Ceramide sensitizes astrocytes to oxidative stress: protective role of cannabinoids

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Cannabinoids induce apoptosis on glioma cells via stimulation of ceramide synthesis *de novo*, whereas they do not affect viability of primary astrocytes. In the present study, we show that incubation with Δ^9-tetrahydrocannabinol did not induce accumulation of ceramide on astrocytes, although incubation of these cells in a serum-free medium (with or without cannabinoids) led to stimulation of ceramide synthesis *de novo* and sensitization to oxidative stress. Thus treatment with H_2O_2 induced apoptosis of 5-day-serum-deprived astrocytes and this effect was abrogated by pharmacological blockade of ceramide synthesis *de novo*. The sensitizing effect of ceramide accumulation may depend on p38 mitogen-activated protein kinase activation rather than on other ceramide targets. Finally, a protective role of cannabinoids on astrocytes is shown as a long-term incubation with cannabinoids prevented H_2O_2-induced loss of viability in a CB_1 receptor-dependent manner. In summary, our results show that whereas challenge of glioma cells with cannabinoids induces accumulation of *de novo*-synthesized ceramide and apoptosis, long-term treatment of astrocytes with these compounds does not stimulate this pathway and also abrogates the sensitizing effects of ceramide accumulation.

Key words: apoptosis, astrocytes, cannabinoids, ceramide, glioma, serine palmitoyltransferase.

INTRODUCTION

The effects exerted by marijuana and their derivatives through THC (Δ^9-tetrahydrocannabinol) and other cannabinoid constituents have been known for many years. However, the molecular basis of these actions were not understood until the discovery of an endogenous cannabinoid system comprising two plasma membrane $G_{i/o}$-coupled cannabinoid receptors (CB_1 [1] and CB_2 [2]) and a family of endogenous ligands for those receptors [3,4]. CB receptors mediate cannabinoid effects by coupling with different signalling pathways. Both the CB_1 and CB_2 receptors signal inhibition of adenylate cyclase [5] and stimulation of ERK (extracellular-signal-regulated kinase) [6], whereas the CB_1 receptor is also coupled with modulation of Ca^{2+} and K^+ channels [5], stimulation of the stress-activated p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase) [7], stimulation of the focal adhesion kinase [8], hydrolysis of sphingomyelin [9] and stimulation of PI3K/PKB (phosphatidylinositol 3-kinase/protein kinase B) [10].

One of the most exciting areas of research in this field is the study of the potential application of cannabinoids as therapeutic agents [11,12]. Among these possible applications, cannabinoids are being tested as therapeutic agents on neurological and neurodegenerative disorders [13,14]. Neuroprotection by cannabinoids has been related to CB_1-mediated inhibition of voltage-sensitive Ca^{2+} channels to reduce Ca^{2+} influx, glutamate release and excitotoxicity [12,15], and to the ability of cannabinoids to act as antioxidants [16,17]. Canabinoids have also been shown to protect glial cells from apoptosis through stimulation of the PI3K/PKB pathway [18,19].

In addition, cannabinoids are being investigated as potential antitumoural drugs [20]. Challenge with cannabinoids induces apoptosis on glioma cells via stimulation of *de novo* synthesis of ceramide [21,22], a sphingolipid-based lipid that regulates a variety of cellular processes including differentiation, proliferation and apoptosis [23]. Thus ceramide has been shown to mediate the action of oxidative stress in several cell types [24–26].

Therefore cannabinoids exert opposite effects on the survival of transformed and non-transformed glial cells. However, the molecular basis of this dual action is as yet unknown. In the present study, we investigated whether the opposite response to cannabinoids of glioma cells and astrocytes could be based on differences in the regulation of the pathway of *de novo* ceramide synthesis.

MATERIALS AND METHODS

Cell culture

The rat glioma C6.9 line (15–25 passages) was cultured as described previously [27]. Cortical astrocytes were prepared from 24 to 48 h Wistar rats and cultured in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1, v/v) supplemented with 0.5 % (w/v) glucose, 5 mg/ml streptomycin, 5 units/ml penicillin and 10 % foetal calf serum as described previously [28]. After 21 days, serum deprivation was performed by removing serum-containing medium and replacing it by a chemically defined serum-free medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1, v/v), supplemented with 5 mg/ml streptomycin, 5 μg/ml insulin,

Abbreviations used: ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; LCB1, long-chain base subunit 1; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SPT, serine palmitoyltransferase; THC, Δ^9-tetrahydrocannabinol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

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50 μg/ml transferrin, 20 nM progesterone, 50 μM putrescine and 30 nM sodium selenite.

Apoptosis and cell viability

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] test. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining was performed as described previously [22]. Briefly, cells were fixed for 20 min at room temperature (25 °C) in PBS containing 4 % (w/v) paraformaldehyde and 5 % (w/v) sucrose, permeabilized with 0.05 % Triton X-100 in PBS for 5 min, and blocked with 0.5 % BSA in PBS for 30 min. DNA ends were subsequently labelled for 2 h at 37 °C in the following reaction mixture: Tris-buffered saline (pH 7.2), 2 mM $CoCl_2$, 0.1 unit/μl terminal deoxynucleotidyl transferase (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) and 2.5 pmol/μl biotin-16-dUTP (Roche, Basel, Switzerland). Finally, cells were incubated for an additional 2 h with 3.5 μg/ml streptavidin Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) in PBS with 0.1 % BSA.

Ceramide levels

Ceramide levels were determined as described previously [22]. Briefly, after incubation of the cells under different conditions, lipids were extracted, saponified and incubated with diacylglycerol kinase from *Escherichia coli* in the presence of [γ-^{32}P]ATP. Finally, ceramide 1-phosphate was resolved by TLC.

SPT (serine palmitoyltransferase) assay

SPT activity was determined in digitonin-permeabilized C6.9 cells and astrocytes as described previously [22,29]. Briefly, reactions were started by the addition of an assay mixture containing 8.75 μg/ml (for glioma cells) or 15 μg/ml (for astrocytes) digitonin, 100 mM Hepes (pH 8.3), 200 mM sucrose, 2.5 mM EDTA, 5 mM dithioerythritol, 50 μM pyridoxal phosphate, 1.0 mg/ml BSA, 0.3 mM palmitoyl-CoA and 0.25 mM L-[U-^{14}C]serine (3 μCi/assay). After 30 min (for glioma cells) or 45 min (for astrocytes), reactions were stopped with 0.5 M NH_4OH, and the product [^{14}C]ketosphinganine was extracted with choloroform/methanol/1 % NaCl.

Western-blot analysis

Western-blot analyses were performed with antibodies that recognize PKB, PKB phosphorylated on Ser-473, ERK, p38 MAPK, p38 MAPK phosphorylated on Thr-180/Tyr-182 (Cell Signalling, Beverley, MA, U.S.A.), ERK phosphorylated on Thr-202/Tyr-204 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), LCB1 (long-chain base subunit 1) SPT subunit (BD Biosciences–PharMingen, Franking Lakes, NJ, U.S.A.), JNK phosphorylated on Thr-183/Tyr-185 (Promega, Madison, WI, U.S.A.), α-tubulin and catalase (Sigma, St. Louis, MO, U.S.A.).

Statistics

Results shown represent means $\pm$ S.D. Statistical analysis was performed by ANOVA with a *post hoc* analysis by the Student–Neuman–Keuls test.

RESULTS AND DISCUSSION

Cannabinoid treatment does not stimulate ceramide synthesis *de novo* in astrocytes

Unlike glioma cells, which undergo apoptosis when challenged with cannabinoids [21,27], the viability of astrocytes (non-transformed glial cells) remains unaffected after treatment with these compounds [27]. Since cannabinoid induced apoptosis of glioma cells relies, at least partially, on the accumulation of *de novo*-synthesized ceramide [21,22], we investigated whether the different response to cannabinoids of transformed and non-transformed glial cells could be based on differences in this pathway. We compared the effects of 5-day THC treatment on ceramide levels and the activity of SPT (the enzyme that catalyses the rate-limiting step of sphingolipid biosynthesis [30]) in C6.9 glioma cells and primary astrocytes. As shown in Figure 1(A), incubation with THC led to a 4-fold increase of ceramide levels in C6.9 cells, whereas no significant differences were observed between THC- and vehicle-treated astrocytes. Similarly, treatment with THC induced more than 5-fold stimulation of SPT activity in C6.9 cells but did not affect enzyme activity in astrocytes (Figure 1B). These results indicate that one of the reasons for the different response to cannabinoids of transformed and non-transformed glial cells may be the lack of stimulation of the ceramide synthesis pathway in the latter.

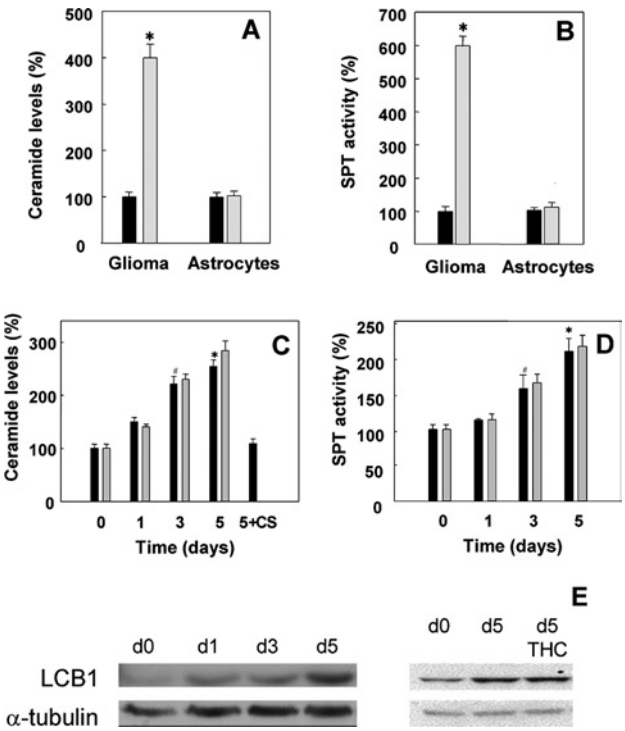

Figure 1 Astrocytes and glioma cells respond differently to cannabinoids

C6.9 glioma cells or astrocytes were incubated in serum-free medium in the presence of 1 μM THC (shaded bars) or vehicle (black bars) for 5 days and ceramide levels (**A**) or SPT activity (**B**) were determined. Results correspond to six different experiments and show percentage of ceramide level or SPT activity with respect to the vehicle-treated cells. $^*P < 0.01$, significantly different from the vehicle-treated cells. Ceramide levels (**C**) or SPT activity (**D**) of THC (shaded bars) or vehicle (black bars)-treated astrocytes was determined at different days of incubation. Results correspond to four different experiments and show percentage of ceramide levels or SPT activity relative to zero time cells. CS, L-cycloserine (used at 0.5 mM). $^*P < 0.01$ and $^\#P < 0.05$, significantly different from zero time cells. (**E**) Astrocytes were incubated in serum-free medium for the indicated times in the presence or absence of 1 μM THC, cells were lysed and Western-blot analyses were performed with anti-LCB1 or anti-α tubulin antibodies. Representative blots of six (left) or three (right) experiments are shown.

Serum deprivation sensitizes astrocytes to oxidative stress via ceramide accumulation

Although cannabinoids did not stimulate ceramide synthesis in astrocytes, we observed that incubation of these cells in a

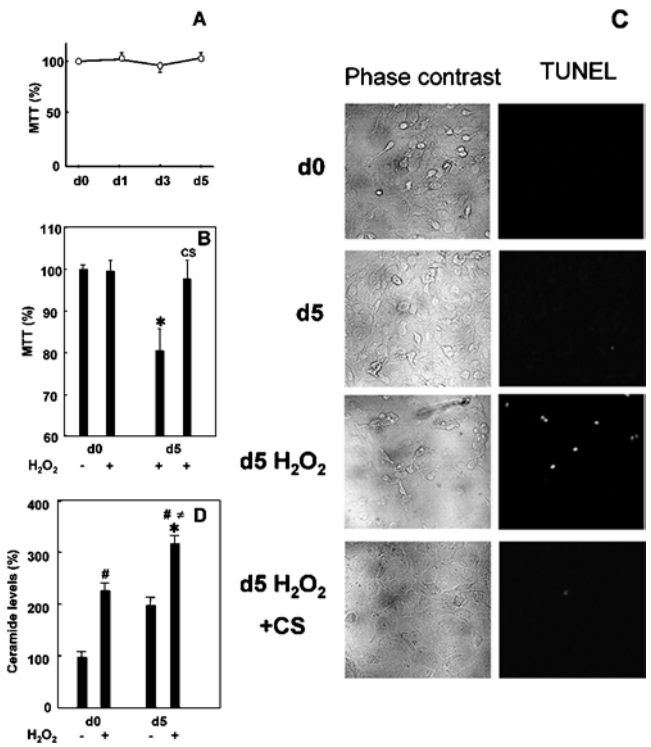

Figure 2 Serum deprivation sensitizes astrocytes to oxidative stress

Astrocytes were incubated in serum-free medium and cell viability was determined by the MTT test at the indicated times. (**A**) Results correspond to six different experiments and show the percentage of MTT reduction related to zero time cells. (**B**) Astrocytes were incubated for 1 h (d0) or 5 days (d5) in the presence or absence of 0.5 mM L-cycloserine (CS) in serum-free medium and treated with or without 100 μM H_2O_2 for 3 h further. The medium was subsequently replaced with fresh serum-containing (d0) or serum-free (d5) medium and, after 24 h, cell viability was determined by the MTT test ($^*P < 0.01$, significantly different from the vehicle-treated cells, $n = 6$). (**C**) Cells were fixed and TUNEL was performed. Representative photographs of three TUNEL staining experiments are shown. (**D**) Astrocytes were incubated for 1 h (d0) or 5 days (d5), treated with or without 100 μM H_2O_2 for a further 3 h, and ceramide levels were determined. Results correspond to three different experiments and show percentage of ceramide levels relative to d0 vehicle-treated cells. $^*P < 0.05$, significantly different from d0 H_2O_2-treated cells; $^{\#}P < 0.01$, significantly different from d0 vehicle-treated cells; and $^{\neq}P < 0.01$, significantly different from d5 vehicle-treated cells.

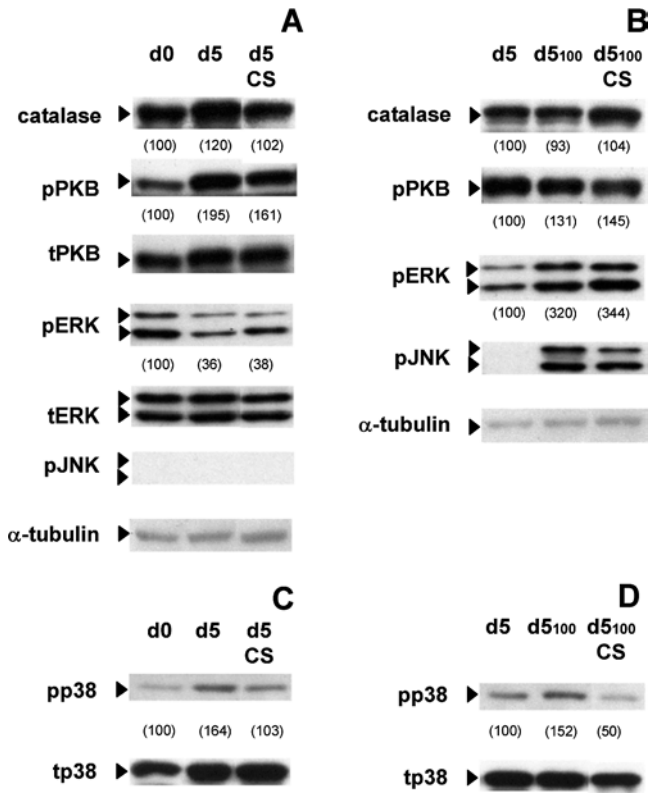

Figure 3 L-Cycloserine prevents the effects of serum deprivation and H_2O_2 treatment on p38 MAPK activation

(**A**, **C**) Astrocytes were cultured in serum-containing medium (d0) or incubated for 5 days in serum-free medium in the presence or absence of 0.5 mM L-cycloserine (CS) and lysed. (**B**, **D**) Alternatively, astrocytes were cultured for 5 days in serum-free medium (with or without 0.5 mM L-cycloserine) and were treated with 100 μM H_2O_2 ($d5_{100}$ or $d5_{100}$CS) or vehicle (d5) for 3 h, the medium was subsequently replaced with fresh serum-free medium and, after 10 min, astrocytes were lysed and Western-blot analyses were performed. The mean value of absorbance (expressed as a percentage) relative to day 0 (**A**, **C**) or day 5 (**B**, **D**) controls of six (**A**) and three (**B**–**D**) experiments are shown in parentheses. Mean absorbance values represent the ratio of the absorbance of each band and the corresponding non-phosphorylated (for PKB, ERK and p38 MAPK) or α-tubulin (for catalase) bands. p-, phospho; t-, total.

chemically defined serum-free medium, both in the presence and in the absence of THC, led to a progressive accumulation of ceramide (Figure 1C) and stimulation of SPT activity (Figure 1D), which reached a 2.5- and 2-fold increase respectively by day 5 of incubation. Ceramide accumulation was prevented by L-cycloserine, an SPT inhibitor (Figure 1C). This indicates that serum deprivation stimulates ceramide synthesis *de novo* in astrocytes. In addition, an increase in LCB1 levels, one of the two subunits of the SPT enzyme in mammals [30], was observed both in the presence and in the absence of THC (Figure 1E), suggesting that SPT up-regulation may be the mechanism linking serum deprivation and stimulation of the ceramide synthesis *de novo*. Nevertheless, we cannot rule out that additional mechanisms involving regulation of SPT or other enzymes of the sphingolipid pathway may also be involved in this process.

Since the accumulation of ceramide described above (Figure 1C) does not affect astrocyte viability by itself (Figure 2A), and ceramide was found to be involved in the response to oxidative stress in different cell types [23–26,31], we pondered over the question whether ceramide may play a role in rendering these cells more sensitive to oxidative stress. To test this hypothesis, we studied the effect of a 3 h incubation with H_2O_2 on cell viability, and apoptosis of control (1 h serum deprived) and 5 days serum-deprived astrocytes. Our results showed that, unlike control cells that were unaffected by the H_2O_2 challenge, a significant increase in cell death (Figure 2B) and apoptosis (Figure 2C) took place when H_2O_2 was added to serum-deprived astrocytes that exhibited ceramide levels remarkably higher than control cells challenged with H_2O_2 or than H_2O_2-untreated serum-deprived astrocytes (Figure 2D). In addition, incubation with L-cycloserine abrogated the effects of H_2O_2 on cell viability (Figure 2B) and apoptosis (Figure 2C). Taken together, these results indicate that *de novo* synthesized ceramide is involved in astrocyte sensitization to oxidative stress as induced by serum deprivation.

Ceramide accumulation on serum-deprived astrocytes may activate p38 MAPK

Since long-term incubation with ceramide has been shown to increase oxidative stress damage via a decrease in catalase levels [26], we first investigated whether this could be the mechanism involved in sensitizing astrocytes to H_2O_2. Incubation of these cells in a chemically defined serum-free medium for 5 days did not lead to a decrease in the levels of this enzyme (Figure 3A),

indicating that, at least in our model, sensitization to oxidative stress may depend on the regulation of additional targets. Therefore we further investigated several kinases that have been implicated in the control of cell fate by ceramide [23,32,33]. Results showed that incubation for 5 days in serum-free medium increased p38 MAPK (Figure 3C) and PKB phosphorylation in parallel with a decrease in ERK phosphorylation (Figure 3A). Nevertheless, abrogation of ceramide accumulation by treatment with L-cycloserine only prevented serum-deprivation-induced p38 MAPK activation (Figure 3C), and it did not significantly affect the changes observed in PKB and ERK (Figure 3A).

We next investigated the response to H_2O_2 of serum-deprived astrocytes. As shown in Figure 3(D), incubation with L-cycloserine prevented H_2O_2-induced p38 MAPK activation but did not modify the activation of PKB, ERK and JNK by this agent (Figure 3B). Taken together, these results suggest that p38 MAPK may be involved in the sensitizing effect of ceramide to oxidative stress in our model.

Cannabinoids protect serum-deprived astrocytes from H_2O_2-induced apoptosis via a CB_1 receptor-dependent mechanism

Cannabinoids have been shown to protect neural cells from different insults, including oxidative damage [14,16,17]. These protective actions have been attributed to both CB_1 receptor-dependent and -independent mechanisms. Therefore we tested whether cannabinoids were capable of preventing the effect of H_2O_2 on serum-deprived astrocytes via any of these two mechanisms. Incubation for 5 days in the presence of WIN-55,212-2 (a synthetic cannabinoid without antioxidant properties) as well as with THC (a phenol-ring-containing cannabinoid with antioxidant properties) prevented the effect of H_2O_2 on cell viability (Figure 4A). In addition, this effect was abrogated by co-incubation with the CB_1 cannabinoid receptor antagonist SR141716 (Figure 4A), suggesting that the protective role of cannabinoids in this model relies on the activation of the CB_1 receptor. On the other hand, the antioxidant properties of THC (but not WIN-55,212-2) were confirmed by the observation that addition of THC 30 min before H_2O_2 challenge was able to prevent the H_2O_2-induced loss in cell viability independent of CB_1 receptor activation, as inferred from the lack of antagonistic effect of SR141716 (Figure 4B).

Since incubation with the cannabinoids only partially abrogated the ability of H_2O_2 to increase ceramide levels on serum-deprived astrocytes (Figure 4C), we next investigated several downstream targets that might be involved in the protective actions of cannabinoids. Incubation for 5 days in the presence of THC or WIN-55,212-2, modified neither the basal activity of different targets [such as PKB, ERK, JNK and p38 MAPK (Figures 5A and 5C)] that have been implicated in the control of cell fate by cannabinoids [6,7,11,21] nor affected the activation of these targets by H_2O_2 (Figures 5B and 5D). Although it cannot be discarded that some of those kinases may be important in triggering survival signals that protect serum-deprived astrocytes from oxidative stress, in our model, long-term treatment with cannabinoids seems to affect a different set of targets responsible for the cyto-protective actions of these compounds.

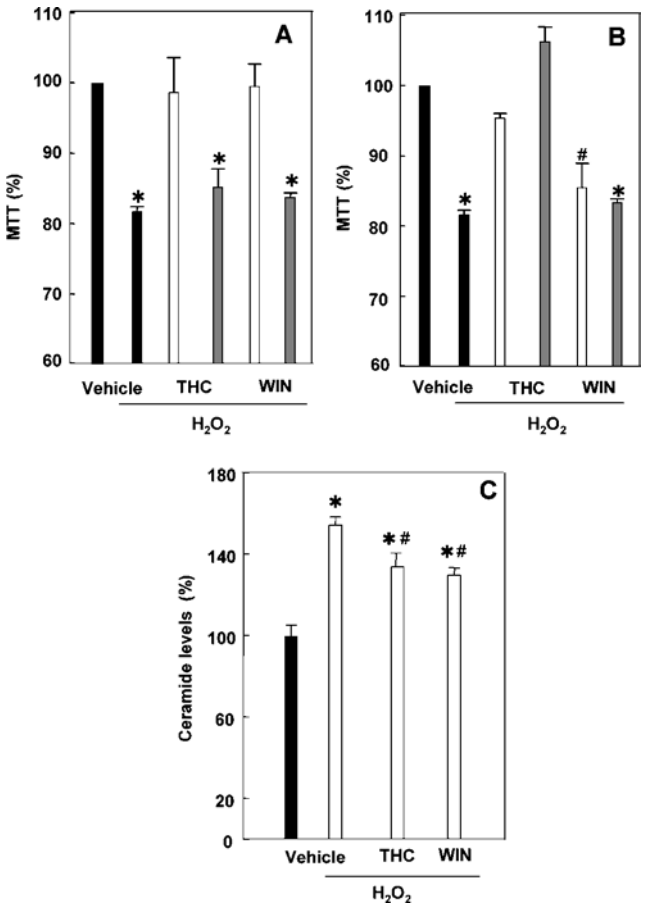

Figure 4 Cannabinoids abrogate the sensitization to oxidative stress of serum-deprived astrocytes

(**A**) Astrocytes were incubated for 5 days in serum-free medium in the presence of vehicle (black bars), 1 μM of the corresponding cannabinoid (open bars) or 1 μM of the corresponding cannabinoid plus 1 μM SR141716 (shaded bars); (**B**) alternatively, after incubation of astrocytes in serum-free medium for 5 days, cells were treated for 30 min with vehicle (black bars), 1 μM cannabinoid (open bars) or 1 μM cannabinoid plus 1 μM SR141716 (shaded bars). In all cases, astrocytes were further treated with or without 100 μM H_2O_2 for 3 h; the medium was replaced with fresh serum-free medium and, after 24 h, cell viability was determined by the MTT test. Results correspond to four different experiments and show the percentage of MTT reduction. $^*P < 0.01$, $^{\#}P < 0.05$, significantly different from vehicle-treated cells. WIN, WIN-55,212-2. (**C**) Astrocytes were incubated for 5 days in serum-free medium in the presence of vehicle or 1 μM of the corresponding cannabinoid and subsequently treated with or without 100 μM H_2O_2 for a further 3 h. Finally, ceramide levels were determined. Results correspond to three different experiments and show percentage of ceramide levels relative to d5 vehicle-treated cells. $^*P < 0.05$, significantly different from vehicle-treated cells; and $^{\#}P < 0.05$, significantly different from vehicle/H_2O_2-treated cells.

Concluding remarks

In line with the established role of cannabinoids as protective agents of the central nervous system, treatment with these compounds has been shown to protect glial cells from various pro-apoptotic stimuli [18,19]. Similarly, in the present study, we show that cannabinoids could also be involved in protecting astrocytes from oxidative damage and that, apart from their antioxidant properties, cannabinoids act via the CB_1 receptor. In contrast, cannabinoid receptor activation leads to apoptosis of glioma cells [21,34]. Although the presence of CB_2 receptors in transformed glial cells [34] but not in astrocytes [35] may, at least partially, justify such a different response, THC-induced apoptosis of C6.9 cells also takes place when the CB_2 receptor is blocked with a selective antagonist [21], indicating that activation of the CB_1 receptor is enough to induce apoptosis. Thus it is conceivable that CB_1 receptor stimulation modulates a different set of targets in astrocytes and glioma cells, one of which seems to be the pathway of *de novo* ceramide synthesis [21,22]. It must be borne in mind that gliomas accumulate a number of alterations that affect specifically the control of cell proliferation. For instance, a high percentage of gliomas display constitutive activation of the PI3K/PKB cascade [36]. Thus the CB_1-dependent short-term activation

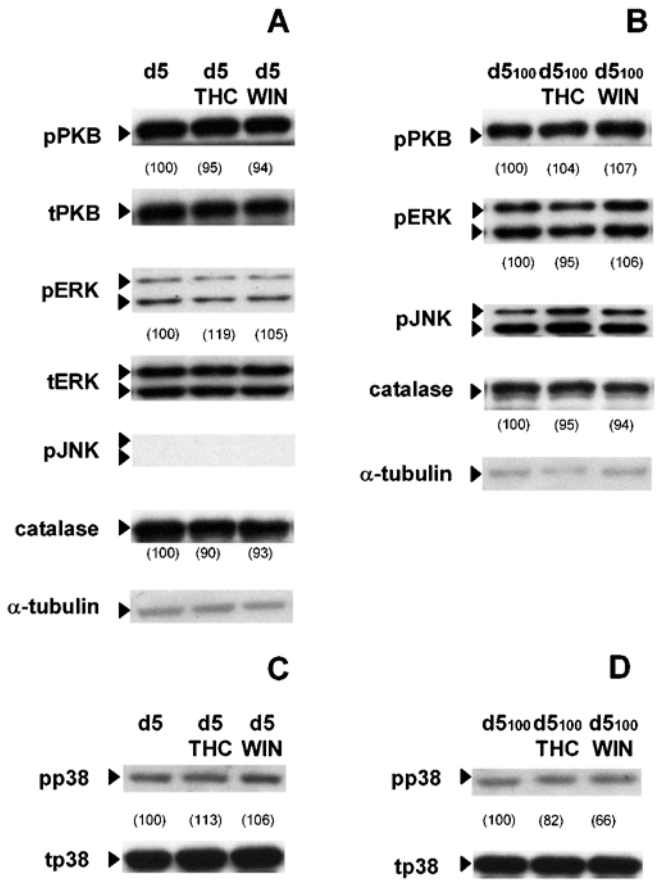

Figure 5 Cannabinoid treatment does not modify serum deprivation or hydrogen peroxide effects on several targets

(**A**, **C**) Astrocytes were incubated for 5 days in serum-free medium in the presence of 1 μM THC (d5THC), 1 μM WIN-55,212-2 (d5WIN) or vehicle (d5) and lysed. (**B**, **D**) Alternatively, vehicle- or cannabinoid-treated astrocytes cultured for 5 days were treated with 100 μM H_2O_2 ($d5_{100}$, $d5_{100}$THC or $d5_{100}$WIN) for 3 h; the medium was replaced with fresh serum-free medium and, after 10 min, cells were lysed and Western-blots analyses were performed. In parenthesis the mean value of absorbance (expressed as a percentage) relative to vehicle- (**A**, **C**) or H_2O_2- (**B**, **D**) treated astrocytes of six (**A**) and three (**B**–**D**) experiments are shown in parentheses. Absorbance values represent the ratio of each band to the corresponding non-phosphorylated (for PKB, ERK and p38 MAPK) or α-tubulin (for catalase) bands. p-, phospho; t-, total.

of PKB observed in astrocytes triggers a protective response in these cells [18] but it may not be of relevance in gliomas. Accordingly, cannabinoid treatment induces long-term ceramide-dependent PKB inhibition [22], as well as ERK, JNK and p38 MAPK activation, in glioma cells [21,22], whereas, as shown here, none of these kinases is activated by cannabinoids in serum-deprived astrocytes. These observations may reflect the lack of stimulation of the ceramide synthesis pathway by these compounds in astrocytes.

Ceramide seems to play an important role in controlling the fate of astrocytes. Results of the present study indicate that *de novo*-synthesized ceramide sensitizes astrocytes to H_2O_2 and suggest that this metabolite may sense the stress level in these cells. Thus it is tempting to speculate that only those stimuli that increase ceramide levels above a theoretical threshold would trigger an apoptotic response in these cells. According to this hypothesis, the combination of 5-day serum deprivation and H_2O_2 treatment, but not either of the two stimuli alone, leads to apoptosis of astrocytes. Nevertheless, different factors such as the intracellular location of the generated ceramide and the activity of several ceramide-metabolizing enzymes [31] may influence the biological activity of this sphingolipid.

In summary, results show that cannabinoids protect astrocytes from ceramide-induced sensitization to oxidative damage and support the idea that these compounds could play a major role in glio protection.

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