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Inductions of the *fatty acid 2-hydroxylase (FA2H)* gene by Δ⁹-tetrahydrocannabinol in human breast cancer cells

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Abstract

To investigate gene(s) being regulated by Δ^9-tetrahydrocannabinol (Δ^9-THC), we performed DNA microarray analysis of human breast cancer MDA-MB-231 cells, which are poorly differentiated breast cancer cells, treated with Δ^9-THC for 48 hr at an IC_{50} concentration of approximately 25 μM. Among the highly up-regulated genes (> 10-fold) observed, *fatty acid 2-hydroxylase (FA2H)* was significantly induced (17.8-fold). Although the physiological role of FA2H has not yet been fully understood, FA2H has been shown to modulate cell differentiation. The results of Oil Red O staining after Δ^9-THC exposure showed the distribution of lipid droplets (a sign of the differentiated phenotype) in cells. Taken together, the results obtained here indicate that *FA2H* is a novel Δ^9-THC-regulated gene, and that Δ^9-THC induces differentiation signal(s) in poorly differentiated MDA-MB-231 cells.

Keywords

Δ^9-tetrahydrocannabinol; Fatty acid 2-hydroxylase; PPARα human breast cancer cells; Differentiation

INTRODUCTION

Δ^9-Tetrahydrocannabinol (Δ^9-THC), a major component of the drug-type cannabis plant, exhibits a variety of biological effects, such as anti-cancer and anti-arteriosclerosis (Pertwee *et al.*, 2010). Among these effects, Δ^9-THC has a differentiation-inducing potential on cells, such as cultured 3T3-L1 fibroblasts into adipocytes (O'Sullivan *et al.*, 2005) via peroxisome

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proliferator-activated receptor γ (PPARγ). Transcription factors have been shown to be key determinants in differentiation processes (Conzen, 2008; Peters *et al.*, 2012). Representatives of several transcription factor families have been implicated in these processes, including CCAAT/enhancer binding proteins (C/EBPα/β/δ) and PPARγ (Conzen, 2008; Peters *et al.*, 2012). In addition to PPARγ, two other PPAR isoforms have been identified: PPARα and PPARβ. When activated by their ligands, PPARs translocate to the nucleus, leading to the induction of the target genes involved in cellular differentiation processes. However, interplay between Δ^9-THC and PPARγ in poorly differentiated breast "cancer" MDA-MB-231 cells and the genes responsible for the process have not been fully resolved, and the possible involvement of the other two PPAR isoforms has not been addressed.

Using DNA microarray analysis, the *fatty acid 2-hydroxylase (FA2H)* gene was shown to be significantly induced (17.8-fold) in Δ^9-THC-treated MDA-MB-231 cells. Although the physiological role of FA2H has not yet been fully understood, FA2H has been shown to modulate cell differentiation involving keratinocytes, adipocytes, and Schwann cells (Hama, 2010). The expression of FA2H is highly variable among cell types, and is inducible during cellular differentiation by certain stimuli (Hama, 2010). The results of Oil Red O staining after Δ^9-THC exposure showed lipid droplet accumulation in cells, which is a sign of the differentiated phenotype. We next investigated the pathway(s) of Δ^9-THC-mediated *FA2H* induction in MDA-MB-231 cells, which are potentially coupled with the differentiation process. The findings of this study show for the first time that i) *FA2H* is a novel Δ^9-THC target gene, and that ii) PPARα/γ, especially PEARα, -mediated pathway(s) are, at least in part, involved in the up-regulation of *FA2H* by Δ^9-THC.

MATERIALS AND METHODS

Materials and cell culture

Δ^9-THC was isolated and purified from drug-type cannabis leaves according to the methods described elsewhere (Aramaki *et al.*, 1968). The purity of Δ^9-THC was found to be at least above 98% by gas chromatography (Takeda *et al.*, 2008). GW7647, GSK0660, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). MK886 and nTZDpa were purchased from Santa Cruz Biotechnology (Santa. Cruz, CA, USA). GW9662 was purchased from Wako Chemical (Osaka, Japan). All other reagents were of analytical grade commercially available and used without further purification. Cell culture conditions and methods were based on procedures described previously (Takeda *et al.*, 2011). Briefly, human breast cancer MDA-MB-231 and MCF-7 cell lines (obtained from the American Type Culture Collection, Rockville, MD, USA) were routinely grown in phenol red-containing minimum essential medium alpha (Invitrogen, Carlsbad, CA, USA), supplemented with 10 mM HEPES, 5% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2–95% air-humidified incubator. Before chemical treatments, the medium was changed to phenol red-free minimum essential medium alpha (Invitrogen) supplemented with 10 mM HEPES, 5% dextran-coated charcoal-treated serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Control incubations contained equivalent additions of solvents.

Preparation of total RNA and DNA microarray analyses

Total RNA was collected from 25 μM Δ^9-THC or vehicle-treated MDA-MB-231 cells 48 hr after exposure using the RNeasy kit (Qiagen, Inc. Hilden, Germany), and was purified using RNeasy/QIAamp columns (Qiagen, Inc.). The specific gene expression pattern in MDA-MB-231 cells was examined by DNA microarray analysis in comparison with vehicle-controls. Total RNA was extracted from both cells, and cDNA synthesizing and cRNA labeling were conducted using a Low RNA Fluorescent Linear Amplification Kit (Agilent, Palo Alto, CA, USA). Overall changes in gene expression were evaluated using two-color microarray-based gene expression analysis (Hwang *et al.*, 2011; Takeda *et al.*, 2011; Toyama *et al.*, 2011). Labeled cRNA (Cy3 to controls, Cy5 to Δ^9-THC) was hybridized to human oligo DNA microarray slides (Agilent) that carried spots for human genes. Specific hybridization was analyzed using a Microarray scanner (Agilent) and evaluated as a scatter-plot graph for gene expression. Hokkaido System Science (Sapporo, Japan) provided assistance with the experiments.

Analysis of FA2H mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from MDA-MB-231 and MCF-7 cells using the RNeasy kit (Qiagen, Inc.) and was purified using RNeasy/QIAamp columns (Qiagen, Inc.), and the following cDNA (cDNA) synthesis, RT, and PCR were performed using the SuperScript One-Step RT-PCR System with Platinum *Taq* polymerase (Invitrogen). The primers used were FA2H (sense) 5′-AAC GAG CCT GTA GCC CTT GA-3′ and FA2H (antisense) 5′-ACT GCC ACC GTG TAC TCT GTT-3′. Primers for the PCR of β-actin were taken from a previously published study (Takeda *et al.*, 2011). The PCR of FA2H and β-actin was performed under conditions that produced 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.

Oil-Red O staining

MDA-MB-231 cells were treated with vehicle (control), 25 μM nTZDpa, or 25 μM Δ^9-THC for 216 hr. Oil-Red O staining for the detection of lipid droplets was performed as described previously (Tontonoz *et al.*, 1994) and images were obtained as described previously (Takeda *et al.*, 2011).

RESULTS AND DISCUSSION

We first determined an IC_{50} concentration 48 hr after exposure of MDA-MB-231 cells to Δ^9-THC. Based on this information (*i.e.*, 25 μM), to obtain genes that are regulated by Δ^9-THC, we performed DNA microarray analysis of MDA-MB-231 cells 48 hr after exposure to Δ^9-THC (25 μM) or nTZDpa (25 μM), an agonist specific for PPARγ. As shown in Fig. 1A, among the genes involved in cellular differentiation, the *FA2H* gene was substantially up-regulated by Δ^9-THC (*i.e.*, 17.8-fold), although other genes (*C/EBPα* and *C/EBPβ*) were similarly regulated by both Δ^9-THC and nTZDpa. Using RT-PCR analysis, the *FA2H* gene

was shown to be up-regulated by 5 μM or 25 μM Δ^9-THC in a concentration-dependent manner (Fig. 1A, *inset*). The exposure time of 25 μM Δ^9-THC for the maximal induction of *FA2H* was 48 hr. Among the Δ^9-THC-regulated genes, *FA2H* was found to be stimulated more than 10-fold (data not shown). The *FA2H* gene is known to be inducible during differentiation (Hama, 2010). If this phenomenon is true for Δ^9-THC, MDA-MB-231 cells treated with Δ^9-THC would exhibit features typical of cellular differentiation. We next analyzed whether Δ^9-THC induced the accumulation of cytosolic lipid droplets, a maker of differentiation. Oil Red O staining of Δ^9-THC-treated cells for 216 hr clearly indicated that the number of positively stained cells was higher than that of vehicle-treated cells (Fig. 1B, a vs. c/d). An enlarged image (the '*inset*' in Fig. 1B, d) clearly exhibited cytosolic lipid droplets around the nucleolus. As expected, nTZDpa-treated cells were stained by Oil Red O while Δ^9-THC-treated cells were unequivocally stained. In addition, differences were observed in the cell morphologies induced by Δ^9-THC and nTZDpa (Fig. 1B, b vs. c). Thus, it is suggested that Δ^9-THC induces a differentiation phenotype potentially coupled with *FA2H* induction, whereas its differentiation pathways may be different from those of nTZDpa.

To analyze the Δ^9-THC-mediated induction pathways of *FA2H*, we first investigated the effects of PPARα/β/γ isoform-selective antagonists on the Δ^9-THC-mediated up-regulation of *FA2H*. Δ^9-THC-stimulated *FA2H* expression was markedly abrogated by antagonists specific for PPARα (MK886), followed by PPARγ (GW9662) to a lesser extent (Fig. 2A, lane 2 vs. 3 and 5), while failure to abrogate was detected with a PPARβ (GSK0660)-selective antagonist (Fig. 2A, lane 2 vs. 4). To further support the evidence obtained in Fig. 2A, we focused on PPARα/γ and investigated whether or not agonists selective for PPARα (GW7647) and PPARγ (nTZDpa) induce *FA2H* in MDA-MB-231 cells. A massive induction of *FA2H* was observed with the GW7647 (25 μM) treatment as well as the Δ^9-THC (25 μM) treatment relative to that with nTZDpa. In addition, the induction of *FA2H* by Δ^9-THC was also detected in human breast cancer MCF-7 cells (data not shown). Different from PPARγ, the physiological role of PPARα in breast cancer is largely unknown (Conzen, 2008); therefore, the potential involvement of PPARα in the induction of *FA2H* mediated by Δ^9-THC in breast cancer cells is suggested here.

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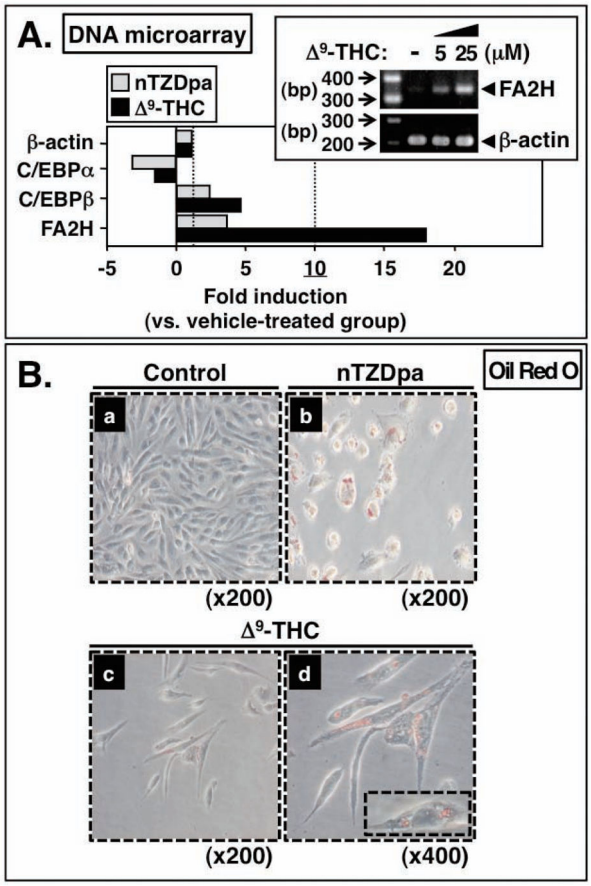

Fig. 1.

Comparison of the biological activities of Δ^9-THC and nTZDpa. (A) Results of DNA microarray analysis. Data are expressed as fold induction vs. vehicle-treated groups. MDA-MB-231 cells were treated with vehicle or 25 μM Δ^9-THC or 25 μM nTZDpa for 48 hr, followed by mRNA isolation. Details of the microarray conditions are described in the Materials and Methods. *Inset* figure, RT-PCR analysis of *FA2H* mRNA levels after 48 h exposure of MDA-MB-231 cells to 5 or 25 μM Δ^5-THC. β-actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. (B) Results of Oil Red O-staining. MDA-MB-231 cells were treated with vehicle (control; a), 25 μM nTZDpa (nTZDpa; b), or 25 μM Δ^9-THC (Δ^9-THC; c) for 216 hr, followed by Oil Red O-staining to identify lipid droplets. Images were taken under phase-contrast microscopy at x200 (panels; a–c). Panel d was taken at x400. *Inset* figure of the panel exhibits the cytosolic accumulation of lipid droplets.

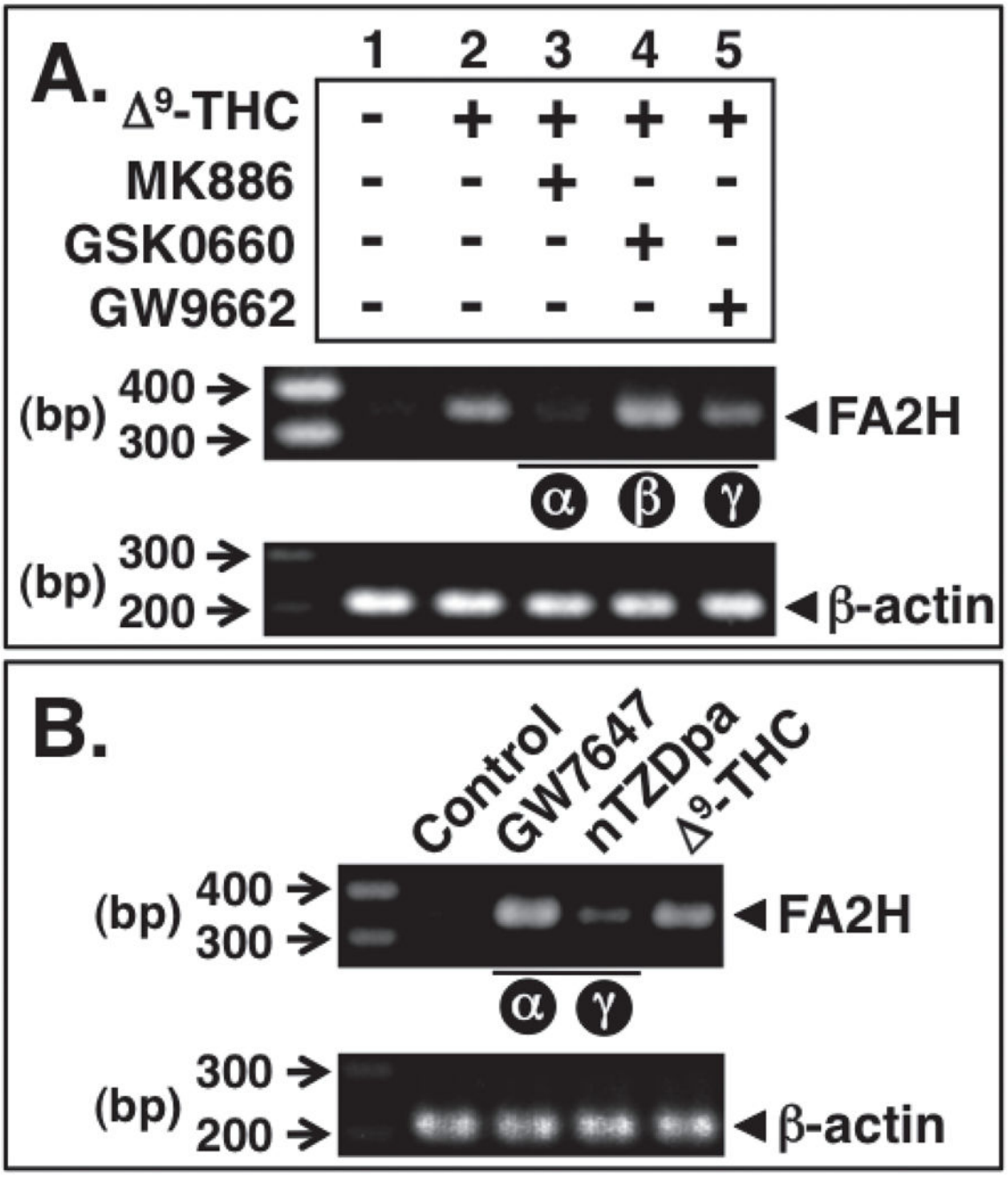

Fig. 2.

Involvement of PPARα/γ in the Δ^9-THC-mediated up-regulation of *FA2H*. (A) Effects of PPARα/β/γ antagonists (MK866/GSK0660/GW9662, respectively) on the Δ^9-THC-induced up-regulation of *FA2H*. RT-PCR analysis of *FA2H* mRNA levels was performed after 48 hr exposure of MDA-MB-231 cells to 25 μM Δ^9-THC. Each antagonist specific for PPARs was pre-treated for 2 hr. β-actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded. (B) Effects of PPARα/γ agonists (GW7647/nTZDpa, respectively) on the expression of *FA2H*. RT-PCR analysis of *FA2H* mRNA levels was

performed after 48 hr exposure of MDA-MB-231 cells to 25 μM GW7647 or nTZDpa. Each β-actin was used as an RNA normalization control. A 100-bp DNA ladder marker was also loaded.