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p38 MAPK is involved in CB_2 receptor-induced apoptosis of human leukaemia cells

Blanca Herrera, Arkaitz Carracedo, María Diez-Zaera, Manuel Guzmán, Guillermo Velasco*

Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, clJosé Antonio Novais s/n, 28040 Madrid, Spain

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Abstract Cannabinoids have been shown to inhibit the growth of a broad spectrum of tumour cells. However, the molecular mechanisms involved in that effect have not been completely elucidated. Here, we investigated the possible involvement of mitogen-activated protein kinases (MAPKs) in CB_2 receptor-induced apoptosis of human leukaemia cells. Results show that stimulation of the CB_2 receptor leads to p38 MAPK activation and that inhibition of this kinase attenuates CB_2 receptor-induced caspase activation and apoptosis. These findings support a role for p38 MAPK in CB_2 receptor-induced apoptosis of human leukaemia cells.

Keywords: Cannabinoids; Apoptosis; p38 MAPK; CB_2 receptor

1. Introduction

Δ^9-tetrahydrocannabinol (THC) exerts a wide variety of biological effects by mimicking endogenous substances – the endocannabinoids anandamide [1] and 2-arachidonoylglycerol [2] – that bind to and activate specific cannabinoid receptors. So far, two cannabinoid-specific $G_{i/o}$ protein-coupled receptors, CB_1 and CB_2, have been cloned and characterized from mammalian tissues [3]. The CB_1 receptor is particularly abundant in discrete areas of the brain. In contrast, the CB_2 receptor was initially described to be present in the immune system [4], although recently it has been shown that expression of this receptor also occurs in cells from other origins [5–7].

One of the most exciting areas of research in the cannabinoid field is the study of the therapeutic value of these compounds [8,9]. Among these possible applications, cannabinoids are being investigated as potential antitumoural drugs [10]. The antitumoural actions of cannabinoids rely, at least in part, on the ability of these compounds to induce apoptosis on a wide spectrum of tumour cells [10]. Both CB_1 and CB_2 receptors have been shown to mediate these cannabinoid actions [10]. In particular, the CB_2 receptor plays a major role in the pro-apoptotic effect of cannabinoids in gliomas [11], skin carcinomas [6] and lymphomas [12], although the molecular mechanisms responsible for this effect remain unravelled.

The CB_1 receptor modulates several mitogen-activated protein kinase (MAPK) pathways that are involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase (ERK) [13], c-Jun N-terminal kinase (JNK) and p38 MAPK [14–16]. In the present study, we investigated the potential involvement of these signalling routes in the mechanism of CB_2 receptor-induced apoptosis.

2. Materials and methods

2.1. Reagents

THC, the CB-receptor antagonists (SR141716 and SR144528) and JWH-133 were kind gifts from Dr. Javier Fernández-Ruiz (Complutense University, Madrid), Sanofi-Aventis (Montpellier, France) and Dr. John W. Huffman (Clemson University, SC), respectively. The fluorescent probes Hoechst 33258 and tetramethylrhodamine metyl ester (TMRM) as well as annexin V-fluorescein isothiocyanate (FITC) were from Molecular Probes (Leiden, The Netherlands). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-phospho-ERK, anti-phospho-JNK, anti-caspase 3 and anti-poly(ADP-ribose) polymerase (PARP) antibodies were from Cell Signalling (Berverly, MA). Anti-α-tubulin was from Sigma Chemical Co. (St. Louis, MO), anti-BH3 interacting-domain death-agonist (Bid) antibody was from R&D Systems (Minneapolis, MN), and anti-cytochrome *c* and anti-Bcl-x antibodies were from BD Pharmingen (San Diego, CA). PD169316 was from Calbiochem (Darmstadt, Germany).

2.2. Cell culture and viability

Exponentially growing Jurkat cells were cultured in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum, 10 mM HEPES, 5 U/mL penicillin and 5 mg/mL streptomycin. Jurkat cells stably transfected with Bcl-x_L expression vector [17] were maintained in the same medium supplemented with 1 mg/mL G418. Cells were transferred to a serum-free medium 30 min before performing the different treatments.

2.3. Nuclear DNA content

Cells were collected by centrifugation at $1500 \times g$ for 5 min, washed once, and incubated for 30 min at room temperature in PBS containing 1% (w/v) BSA, 30% ethanol and 5 μg/mL Hoechst 33342. Fluorescence intensity was analyzed using a LSR flow cytometer (Becton Dickinson, San Jose, CA).

2.4. Quantitative real time PCR

Total RNA was isolated using the RNeasy Protect kit (Qiagen, Hilden, Germany) including a DNase digestion step using the RNase-free DNase kit (Qiagen). cDNA was obtained using the

*Corresponding author. Fax: +34 913944672.
E-mail address: gvd@bbm1.ucm.es (G. Velasco).

Abbreviations: Bid, BH3 interacting-domain death-agonist; tBid, truncated Bid; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; THC, Δ^9-tetrahydrocannabinol; TMRM, tetramethylrhodamine methyl ester; $\Delta\psi_m$, mitochondrial-inner-membrane transmembrane potential

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Transcriptor (Roche, Basel, Switzerland). Taqman probes for human CB_1 and CB_2 were obtained from Applied Biosystems (Foster City, California). Experiments were routinely performed including a negative control (no cDNA) as well as a positive control for CB_1 receptor mRNA expression (cDNA obtained from RNA of the human astrocytoma cell line U373MG).

2.5. Apoptosis

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

2.6. Mitochondrial transmembrane potential

Cells (approximately 0.55×10^6 cells per assay) were collected by centrifugation at $1500 \times g$ for 5 min, washed once, and incubated for 30 min at room temperature with binding buffer (see above) plus 0.25 μM TMRM and 3 μL annexin V-FITC. After incubation for 1 additional minute with 1 μM Hoechst 33258 – in order to discriminate living cells – fluorescence intensity was analyzed using a LSR flow cytometer. Ten thousand cells were recorded in each analysis.

2.7. Mitochondria- and cytosol-enriched fractions

They were isolated as described in [18].

2.8. Western blot

Samples (15–45 μg protein/condition) were subjected to SDS/PAGE in 10% or 15% polyacrylamide gels and transferred to PVDF membranes. Western blot analysis was performed using the corresponding antibodies.

2.9. Caspase activity

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

2.10. Statistics

Results shown represent means ± S.D. In statistical analyses of Western blot bands, S.D. values are omitted for clarity. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student–Neuman–Keuls test.

3. Results and discussion

3.1. p38 MAPK is involved in CB_2 receptor-induced apoptosis

THC has been shown to induce apoptosis of human leukaemia cells via the CB_2 receptor [12]. We confirmed by real time quantitative PCR that CB_2 was the unique cannabinoid receptor expressed by these cells (CB_2 amplification was evident after 22 cycles whereas no amplification of CB_1 receptor mRNA was detected after 45 cycles) and that THC-induced cell death was prevented by the CB_2-selective antagonist SR144528 but not by the CB_1-selective antagonist SR141716 (Fig. 1A). Here, we investigated whether coupling of the CB_2 receptor to MAPKs participates in this effect. As shown in Fig. 1B, incubation with THC led to activation of ERK, JNK and p38 MAPK. Pharmacological inhibition of p38 MAPK but not of ERK or JNK significantly prevented THC-induced apoptosis of Jurkat cells (Fig. 1C, E and F). In line with that observation, THC treatment activated p38 MAPK and SR144528 but not SR141716 prevented this effect (Fig. 1D). The CB_2-selective agonist JWH-133 [19] at 10 μM reduced cell viability by 39 ± 10% (6 h, $n = 4$) and slightly activated p38 MAPK (Fig. 1D), but for unknown reasons its potency of action was notably lower than that of THC.

In order to study the functional role of p38 MAPK in THC-induced apoptosis, we sought for a system in which this process was blunted. For this purpose, we used Jurkat cells overexpressing the pro-survival protein Bcl-x_L [17]. As shown in Fig. 2A, Bcl-x_L overexpressing Jurkat cells were resistant to THC-induced apoptosis. Moreover, THC treatment did not stimulate p38 MAPK in Bcl-x_L overexpressing cells and the amount of phosphorylated p38 MAPK was much lower in Bcl-x_L overexpressing than in control cells (Fig. 2B). Taken together, these results indicate that activation of p38 MAPK kinase plays a significant role in CB_2 receptor-induced apoptosis of Jurkat cells.

3.2. p38 MAPK is involved in CB_2 receptor-induced caspases 3 and 8 activation

We have recently observed that THC treatment of Jurkat cells leads to a CB_2 receptor-dependent loss of mitochondrial-inner-membrane transmembrane potential ($\Delta\psi_m$) and cytochrome *c* release that lead in turn to caspases 3 and 8 activation and apoptosis (manuscript in preparation). We therefore investigated the involvement of p38 MAPK in these events. Pharmacological inhibition of p38 MAPK did not prevent THC-induced loss of $\Delta\psi_m$ (Fig. 3A) and only slightly attenuated mitochondrial cytochrome *c* release [as a control, pharmacological inhibition of the mitochondrial respiratory chain with rotenone completely prevented mitochondrial cytochrome *c* release (Fig. 3B) – but did not affect p38 MAPK phosphorylation (Fig. 3C)]. In contrast, incubation with PD169316 significantly prevented caspases 3 (Fig. 4A and B) and 8 (Fig. 4D and E) activation as well as cleavage of the caspase 3 selective substrate PARP (Fig. 4C) and the caspase 8 substrate Bid (Fig. 4F). The notion that p38 MAPK may play a role in caspase activation is in line with previous observations in other cellular systems [20] and suggest that, upon cannabinoid challenge, p38 MAPK is involved in regulating the activation of caspases 3 and 8 rather than in acting as a primary regulator of the mitochondrial events that trigger cytochrome *c* release.

3.3. Concluding remarks

Activation of cannabinoid receptors has been shown to induce apoptosis in different tumour cells. Nevertheless, the molecular mechanisms involved in this effect are not completely understood yet. It has been recently reported that activation of the CB_2 receptor induces apoptosis in the human leukaemia cell line Jurkat [12]. Here, we show that activation of p38 MAPK participates in this effect. Cannabinoid receptors induce apoptosis via sustained ERK activation [5] and Akt inhibition [21]. JNK has also been implicated in the pro-apoptotic effect of cannabinoids [22]. However, although p38 MAPK is coupled to CB_1 [14,15] and CB_2 [23] receptors, and this kinase has been repeatedly shown to participate in programmed cell death [24], this is the first time in that p38 MAPK is shown to participate in cannabinoid receptor-induced

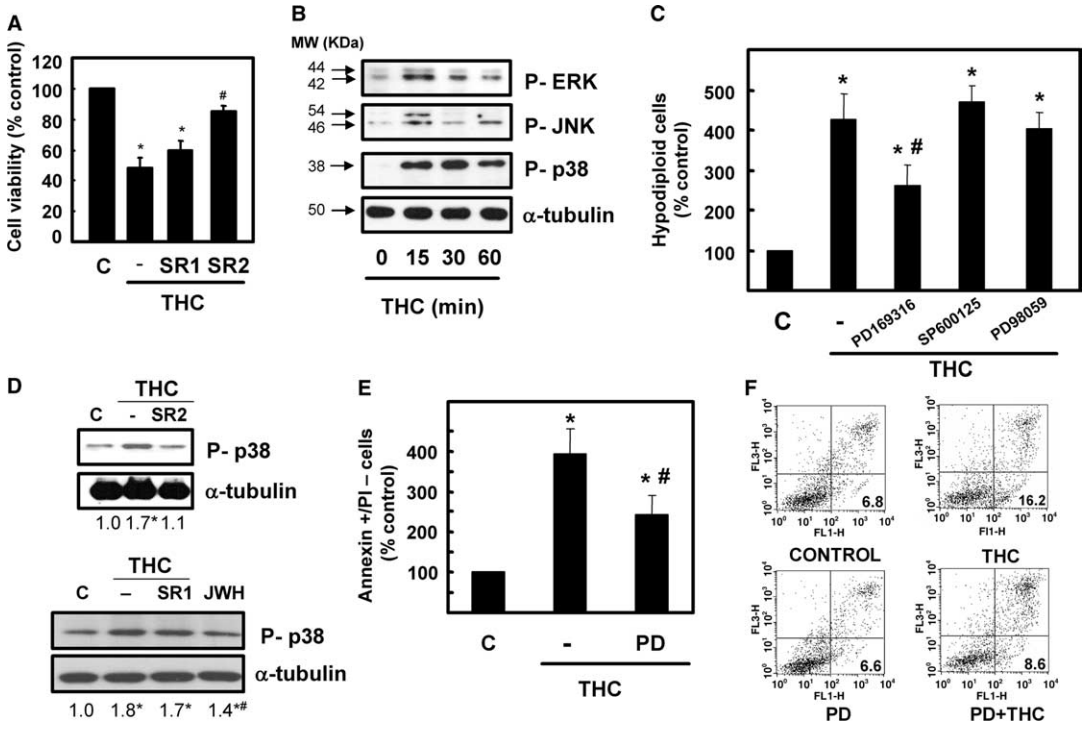

Fig. 1. p38 MAPK is involved in CB_2 receptor-induced apoptosis. Panel A: Cells were pre-incubated with vehicle (−), 1 μM SR141716 (SR1) or 2 μM SR144258 (SR2) for 20 min, further treated with vehicle (C) or 1.5 μM THC for 2 h, and cell viability was determined by Trypan blue exclusion. Results correspond to 4 different experiments and are expressed as the percentage of viable cells relative to vehicle-treated cells. Panel B: Cells were incubated with 1.5 μM THC for the times indicated, cell extracts were obtained and phospho-ERK, phospho-JNK, phospho-p38 MAPK and α-tubulin content was analyzed by Western blot. Panel C: Cells were pre-incubated with vehicle (−), 0.4 μM PD169316 (a selective p38 MAPK inhibitor), 10 μM SP600125 (a selective JNK inhibitor) or 25 μM PD98059 (a selective ERK pathway inhibitor) for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 1 h. Apoptosis was then analyzed by flow cytometry. Results correspond to 6 different experiments and are expressed as the percentage of hypodiploid cells relative to vehicle-treated cells. Panel D: Cells were pre-incubated with vehicle (−), 1 μM SR141716 (SR1) or 2 μM SR144258 (SR2) for 20 min, and further treated with vehicle (C), 1.5 μM THC or 10 μM JWH-133 (JWH) for 15 min. Cell extracts were then obtained and phospho-p38 an α-tubulin content was analyzed by Western blot. Representative experiments and optical density values relative to loading controls are shown. Panels E and F: Cells were pre-incubated with vehicle (−) or 2 μM PD169316 for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 1 h. Apoptosis was then determined by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of annexin V-positive/propidium iodide (PI)-negative cells relative to vehicle-treated cells (panel E). A dot plot of a representative experiment is shown. Values in the lower-right corner of each plot indicate the percentage of annexin V-positive/PI-negative cells (panel F). *Significantly different ($P < 0.01$) from vehicle-treated cells. #Significantly different ($P < 0.01$) from cells treated only with THC.

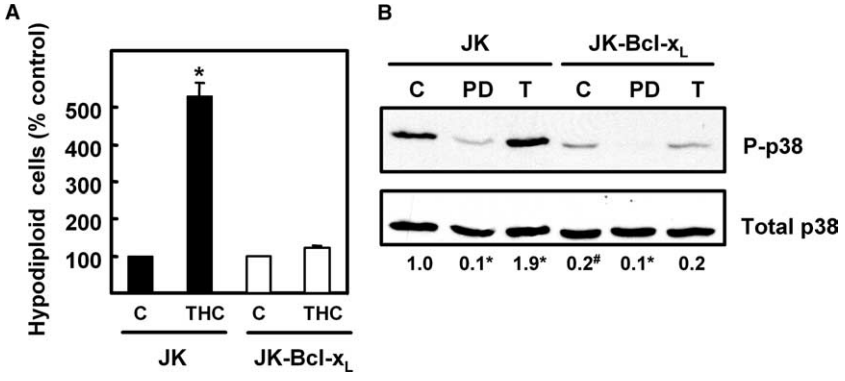

Fig. 2. THC does not stimulate p38 MAPK in Bcl-x_L overexpressing cells. Panel A: Control (JK) and Bcl-x_L overexpressing (JK-Bcl-x_L) Jurkat cells were treated with vehicle or 1.5 μM THC for 1 h, and nuclear DNA content was analyzed by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of hypodiploid cells relative to the corresponding vehicle-treated cells. Panel B: JK and JK-Bcl-x_L cells were pre-incubated with vehicle or 0.4 μM PD169316 for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 15 min. Cell extracts were then obtained and phospho-p38 and total p38 content was analyzed by Western blot. Optical density values relative to loading controls are shown. A representative experiment of 3 is shown. *Significantly different ($P < 0.01$) from the respective vehicle-treated cells. #Significantly different ($P < 0.01$) from control-JK cells.

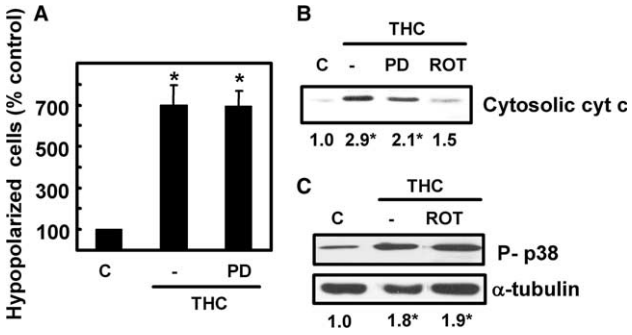

Fig. 3. p38 MAPK does not affect apoptotic mitochondrial events. Panel A: Cells were pre-incubated with vehicle (−) or 2 μM PD169316 for 20 min, further treated with vehicle (C) or 1.5 μM THC for 15 min, and mitochondrial transmembrane potential changes were analyzed by flow cytometry. Results correspond to 3 different experiments and are expressed as the percentage of hypopolarized cells relative to vehicle-treated cells. Panels B and C: Cells were pre-incubated with vehicle (−), 2 μM PD169316 or 20 μM rotenone (ROT) for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 15 min. Cytosolic fractions were isolated and cytochrome *c* (cyt *c*) content was analyzed by Western blot (panel B) or cell extracts were obtained and phospho-p38 an α-tubulin content was analyzed by Western blot (panel C). A representative experiment of 3 is shown in each panel. Optical density values relative to loading controls are shown. *Significantly different ($P < 0.01$) from vehicle-treated cells.

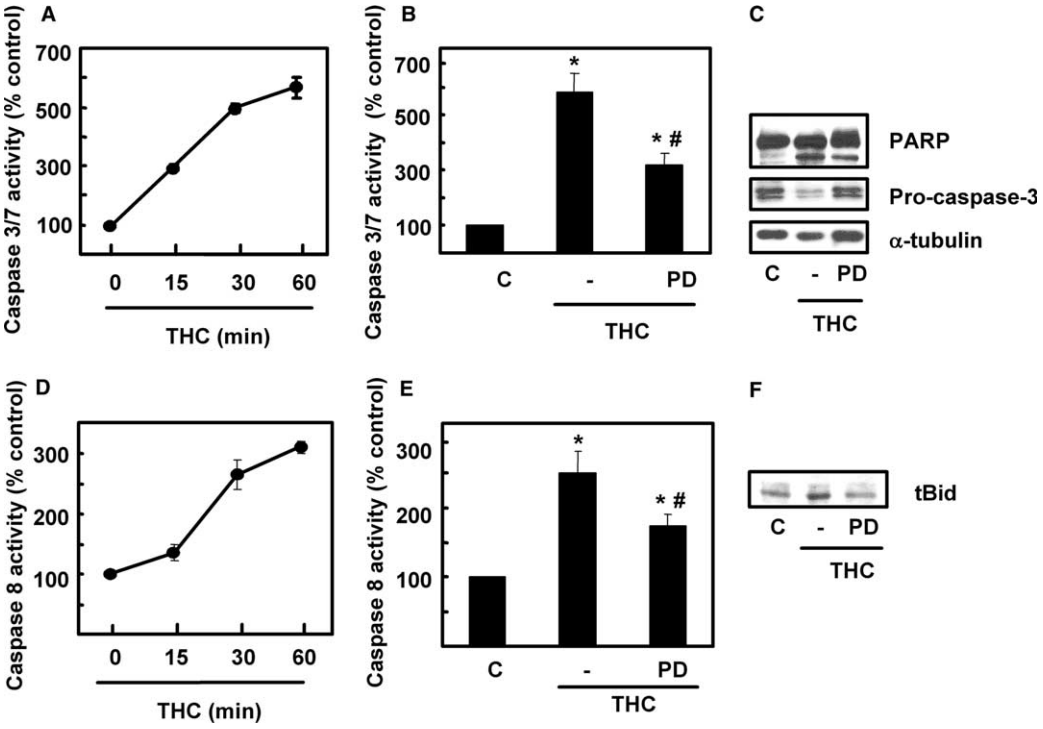

Fig. 4. p38 MAPK is involved in THC-induced caspases 3 and 9 activation. Panels A and D: Cells were treated with 1.5 μM THC for the indicated times and DVEDase (caspase 3/7, panel A) and LETDase (caspase 8, panel D) activities were assayed at each time point. Results correspond to 4 different experiments and are expressed as the percentage of caspase 3 or 8 activity relative to vehicle-treated cells. Panels B and E: Cells were pre-incubated with vehicle (−) or 2 μM PD169316 for 20 min, further treated with vehicle (C) or 1.5 μM THC for 1 h, and DVEDase (caspase 3/7, panel B) and LETDase (caspase 8, panel E) activities were assayed. Results correspond to 4 different experiments and are expressed as the percentage of caspase activity related to vehicle-treated cells. Panels C and F: Cells were pre-incubated with vehicle (−) or 2 μM PD169316 for 20 min, and further treated with vehicle (C) or 1.5 μM THC for 1 h (panel C) or 30 min (panel F). Cell extracts were obtained and PARP, procaspase 3 and α-tubulin (panel C) or truncated Bid (tBid, panel F) were determined. A significant experiment of 2 is shown. *Significantly different ($P < 0.01$) from vehicle-treated cells. #Significantly different ($P < 0.01$) from cells treated only with THC.

apoptosis. This effect could be cell-specific since cannabinoid-induced apoptosis of glioma [5] and dendritic [25] cells does not depend on p38 MAPK activation. In contrast, ERK and JNK are not apparently involved in THC-induced apoptosis of Jurkat cells. Nevertheless, further research is still necessary to elucidate the molecular bases of the activation of the different MAPK cascades in the context of CB_2 receptor-induced apoptosis.

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