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Advances in Brief

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

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Abstract

Cannabinoids, the active components of marijuana and their endogenous counterparts were reported as useful analgetic 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, Δ^9-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 Δ^9-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, T47D, and DU145 cells (5). Interestingly, cannabinoid-induced inhibition of proliferation in breast cancer cells results from cycle arrest at the G_1-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, Derocq *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 CB_2 receptor is predominantly expressed in immune cells (12–14). Both cannabinoid receptors are coupled to heterotrimeric $G_{i/o}$-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).

The aim of this study was to identify critical elements that link the cannabinoid receptors to activation of the ERK/MAPK and the Akt/PKB pathway. Hence, we tested whether cannabinoid receptors transactivate 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

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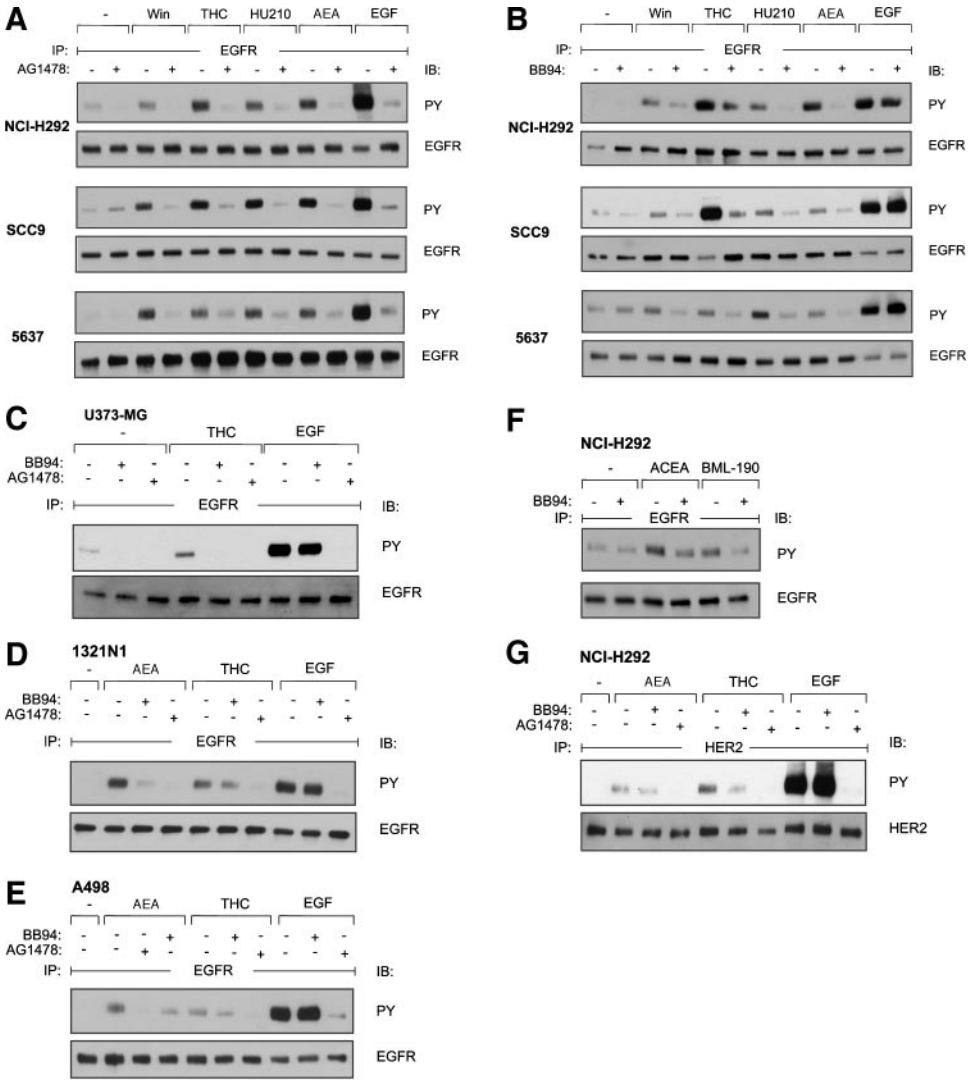


Fig. 1. EGFR signal transactivation requires EGFR tyrosine kinase activity and a metalloprotease activity. *A*, serum-starved NCI-H292 cells, SCC9 cells, and 5637 cells were preincubated with EGFR-specific tyrphostin AG1478 (250 nM, 20 min) or vehicle (DMSO) and were treated with Win55,212-2 (*Win*; 10 μM), Δ9-tetrahydrocannabinol (*THC*; 1 μM), HU210 (50 nM), anandamide [AEA (*AN*); 10 μM], and epidermal growth factor (EGF) (5 ng/ml) for 3 min. After lysis, EGFR was immunoprecipitated (*IP*) using anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by (*IB*) with anti-phosphotyrosine (*PY*) antibody, followed by reprobing of the same filter with anti-EGFR antibody. *B*, serum-starved NCI-H292 cells, SCC9 cells, and 5637 cells were preincubated with metalloprotease inhibitor batimastat (*BB94*; 5 μM, 20 min) or vehicle (DMSO), stimulated for 3 min, and analyzed as described in *A*. *C*, *D*, and *E*, Serum-starved U373-MG, 1321N1, and A498 cells were preincubated with inhibitors and were stimulated for 3 min as indicated, and were analyzed as described in *A*. *F*, serum-starved NCI-H292 cells were preincubated with BB94 (5 μM, 20 min) and were stimulated with arachidonyl-2′-chloroethylamide (ACEA) (200 nM) or BML-190 (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), tyrphostin AG1478 (250 nM, 20 min), or vehicle (DMSO) and stimulated with anandamide (*An*; 10 μM) or THC (1 μM) for 3 min. After lysis, HER2 was immunoprecipitated and assayed for HER2 tyrosine phosphorylation content.

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), Crm197 (Quadratech 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 from TOCRIS (Bristol, United Kingdom).

Protein Analysis. Cells were lysed and proteins immunoprecipitated as described previously (19). After SDS-PAGE, proteins were transferred to nitrocellulose membrane. Western blots were performed according to standard methods. The antibodies against human EGFR (108.1), SHC, and HER2/neu have been characterized before (19). Phosphotyrosine was detected with the 4G10 monoclonal antibody (UBI, Lake Placid, NY). Polyclonal anti-phospho-p44/p42 (Thr202/Tyr204) MAPK antibody and anti-phospho-Akt (Ser473) antibody were purchased from New England Biolabs (Beverly, MA). Polyclonal anti-Akt1/2 and anti-ERK2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-TACE antibody was from Chemicon (Harrow, United Kingdom).

Apoptosis Assay. NCI-H292 lung cancer cells were seeded and grown for 20 h. On serum starvation for 24 h, cells were treated with THC as indicated in Fig. 3*C* for 6 h. Cells were collected in assay buffer (1% sodium citrate, 0.1% Triton X-100) containing propidium iodine and were incubated at 4°C for 3 h. Nuclear DNA staining was analyzed on a Becton Dickinson FACScalibur flow cytometer.

For statistical analysis, Student's *t* test was used to compare data between two groups. Values are expressed as mean $\pm$ SD of at least triplicate samples. $P < 0.05$ was considered statistically significant.

RNA Interference and Reverse Transcription-PCR Analysis. Transfection of 21-nucleotide siRNA duplexes (Dharmacon Research, Lafayette, CO) for targeting endogenous genes was carried out using OligofectAMINE (Invitrogen) and 4.2 μg small interfering RNA (siRNA) duplex per 6-well plate as described previously (19). Transfected SCC-9 cells and NCI-H292 cells were serum starved and assayed 4 days and 3 days after transfection, respectively. Highest efficiencies in silencing target genes were obtained by using mixtures of siRNA duplexes targeting different regions of the gene of interest. The sequences of siRNA used were described previously (19). Specific silencing of targeted genes was confirmed by Western blot (TACE) and reverse transcription-PCR analysis. RNA, isolated using RNeasy Mini kit (Qiagen, Hilden, Germany), was reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim, Germany). PuReTaq Ready-To-Go PCR Beads (Amer-

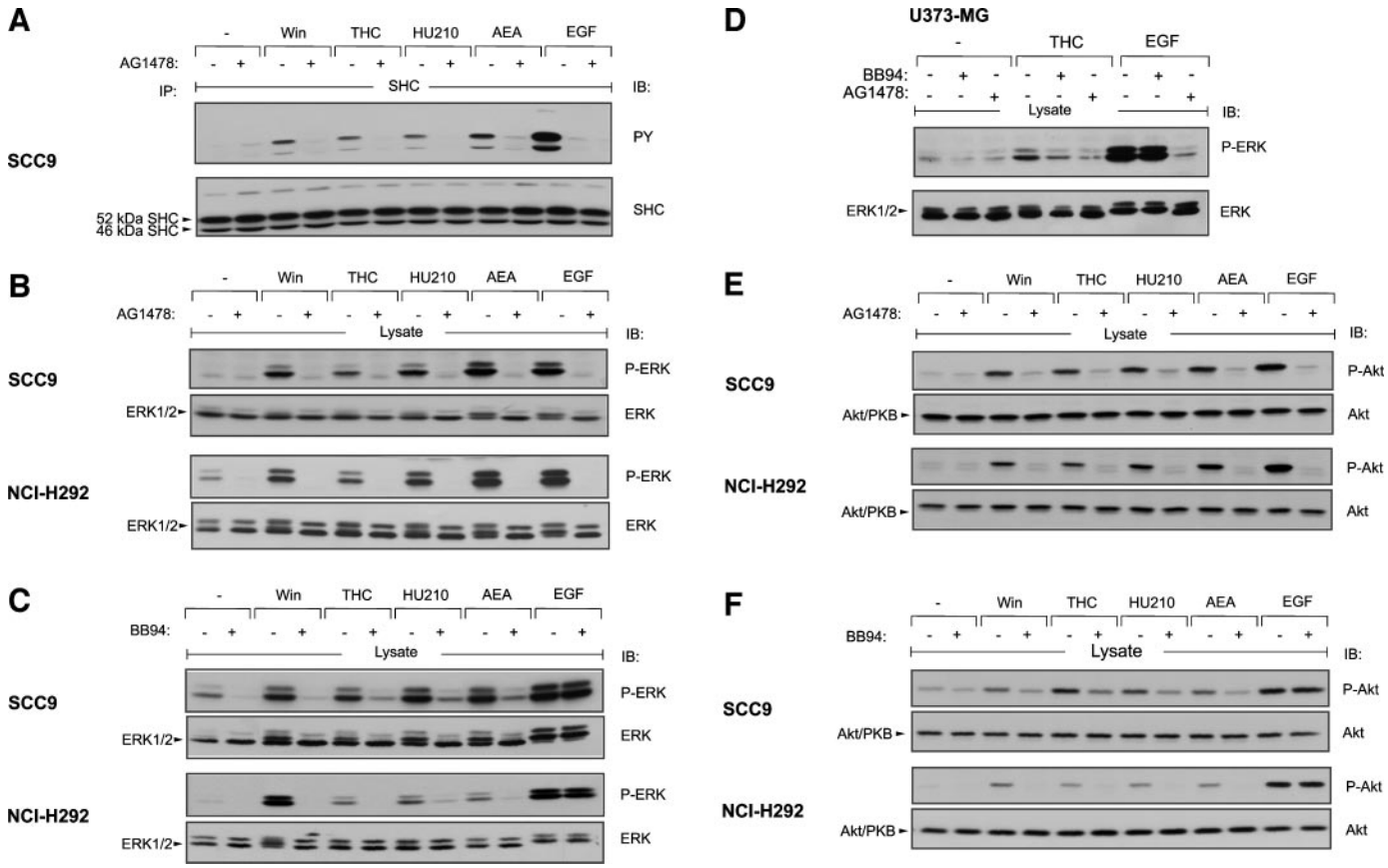


Fig. 2. Cannabinoid-induced SHC tyrosine phosphorylation and activation of extracellular signal-regulated kinase 1/2 (*ERK1/2*) and Akt/PKB pathways is EGFR- and metalloprotease-dependent. *A*, serum-starved SCC9 cells were treated as described in Fig. 1 *A*, precipitated SHC was immunoblotted with αPY antibody followed by reprobing of the same filters with anti-SHC antibody. *B* and *C*, serum- starved SCC9 cells and NCI-H292 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and were stimulated for 7 min as indicated. Phosphorylated ERK1/2 was detected by immunoblotting with phospho-specific ERK1/2 (P-ERK) antibody. The same filters were reprobed with anti-ERK antibody. *D*, serum-starved U373-MG cells were preincubated with inhibitors and stimulated for 7 min as indicated and were analyzed as described in *B*. *E* and *F*, serum-starved SCC9 cells and NCI-H292 cells were preincubated with AG1478 (250 nM, 20 min) or BB94 (5 μM, 20 min), respectively, and stimulated for 7 min as indicated. Activated Akt/PKB was detected by immunoblotting with phospho-specific Akt/PKB (P-Akt) antibody. The same filters were reprobed with anti-Akt/PKB antibody. *Win*, Win55,212-2; *THC*, Δ^9-tetrahydrocannabinol; *AEA*, anandamide; *EGF*, epidermal growth factor; *IB*, immunoblotting; *PY*, anti-phosphotyrosine; *P-ERK*, phospho-specific ERK1/2; *kDa*, M_r in thousands; *P-Akt*, phospho-specific Akt/PKB.

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 $1,5 \times 10^4$ cells/well. On serum deprivation for 48 h, cells were subjected to preincubation and stimulation as indicated in Fig. 3*A*. 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-bottomed 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-y1)-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), 1321N1 (astrocytoma), and A498 (kidney cancer) 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 batimastat (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. 1*F*). 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. 1*G*). 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

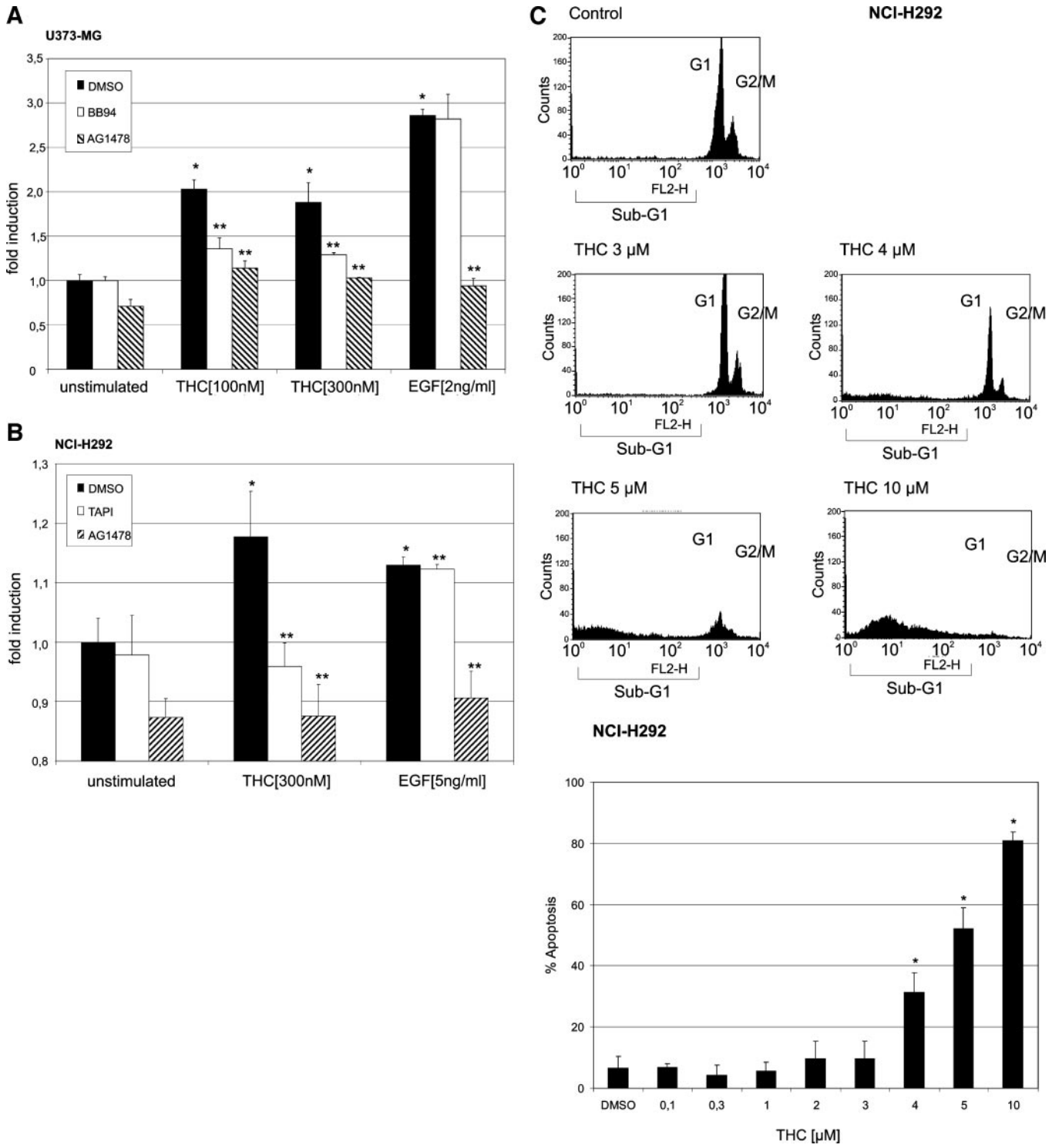


Fig. 3. Δ^9-Tetrahydrocannabinol (THC)-induced proliferation of U373-MG and NCI-H292 cells depends on EGFR transactivation. *A*, U373-MG cells were treated with inhibitors as indicated and incubated in the presence or absence of ligands (*THC*, 100 nM/300 nM; *EGF*, 2 ng/ml) for 18 h. Cells were then pulse labeled with [^{3}H]thymidine, and thymidine incorporation was measured by liquid scintillation counting. Quantitative analysis is from four independent experiments. *, $P < 0.01$ for the difference between DMSO *versus* THC and EGF; **, $P < 0.01$ for the difference between agonists *versus* inhibitors + agonists. *B*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Serum-starved NCI-H292 cells were treated with AG1478 (250 nM) and the metalloprotease inhibitor TAPI (5 μM) and were incubated for 24 h in the presence or absence of ligands (THC 300 nM, EGF 5 ng/ml). *Bars*, ± SD of absorbance at 570 nm of three independent experiments. *, $P < 0.01$ for the difference between DMSO *versus* THC and EGF; **, $P < 0.01$ for the difference between agonists *versus* inhibitors + agonists. *C*, serum-starved NCI-H292 cells were treated with different concentrations of THC as indicated for 6 h. After collection of the cells in assay buffer, nuclei were stained with propidium iodide and were analyzed by flow cytometric analysis. Quantification of experiments was performed in quadruplicate; *bars*, ±SD. *, $P < 0.001$ for the difference between DMSO *versus* THC; FL2-H, fluorescence channel 2-height.

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.

2*A* demonstrates that treating cells with Win55,212-2, THC, HU210, and AEA resulted in EGFR-dependent tyrosine phosphorylation of SHC. We further analyzed ERK1/2 activation by immunoblot using activation state-specific MAPK antibodies. As shown in Fig. 2, *B–D*, treating NCI-H292, SCC9, and U373-MG cells with cannabinoids potently activated ERK1/2 to a similar extent as EGF (5 ng/ml) stimulation. Preincubation with AG1478 or BB94 completely abrogated cannabinoid-induced MAPK activation, indicating that, in these human carcinoma cells, cannabinoid-induced ERK1/2 activation completely depends on EGFR signal transactivation (Fig. 2, *B–D*).

In addition to ERK stimulation, a variety of GPCR agonists were shown to activate the survival mediator Akt/PKB (18, 19). Because cannabinoids were known to activate Akt/PKB in astrocytoma cells (22), we addressed the question as to whether cannabinoids activate Akt/PKB in human cancer cells depending on EGFR function. Indeed, activation of Akt/PKB by all four cannabinoids was completely blocked by the selective EGFR inhibitor AG1478 and by the metalloprotease inhibitor BB94 (Fig. 2, *E* and *F*).

Taken together, tyrosine phosphorylation of SHC and activation of the Akt/PKB and the ERK/MAPK pathway after cannabinoid treatment of different human cancer cell lines critically depend on metalloprotease-mediated EGFR signal transactivation.

EGFR Mediates Cannabinoid-Induced Proliferation. Because ERK1/2 are generally known to mediate proliferation in a variety of cells, we addressed the question as to whether cannabinoids induce proliferation of cancer cells. To determine the proliferation rate in response to THC stimulation, we measured DNA synthesis and the turnover of MTT. As shown in Fig. 3*A*, THC at concentrations as low as 100 nM was capable of increasing the DNA synthesis of U373-MG cells. To investigate the involvement of the EGFR signal transactivation pathway for cannabinoid-induced DNA synthesis, we preincubated the cells with AG1478 and the metalloprotease inhibitor BB94. The increased DNA synthesis induced by THC was blocked in the presence of either AG1478 or BB94 (Fig. 3*A*). Furthermore, in the lung cancer cell line NCI-H292, THC (at 300 nM) was as potent as EGF (at 5 ng/ml) in increasing proliferation (Fig. 3*B*). THC-induced cell proliferation of NCI-H292 cells was completely blocked by preincubation with AG1478 and the metalloprotease inhibitor TAPI. In contrast to these observations, several earlier studies reported the induction of apoptosis on treatment of cells with higher concentrations of THC. Therefore, we tested whether, and in particular at what concentrations, THC would be able to trigger cell death of NCI-H292 cells. As shown in Fig. 3*C*, micromolar concentrations of THC were capable of inducing apoptosis, whereas submicromolar concentrations did not affect cell survival.

These results demonstrate that pharmacologically active concentrations of THC are capable of promoting the cell proliferation of human carcinoma cells and identify EGFR signal transactivation as the underlying molecular mechanism.

TACE/ADAM17 Mediates Cannabinoid-Induced EGFR Activation. Our observations clearly showed that cannabinoid receptors transactivate the EGFR in a wide variety of human cancer cell lines involving metalloprotease activity (Fig. 1, *A–E*). We have previously shown the critical involvement of the metalloprotease-disintegrin TACE in proAmphiregulin shedding after GPCR stimulation (19). Therefore, we raised the question as to whether TACE is required for cannabinoid-induced EGFR signal transactivation. We blocked endogenous TACE expression using a siRNA approach. TACE mRNA and protein was effectively and specifically reduced by transfecting siRNAs into NCI-H292 cells (Fig. 4, *A* and *B*). Inhibition of TACE expression but not of the related ADAM12 completely suppressed cannabinoid-induced EGFR tyrosine phosphorylation in NCI-H292 and SCC9 cells (Fig. 4*C*). Likewise, the cannabinoid-induced phosphorylation 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. 4*D*). As expected, suppression of neither protease had an affect on signaling events induced by direct EGF stimulation of the EGFR.

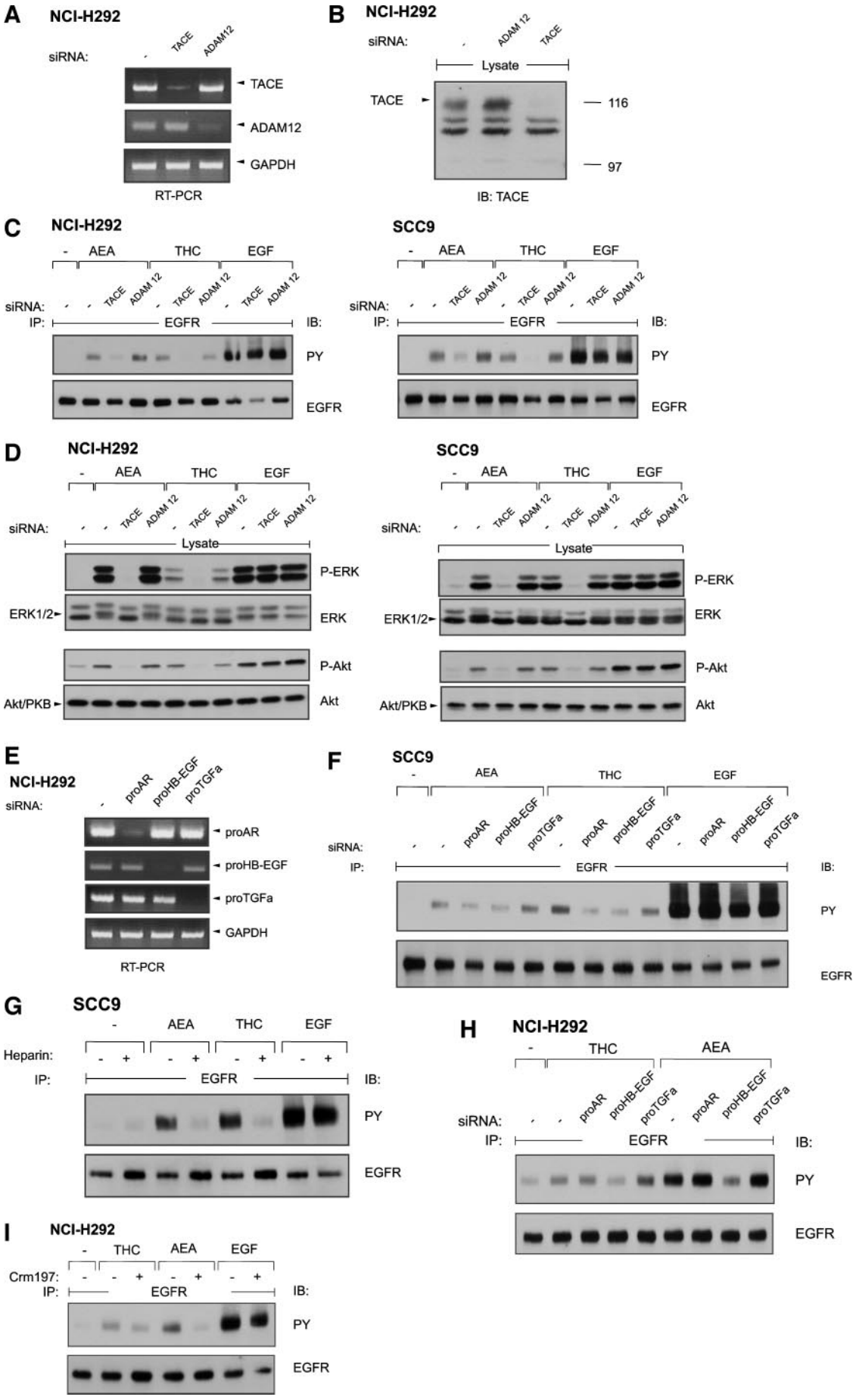
Ectodomain Shedding of proHB-EGF and proAR Mediates Cannabinoid-Induced EGFR Activation. Among the different EGF-like precursors, proAR, proHB-EGF, and proTGFα are predominantly expressed in NCI-H292 and SCC9 cells as indicated by cDNA microarray analysis (data not shown; Ref. 19). To investigate which ligand is involved in the EGFR 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. 4*E*; Ref. 19). AEA- and THC-induced tyrosine phosphorylation of the EGFR in SCC9 cells required proAR as well as proHB-EGF expression (Fig. 4*F*). 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. 4*G*). In contrast, in NCI-H292 cells, cannabinoid-induced transactivation of the EGFR did solely depend on proHB-EGF (Fig. 4*H*). 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. 4*I*).

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



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. 2*E*). 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 proTGFα 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 nM) 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 (35–350 nM; 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

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Fig. 4. Tumor necrosis factor α-converting enzyme (TACE) mediates cannabinoid-induced EGFR transactivation and extracellular signal-regulated kinase 1/2 (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 Δ^9-tetrahydrocannabinol (*THC*; 1 μM), anandamide (*An*; 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. *P-ERK*, phospho-specific ERK1/2; *P-Akt*, phospho-specific Akt/PKB. *E*, NCI-H292 cells were transfected with siRNAs raised against proAR, proHB-EGF, and proTGFα. Gene expression was analyzed by reverse transcription-PCR (*RT-PCR*). *F*, SCC9 cells were transfected with siRNAs raised against proAR, proHB-EGF, and proTGFα; stimulated with THC (1 μM) and AEA (10 μM) for 3 min; and assayed for EGFR tyrosine phosphorylation content. *G*, serum-starved SCC9 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; *PY*, anti-phosphotyrosine; *IP*, immunoprecipitated.

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