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⁹-Tetrahydrocannabinol Disrupts Estrogen-Signaling through Up-Regulation of Estrogen Receptor β (ER β)

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Abstract

⁹-Tetrahydrocannabinol (⁹-THC) has been reported as possessing antiestrogenic activity, although the mechanisms underlying these effects are poorly delineated. In this study, we used the estrogen receptor α (ER α)-positive human breast cancer cell line, MCF-7, as an experimental model and showed that ⁹-THC exposures markedly suppresses 17 β -estradiol (E2)- induced MCF-7 cell proliferation. We demonstrate that these effects result from ⁹-THC's ability to inhibit E2-liganded ER α activation. Mechanistically, the data obtained from biochemical analyses revealed that (i) ⁹-THC up-regulates ER β , a repressor of ER α , inhibiting the expression of E2/ER α -regulated genes that promote cell growth and that (ii) ⁹-THC induction of ER β modulates E2/ER α signaling in the absence of direct interaction with the E2 ligand binding site. Therefore, the data presented support the concept that ⁹-THC's antiestrogenic activities are mediated by the ER β disruption of E2/ER α signaling.

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INTRODUCTION

Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), a major component of marijuana, exhibits a variety of pharmacological and toxicological effects.¹⁻⁴ Among Δ^9 -THC's biological activities, its recognized antiestrogenic activity has been the subject of several investigations. An early hypothesis suggested that Δ^9 -THC may bind directly to the estrogen receptor α (ER α), thus interfering with 17 β -estradiol (E2) ligand binding. However, this mode of action now appears unlikely, and the molecular mechanism(s) of Δ^9 -THC-mediated antiestrogenic effects are still under debate.^{5,6} Since Δ^9 -THC is used both recreationally and medicinally for the treatment of pain and nausea in cancer patients undergoing chemotherapy in the United States and other countries (i.e., medical marijuana), it is important to ascertain the mechanistic basis of Δ^9 -THC's E2 signaling disruption.

The proliferation of the ER α -positive breast cancer MCF-7 cell line is stimulated by E2, resulting from the activation of E2/ER α signal transduction pathways. In 1996, Kuiper et al. identified a second ER, ER β .⁷ In contrast to ER α , the physiological role of ER β is not fully understood, although ER β is recognized as a repressor of ER α 's activity, both through its ability to heterodimerize with the α isoform and its direct function as a ER β /ER β homodimer.⁷⁻¹² In addition, it has been suggested that ER β may act as a tumor suppressor and that the loss of ER β promotes breast carcinogenesis.^{9,13} Although Δ^9 -THC effects have been studied in the MCF-7 cell model, to the best of our knowledge no mechanistic data are available to account for Δ^9 -THC's antiestrogenic action. Here, we report that Δ^9 -THC disrupts E2/ER α signaling in MCF-7 cells through up-regulation of ER β expression, resulting in altered proliferative responses, and that these effects occur in the absence of direct interaction of Δ^9 -THC with ER β 's ligand binding site.

MATERIALS AND METHODS

Materials

⁹-THC was isolated from the drug-type cannabis leaves according to the established methods.^{3,4,14} ⁹-THC-11-oic acid was provided by the National Institute on Drug Abuse (NIDA, Bethesda, MD, USA). The purity of ⁹-THC was determined as >98% by gas chromatography.¹⁴ 17 β -Estradiol (purity: >99%) was purchased from Nakarai Tesque (Kyoto, Japan). ICI 182,780 (purity: >98%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DPN (purity: >99%) and PHTPP (purity: >99%) were purchased from Tocris Bioscience (Ellisville, MO, USA). All other reagents and chemicals used were of the highest grade available.

Cells and Cell Cultures

Cell culture conditions and methods were based on procedures described previously.^{15,16} Briefly, the human breast cancer cell lines, MCF-7 and MDA-MB-231 (obtained from the American Type Culture Collection, Rockville, MD, USA), were routinely grown in phenol red-containing minimum essential medium α (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin, at 37 °C in a 5% CO₂-95% air-humidified incubator. Before chemical treatments, the medium was changed to phenol red-free minimum essential medium α (Invitrogen, Carlsbad, CA, USA) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum (DCC-serum), 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. Cultures of approximately 60% confluence (subconfluence) in a 100-mm Petri dish were used to seed for the experiments assessing cell viability, cell morphology, microarray transcription profiling, mRNA expression, and transfection analysis (dual-luciferase reporter assay).

Cell Viability Analysis

In the cell viability studies, the cells were seeded into 96-well plates at a density of ~5000 cells/well, and ⁹-THC ranging from 1 μ M to 50 μ M with or without E2 (100 pM) was introduced 4 h after plating. After 48 h of incubation, cell viability was analyzed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS reagent; Promega, Madison, WI, USA), according to the manufacturer's instructions. Test chemicals were prepared in appropriate organic solvents including DMSO or ethanol. Control incubations contained equivalent additions of solvents with no measurable influence of vehicle observed on cell viability at the final concentrations used.

Cell Morphology Studies

For morphological examination of the MCF-7 cells, images were obtained using a Leica DMIL inverted microscope (Leica Microsystems, Wetzlar, Germany) and captured with a Pixera Penguin 600CL Cooled CCD digital camera (Pixera Co., Los Gatos, CA, USA). Data were processed using Pixera Viewfinder 3.0 software (Pixera Co., Los Gatos, CA, USA). The breast cancer cells were plated in 6-well plates. Three areas with approximately equal cell densities were identified in each well, and images of each of these areas were captured.

DNA Microarray Analysis

Total RNA was collected from 25 μ M 9 -THC or vehicle-treated MCF-7 cells 48 h after exposure by using the RNeasy kit (Qiagen, Inc. Hilden, Germany) and was purified using RNeasy/QIAamp columns (Qiagen, Inc. Hilden, Germany). The specific gene expression pattern in the MCF-7 cells was examined by DNA microarray analysis in comparison with vehicle-controls. From both cells, total RNA was extracted, and cDNA synthesizing and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification kit (Agilent, Palo Alto, CA, USA). Labeled cRNA (Cy3 to controls, Cy5 to 9 -THC samples) was hybridized to human oligo DNA microarray slides (Agilent, Palo Alto, CA) that are spotted with human genes. Specific hybridization was analyzed using a Microarray scanner (Agilent, Palo Alto, CA, USA) and evaluated as a scatter-plot graph for gene expression. Hokkaido System Science (Sapporo, Japan) provided assistance with these experiments.

Analysis of CDC2, egr-1, ER α , ER β , and Ki-67 mRNA Levels by Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from MCF-7 and MDA-MB-231 cells using the RNeasy kit (Qiagen, Inc. Hilden, Germany) and purified with RNeasy/QIAamp columns (Qiagen, Inc. Hilden, Germany), and the following cDNA (cDNA) synthesis, RT and PCR, were performed using the Superscript One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA). The primers used were as follows: CDC2 (sense), 5'-TCA GTC TTC AGG ATG TGC TT-3'; CDC2 (antisense), 5'-GCA AAT ATG GTG CCT ATA CTC C-3'; egr-1 (sense), 5'-AAG GCC CTC AAT ACC AGC TAC-3'; egr-1 (antisense), 5'-CAT CGC TCC TGG CAA ACT TTC-3'; ER α (sense), 5'-ATC TGC CAA GGA GAC TCG CTA-3'; ER α (antisense), 5'-TCG GTC TTT TCG TAT CCC AC-3'; ER β (sense), 5'-CCT CCT ATG TAG ACA GCC ACC A-3'; ER β (antisense), 5'-TGG CGC AAC GGT TCC CAC TAA-3'; Ki-67 (sense), 5'-TAT CCA GCT TCC TGT TGT GTC-3'; and Ki-67 (antisense), 5'-CTG GCT CCT GTT CAC GTA TTT-3'. Primers for PCR of β -actin were taken from previously published work.¹⁵ PCR of CDC2, egr-1, ER α , ER β , Ki-67, and β -actin was performed under conditions producing template quantity-dependent amplification over 40 cycles. PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate EDTA buffer and stained with ethidium bromide. When the RT reaction was omitted, no signal was detected in any of the samples. β -Actin was used as an internal control for RT-PCR.

Construction of Human ER α and ER β Expression Plasmids

To construct human ER α and ER β expression plasmids, we first obtained cDNAs of human ER α (catalog #: RC213277) and ER β (catalog #: RC218519) from OriGene (Rockville, MD, USA). Because these cDNA constructs were inserted into an expression plasmid (pCMV6) with a Myc-DDK tag sequence, this sequence (93 bp) was deleted from the constructs, and a stop codon (TGA) was added to the 3'-end of the open reading frame. The nucleotide sequences of the resulting clones for ER α and ER β in pCMV6-ER α and pCMV6-ER β , respectively, were validated by DNA sequencing.

Transfection and Luciferase Reporter Assay

Transfections of each expression plasmid were performed using Lipofectamine LTX and PLUS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Maximal transcriptional efficiencies for the use of the ER α and ER β expression plasmids (pCMV6-ER α and pCMV6-ER β) were determined as 100 ng in the transfections. DNA mixtures of 300 ng of (ERE)₃-Luc plasmid (a kind gift from Dr. Mori) that contains the ERE (estrogen-responsive element) were cotransfected with 20 ng of *Renilla* luciferase reporter plasmid (pRL-TK) driven by the herpes simplex virus thymidine kinase promoter in 24-well plates. All plasmid concentrations were equalized with the parental pCMV6 vector. After 24 h, the medium was changed to medium supplemented with 10 mM HEPES, 5% DCC-serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin, and the transfected cells were treated with ⁹-THC, E2, DPN, or vehicle for 4 or 24 h. For the cotreatment with antagonists specific for ER α (ICI 182,780) or ER β (PHTPP), these antagonists were pretreated for 1 h in advance of ⁹-THC addition. Cells were then harvested and lysed in passive lysis buffer (Promega, Madison, WI, USA). Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The *Renilla* luciferase activity was used to normalize the firefly luciferase activity of each sample. All of the transfection experiments were performed in quintuplicate.

Antibodies and Western Immunoblot Analysis

Antibodies specific for ER β (ab3576; Abcam, Cambridge, MA, USA) and β -actin (A5060; Sigma Co., St. Louis, MO, USA) were used. Whole cell extracts were prepared as previously described.¹⁷ SDS-polyacrylamide gel electrophoresis/Western immunoblot analysis was performed based on procedures described previously.¹⁷ Equal amounts of protein for each sample were confirmed by probing with β -actin. Cell extracts prepared with human ER β cDNA-transfected cells were used as a positive control.

Fluorescence Polarization Assays for Measuring Ligand Binding to ER α and ER β

The possible binding of ⁹-THC to ER α /ER β was measured using PolarScreen Estrogen Receptor Competitor Assays from Life Technologies (Carlsbad, CA, USA) (Part # P2698 for ER α ; Part # P2700 for ER β) according to the manufacturer's instructions and the report by Powell et al.¹¹

Data Analysis

IC₅₀ values were determined using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA, USA), according to analyses described previously.¹⁵ Differences were considered significant when the *p* value was calculated as <0.05. Statistical differences between two groups were calculated by the Student's *t* test. Other statistical analyses were performed by Scheffe's *F* test, a post hoc test for analyzing results of ANOVA testing. These calculations were performed using Statview 5.0 J software (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

⁹-THC-induced growth-suppressive effects on cells, decreased MCF-7 cell viability and produced alterations in cell morphology, effects that were remarkably enhanced in the

presence of physiological concentrations of E2 (100 pM) (Figure 1A and B; IC₅₀ value = 34.5 μ M vs 10.4 μ M). In an earlier report by von Bueren et al., E2-induced MCF-7 cell proliferation was inhibited by ⁹-THC in the same concentration range as that used in this study, although the molecular mechanism(s) of this interaction were not ascertained.¹⁸ Since the majority of breast cancers are ER α -positive and depend on estrogen for their growth,¹⁹ we examined the effects of ⁹-THC on ER α -mediated transcriptional activation. ⁹-THC clearly interfered with ER α -mediated transcriptional activation, with both basally produced E2 and in the presence of additions of E2 (100 pM), additions that would otherwise lead to stimulation of MCF-7 cell growth (Figure 1C). These findings suggest the basis of a mechanism, indicating a previously unrecognized interaction between ⁹-THC and E2 in ER α -positive MCF-7 cells.

We next analyzed whether CDC2, one of the downstream targets of E2/ER α involved in the cell growth,¹³ was affected by ⁹-THC. CDC2 expression was detected in the control as well as in the E2-treated groups; however, ⁹-THC additions almost completely abrogated the expression of CDC2, even in the presence of E2 (100 pM) (Figure 2, upper panel). Thus, ⁹-THC exhibited antiestrogenic activity *in vitro*, apparently through the inhibition of E2/ER α signaling pathways. To better assess ⁹-THC's potential antiestrogenic mechanism(s), DNA microarray analysis was performed. Among the genes regulated >5-fold by ⁹-THC, a significant up-regulation of ER β (>9.6-fold) was observed, whereas ER α expression was not influenced (Figure 3A). ⁹-THC up-regulation of ER β was also verified by both RT-PCR (Figure 3B) and by Western immunoblot analysis (Figure 3C). As the results demonstrate, a clear concentration-dependent up-regulation of ER β mRNA and protein resulted in MCF-7 cells following ⁹-THC treatments (Figure 3B and C, respectively).

Inversely related to the ER β results, the same ⁹-THC exposures resulted in concentration-dependent inhibition of ER α -mediated transcriptional activities (Figure 4A). Furthermore, overexpression of ER β significantly reduced the reporter gene activity of ER α , and this inhibition was additionally down-regulated by ⁹-THC. It was reasoned that if ⁹-THC is abrogating E2/ER α activity coupled with cell proliferation, then Ki-67, a general proliferation marker up-regulated by E2 in breast cancer,^{9,20} should also demonstrate coordinate down-regulation, in a manner similar to that of the CDC2 results (Figure 2). A positive signal for Ki-67 was detected under basal culture conditions in the MCF-7 cells; however, this expression was decreased by ⁹-THC exposures with a concentration dependency (Figure 3B). It should be noted that an inverse correlation between ER β and Ki-67 expression was observed (Figure 3B). We further analyzed whether expression levels of *egr-1*, a tumor suppressor in human breast cancer cells²¹ and a downstream target of ER β ,²² was affected by ⁹-THC. Although the basal expression of *egr-1* was very low in the control and 100 pM E2-treated samples, *egr-1* was up-regulated by ⁹-THC or by ⁹-THC + E2 treatments (Figure 2, middle panel). These data implicate ER β as a likely target of ⁹-THC's antiestrogenic action on ER α .

To further strengthen the findings suggesting that ⁹-THC inhibits ER α via a targeted action on ER β , we further examined the effect of ICI 182,780, a selective ER α antagonist, on ⁹-THC-mediated ER α inhibition in MCF-7 cells. Although ICI 182,780 alone further inhibited

the 9 -THC suppressed level of ER α activity, in a concentration-dependent manner, ER α was additionally inhibited following the transfection of an ER β expression construct (Figure 4B). It is noteworthy that 9 -THC itself failed to activate and/or bind to ER α (Figure 4C, data not shown).^{5,6} Although very high concentrations of E2 (>1 nM) can activate ER β ,²³ in our cell culture models, E2 (100 pM) preferably activated ER α and not ER β .

ICI 182,780 itself is reported to evoke ER α and ER β heterodimerization when these receptors are coexpressed.¹⁰ 9 -THC's up-regulation of ER β expression might provide a similar scaffold to ER α , leading to inhibition of ER α -positive breast cancer cell growth (see Figures 3 and 4B). Although the current study did not assess potential ER β /ER β homodimer vs ER α /ER β heterodimer complexes, it is possible that receptor homodimer interactions may additionally contribute to 9 -THC's antiproliferative activities in MCF-7 cells.

It has been reported that, in addition to the ER α /ER β heterodimer, ER β (possibly homodimer) may also exhibit antiproliferative effects on breast cancer cells.^{9,13,24,25} Since MCF-7 cells express both ER α and ER β , to assess the effect of 9 -THC on the ER β activity more directly, we used the human breast cancer MDA-MB-231 cell line, which exhibits very low basal expression of ER β and lacks ER α expression (see Figure 5A, inset). Although the basal transcriptional activity was not substantively affected by transfection of an ER β cDNA expression construct alone, when combined, 9 -THC additions resulted in a concentration-dependent and highly significant activation of ER β -mediated transcriptional activation (Figure 5A). Furthermore, PHTPP, a selective ER β antagonist, effectively abrogated 9 -THC's activation of ER β , while ICI 182,780 was ineffective in these experiments (Figure 5B).

ER β -mediated luciferase gene reporter activity was detected largely at 24 h but not at 4 h after the addition of 9 -THC, whereas DPN (a selective ER β agonist) activation was detected by 4 h post-exposure (data not shown). These results may imply that 9 -THC is producing secondary effects. 9 -THC exposures on ER β -regulated genes were therefore examined at a 4 h time point. Twenty four hours after ER β cDNA transfection into MDA-MB-231 cells, 5, 10, or 25 μ M 9 -THC was added to cells followed by a 4 h incubation and analysis of the ER β target genes, CDC-2 and egr-1. The data obtained indicated that these ER β -regulated genes were not modulated by 9 -THC; neither up-regulated (egr-1) nor down-regulated (CDC2) (CDC2 is known to be down-regulated when ER β is activated)¹³ (Figure 6A). Expression time-course data of ER β in MDA-MB-231 cells treated with 25 μ M 9 -THC revealed that ER β was up-regulated as a function of time (i.e., at 24–96 h) (Figure 6B). Also, the basal transcriptional activity of ER β tended to be stimulated at 24 h with 24 μ M 9 -THC (Figure 5A). 9 -THC also up-regulated ER β protein in these cells (data not shown).

As shown clearly in Figure 7A and strikingly different from the results described in Figure 1B for MCF-7 cells, at 48 h in the presence of 100 pM E2, 9 -THC did not exhibit antiproliferative effects on the ER α -deficient MDA-MB-231 cells. Thus, it is suggested that 9 -THC requires ER α for its antiproliferative effects in the presence of E2, emphasizing possible ER β -mediated E2/ER α inhibition.

To obtain evidence whether 9 -THC directly binds to the ligand binding domain (LBD) of ER β , we performed Fluorescence Polarization (FP) Competition Binding Assays, as reported by Powell et al.^{11,12} As is shown in Figure 7B, 9 -THC ranging from 0.0001 μ M to 1000 μ M was not able to compete with fluorescently labeled E2 (4.5 nM) for binding to ER β . As expected, this inactivity was also seen in the case with ER α (data not shown).

Because we have reported that 9 -THC-11-oic acid, a major metabolite of 9 -THC in human, is an inhibitor of 15-lipoxygenase as an active metabolite,³ we tested the possibility that 9 -THC-11-oic acid may exert antiestrogenic effects as seen in the case of 9 -THC. However, no activity was observed with this latter compound (Takeda et al., unpublished observation).

It is of interest that some phytoestrogens have been reported to induce ER α /ER β heterodimer formation and that this combination contributes to growth-suppressive effects.¹²

9 -THC might induce ER α /ER β heterodimerization in cells that coexpress ER α and ER β , although 9 -THC was not shown to be a ligand for ER β as well as for ER α . If this dimerization is important for 9 -THC's action observed in the MCF-7 cells, 9 -THC might first induce ER β and next recruit coregulator(s) and/or induce molecule(s) involved in the formation of the ER α /ER β heterodimer.²⁶

Although the concentrations of 9 -THC used in this study seem to be high,²⁷ they might reflect the therapeutically relevant situation after its 9 -THC treatment since it is reported that 9 -THC can be accumulated up to 20-fold in some tissues (i.e., fat tissue) after marijuana smoking.^{28,29} Importantly, although the benefits of 9 -THC are apparent as an adjuvant in cancer chemotherapy, the results reported here indicate that 9 -THC may exhibit endocrine-disrupting effects as an antiestrogen. Clearly, additional studies are needed to demonstrate the potential impact of the therapeutically relevant concentrations of 9 -THC with *in vivo* models. Interestingly, ER α /ER β homodimer-selective estrogen receptor modulators (SERMs) are described as a class of compounds acting on the ERs to decrease E2-dependent disease. In these respects, it will be important to definitively investigate the effect of 9 -THC on the protein-protein interaction between ER α and ER β , perhaps using an *in situ* model such as the highly sensitive BRET (bioluminescence resonance energy transfer) assay.¹⁰ Although 9 -THC did not exhibit binding capacity for the ERs in our studies, it is possible that this cannabinoid may be categorized in the SERMs category based on its potential to modulate ER interactions.

In conclusion, our studies demonstrate that 9 -THC inhibits E2/ER α signaling by up-regulating ER β , the induced levels of ER β likely serve as the basis for 9 -THC's abrogation of E2/ER α , and that 9 -THC's antiproliferative effects on breast cancers may be modulated by expression levels of ER α in the presence of E2 (Figures 1B and 7A).

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ABBREVIATIONS

⁹-THC	⁹ -tetrahydrocannabinol
ERα/β	estrogen receptor α/β
E2	17 β -estradiol
FP	fluorescence polarization
LBD	ligand binding domain

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Figure 1.

Δ^9 -THC abrogates E2/ER α signaling in MCF-7 cells. (A) MCF-7 cells were treated with vehicle (control), 25 μ M Δ^9 -THC (Δ^9 -THC), 100 pM E2 (E2), and 25 μ M Δ^9 -THC/100 pM E2 (Δ^9 -THC + E2) for 48 h prior to the examination of cellular morphology. Representative data images are shown. Images were taken with $\times 200$ magnification. (B) ER α -positive MCF-7 cells were exposed for 48 h to Δ^9 -THC ranging from 1 μ M to 50 μ M in the presence or absence of E2 (100 pM). After the treatments, cell viability was measured according to the methods described in Materials and Methods. Data are expressed as the percent of vehicle-treated group (indicated as 0), as the mean $\pm$ SD ($n = 6$). (C) MCF-7 cells were transiently transfected with a luciferase reporter gene construct containing three copies of a consensus estrogen-responsive element (ERE). After transfection, cells were treated with vehicle (–/–), Δ^9 -THC (25 μ M), E2 (100 pM), or Δ^9 -THC (25 μ M) + E2 (100 pM). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of vehicle-treated control (indicated as –/–), as the mean $\pm$ SD ($n = 5$).

*Significantly different ($p < 0.05$) from the E2-treated group.

Figure 2.

Effect of Δ^9 -THC on the E2-regulated target genes. RT-PCR (upper panel, CDC2; middle panel, egr-1) analyses of each gene level in MCF-7 cells were performed 48 h after treatment with 25 μ M Δ^9 -THC or vehicle in the presence or absence of 100 pM β -Actin was used an internal loading control. A 100-bp DNA ladder marker for RT-PCR was also loaded. A representative data image is shown.

Figure 3.

9 -THC up-regulates ER β . (A) Results of DNA microarray analysis. Data are expressed as fold induction vs vehicle-treated groups. MCF-7 cells were treated with vehicle or 25 μ M 9 -THC for 48 h, followed by mRNA isolation. Details of microarray conditions are described under Materials and Methods. (B) RT-PCR analysis of ER α , ER β , and Ki-67 transcript levels after treatment with 5 or 25 μ M 9 -THC or without 9 -THC (indicated as -). β -Actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. A representative data image is shown. (C) Western immunoblot analysis of ER β . MCF-7 cells were treated with 5 μ M or 25 μ M 9 -THC (indicated as 5 or 25) or

vehicle (indicated as –) for 48 h. The cell lysates derived from transient transfection of human ER β cDNA-expression plasmid (transfected plasmids: 0.0025, 0.25, and 2.5 μ g) were also loaded. Total cell lysates were prepared, and Western immunoblot analyses were performed using antibodies specific for ER β and β -actin, respectively. The band intensity of ER β (–/– lane as 1.0), which was quantified by using NIH Image, version 1.61, software, is shown beneath the blot image. β -Actin was used as an internal loading control.

Figure 4.

⁹-THC inhibits ER α -mediated transcriptional activities in concert with overexpression of ER β or addition of ICI 182,780. (A) MCF-7 cells were transiently transfected with a luciferase reporter gene construct-containing three copies of an ERE and expression plasmid for ER β . After transfection, cells were treated with vehicle (–/–) or ⁹-THC (5 or 25 μ M). ER β transfection (–) indicates mock-transfected groups. After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of vehicle-treated control (indicated as –/–), as the mean $\pm$ SD ($n = 5$). *Significantly different ($p < 0.05$) from the vehicle-treated group. (B) MCF-7 cells were transiently transfected with a luciferase reporter gene construct containing three copies of an ERE and expression plasmid for human ER β . After transfection, cells were treated with vehicle (–/–/–) or ⁹-THC (25 μ M) in the presence or absence of ICI 182,780 (ICI, 0.1–2.5 μ M). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of vehicle-treated control (indicated as –/–/–), as the mean $\pm$ SD ($n = 5$). N.D., not detectable due to complete

inhibition. (C) Effects of 9 -THC and E2 on ER α - or ER β -mediated transcription activities. ER α -negative MDA-MB-231 cells were transiently transfected with a luciferase reporter gene construct-containing three copies of ERE and expression plasmids for human ER α or ER β . After transfection, cells were treated with vehicle (-/-/-) or E2 (100 pM) or 9 -THC (25 μ M). ER α/β transfection (-) indicates mock-transfected groups. After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of Vehicle-treated control (indicated as -/-/-), as the mean $\pm$ SD ($n = 5$), *Significantly different ($p < 0.05$) from the vehicle-treated group.

Figure 5.

Δ^9 -THC behaves as an activator for ER β in ER α -negative MDA-MB-231 cells. (A) MDA-MB-231 cells were transiently transfected with a luciferase reporter gene construct containing three copies of an ERE and expression plasmid for human ER. After transfection, cells were treated with vehicle (–/–) or Δ^9 -THC (5, 10, or 25 μ M). ER β transfection (–) indicates mock-transfected groups. After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of vehicle-treated control (indicated as –/–), as the mean $\pm$ SD ($n = 5$). *Significantly different ($p < 0.05$) from the vehicle-treated group. Inset: RT-PCR analysis of ER α and ER mRNA basal levels in MDA-MB-231 cells. β -Actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. (B) MDA-MB-231 cells were transiently transfected with a luciferase reporter gene construct containing three copies of an ERE and expression plasmid for human ER β . After transfection, cells were treated with vehicle (–/–/–) or Δ^9 -THC (25 μ M) in the presence or absence of ICI 182,780 (ICI, 2.5 μ M) or PHTPP (2.5 μ M). After 24 h, cells were harvested and assayed for luciferase activity, and all transfections were normalized for efficiency using the internal *Renilla* control plasmid. Data are expressed as the percent of vehicle-treated control (indicated as –/–/–), as the mean $\pm$ SD ($n = 5$). *Significantly different ($p < 0.05$) from the vehicle-treated group (–/–/–). **Significantly different ($p < 0.05$) from the Δ^9 -THC/ICI-treated groups (–/–/–). N.S., not significant.

Figure 6.

Effect of Δ^9 -THC on the ER β -regulated target genes. (A) MDA-MB-231 cells were transiently transfected with an expression plasmid for ER β . Twenty-four hours after transfection, cells were treated with vehicle (–/–) or Δ^9 -THC (5, 10, or 25 μ M). ER β transfection (–) indicates mock-transfected groups. After 4 h, RT-PCR (upper panel, CDC2; middle panel, egr-1) analyses of each gene level were performed. β -Actin was used as an internal loading control. A 100-bp DNA ladder marker for RT-PCR was also loaded. A representative data image is shown. (B) RT-PCR analyses of ER β . Time course analysis (0, 4, 24, 72, or 96 h) of ER β transcript levels in MDA-MB-231 cells after treatment with 25 μ M Δ^9 -THC or without Δ^9 -THC was performed. β -Actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. A representative data image is shown.

Figure 7.

Interaction of Δ^9 -THC and E2. (A) MDA-MB-231 cells were exposed for 48 h to Δ^9 -THC ranging from 1 μ M to 25 μ M in the presence of E2 (100 pM). After the treatments, cell viability was measured according to the methods described in Materials and Methods. Data are expressed as the percent of vehicle-treated group (indicated as 0), as the mean $\pm$ SD ($n = 6$). (B) Fluorescence polarization competition binding assays for $ER\beta$. Effect of 0.0001 – 1000 μ M Δ^9 -THC on the fluorescently labeled E2 (4.5 nM) binding to $ER\beta$. The assays were performed according to the methods described in Materials and Methods and the Powell et al. reports.^{11,12}