Δ9-Tetrahydrocannabinol induces apoptosis in human prostate PC-3 cells via a receptor-independent mechanism

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Abstract The effect of Δ9-tetrahydrocannabinol (THC), the major psycho-active component of marijuana, in human prostate cancer cells PC-3 was investigated. THC caused apoptosis in a dose-dependent manner. Morphological and biochemical changes induced by THC in prostate PC-3 cells shared the characteristics of an apoptotic phenomenon. First, loss of plasma membrane asymmetry determined by fluorescent anexin V binding. Second, presence of apoptotic bodies and nuclear fragmentation observed by DNA staining with 4′,6-diamino-2-phenylindole (DAPI). Third, presence of typical 'ladder-patterned' DNA fragmentation. Central cannabinoid receptor expression was observed in PC-3 cells by immunofluorescence studies. However, several results indicated that the apoptotic effect was cannabinoid receptor-independent, such as lack of an effect of the potent cannabinoid agonist WIN 55,212-2, inability of cannabinoid antagonist AM 251 to prevent cellular death caused by THC and absence of an effect of pertussis toxin pre-treatment.
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Key words: Cannabinoid; Cannabinoid receptor; Apoptosis; Prostate cell

1. Introduction

Marijuana cannabinoids exert a wide spectrum of psycho-active and immuno-active effects, such as the coordination of movement, short-term memory and titration of mood and emotions. Cannabinoids have also been shown to be involved in immune homeostasis and control [1]. It is known that cannabinoids bind receptors in the brain and that these receptors appear also to be in tissues outside the central nervous system. Two cannabinoid receptor subtypes have been identified, designated CB_1 and CB_2 [2]. CB_1 is predominantly expressed in brain although it has also been detected in reproductive organs such as uterus [3] and testis [4], while CB_2 has been predominantly found in immune tissues. Cannabinoids binding to receptor inhibits adenylate cyclase and the accumulation of cAMP. In contrast, cannabinoid activation of arachidonic acid release by stimulation of phospholipase C and release of the calcium concentration from intracellular stores are receptor-independent effects. The availability of the major active psychotropic component of marijuana, Δ9-tetrahydrocannabinol (THC), in its pure form and the recent discovery of the endogenous ligand for cannabinoids receptors [5] have generated a lot of studies on cannabinoids and their cellular effects. Cannabinoids have been shown to induce apoptosis in mononuclear cells [6], macrophages, lymphocytes [7] and glial cells [8] and they activate cell growth in hematopoietic cell lines [9]. However, few studies have been performed in tissues outside the central nervous system and immune cells. The effects of cannabinoids in peripheral tissues remain still unelucidated. Although endogenous cannabinoids, like anandamide, are principally synthesized in brain, recent reports indicate that they are also produced in testis [10]. The fact that anandamide is produced in testis and that CB_1 receptor is also expressed in reproductive organs prompted us to investigate the effect of cannabinoids in prostate cells.

The in vitro study of prostate cancer has been limited by the availability of cell lines. Both normal and tumor-derived prostate epithelial cells have been extremely difficult to growth in culture. PC-3 cells are an androgen refractory human epithelial cell line derived from a prostatic metastasis to the bone. In this study, we have observed that THC induces apoptosis in PC-3 cells. The cannabinoid effect was compared to that produced by ceramide, used as positive control for apoptotic induction.

Cells undergoing apoptosis display several morphological and biochemical alterations, including a reduced cellular volume, condensed chromatin in the nucleus, changes in the distribution of plasma membrane phospholipids and cleavage of chromatin into oligonucleosome length DNA fragments ('DNA ladders') by nuclease, although this fragmentation of internal nucleosomal DNA is not always present in apoptotic cells [11]. The biochemical signals that regulate apoptosis are not well defined but recent evidence has implicated ceramide as an important second messenger regulating cell death [12] as well as other stress responses like induction of nerve growth factor synthesis by astrocytes [13]. Ceramide has been shown to activate several kinases and phosphatases including the ζ isoform of protein kinase C (PKC ζ) [14]. Ceramide is generated from sphingomyelin hydrolysis by a PLC-type sphingomyelinase. Sphingomyelin breakdown has been shown to occur in multiple cell types in response to a variety of extracellular mediators and play an important role in the regulation of cell death, differentiation and senescence. In prostate cells, ceramide has been shown to mediate apoptosis induced by suramin [15] and by PKC [16].

2. Materials and methods

2.1. Materials

THC and 3-4,5-dimethylthiazole-2,5-diphenyltetrazolium bromide thiazole blue (MTT) were from Sigma (St. Louis, USA). WIN 55,212-2 and AM 251 were from Tocris (Bristol, UK). D-Erythro-*N*-acetylsphingosine (C2-ceramide), 4′,6-diamino-2-phenylindole (DAPI) and annexin V-FITC were from Calbiochem (La Jolla, CA, USA). Anti-cannabinoid CB1 receptor polyclonal antibody was from Chemicon International (Temecula, CA, USA). Fluorescein-conjugated donkey anti-rabbit IgG was from Amersham (Little Chaltfont, UK). Other agents were from Sigma (St. Louis, USA).

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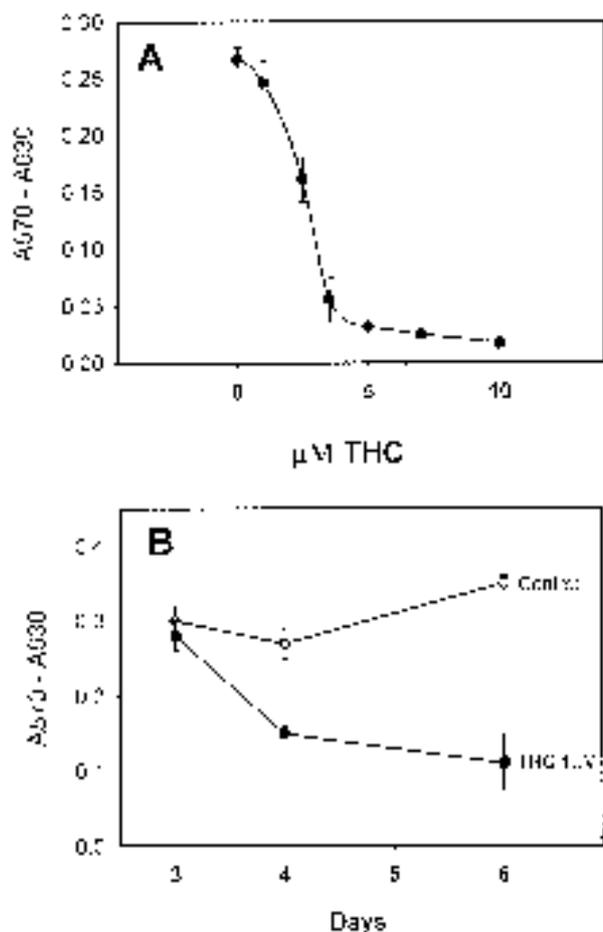E-mail: bqidm@bioqui.alcala.es

Fig. 1. Inhibition of human prostate PC-3 cells mitochondrial oxidative metabolism by THC. A: Cells were cultured in serum-free medium in the presence of increasing concentrations of THC for 3 days. B: Cells were cultured in serum-free medium in the absence (○) or presence (●) of 1 μM THC for the times indicated. Each point represents triplicate determinations and results are based on at least three independent experiments.

2.2. Cell cultures

PC-3 cells were routinely grown in RPMI 1640 medium supplemented with 10% fetal calf serum. 24 h before the experiment, the serum-containing medium was removed and cells were transferred to a chemically defined medium consisting of RPMI 1640 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite.

2.3. MTT assay

After treatment, cells were incubated with 5 μg/ml MTT for 1 h at 37°C and then, formazan crystals were solubilized by addition of 60 mM HCl in isopropanol for 24 h at 37°C. The absorbance at 570 and 630 nm was then monitored.

2.4. Anexin V-FITC binding

Cells were grown in glass coverslips and were made quiescent by serum starvation. After treatment, cells were washed in phosphate-buffered saline (PBS) and then with binding buffer (50 mM HEPES pH 7.4, 750 mM NaCl, 12.5 mM $CaCl_2$, 5 mM $MgCl_2$ and 20% BSA). Recombinant anexin V conjugated with fluorescein isothiocyanate (200 μg/ml) was then added for 30 min. After washing with binding buffer, 10 μl propidium iodide (PI) was added and then, cells were observed by fluorescence microscopy [17].

2.5. DAPI staining of nuclei

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome DAPI. PC-3 cells were grown in glass coverslips and after treatment, coverslips were incubated with a drop of 1 μg/ml

DAPI for 20 min. Coverslips were then washed in PBS, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and subjected to fluorescence microscopy.

2.6. DNA fragmentation

DNA from prostate cancer PC-3 cells was prepared by modification of a previous method [18]. Cells were cultured in plates and treated according to the experiment. After treatment, cells (floating plus attached) were washed in PBS and pelleted. Cells were lysed in 250 μl of STE (250 mM sucrose, 5 mM Tris-HCl, pH 8.0, 1 mM EDTA), 1.56 ml TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 125 μl 25% (w/v) sodium dodecyl sulfate overnight at 37°C. After treatment with 50 μg/ml RNAse A for 1 h at 37°C, 8 M potassium acetate was added to obtain a final concentration of 1.33 M and then lysate was extracted with chloroform:isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase with ethanol and an aliquot containing 4 μg DNA was subjected to electrophoresis in 2% agarose gels.

2.7. Immunofluorescence of CB_1 receptor

PC-3 cells were seeded in glass coverslips and grown in serum-containing medium for 2 days. Afterwards, coverslips were washed in PBS and immersed in 3.7% formaldehyde for 5 min after which they were rinsed in PBS and subsequently incubated in 0.05% Triton X-100 for 5 min. After washing in PBS, coverslips were incubated with rabbit anti-cannabinoid CB_1 receptor (1:10 dilution) for 120 min in a humid chamber. Afterwards, coverslips were rinsed extensively in PBS and incubated with the secondary antibody fluorescein-conjugated anti-rabbit IgG (1:25 dilution) for 60 min. Coverslips were then washed in PBS, mounted with Vectashield and observed by fluorescence microscopy.

3. Results and discussion

3.1. THC and C_2-ceramide induce apoptosis in prostate PC-3 cells

The study of the action of cannabinoids in prostate tumor cells was initiated by analyzing the effect of the major active component of marijuana, THC, in PC-3 cells viability. PC-3 cells were treated with different doses of THC and cellular viability was determined by the ability of cells to hydrolyze tetrazolium salts, which is dependent on mitochondrial oxidative metabolism. Treatment of cells with THC resulted in a dramatic drop of cellular viability in a dose-dependent manner (Fig. 1A). Cell death occurred after 2–3 days of the addition of THC and a maximal effect was observed at 6 days
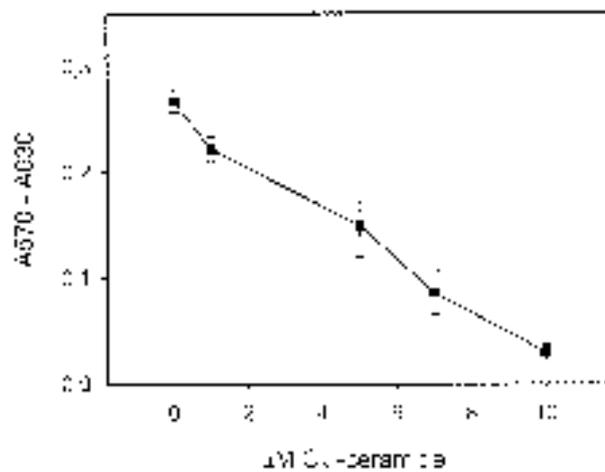

Fig. 2. Inhibition of human prostate PC-3 cells mitochondrial oxidative metabolism by C_2-ceramide. Cells were incubated in serum-free medium in the presence of increasing concentrations of C_2-ceramide for 3 days. Each point represents triplicate determinations and results are based on two independent experiments.
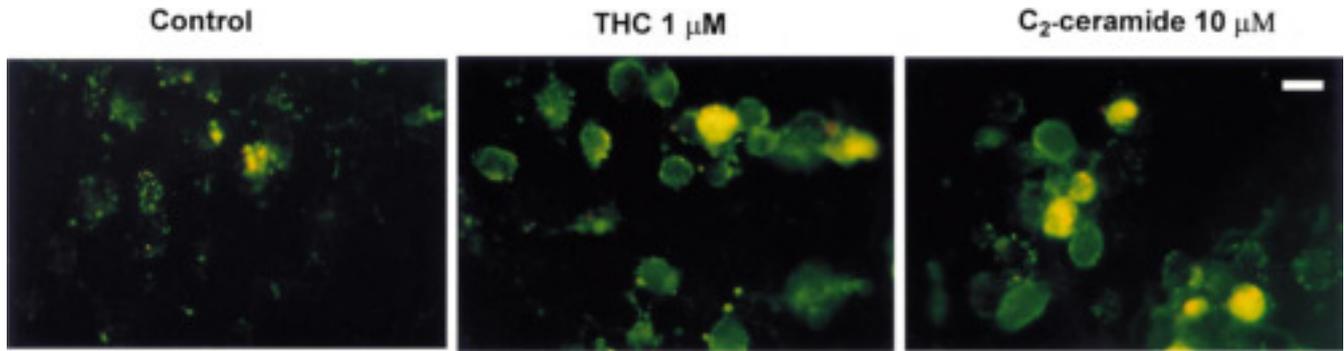
Fig. 3. Anexin V-FITC binding to PC-3 cells. Cells were cultured in serum-free medium and treated for 6 days with A: DMSO (0.1%), B: 1 μM THC, C: 10 μM C_2-ceramide. Micrographs are representative of three independent experiments (bar = 10 μm).
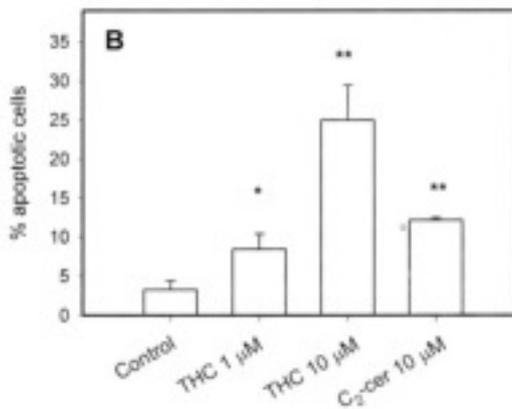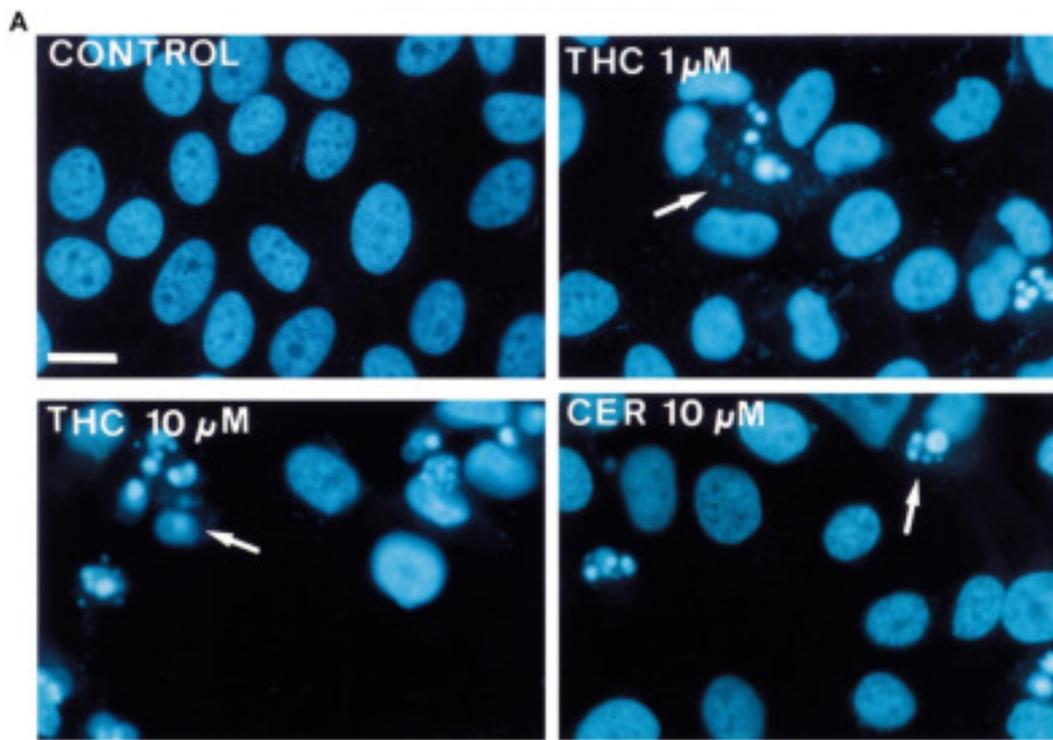

Fig. 4. Nuclear fragmentation observed by DNA staining with DAPI. A: Human prostate PC-3 cells were cultured in serum-free medium and exposed to vehicle, 1 μM THC, 10 μM TCH and 10 μM C_2-ceramide and stained with DAPI. Condensed and fragmented nuclei (arrow) were observed in cells treated with THC and with C_2-ceramide. Micrographs are representatives of three independent experiments (bar = 10 μm). B: Quantitative analysis of three independent experiments. Significantly different (*$P < 0.05$, **$P < 0.01$) versus incubation with vehicle.
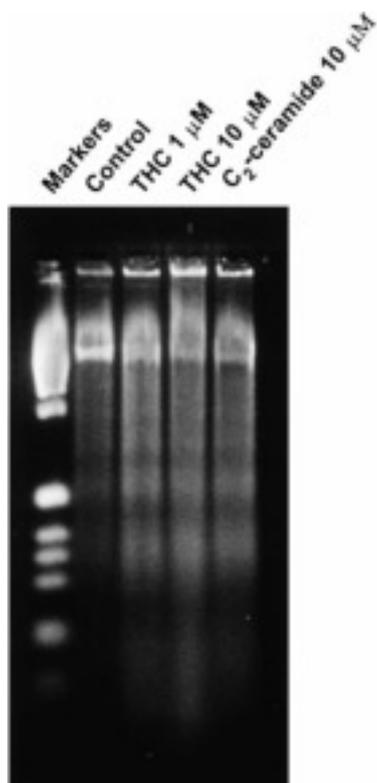
Fig. 5. DNA fragmentation induced by THC and C_2-ceramide. Human prostate PC-3 cells were exposed to vehicle, 1 μM THC, 10 μM TCH and 10 μM C_2-ceramide for 7 days. The result shown is a representative of four other independent experiments.

(Fig. 1B). We then compared the effect produced by THC with that induced by ceramide, which has been shown to induce apoptosis in many cells including prostate cells [15]. In our cell type, addition of the short chain cell-permeable analog of ceramide, C_2-ceramide, also reduced the cellular viability. The effect was maximal at 10 μM C_2-ceramide (Fig. 2).

An early and critical event in apoptosis involves changes on the cell surface as exposure of phosphatidylserine that can be detected by binding of fluorescent annexin V. To characterize the cellular death process caused by THC, PC-3 cells were treated with the agent and binding of FITC-annexin V was detected by fluorescence microscopy. A simultaneous label of cellular DNA with PI was performed to discriminate between apoptotic cells ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$) [15] (data not shown). Results revealed that treatment with 10 μM THC and 10 μM ceramide induce changes in plasma membrane asymmetry from 3 days and were maintained until 6 days, assessed by an increase in membrane fluorescence (Fig. 3 and data not shown).

To further analyze whether the reduction in cell viability by THC and ceramide is due to apoptosis, PC-3 cells were treated with 1 μM THC, 10 μM THC and 10 μM C_2-ceramide for different days and then, cells were stained with DAPI, which enters the cells and binds to fragmented DNA. Nuclear changes were observed by fluorescence microscopy. Results in Fig. 4A show apoptotic bodies in cells treated with THC and C_2-ceramide but not in control cells. Quantitative analysis is shown in Fig. 4B. THC induced a dose-response increase in apoptotic cells. The increase is higher than that observed in ceramide-treated cells used as a positive control (Fig. 4).

To confirm whether THC induced internucleosomal cleavage of DNA, DNA from treated cells was isolated and resolved by agarose electrophoresis. THC induces a dose-dependent DNA fragmentation into typical 180 bp multiples, giving rise to the ladder pattern characteristic of cell death by apoptosis (Fig. 5). Ceramide-treated cells also showed the ladder pattern (Fig. 5).

Results shown here indicate that the cellular death process induced by THC in prostate tumor cells PC-3 shares the biochemical and morphological changes characteristic of apoptotic cells. These changes are comparable to those induced by ceramide.

3.2. THC-induced apoptosis in PC-3 cells occurs via a receptor-independent pathway

THC binds to two types of receptors, CB_1 and CB_2, which inhibit adenylate cyclase via a pertussis toxin (PTX)-sensitive G protein. CB_1 receptor mRNA has been described in human
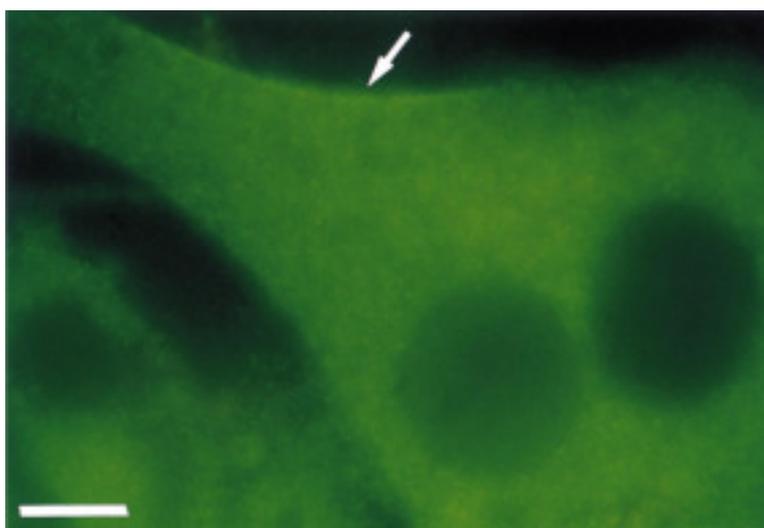

Fig. 6. Immunofluorescence of cannabinoid receptor CB_1 in human prostate PC-3 cells. The micrograph shown is a representative of two other experiments (bar = 5 μm).

prostate [19] although receptor protein has not been characterized in prostate. Immunofluorescence studies show that CB$_1$ receptor is expressed in PC-3 cells (Fig. 6). Therefore, we then studied whether the apoptosis induced by THC in prostate PC-3 cells was mediated by this receptor. We first analyzed the effect of the potent synthetic ligand aminoalkyndole, WIN 55,212-2 [19], which displays a high affinity for both cannabinoid receptors. Neither 0.5 nor 1 μM WIN 55,212-2 had any effect on PC-3 cell viability (Fig. 7). Moreover, the effect produced by THC was not prevented by the antagonist AM 251 (Fig. 6). This results suggests a receptor-independent mechanism for THC-induced apoptosis in PC-3 prostate cells. Cannabinoid receptors are coupled to a hetero-trimeric G$_i$ protein that can be inactivated by pre-treatment with PTX. We therefore compared the apoptotic effect of THC in the presence or absence of PTX. Pre-treatment of PC-3 cells with 100 ng/ml PTX had no effect on apoptosis induced by THC (Fig. 8), reinforcing the notion of a cannabinoid receptor-independent phenomenon.

Thus, in this study, we have shown that the major active component of marijuana, THC, induced prostate PC-3 cell death by an apoptotic process. The apoptotic death caused by THC treatment was even higher than that produced by ceramide. In spite of the presence of cannabinoid receptor CB$_1$ in prostate PC-3 cells, THC exerts its action via a non-receptor-mediated pathway. Our results are in concordance with previous studies which demonstrate in other cell types, a cannabinoid induction of apoptosis by a receptor-independent mechanism [7,8]. Cannabinoids also regulate hemato-poietic cell growth independently of receptor [9]. Whatever the precise mechanism underlying the non-receptor-mediated effects of cannabinoids, these molecules regulate fundamental cellular functions like proliferation and cell death. The apoptotic effect of THC described in this study might provide the basis for future therapeutic application of cannabinoids. Further studies will determinate whether cannabinoids exert other effects in prostate cells.
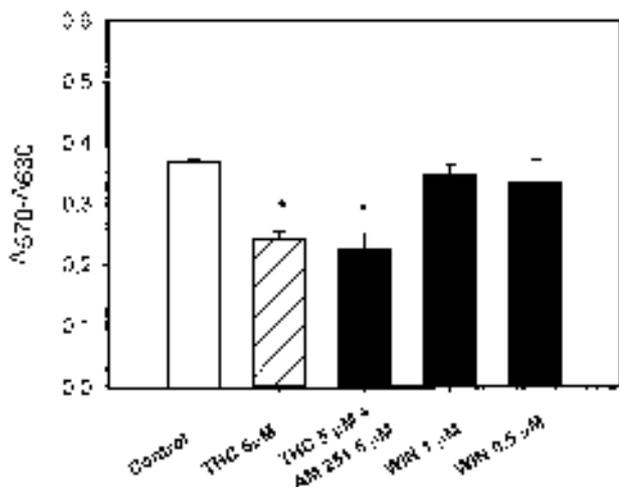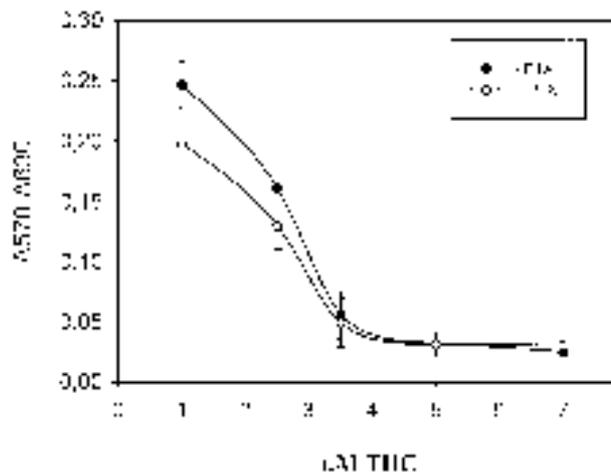

Fig. 8. Absence of an effect of PTX on THC-induced cell death. PC-3 cells were cultured in serum-free medium, pre-incubated for 30 min with 100 ng/ml PTX and treated with different doses of THC for 3 days. Mitochondrial oxidative metabolism was determinate by a MTT assay. Each point represents triplicate determinations and results are based on two independent experiments.

References

[1] Klein, T.W., Newton, C. and Friedman, H. (1998) Immunol. Today 19, 373–381.
[2] Felder, C.C. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 179–200.
[3] Das, S.K., Paria, B.C., Chakraborty, I. and Dey, S.K. (1995) Proc. Natl. Acad. Sci. USA 92, 4332–4336.
[4] Gérard, C.M., Mollereau, C., Vassart, G. and Parmentier, M. (1991) Biochem. J. 279, 129–134.
[5] Mechoulam, R., Fride, E. and Di Marzo, V. (1998) Eur. J. Pharmacol. 359, 1–18.
[6] Schwarz, H., Blanco, F.J. and Lotz, M. (1994) J. Neuroimmunol. 55, 107–115.
[7] Zhu, W., friedman, H. and Klein, T. (1998) J. Pharmacol. Exp. Ther. 286, 1103–1109.
[8] Sánchez, C., Galve-Roperh, I., Canova, C., Brachet, P. and Guzmán, M. (1998) FEBS Lett. 436, 6–10.
[9] Derocq, J.M., Bouaboula, M., Marchand, J., Rinaldi-Carmona, M., Ségui, M. and Casellas, P. (1998) FEBS Lett. 425, 419–425.
[10] Schmid, P.C., Schwindenhammer, D., Krebsbach, R.J. and Schmid, H.H.O. (1998) Chem. Phys. Lipids 92, 27–35.
[11] Pallardy, M., Perrin-Wolff, M. and Biola, A. (1997) Toxicol. In Vitro 11, 573–578.
[12] Kolesnick, R.N. and Krönke, M. (1998) Annu. Rev. Physiol. 60, 643–665.
[13] Galve-Roperh, I., Haro, A. and Díaz-Laviada, I. (1997) Mol. Brain Res. 52, 90–97.
[14] Galve-Roperh, I., Haro, A. and Díaz-Laviada, I. (1997) FEBS Lett. 415, 271–274.
[15] Gill, J.S. and Windebank, A.J. (1997) Cancer Lett. 119, 169–176.
[16] Garzotto, M., White-Jones, M., Jiang, Y., Ehleiter, D., Liao, W., Haimovitz-Friedman, A., Fuks, Z. and Kolesnick, R. (1998) Cancer Res. 58, 2260–2264.
[17] Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) J. Immunol. Methods 184, 39–51.
[18] Román, I.D., Rodríguez-Henche, N., Fueyo, J.A., Zueco, J.A., Menor, C., Prieto, J.C. and Guijarro, L.G. (1998) Arch. Toxicol. 72, 559–565.
[19] Galiégue, S., Mry, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G. and Casellas, P. (1995) Eur. J. Biochem. 232, 54–61.

Fig. 7. Comparison of THC and WIN 55,212-2 on mitochondrial oxidative metabolism effect. PC-3 cells were incubated in the presence of 5 μM THC, 5 μM THC plus 5 μM AM 251 or different doses of WIN 55,212-2 for 3 days and mitochondrial oxidative metabolism was determinated by a MTT assay. Each point represents triplicate determinations and results are based on two independent experiments. *, Significantly different ($P < 0.05$) versus incubations with vehicle.