Abstract

It has been proposed that cannabinoids are involved in the control of cell fate. Thus, these compounds can modulate proliferation, differentiation, and survival in different manners depending on the cell type and its physiopathologic context. However, little is known about the effect of cannabinoids on the cell cycle, the main process controlling cell fate. Here, we show that Δ⁹-tetrahydrocannabinol (THC), through activation of CB₂ cannabinoid receptors, reduces human breast cancer cell proliferation by blocking the progression of the cell cycle and by inducing apoptosis. In particular, THC arrests cells in G₂-M via down-regulation of Cdc2, as suggested by the decreased sensitivity to THC acquired by Cdc2-overexpressing cells. Of interest, the proliferation pattern of normal human mammary epithelial cells was much less affected by THC. We also analyzed by real-time quantitative PCR the expression of CB₁ and CB₂ cannabinoid receptors in a series of human breast tumor and nontumor samples. We found a correlation between CB₂ expression and histologic grade of the tumors. There was also an association between CB₂ expression and other markers of prognostic and predictive value, such as estrogen receptor, progesterone receptor, and ERBB2/HER-2 oncogene. Importantly, no significant CB₂ expression was detected in nontumor breast tissue. Taken together, these data might set the bases for a cannabinoid therapy for the management of breast cancer. (Cancer Res 2006; 66(13): 6615-21)

Introduction

There are very few critical decisions that cells must take during their lifetime. Basically, these are whether to proliferate, differentiate, or die. A tight regulation of the cell cycle is crucial to control all these decisions, and its deregulation has devastating consequences, such as cancer (1). It has been proposed that cannabinoids, the active components of Cannabis sativa, play a role in the control of the aforementioned decisions. For example, they can modulate survival, proliferation, and differentiation depending on the cell type and its physiopathologic context (2, 3). Among the ~70 cannabinoids synthesized by C. sativa, Δ⁹-tetrahydrocannabinol (THC) is the most important in terms of potency and abundance (4). THC exerts a wide variety of biological effects by mimicking endogenous compounds, the endocannabinoids anandamide and 2-arachidonoylglycerol, which activate specific cannabinoid receptors. Thus far, two G protein-coupled cannabinoid-specific receptors have been cloned from mammalian tissues: CB₁ abundantly expressed in the brain and at many peripheral sites, and CB₂, almost exclusively expressed in the immune system (5). Engagement of these receptors by THC or endocannabinoids affects several signaling pathways, some of them directly involved in the control of cell fate. For instance, cannabinoids modulate mitogen-activated protein kinases and the phosphatidylinositol 3-kinase/Akt survival pathway, which have a prominent role in the control of cell growth and differentiation (6). Due to the growing evidence that cannabinoids participate in the control of cell fate and to the fact that the cell cycle is a key process underlying the regulation of survival/proliferation/differentiation decisions, we decided to study the effect of THC on the cell cycle and the mechanism of cannabinoid action on this process. Because breast tumors are one of the most common human neoplasias and one of the leading causes of death among Western women (7), we decided to focus our studies on this particular type of cancer.

Materials and Methods

Cell culture and viability. EVSA-T, MDA-MB-231, MDA-MB-468, and SKBr3 cells were kindly given by Dr. López-Rivas [Centro Andaluz de Biología del Desarrollo, Consejo Superior de Investigaciones Científicas (CSIC), Sevilla, Spain], and MCF-7 and T-47D cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in RPMI 1640 (EVSA-T, MDA-MB-231, MCF-7, and T-47D) or DMEM (MDA-MB-468 and SKBr3) supplemented with 10% fetal bovine serum (FBS), 5 units/mL penicillin, and 5 mg/mL streptomycin. Human mammary epithelial cells (EMEC) were kindly given by Dr. Lacal (Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain) and grown in mammary epithelial growth medium (Cambrex, East Rutherford, NJ) according to the manufacturer’s instructions. Cannabinoid ligands were prepared in DMSO. Control incubations had the corresponding DMSO content. No significant influence of DMSO was observed on cell viability at the final concentration used (0.1-0.2%, v/v). Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (Sigma, St. Louis, MO) according to the manufacturer’s instructions.

Western blot analysis. Samples were subjected to SDS-PAGE, and proteins were transferred onto polyvinylidene fluoride membranes. Blots were incubated with the following antibodies: anti-phosphorylated Cdc2 (Tyr15), anti-cyclin B1, anti-Cdc2/C, anti-p27, anti-caspase-3, and anti-poly(ADP-ribose) polymerase (PARP) (Cell Signaling, Beverly, MA); anti-Cdc2 and anti-p21 (Santa Cruz, Santa Cruz, CA); anti-Wee1 (BioVision, Mountain View, CA); anti-survivin (R&D Systems, Minneapolis, MN); and anti-α-tubulin as loading control (Sigma). Luminograms were obtained with an enhanced chemiluminescence detection kit (Amersham Life Sciences, Arlington Heights, IL), and densitometric analysis was done with Multi-analyser software (Bio-Rad, Hercules, CA).

Cell cycle analysis. Cells were permeabilized and fixed in 1% (w/v) bovine serum albumin and 30% ethanol-PBS and labeled with 5 μM Hoechst 33342 (Molecular Probes, Leiden, the Netherlands). Fluorescence intensity was analyzed using a LSR flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells per analysis were recorded.
Apoptosis. Cells were incubated in binding buffer [10 mmol/L HEPES (pH 7.4), 2.5 mmol/L CaCl$_2$, 140 mmol/L NaCl] supplemented with Annexin V-FITC (Molecular Probes). Propidium iodide (PI; Sigma) was added 1 minute before sample analysis. Fluorescence intensity was analyzed using a FACS Scalibur flow cytometer (Becton Dickinson). For triple staining experiments, cells were labeled with Hoechst 33342, and apoptosis was analyzed as described above. Ten thousand cells per analysis were recorded.

Caspase-3 activity. Caspase-3/7 activity was determined with a luminogenic substrate (Caspase-Glo, Promega, Madison, WI) according to the manufacturer’s instructions. Luminescence was determined in a Microplate Fluorescence Reader (BMG Labtech, Offenburg, Germany).

Tissue samples. Samples were obtained from the Centro Nacional de Investigaciones Oncológicas Tumor Bank (Madrid, Spain). Histologic grade was assessed according to Elston and Ellis criteria. Immunohistochemical staining for prognostic and predictive factors was done by the EnVision method with a heat-induced antigen retrieval step. Monoclonal antibodies for estrogen receptor (ER), progesterone receptor (PR), p53 (Novocastra, Newcastle, United Kingdom), and Ki67 (DAKO, Glostrup, Denmark) were used. ERBB2/HER-2 expression was evaluated using Herceptest (DAKO). The percentage of cells with unequivocal nuclear staining for ER, PR, Ki67, and p53 was scored, and a cutoff of 5% was used for positivity for ER and PR and 15% for Ki67 and p53. For ERBB2/HER-2, only cases with 3+ membranous staining were scored as positive.

Confocal microscopy analysis of cannabinoid receptors. Human breast cancer and normal breast 5-µm paraffin-embedded tissue sections were analyzed. Primary antibodies against CB$_1$ and CB$_2$ receptors (Affinity Bioreagents, Golden, CO) were used. Secondary anti-rabbit antibody Alexa Fluor 594 was from Molecular Probes. Cell nuclei were stained with YOYO-1 (Molecular Probes). Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad).

Reverse transcription-PCR analysis. Total RNA was isolated using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany). cDNA was obtained using the RNeasy Protect kit (Qiagen, Hilden, Germany).
Rimonabant, a selective CB1 receptor antagonist, did not block whereas CB1 mRNA was undetectable (Fig. 1D). THC effect in EVSA-T cells (Fig. 1) studied (data not shown). In contrast, SR144528, a selective CB2 receptor antagonist, partially prevented the THC-induced decrease of cell proliferation is due to the blockade of the G_{2}-M transition. We next sought to examine whether an alteration of EVSA-T cell cycle underlies THC antiproliferative effect. The cannabinoid increased the number of cells in the G_{0}-G_{1} compartment and, in parallel, decreased the number of cells in S phase (Fig. 2A and C). At the highest concentration tested (5 μmol/L), THC also produced the following: (a) an increase in the number of cells in G_{2}-M phases and (b) the appearance of a population of hypodiploid cells (Fig. 2A and C). The latter two effects were prevented by SR144528 (Fig. 2C). Importantly, THC did not alter the cell cycle profile of HMEC cells (Fig. 2B).

To analyze the precise mechanism of THC action, we first studied the expression of several proteins involved in the G_{2}-M transition. THC decreased the total levels of Cdc2 [p34, cyclin-dependent kinase (CDK) 1] (Fig. 3A), the major CDK controlling the entrance of cells in mitosis after completing G_{2} events (8), and SR144528 (2 μmol/L) completely prevented this effect [relative optical density (OD) after 16 hours of treatment relative to vehicle: SR144528+THC, 105 ± 4]. The expression of cyclin B1, the positive regulatory subunit of Cdc2 (9), did not significantly change on cannabinoid challenge (data not shown). The levels of p21, a CDK inhibitor known to prevent Cdc2-cyclin B activation (8), were enhanced by THC (Fig. 3A). It has been recently proposed that p27, a CDK inhibitor traditionally associated to the regulation of G_{1}-S transition, can also inhibit Cdc2 at G_{2}-M (10). In our system, however, THC did not modify p27 levels (data not shown).

To be active, Cdc2 has to be dephosphorylated in the Tyr^{15} residue (11). THC treatment decreased the amount of phosphorylated Tyr^{15}, Cdc2 to a lower extent than total Cdc2 levels (Fig. 3A), indicating that the ratio inactive/active Cdc2 was augmented by THC. Phosphorylation of Cdc2 in Tyr^{15} is controlled by the Wee1/Mik1 family of protein kinases and by the phosphatase Cdc25C (11). Our results show that THC enhances Wee1 and reduces Cdc25C protein levels (Fig. 3A).

In view of the aforementioned results, it is conceivable that THC exposure prevents EVSA-T cells to reach the required levels of active Cdc2 to enter mitosis. To test whether Cdc2 down-regulation is important in the growth-inhibiting effect of THC, we heterologously expressed this kinase. As shown in Fig. 3B, cells overexpressing Cdc2 became significantly more resistant to THC.

### Results

**THC inhibits proliferation of human breast cancer cells.**

Several human breast cell lines were incubated with THC, and viable cell numbers were estimated. THC decreased proliferation in all the tumor cells tested (Fig. 1A). Among the tumor cells, those with more aggressive phenotype (ER−) were more sensitive to THC (Fig. 1B). Remarkably, nontumor HMEC cells were the most resistant to cannabinoid treatment (IC_{50} >12 μmol/L; Fig. 1A). Rimonabant, a selective CB1 receptor antagonist, did not block THC effect in EVSA-T cells (Fig. 1C) or any of the other cell lines studied (data not shown). In contrast, SR144528, a selective CB2 receptor antagonist, partially prevented the THC-induced decrease of EVSA-T cell proliferation (Fig. 1C). Both reverse transcription-PCR (RT-PCR; Fig. 1D) and real-time quantitative PCR experiments (Fig. 1B) confirmed the expression of CB2 mRNA in this cell line, whereas CB1 mRNA was undetectable (Fig. 1B and D).

**THC-induced decrease of cell proliferation is due to the blockade of the G_{2}-M transition.**

### Figure 2. Effect of THC on cell cycle dynamics. Cell cycle profiles of EVSA-T (A) and HMEC cells (B) incubated with 5 μmol/L THC or the corresponding vehicle for 48 hours. Representative experiments (n ≥ 3). C, percentage of EVSA-T cells (mean ± SE) in every phase of the cell cycle after 48 hours of incubation with the indicated compounds. Where indicated, rimonabant (SR1; 1 μmol/L) or SR144528 (SR2; 2 μmol/L) was added 1 hour before THC. Significant differences from control (∗, P < 0.05; **, P < 0.01) or the corresponding concentration of THC alone (∗, P < 0.05).

### Control of Cell Cycle by Cannabinoids

**Statistical analysis.** ANOVA with a post hoc analysis by the Student’s-Newman-Keuls’ test was routinely used. For cannabinoid receptor expression in human samples, data were log transformed to achieve normality in the distribution. An F-test was subsequently done to compare different variances was applied using the Welch modification.

### Figure 3. Expression of CDKs and phosphokinases in response to THC. A, expression of Cdc2, phosphorylated Cdc2 in Tyr^{15} and Cdc2-cyclin B1 complex (p34) (Fig. 3A). B, expression of p21 (Fig. 3A), C, expression of Wee1 (Fig. 3A). D, expression of cdc25C (Fig. 3A). Significant differences from control (∗, P < 0.05; **, P < 0.01) or the corresponding concentration of THC alone (∗, P < 0.05).
THC-induced cell cycle arrest is associated with apoptosis.

We next tried to elucidate whether THC-induced inhibition of proliferation was associated with cell death. The cannabinoid induced apoptosis, a process that was prevented by SR144528 (Fig. 4A). THC also induced a two-fold increase in caspase-3 activity, an effect that was prevented by SR144528 (Fig. 4B).

Likewise, we observed reduced levels of both pro-caspase-3 (the inactive precursor of caspase-3) and PARP (a caspase-3 substrate) in cannabinoid-treated cells (Fig. 4C). We subsequently addressed the question of whether apoptotic cells were those arrested in G2-M by THC. We conducted triple staining experiments to analyze the percentage of apoptotic cells in every phase of the cell cycle. As shown in Fig. 5A, THC induced apoptosis in all the cell cycle phases, but the majority of apoptotic cells were in the G2-M compartment.

It is known that survivin, a member of the inhibitor of apoptosis family, can be phosphorylated at Thr^34 by Cdc2. This phosphorylation results in enhanced stability of survivin and the consequent inhibition of caspase activity (12). Survivin was highly expressed in all the breast cancer cell lines tested but was hardly detectable in HMEC (Fig. 5B), in agreement with previous reports showing a sharp differential expression in cancer (high levels) versus normal (undetectable levels) tissues (12). Moreover, THC decreased survivin levels in EVSA-T cells (Fig. 5C), which may explain why THC-induced Cdc2 inactivation results in apoptosis. In fact, when Cdc2 levels were enhanced by overexpression, survivin decrease on cannabinoid treatment was attenuated (relative optical density after 16 hours of treatment for pIRESpuro2-transfected cells: vehicle, 100 ± 6; 5 μmol/L THC, 31 ± 4. For pIRESpuro2-Cdc2-transfected cells: vehicle, 100 ± 5; 5 μmol/L THC, 74 ± 3; Fig. 5D).

Cannabinoid receptors are expressed in human breast tumors. The presence of CB1 and CB2 receptors in human breast tumors was evaluated by real-time quantitative PCR and confocal microscopy. Lower levels of CB1 mRNA were detected in tumors of low-medium (grade 1-2) and high (grade 3) histologic grade compared with normal, noncancerous breast tissue (grade 1-2 versus noncancerous breast tissue (P = 0.008); grade 3 versus noncancerous breast tissue (P = 0.0007); Fig. 6A). CB2 expression was higher than CB1 expression in all the tumors analyzed (P = 0.00002) and seemed to correlate with their histologic grade (grade 1-2 versus grade 3; P = 0.04; Fig. 6A). Of interest, CB2 transcripts were hardly detectable in normal breast tissue (Fig. 6A). Moreover, the expression of CB2 showed an association with molecular markers of prognostic value. Thus, ER– tumors expressed more CB2 mRNA than ER+ tumors (Fig. 6B). CB2 expression was also higher in PR– than in PR+ samples. ER–/ PR– tumors (response rate <10% to conventional therapies)
expressed more CB₂ mRNA than ER+/PR+ tumors (60-70% response rate; ref. 7; Fig. 6B). ERBB2/HER-2-positive tumors (with worse survival prediction at least in node-positive patients; ref. 7) expressed higher levels of CB₂ mRNA than ERBB2/HER-2-negative tumors (Fig. 6B). Confocal microscopy experiments confirmed the presence of CB₁ and CB₂ proteins in tumor cells (Fig. 6C). None of the receptor proteins was detected in normal ducts.
Discussion

There is scant information on the effect of cannabinoids on the cell cycle. It has been shown previously that the endogenous cannabinoid anandamide arrests the cycle of hepatoma HepG2 cells (13), epidermal growth factor–stimulated PC3 prostate cancer cells (14), and the breast cancer cell line EFM-19 (15) at the G1-S transition. The same effect was exerted by a metabolically stable analogue of anandamide, met-F-anandamide, on KiMol thyroid carcinoma cells (16). Results presented herein (a) show that the plant-derived cannabinoid THC is able to block the progression of breast cancer cell cycle and (b) provide a mechanism for this action. Of interest, nontumor mammary epithelial cells were rather insensitive to THC, suggesting that cannabinoids would fulfill one of the demanded requirements of any compound intended to be used in cancer therapy: the selectivity for tumor cells. In our experimental model, THC induces a CB2-mediated cell cycle arrest at the G2-M transition via Cdc2 down-regulation. We are nonetheless aware that our observation that THC effects are significantly but not completely prevented by the CB2 selective antagonist also points to the existence of CB2-independent...
processes in cannabinoid antiproliferative action. Most cancer cells evade antitumor signals because they have defective G1 checkpoints, which makes the G2 checkpoint an attractive target for cancer therapy (11). Since its discovery in 1986 (17), it has been clear that Cdc2 is essential for cell cycle progression (18). In fact, Cdc2-deficient mice die at very early stages of embryonic development (18). The pivotal importance of this particular protein could explain why, in our system, a somewhat moderate decrease of total Cdc2 levels (~40%) results in large changes in cell viability. In addition, our data indicate that THC increases the inactive/active Cdc2 ratio, supporting that the cannabinoid decreases not only total Cdc2 levels but also enzyme-specific activity.

It is important to point out that, although the antiproliferative effect of cannabinoids on different tumor cells has been extensively confirmed both in vitro and in vivo (6), a recent report indicates that THC may enhance breast cancer cell growth under certain circumstances. In that study, the authors showed a direct association between the degree of sensitivity to a tumor to THC and the level of CB1 and CB2 expression. Thus, THC has antiproliferative effect in tumors expressing cannabinoid receptors, whereas those with low to no expression suffer increased growth and metastasis due to THC-induced suppression of the antitumor immune response (19). Results presented herein are not in disagreement with that report, as EVSA-T cells, which are very sensitive to THC, express high levels of CB2.

By real-time quantitative PCR experiments, we have observed a correlation between CB2 expression and the histologic grade of breast tumors. Moreover, CB2 expression is higher in tumors with predicted low response to conventional therapies, for instance ER−/PR− tumors, which are weakly responsive to adjuvant tamoxifen (7). In addition, hardly detectable levels of CB2 were found in normal breast tissue. Because both cell cycle arrest and apoptosis induced by THC are CB2-mediated effects, it is tempting to speculate that the tumors with poor prognosis (i.e., those resistant to conventional therapies and, according to our data, expressing the highest levels of CB2 receptor), may be the most responsive to cannabinoids. In addition, the psychotropic effects of cannabinoids are mediated by CB1, but not CB2 receptors, and, therefore, a cannabinoid-based therapy selectively targeting CB2 receptors would be devoid of the side effects associated to cannabis consumption (20).

Breast cancer is the most common malignant disease among Western women. Although the rates of mortality of breast cancer patients have decreased as a result of early diagnosis by mammograms, certain breast tumors remain resistant to conventional therapies, and current treatments have side effects that substantially affect the patient’s quality of life (21). Our findings might set the basis for new strategies for the management of breast cancer.

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