

Δ^9 -Tetrahydrocannabinol increases nerve growth factor production by prostate PC-3 cells

Involvement of CB1 cannabinoid receptor and Raf-1

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Cannabinoids, the active components of marijuana, exert a variety of effects in humans. Many of these effects are mediated by binding to two types of cannabinoid receptor, CB1 and CB2. Although CB1 is located mainly in the central nervous system, it may also be found in peripheral tissues. Here, we study the effect of cannabinoids in the production of nerve growth factor by the prostate tumor cell line PC-3. We show that addition of Δ^9 -tetrahydrocannabinol to PC-3 cells stimulated nerve growth factor production in a dose-dependent and time-dependent manner. Maximal effect was observed at 0.1 μM Δ^9 -tetrahydrocannabinol and 72 h of treatment. Stimulation was

reversed by the CB1 antagonists AM 251 and SR 141171A. Pre-treatment of cells with pertussis toxin also prevented the effect promoted by Δ^9 -tetrahydrocannabinol. These results indicate that Δ^9 -tetrahydrocannabinol stimulation of nerve growth factor production in these cells was mediated by the cannabinoid CB1 receptor. The implication of Raf-1 activation in the mode of action of Δ^9 -tetrahydrocannabinol is also suggested.

Keywords: cannabinoids; nerve growth factor; prostate; Raf-1.

Cannabinoids, the active components of marijuana, exert a wide variety of effects in humans including euphoria, antinociception and anticonvulsive effects. Many of these effects have been shown to occur through binding to cannabinoid receptors. To date, two cannabinoid receptors have been cloned in humans, the central cannabinoid receptor CB1 [1] and the peripheral receptor CB2 [2]. The primary structure of both receptors is consistent with the seven transmembrane helical structure that characterizes G protein-coupled receptors [3]. Signal transduction occurs via $G_{i/o}$ proteins which mediate inhibition of adenylyl cyclase [4], modulation of N-type Ca^{2+} channels [5], activation of mitogen-activated protein kinase and expression of krox-24 [6]. Although CB1 is expressed predominantly in the central nervous system it has also been found in other peripheral tissues such as the reproductive system and the gastrointestinal tract [7–9]. The second cannabinoid receptor subtype CB2 is expressed predominantly in the immune system.

Despite the widespread use of marijuana by adolescents, very little is known about the effects of cannabinoids on the reproductive system. It has been shown that cannabinoids have inhibitory effects on the regulation of reproduction [10] and testosterone secretion [11] but the molecular mechanisms responsible for this action remain unclear. Other adverse effects of cannabinoids include a possible

increased risk of prostate cancer for male users [12]. However, no *in vitro* experiments have been performed to study the direct effect of cannabinoids in the human prostate.

Malignant epithelial cells of the human prostate exhibit an enhanced capacity for expression of growth factors including nerve growth factor (NGF), to escape a paracrine dependence on stromal cell-derived growth factors [13]. The mature and biologically active form of NGF, β -NGF, has been shown to stimulate the growth of prostate epithelial cells [14,15] and to increase the *in vitro* invasion of prostate cancer cells through a reconstituted basement membrane [16]. The NGF receptor also plays a role in the neoplastic progression of the human prostate. A reduction in the expression of the low-affinity NGF receptor ($p75^{\text{NTR}}$) during malignant transformation of the human prostate has been described [17] suggesting that $p75^{\text{NTR}}$ could be a negative regulator of the human prostate epithelial cell growth [18].

In this study, we investigated the effect of the major psychoactive natural cannabinoid Δ^9 -tetrahydrocannabinol (THC) on NGF production by the malignant epithelial human prostate cell line PC-3. We have previously shown that PC-3 cells express central cannabinoid receptor CB1 [19]. Here we show that THC has a biphasic effect on NGF production by prostate cells. At nanomolar concentrations THC has a stimulant effect, whereas at micromolar concentrations it has an inhibitory effect. The stimulatory effect of THC is mediated by cannabinoid receptor CB1. An involvement of Raf-1 activation is also suggested.

MATERIALS AND METHODS

Materials

Monoclonal anti-NGF was from Chemicon International (Temecula, CA, USA), β -Galactosidase-conjugated anti-NGF

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Abbreviations: CB1, central cannabinoid receptor; MAP, mitogen activated protein; NGF, nerve growth factor; PTX, pertussis toxin; THC, Δ^9 -tetrahydrocannabinol.

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was from Boehringer Mannheim (Germany) and monoclonal anti-Raf-1 was from Transduction Laboratories (UK). Fluorescein-conjugated sheep anti-(mouse IgG) was from Amersham (Little Chalfont, UK).

SR 141716A was kindly donated by Sanofi Recherche (Montpellier, France). THC was from Sigma (St Louis, MO, USA). HU-210 and AM 251 were from Tocris Cookson (Bristol, UK). Other agents were from Sigma.

Cell culture

Human prostate malignant epithelial PC-3 cells were grown routinely in RPMI-1640 medium supplemented with 10% fetal bovine serum. Twenty-four hours before the experiment, the serum-containing medium was removed and cells were transferred to a chemically defined medium consisting of RPMI-1640 supplemented with $5 \mu\text{g}\cdot\text{mL}^{-1}$ insulin, $5 \mu\text{g}\cdot\text{mL}^{-1}$ transferrin and $5 \text{ ng}\cdot\text{mL}^{-1}$ sodium selenite.

NGF determination

After the different treatments performed in triplicate, cell supernatants were collected and diluted in 1 vol. NaCl/P_i containing 0.1% Tween 20 and 0.5% gelatin. NGF released by cells was determined using double-site enzyme-linked immunosorbant assay (ELISA), with monoclonal anti-NGF coupled or not to β -galactosidase as described previously [20].

Identification of Raf-1 by immunoblotting

PC-3 cells, treated as described in the legend to Fig. 4, were scraped into 1 mL of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol containing $1 \mu\text{g}\cdot\text{mL}^{-1}$ leupeptin, $1 \mu\text{g}\cdot\text{mL}^{-1}$ aprotinin, $10 \mu\text{g}\cdot\text{mL}^{-1}$ soybean trypsin inhibitor and 1 mM phenylmethanesulfonyl fluoride). Cells were frozen, then thawed and subsequently disrupted by sonication after which they were centrifuged at $100\,000\text{ g}$ for 1 h. The supernatant was used as the cytosolic fraction and the pellet was resuspended in lysis buffer and used as the particulate fraction. The amount of protein in each sample was determined using the protein assay kit (Bio-Rad, Richmond, CA, USA) and equivalent amounts were analyzed by Western blot as described previously [21] using a primary monoclonal antibody against Raf-1.

RESULTS

Enhanced secretion of NGF by prostate PC-3 cells after treatment with exogenous THC

In order to study the effect of cannabinoids on NGF production by human prostate PC-3 cells, we treated cells with the principle active component of marijuana, THC. Treatment of cells with THC resulted in an increase in cell-secreted NGF that was maximal at $0.1 \mu\text{M}$ THC (4.3-fold increase), although significantly different from $0.01 \mu\text{M}$ THC ($p < 0.001$ by Student's *t*-test) (Fig. 1A). We had previously shown that THC may induce apoptotic death in PC-3 cells at micromolar concentrations [19], therefore we tested higher doses of THC and measured the NGF produced. At $5 \mu\text{M}$ THC an inhibition of cell-secreted

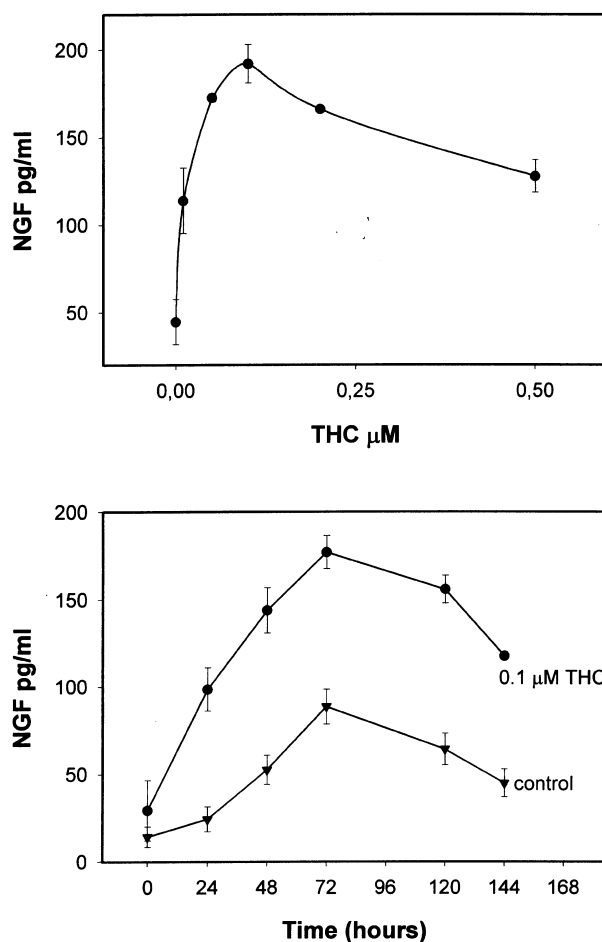


Fig. 1. Effect of THC treatment on NGF production by prostate PC-3 cells. (A) Dose-response induction. Cells were incubated with increasing concentrations of THC for 72 h. (B) Time-course of NGF secretion after treatment with THC. Cells were treated with vehicle alone (∇) or with $0.1 \mu\text{M}$ THC ($\bullet$). Extracellular NGF was assayed using a double-site ELISA. Data are given as mean $\pm$ SD of three independent experiments performed in triplicate.

NGF was observed compared with controls (Table 1). As this dose of THC may cause apoptosis, the inhibition observed could be caused by a decrease in cell number. However, results normalized with total protein

Table 1. Effect of THC and the CB1 receptor antagonist SR 141716A on NGF production by prostate PC-3 cells. Cells were incubated with THC and with or without $1 \mu\text{M}$ SR 141716A (SR1) for 72 h. Extracellular NGF was measured by double-site ELISA. Results are given as means $\pm$ SD of two experiments performed in triplicate.

Treatment	NGF ($\text{pg}\cdot\text{mL}^{-1}$)
None	71.14 ± 0.8
THC $0.1 \mu\text{M}$	140.7 ± 12^a
THC 0.1:M + SR1	99.51 ± 5.2
THC 5:M	51.78 ± 12.6^b
THC 5:M + SR1	48.77 ± 6.1^b

^a $P < 0.0001$ compared with no treatment by Student's *t*-test.

^b $P < 0.01$

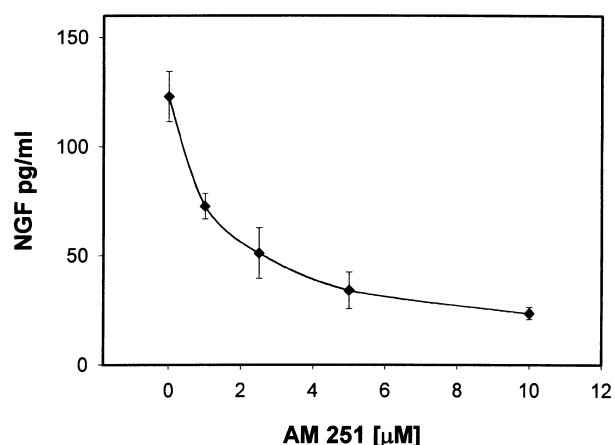


Fig. 2. Dose–response inhibition of THC induction of NGF secretion by the antagonist AM 251. PC-3 cells were treated with 0.1 μM THC and increasing concentrations of AM 251 for 72 h. Extracellular NGF was then measured by double-site ELISA. Data are given as mean ± SD of a representative experiment performed in triplicate. Identical results were obtained in other two independent experiments.

concentration in the supernatant were essentially the same (data not shown).

Time-course experiments revealed that the maximal NGF concentration of the supernatant peaked at 72 h of treatment (Fig. 1B), so all later experiments were performed at this time.

Increasing effect of THC is mediated by CB1 receptor

THC binds to both cannabinoid receptors CB1 and CB2 with similar affinities [22] and exerts a biological effect by binding to both receptors. To investigate whether the effect elicited by THC was mediated by its binding to the central

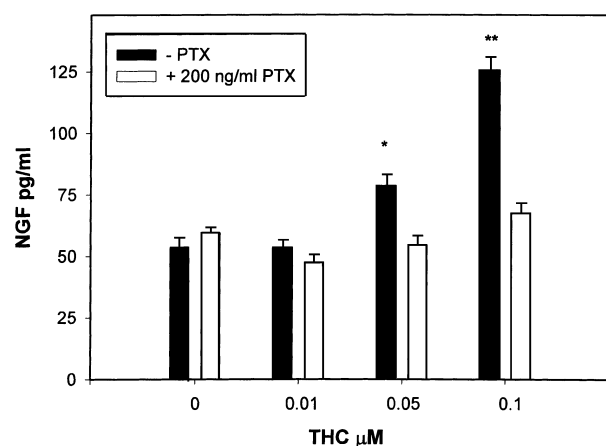


Fig. 3. Inhibition of the stimulant effect of THC by pretreatment with pertussis toxin. PC-3 cells were pre-incubated (white bars) or not (black bars) with 200 ng·mL⁻¹ PTX for 30 min and then incubated with different doses of THC for 72 h. Extracellular NGF was then measured by double-site ELISA. Data are given as mean ± SD of two independent experiments performed in triplicate. (**P* < 0.05; ***P* < 0.01 compared with no addition by Student's *t*-test)

Table 2. Effect of the MAP kinase inhibitor PD 98059 on THC-stimulated NGF production in PC-3 cells. Cells were preincubated or not with 1:1M PD 98059 for 30 min and then, treated with vehicle or 0.1:1M THC for 72 h. Extracellular NGF was measured by double-site ELISA. Results are means ± S.D. of two experiments performed in triplicate.

Treatment	NGF (pg·mL ⁻¹)
None	77.0 ± 3.8
THC	198.3 ± 10.2 ^a
THC + PD 98059	68.16 ± 5.2
PD 98059	64.61 ± 4.6

^a *P* < 0.01 compared with no treatment by Student's *t*-test.

cannabinoid receptor, we used the antagonist of the CB1 receptor SR 141716A [23] and its analog AM 251 [24], which has been shown to bind to the CB1 receptor in rodent brains [25]. Pre-treatment of cells with 1 μM SR 141716A prevented the effect elicited by 0.1 μM THC but did not affect the inhibition produced by 5 μM THC, as shown in Table 1. Addition of AM 251 to PC-3 cells abolished the induction of NGF secretion elicited by 0.1 μM THC with an IC₅₀ of 3 μM (Fig. 2).

Because cannabinoid receptors are coupled to pertussis toxin (PTX)-sensitive G_{i/o} proteins, treatment of cells with PTX should prevent the effect produced by THC. The results shown in Fig. 3 indicate that pretreatment for 30 min with PTX abolished the stimulant effect induced by THC. Therefore, this result shows that the enhanced NGF secretion produced by cannabinoids in prostate PC-3 cells was mediated by cannabinoid receptor CB1.

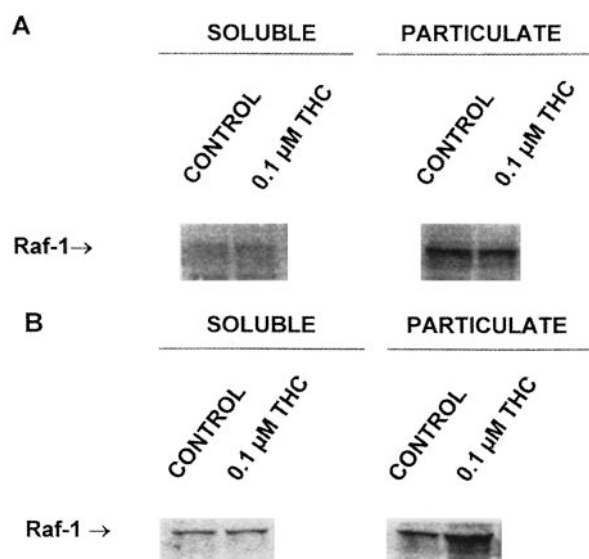


Fig. 4. Immunoblot analysis of Raf-1 in particulate and soluble fraction. PC-3 cells were treated for 1 (A) or 72 h (B) with 0.1 μM THC and cells were then lysed and particulate and soluble fractions were separated by centrifugation. Proteins were resolved by electroblotting and Raf-1 was visualized using monoclonal anti-Raf-1. One representative immunoblot of two is shown.

Involvement of Raf-1 activation in the induction of NGF secretion by THC

We further investigate the signaling pathways involved in the action of cannabinoids in prostate cells. We have previously shown that the mitogen-activated protein (MAP) kinase cascade is involved in signaling pathways that regulate NGF synthesis in astrocytes [26], therefore we decided to study the role of this pathway in the action of cannabinoids using the selective inhibitor of MAP kinase kinase (MEK or MAPK/ERK kinase), PD 98059 [27]. Table 2 shows that the stimulant effect induced by THC was prevented by PD 98059, suggesting involvement of the MAP kinase pathway in the action of cannabinoids.

PD 98059 acts by binding to inactivated MEK and inhibiting its activation by Raf-1 or MEK kinase. We then examined the activation of Raf-1 by THC in prostate cells. When Raf-1 is activated it binds to the GTP-bound form of Ras. The interaction with activated Ras localizes Raf-1 to the plasma membrane and is often the first step in Raf-1 activation [28]. Therefore, translocation of Raf-1 to the plasma membrane is an indicator of its activation. To examine the effect of THC on Raf-1 localization Western blot experiments were undertaken. Cells were treated with THC and, after separation of particulate and soluble fractions, Raf-1 was detected by Western blotting. The results shown indicate that short-term treatment with THC (until 1 h) did not change the distribution of Raf-1 compared with controls (Fig. 4A). However, treatment times (72 h) increased the amount of Raf-1 localized in the particulate fraction and diminished that localized in the soluble fraction (Fig. 4B).

DISCUSSION

This study shows that the exogenous addition of sub-micromolar doses of the cannabinoid THC induced a time-dependent and dose-dependent increase in cell-secreted NGF by prostate tumor PC-3 cells. The fact that the stimulant effect of THC was inhibited by either CB1 receptor antagonists AM 251 and SR 141716A or pretreatment with PTX, supports the notion that the effect was mediated by the CB1 cannabinoid receptor. The maximal dose of THC that produced an increase in NGF secretion was 0.1 μM . Higher doses, 5 μM THC, inhibited basal production of NGF by prostate cells via a SR 141716A-resistant pathway. We have previously described that THC promotes apoptosis in prostate PC-3 cells at micromolar concentrations via a receptor-independent mechanism [19]. These results suggest a biphasic effect of THC on prostate PC-3 cells. At nanomolar THC concentrations, NGF production by PC-3 cells is stimulated, but at micromolar THC concentrations, NGF is decreased and apoptosis takes place. A biphasic effect of THC on hormone secretion has been demonstrated in animals [29,30]. Administration of low doses of THC to male mice caused a rapid and sustained increase in plasma testosterone, whereas higher doses decreased plasma testosterone concentrations [29]. Cannabinoid CB1 receptor agonists also induced a biphasic response on prolactin secretion in rats [30].

The fact that maximal stimulation occurs after 72 h of treatment is in agreement with the notion that NGF is coded by a late expression gene and is in concordance with

previous results obtained in our laboratory showing a long-term induction of NGF synthesis in astrocytes by other compounds [26,31].

NGF immunoreactive proteins have been shown to be produced by stromal and epithelial cell lines derived from human prostate [13,32] and to induce epithelial cell growth [14,15]. Androgen-refractory cancer cell seems to produce an autocrine NGF-like protein to escape a paracrine dependence on stroma cell-derived NGF [13]. However, the mature form of β -NGF does not appear to be expressed by human prostate stroma cells, unlike the long and short forms of precursor NGF [32]. In this study, PC-3 cell-secreted NGF was measured using double-site ELISA and we therefore cannot distinguish between the precursor and the mature form.

Raf-1 is a member of the MAP kinase cascade that is activated upon the binding of many factors to their receptors. Activation of Raf-1 and the MAP kinase cascade by cannabinoids has been shown to occur in cell lines and astrocytes [6,33–35]. We have shown that long-term treatment of human prostate PC-3 cells with THC induced localization of Raf-1 to the membrane, one of the first steps in Raf-1 activation. The long-term effect observed with THC is in agreement with the notion that activation of the MAP kinase pathway may be an important step in the stimulation of NGF production by cannabinoids in prostate cells. Further support for this comes from our results concerning the inhibition of NGF production observed when cells are treated with the MAP kinase inhibitor PD 98059. The mechanism by which THC activates Raf-1 and stimulates production of NGF remains to be clarified. Involvement of the MAP kinase cascade in the control of NGF production by prostate cells is in concordance with the implication of the MAP kinase pathway in cell proliferation. Recent investigations have shown that the ERK cascade and Raf-1 long-term activation mediate the antiproliferative effect of THC in other cell types [33]. However, in that study, the THC doses used were higher and the time necessary to induce death was longer than in our case.

Autonomous production of NGF by prostate epithelial tumor cells has an important role in tumor growth and malignant progression [14–16]. Our results suggest that cannabinoids may play a role in the regulation of NGF synthesis by prostate cells. Studies performed by other authors indicate that endocannabinoids may suppress NGF Trk receptors in prostate DU-145 tumor cells leading to an inhibition of cell proliferation [36]. Taken together, these results suggest that cannabinoids may have a biological role in the regulation of prostate growth. The stimulatory effect on prostate cell NGF production exerted by the cannabinoids employed in this study might provide a field for future investigations. Our results may have important implications not only for the possible development of new antitumor drugs, but also for understanding the role played by cannabinoids in the regulation of prostate cell functions.

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