Cannabinoids Induce Cancer Cell Proliferation via Tumor Necrosis Factor α-Converting Enzyme (TACE/ADAM17)-Mediated Transactivation of the Epidermal Growth Factor Receptor

Stefan Hart, Oliver M. Fischer, and Axel Ullrich

Department of Molecular Biology, Max-Planck-Institute of Biochemistry, Martinsried, Germany

Abstract

Cannabinoids, the active components of marijuana and their endogenous counterparts were reported as useful anecdotal agents to accompany primary cancer treatment by preventing nausea, vomiting, and pain and by stimulating appetite. Moreover, they have been shown to inhibit cell growth and to induce apoptosis in tumor cells. Here, we demonstrate that anandamide, Δ9-tetrahydrocannabinol (THC), HU-210, and Win55,212-2 promote mitogenic kinase signaling in cancer cells. Treatment of the glioblastoma cell line U373-MG and the lung carcinoma cell line NCI-H292 with nanomolar concentrations of THC led to accelerated cell proliferation that was completely dependent on metalloprotease and epidermal growth factor receptor (EGFR) activity. EGFR signal transactivation was identified as the mechanistic link between cannabinoid receptors and the activation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 as well as prosurvival protein kinase B (Akt/PKB) signaling. Depending on the cellular context, signal cross-communication was mediated by shedding of proAmphiregulin (proAR) and/or proHeparin-binding epidermal growth factor-like growth factor (proHB-EGF) by tumor necrosis factor α converting enzyme (TACE/ADAM17). Taken together, our data show that concentrations of THC comparable with those detected in the serum of patients after THC administration accelerate proliferation of cancer cells instead of apoptosis and thereby contribute to cancer progression in patients.

Introduction

Cannabinoids have been used in medicine for more than a century. Recently interest in their therapeutic value has been fuelled by suggestions to apply these drugs in cancer treatment to improve analgesia and to relieve insomnia (1). Because of their neuroprotective properties, cannabinoids have also been proposed to be useful drugs for the therapy of neurodegenerative diseases like Parkinson’s disease. Huntington disease, and multiple sclerosis (2). Orally applicable Δ9-tetrahydrocannabinol (THC; Dronabinol, Marinol) and its synthetic derivative Nabilone (Cesamet) have been approved by the United States Food and Drug Administration to stimulate the appetite of patients with AIDS and to reduce the nausea of cancer patients undergoing chemotherapy (1, 3, 4).

Moreover, recent investigations propose that drugs activating the endogenous cannabinoid system might be used in cancer therapy to slow down or block cancer growth (4). The endogenous cannabinoid anandamide (AEA) acts antiproliferatively in MCF-7, EFM-19, SCC-9 (squamous cell carcinoma), 5637 (bladder carcinoma), U373-MG (glioblastoma), 1321N1 (astrocytoma), and A498 (kidney cancer) cells with cannabinoids such as THC, AEA, HU-210, and Win55,212-2. THC comparable with those detected in the serum of patients after THC administration accelerate proliferation of cancer cells instead of apoptosis and thereby contribute to cancer progression in patients. The aim of this study was to identify critical elements that link the cannabinoid receptors to activation of the EGFR in cancer cell lines, thereby activating downstream mitogenic signaling events.

Our results demonstrate that treatment of NCI-H292 (lung cancer), SCC-9 (squamous cell carcinoma), 5637 (bladder carcinoma), U373-MG (glioblastoma), 1321N1 (astrocytoma), and A498 (kidney cancer) cells with cannabinoids such as THC, AEA, HU-210, and Win55,212-2 leads to rapid EGFR tyrosine phosphorylation, phosphorylation of the adaptor protein Src homology 2 domain-containing (SHC), and downstream activation of ERK1/2 and Akt/PKB. EGFR transactivation is specifically mediated by cannabinoid-induced cleavage of proAmphiregulin (proAR) and/or proHeparin-binding epidermal growth factor-like growth factor (proHB-EGF) at the cell surface by tumor necrosis factor α-converting enzyme (TACE/ADAM17). Importantly, THC induced EGFR- and metalloprotease-dependent cancer cell proliferation. Thus, this cross-communication of CB1/CB2 receptors and the EGFR provides a molecular explanation of how the G1-S phase transition and is independent of apoptosis (3, 5). Furthermore, depending on drug concentration, the timing of drug delivery, and cellular context, cannabinoids may either inhibit or stimulate the function of immune cells. Although high concentrations of cannabinoids block immune cells, Dereq et al. (8) demonstrated proliferation in human B cells after cannabinoid stimulation at nanomolar concentrations (6–8). In addition, murine hematopoietic cells depend on AEA for normal growth in serum-free medium (9).

THC, the endogenous cannabinoid AEA and synthetic cannabinoids like HU-210 and Win55,212-2 interact with specific G protein-coupled receptors (GPCRs). Two subtypes of the cannabinoid receptors, CB1 and CB2, have been cloned and characterized (10, 11). The CB1 receptor, which is responsible for the well-known psychotropic effects of cannabinoids, is highly expressed in the central nervous system, but lower levels are also present in immune cells and peripheral tissues including testis, whereas the CB2 receptor is predominately expressed in immune cells (12–14). Both cannabinoid receptors are coupled to heterotrimeric Gαi-proteins and activate the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (ERK)1/2 and p38 as well as the Akt/PKB survival pathway (5, 15). Extensive research efforts have addressed the question how cannabinoids induce MAPK activation. Thus far, the accumulation of ceramides after cannabinoid stimulation has been implicated in the induction of the ERK/MAPK signal, whereas other reports suggested intracellular ceramide levels not to be required for cannabinoid-induced MAPK activation (5, 12). Previously we and others have shown that a wide variety of GPCR agonists leads to the activation of MAPK via transactivation of the epidermal growth factor receptor (EGFR) (16–19). This mechanistic concept involves the proteolytic processing of a membrane-spanning proEGF-like growth factor by a zinc-dependent metalloprotease of the ADAM family (18–21).

Received 11/28/03; revised 1/27/04; accepted 2/4/04.

Grant support: O. Fischer has been supported by a Boehringer Ingelheim Fonds Ph.D. scholarship.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Axel Ullrich, Department of Molecular Biology, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18A, D-82152 Martinsried, Germany. E-mail: ullrich@biochem.mpg.de.
cannabinoid receptors are linked to MAPK and Akt/PKB activation in a wide variety of human cancer cell lines.

In the light of these results, the use of cannabinoids in cancer therapy has to be reconsidered, because relatively high concentrations of THC induce apoptosis in cancer cells, whereas nanomolar concentrations enhance tumor cell proliferation and may, therefore, accelerate cancer progression in patients.

Materials and Methods

Cell Culture. All of the cell lines (American Type Culture Collection, Manassas, VA) were routinely grown according to the supplier’s instructions. Heparin (Sigma, St. Louis, MO), Cmrl197 (Quadratex Ltd., Epsom Surrey, United Kingdom), batimastat (BB94, British Biotech, Oxford, United Kingdom), TNF-α protease inhibitor (TAPI, Calbiochem), and AG1478 (Alexis Biochemicals) were added to serum-starved cells 20 min before the respective growth factor. Arachidonylethanolamide [also called anandamide (AEA)] and THC were obtained from Sigma, and WIN 55,212-2 mesylate and HU-210 (5 μM) for 3 min, and were analyzed as described in A. G, serum-starved NCI-H292 cells were preincubated with BB94 (5 μM, 20 min), or vehicle (DMSO) and stimulated with anandamide (10 μM) or THC (1 μM) for 3 min. After lysis, HER2 was immunoprecipitated and assayed for HER2 tyrosine phosphorylation content.

...
sham Biosciences, Piscataway, NJ) were used for PCR amplification. Primers (Sigma Ark, Steinheim, Germany) were described previously (19). PCR products were subjected to electrophoresis on a 2.5% agarose gel, and DNA was visualized by ethidium bromide staining.

**[3 H]Thymidine Incorporation Assay.** For the [3 H]thymidine incorporation assay (16), U373-MG cells were seeded into 12-well plates at 1,510^4 cells/well. On serum deprivation for 48 h, cells were subjected to preincubation and stimulation as indicated in Fig. 3A. After 18 h, cells were pulse labeled with [3 H]thymidine (1 mCi/ml) for 4 h, and thymidine incorporation was measured by trichloroacetic acid precipitation and subsequent liquid scintillation counting.

**MTT Assay.** In a 96-well flat-bottom plate (Nunc, Naperville, IL), ~2000 cells/100 μl of cell suspension were seeded. On serum starvation for 24 h, cells were incubated with inhibitors and growth factors as indicated for another 24 h. MTT, a tetrazolium dye [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue, SIGMA, St. Louis, MO] was added to each well to a final concentration of 1 mg/ml MTT. Plates were incubated in the presence of MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which is solubilized (DMSO, acidic acid, SDS), and absorbance was read at 570 nm on a microplate reader.

**Results**

**Cannabinoid-Induced EGFR Signal Transactivation in Human Carcinoma Cells Depends on Metalloprotease Activity.** To address the question whether cannabinoids lead to transactivation of the EGFR in human cancer cell lines, we treated NCI-H292 (lung cancer), SCC-9 (squamous cell carcinoma), 5637 (bladder carcinoma), U373-MG (glioblastoma), A498 (kidney cancer), and 1321N1 (astrocytoma) cells with the synthetic cannabinoids Win55,212-2 and HU210, the endogenous cannabinoid AEA, and the naturally occurring THC. Resulting EGFR tyrosine phosphorylation levels were monitored by immunoblot analysis. As shown in Fig. 1, A–E, cannabinoids rapidly induced EGFR activation within 3 min.

Preincubation of the cells with the metalloprotease inhibitor batimatstat (BB94) or the EGFR kinase-specific inhibitor AG1478 prevented EGFR tyrosine phosphorylation in response to cannabinoid stimulation (Fig. 1, A–E). Stimulation of NCI-H292 cells with receptor subtype-specific agonists arachidonyl-2-chloroethylamide (ACEA) and BML-190 for CB1 and CB2 receptor, respectively, demonstrated that both cannabinoid receptors are capable of transactivating the EGFR (Fig. 1F). Expression of both the CB1 and the CB2 receptor was detected by cDNA microarray and Northern blot analysis in all six cancer cell lines (data not shown). Interestingly, the EGFR relative HER2/neu, which serves as a prognostic marker in many different cancer types, was likewise activated in response to cannabinoid stimulation (Fig. 1G). Both AEA- and THC-induced tyrosine phosphorylation of HER2/neu in NCI-H292 cells depended on metalloprotease and EGFR activity. Therefore, phosphorylation of Her2/neu appears to result from cannabinoid stimulation.
EGFR transphosphorylation. Taken together, these experiments demonstrate that cannabinoids rapidly induce EGFR and Her2/neu signal transactivation in a metalloprotease-dependent manner in different human cancer cell lines.

Cannabinoid-Induced Activation of ERK and the Akt/PKB Survival Pathway Depends on EGFR Function. To assess whether the EGFR links cannabinoids to ERK1/2 activation, we first analyzed the tyrosine phosphorylation content of the adaptor protein SHC. Fig.
phorylation and activation of ERK1/2 and Akt/PKB was abolished in the absence of TACE but was unaffected by the down-regulation of ADAM12 (Fig. 4D). As expected, suppression of neither protease had an effect on signaling events induced by direct EGF stimulation of the EGFR.

**Ectodomain Shedding of proHB-EGF and proAR Mediates Cannabinoid-Induced EGFR Activation.** Among the different EGF-like precursors, proAR, proHB-EGF, and proTGFα are predominantly expressed in NCI-H292 and SCC9 cells as indicated by cDNA microarray analysis (data not shown; Ref. 19). To investigate which ligand is involved in the EGF signal transactivation pathway after cannabinoid stimulation, we transiently transfected siRNAs, and efficient and specific silencing of the endogenous expression of proAR, proHB-EGF, and proTGFα was monitored by reverse transcription-PCR (Fig. 4E; Ref. 19). AEA- and THC-induced tyrosine phosphorylation of the EGFR in SCC9 cells required proAR as well as proHB-EGF expression (Fig. 4F). Suppression of either ligand resulted in partial reduction of cannabinoid-induced EGFR phosphorylation, whereas proTGFα inhibition did not affect EGFR phosphorylation at all. Furthermore, preincubation with heparin, which abrogates both proAR and proHB-EGF function (23, 24), also interfered with cannabinoid receptor-EGFR cross-talk (Fig. 4G). In contrast, in NCI-H292 cells, cannabinoid-induced transactivation of the EGFR did solely depend on proHB-EGF (Fig. 4H). The ability of the diphtheria toxin mutant Crm197, a specific inhibitor of proHB-EGF function (25), to block EGFR phosphorylation in response to THC stimulation substantiated this observation (Fig. 4I).

Together, these results show that cannabinoid-induced EGFR signal transactivation is mediated by specific proteolytic processing of the two heparin-binding EGFR ligands, proAR and proHB-EGF, by one and the same zinc-dependent metalloprotease TACE.

**Discussion**

Cannabinoids were shown to induce apoptosis in cells of the neuronal system including neurons, astrocytes, human grade IV astrocytoma, glioma C6, astrocytoma U373-MG, neuroblastoma N18 TG2, and pheochromocytoma PC12 cells and to inhibit proliferation of MCF-7, EFM-19, T47D, and DU145 cells (5, 26). On the basis of these findings and their analgesic properties, cannabinoids were suggested as useful drugs to support cancer therapy. Here we show that various cannabinoids potently induce mitogenic kinase signaling in different cancer cell lines. Moreover, we demonstrate, in contrast to other studies that used cannabinoids such as THC at micromolar concentrations, that nanomolar concentrations of THC induce proliferation of cancer cells (Fig. 3, A and B). Importantly, the concentration of THC that was used here is more likely to reflect the therapeutically relevant situation detected in serum after drug treatment (27–31).

The binding of cannabinoids to their cognate receptors has been shown to enhance the activity of the MAPKs ERK1/2. The activation of the MAPK pathway in glioma cells on cannabinoid treatment was suggested to involve the activation of Raf1 by increased ceramide levels (32). However, our results identified signal transactivation of the EGFR as the key mechanism linking cannabinoid receptors to MAPK signaling cascades in a wide variety of human cancer cell lines. Activation of ERK1/2 by four different cannabinoids coincides with the phosphorylation of the EGFR and was blocked by a specific inhibitor of the EGFR, AG1478 (Fig. 2, B–D). Although the ability of AG1478 to block cannabinoid-induced MAPK activation was noted before in U373-MG cells by Galve-Roperh et al (22), they excluded the existence of cannabinoid receptor-mediated EGFR transactivation.
**EGFR MEDIATES CANNABINOID-INDUCED CELL PROLIFERATION**

**A** NCI-H292
- siRNA:
  - TACE
  - ADAM12
  - GAPDH
- RT-PCR

**B** NCI-H292
- siRNA:
  - TACE
  - Lysate
  - 116
  - 97
  - IB: TACE

**C** NCI-H292
- siRNA:
  - AEA
  - THC
  - EGF
- IP:
  - EGR
  - PY
  - EGFR

**D** NCI-H292
- siRNA:
  - AEA
  - THC
  - EGF
- IP:
  - ERK1/2
  - ERK
  - P-ERK
  - Akt/PKB
  - P-Akt

**E** NCI-H292
- siRNA:
  - proAR
  - proHB-EGF
  - proTGFa
  - GAPDH
- RT-PCR

**F** SCC9
- siRNA:
  - AEA
  - THC
  - EGF
- IP:
  - EGFR
  - PY

**G** SCC9
- Heparin:
  - IP:
  - + +
  - EGFR

**H** NCI-H292
- siRNA:
  - THC
  - AEA
- IP:
  - EGFR
  - PY

**I** NCI-H292
- Crm197:
  - IP:
  - + +
  - EGFR

Downloaded from cancerres.aacrjournals.org on November 13, 2015. © 2004 American Association for Cancer Research.
because of the inability to detect tyrosine-phosphorylated EGFR, which was probably caused by a lack of sensitivity of detection.

Interestingly, in addition to ERK1/2 activation, Akt/PKB phosphorylation was detected after cannabinoid treatment in an EGFR-dependent manner (Fig. 2E). Such a parallel contiguous activation of ERK1/2 and of Akt/PKB was observed before, e.g., in glioblastoma cells treated with cannabinoid receptor agonists, and was suggested to protect astrocytes from ceramide-induced apoptosis in a dose- and time-dependent manner (32, 33).

Our experimental finding that cannabinoid-induced EGFR cross-talk is established in a variety of human cancer cell lines (Fig. 1, A–E) implicates the EGFR as a central integrator of cannabinoid signaling. The cross-communication between GPCRs and the EGFR involves the proteolytic processing of different membrane-spanning proEGF-like growth factor ligands like proAR, proHB-EGF, and proTGFβ by zinc-dependent metalloproteases like ADAM10, ADAM12, and TACE, depending on the cellular context (18, 19). In human cancer cell lines, we demonstrate that TACE mediates transactivation of the EGFR after cannabinoid stimulation via proteolytic processing of proHB-EGF and/or proAmphiregulin (Fig. 4, F–I). Abrogation of either TACE or the respective proEGF-like growth-factor function completely blocked cannabinoid-induced EGFR tyrosine phosphorylation and subsequent activation of the mitogenic ERK pathway and the prosurvival Akt/PKB pathway (Fig. 4). We previously described the involvement of TACE in EGFR signal transactivation after lysophosphatidic acid (LPA) and carbachol stimulation (19). Moreover, TACE was found to mediate EGFR activation by cigarette smoke via proAR shedding in NCI-H292 cells (34). However, this is the first report demonstrating a TACE- and HB-EGF-dependent EGFR signal transactivation after GPCR stimulation. Interestingly, knockout experiments by Jackson et al. show that newborn mice lacking TACE, HB-EGF, and the EGFR have similar defective valvulogenesis and suggest EGFR activation by TACE-processed proHB-EGF (35). Moreover, our data substantiate the concept that, depending on the cell type and the stimulated GPCR, different ADAM proteases and proEGF-like growth factor ligands are capable of activating the EGFR (18).

Cannabis-based drugs are in phase three clinical trials for treating pain associated with cancer. Furthermore, THC is currently used to treat nausea in cancer patients undergoing extensive chemotherapy (1, 4, 36). In contrast, Grand and Gandhi (37) recently presented a case study of acute pancreatitis induced by cannabis smoking, indicating that cannabinoids may be a risk factor for pancreatic cancer. Smoking of THC is the most effective route of delivery, as THC is rapidly absorbed after inhalation, and the effects become fully apparent within minutes. Pharmacological activity of smoked THC depends on the depth and length of inhalation. Maximum serum concentrations up to 267 ng/ml (850 nmol) are measured after smoking THC (27, 28, 38), whereas maximum serum concentrations of oral or rectal administered THC or its derivatives as a drug are lower (35–300 nmol; Refs. 29, 30, 39). Here we observed a proliferative response of glioblastoma and lung cancer cells at concentrations of 100–300 nm THC, whereas THC at micromolar concentrations induced cell death in agreement with previous observations with neuronal cell types and immune cells (Fig. 3, A–C; Refs. 5, 40–42). These findings indicate that the biological responses to cannabinoids critically depend on drug concentration and cellular context. Taken together, these results have to be taken into account when considering therapeutic applications of cannabinoids. The risk in the medical use of THC or cannabis for the treatment of patients with established tumors is the further acceleration of tumor growth due to the proliferative potential of cannabinoids.

Acknowledgments

We thank U. Eichelsbacher, R. Gautsch, and R. Hornberger for their help with cell culture; T. Kayazeva for cDNA; P. Kayazeva for help with cDNA arrays and Northern blot analysis; and N. Prenzel and M. Buschbeck for critically reading the manuscript.

References


Fig. 4. Tumor necrosis factor-α-converting enzyme (TACE) mediates cannabinoid-induced EGFR transactivation and extracellular signal-regulated kinase 3/2 (ERK1/2) and Akt/PKB activation. A and B, NCI-H292 cells were transfected with TACE or ADAM12 siRNA. Gene expression was analyzed by reverse transcription-PCR or immunoblotting (IB) with TACE antibody. C, NCI-H292 cells and SCC9 cells were transfected with siRNAs raised against TACE and ADAM12, were serum starved, and were stimulated for 3 min with Δ⁹-tetrahydrocannabinol (THC; 1 μM), anandamide (A; 10 μM), and EGF (5 ng/ml), and were assayed for EGFR tyrosine phosphorylation content. D, NCI-H292 cells and SCC9 cells were transfected with siRNAs and stimulated for 7 min with agonists as indicated. Phosphorylated ERK1/2 and activated Akt/PKB were detected by immunoblotting with phospho-specific ERK1/2 (P-ERK) and Akt/PKB (P-Akt) antibodies, respectively. The same filters were reprobed with anti-ERK1/2 antibody and anti-Akt/PKB, respectively. E, P-ERK, phospho-specific ERK1/2, P-Akt, phospho-specific Akt/PKB. F, NCI-H292 cells were transfected with siRNAs raised against proAR, proHB-EGF, and proTGFβ. Gene expression was analyzed by reverse transcription-PCR (RT-PCR). F, SCC9 cells were transfected with siRNAs raised against proAR, proHB-EGF, and proTGFβ; stimulated with THC (1 μM) and AEA (10 μM) for 3 min; and assayed for EGFR tyrosine phosphorylation content. G, serum-starved SC9 cells were preincubated with heparin (100 ng/ml) 15 min, stimulated with THC (1 μM) and AEA (10 μM), and assayed for EGFR tyrosine phosphorylation. H, NCI-H292 cells were transfected with siRNAs as indicated, stimulated with THC (1 μM) and AEA (10 μM), and assayed for EGFR tyrosine phosphorylation. I, serum-starved NCI-H292 cells were preincubated with CRM197 (10 μg/ml) 20 min, stimulated with THC (1 μM) and AEA (10 μM) for 3 min, and assayed for EGFR tyrosine phosphorylation. IB, immunoblotting; PT, anti-phosphorytropic; IP, immunoprecipitated.
Cannabinoids Induce Cancer Cell Proliferation via Tumor Necrosis Factor α-Converting Enzyme (TACE/ADAM17)-Mediated Transactivation of the Epidermal Growth Factor Receptor

Stefan Hart, Oliver M. Fischer and Axel Ullrich