

Fig. 6 Cannabinoid-mediated neuroprotection (CP 55,940) in oxidative stress assays on primary cerebellar granule cell cultures derived from wild-type mice (WT) and homozygous CBN/CBN littermates (CB1-KO). Results are presented as in Fig. 2(d). Data are expressed as mean ± SEM. $^{**}p < 0.01$, as compared to control (Student's *t*-test, $n = 4$). Note the lack of significant differences between the two genotypes.

Several cannabinoids were tested in cell-free biochemical antioxidant assays and, as expected, phenolic compounds (Δ^9-THC, cannabinol, cannabidiol, CP 55,940, HU 210 and AM 404) were found to be potent lipophilic antioxidants. These investigations were extended by *in vitro* oxidative stress toxicity experiments, using clonal neuronal cell lines (HT22 and PC12) and rat primary cerebellar granule cells: antioxidant phenolic cannabinoids were also protective against oxidative stress in the cells. These experiments did not completely rule out the putative participation of CB1 to the neuroprotective effects of cannabinoid compounds. One possibility to analyse the involvement of CB1 in the neuroprotective antioxidant effects of CB1 agonists would be to use the specific CB1 antagonist SR 141716A in the same neurotoxicity assays (Nagayama *et al*. 1999). However, SR 141716A was shown to exert, both *in vivo* and *in vitro*, more complex actions than a simple antagonistic effect at CB1 receptors. In CB1-transfected cells and in endogenously CB1-expressing neuronal cell lines, SR 141716A was shown to act as an inverse agonist (Shire *et al*. 1999; Meschler *et al*.

2000). Furthermore, in primary cerebellar granule cells, SR 141716A was recently shown to have different effects on cannabinoid-mediated inhibition of Ca^{2+} mobility in different subcellular portions of the neurones. A mere antagonistic effect was observed at CB1 receptors located in the neurites, whereas a paradoxical 'agonist-like' inhibition of Ca^{2+} influx was observed in the soma of the neurons (Hillard *et al*. 2000). Therefore, the involvement of CB1 in cannabinoid-mediated neuroprotection was checked by two genetic approaches, i.e. in heterologously CB1-expressing cell lines as compared to control-transfected lines and in primary cerebellar neurones derived from wild-type and CB1-deficient mice (CBN mouse line; G. Marsicano *et al.*, manuscript in preparation). No differences were observed in the neuroprotective activity of the tested drugs in presence or in absence of CB1, neither in the cell line nor in primary cerebellar cultures. These results correlate with the data from Chen and Buck (2000). These authors used a different model of oxidative stress in non-neuronal cell lines and found a CB1-independent protection caused by several cannabinoids. However, they showed the presence of CB1 in their cell lines by RT-PCR and did not provide any data using the same cell type with and without CB1. The present results strongly extend the concept of the antioxidant action of cannabinoids, providing direct evidence for the independence of such activity from CB1.

Oxidative stress is one of the central events onto which many neurodegenerative cascades converge. Therefore, H_2O_2-induced oxidative cell death *in vitro* is a clear paradigm of neurodegeneration that can provide useful information about the neuroprotective aspects of certain pharmacological compounds. Indeed, H_2O_2 is known to be a mediator of oxidative apoptosis in neuronal cells (Behl *et al*. 1994; Maher and Davis 1996; Chun *et al*. 2001). Here, we clearly show that cannabinoids possess protective antioxidant properties that are independent of the presence of the cannabinoid receptor CB1. In addition to the direct antioxidant activity of the phenolic cannabinoids, these compounds may further affect membrane-associated and intracellular signalling mechanisms. For instance, due to their lipophilicity, these compounds could increase the membrane fluidity and may eventually lead to changes in the activity of membrane-bound receptor systems (e.g. neurotransmitter receptors). Moreover, molecular interactions with intracellular signalling processes could be similar to those that are known to be executed by 17β-estradiol, which is another phenolic neuroprotective antioxidant acting independently of its cognate oestrogen receptors (Moosmann and Behl 1999). In addition to its wide range of oestrogen receptor-dependent effects, oestradiol performs various receptor-independent neuromodulatory activities including also the activation of the neuroprotective mitogen-activated protein kinase signalling (Behl and Holsboer 1999), which is known to be also activated by cannabinoids, both in a CB1-dependent (Valjent *et al*. 2001) and CB1-independent manner (Jan and Kaminski 2001). Finally, CB1-dependent effects of cannabinoids could be involved in upstream events that eventually could affect intracellular oxidative pathways. As an example, it is likely that activation of CB1 might inhibit glutamate toxicity by its counteracting hyperpolarizing action (Di Marzo *et al*. 1998; Hampson *et al*. 1998; Piomelli *et al*. 2000; Hampson and Grimaldi 2001). Indeed, CB1 was shown to be involved in some neuroprotective actions of cannabinoids *in vivo* (Nagayama *et al*. 1999) and is up-regulated in particular brain areas during experimental ischaemia in rats (Jin *et al*. 2000). Endocannabinoids are also increased in brain after closed-head injury and can mediate neuroprotective properties in the same paradigm by a CB1-dependent mechanism (Panikashvili *et al*. 2001). These data suggest a general neuroprotective function of the endocannabinoid system. Neuroprotective activities of endocannabinoids appear to be CB1-mediated and do not involve antioxidant properties due to their lack of phenolic moieties, which are the mediators of antioxidant neuroprotection of several exogenous cannabinoid drugs. Therefore, it is tempting to propose potent therapeutic applications for drugs that are able to both sustain the 'endogenous' CB1-mediated neuroprotective activity of endocannabinoids and to provide antioxidant protection. Good candidates are inhibitors of endocannabinoid uptake, such as AM 404 and the newly developed compound VDM 11 (De Petrocellis *et al*. 2000), both of which contain a phenolic residue. In addition, given the 'upon demand' activation of the endocannabinoid system (Di Marzo *et al*. 1998; Piomelli *et al*. 2000), using endocannabinoid uptake inhibitors might diminish the undesirable psychotropic side-effects generally observed after treatment with CB1 agonists. In conclusion, the use of antioxidant cannabinoids or, in particular, the inhibition of endocannabinoid uptake by antioxidant drugs could provide promising avenues for the therapeutic targeting of different aspects of neurodegenerative diseases, by stimulating a self-protective endogenous system of the brain and by counteracting oxidative stress.

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