0026-895X/03/6305-983–992$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:983–992, 2003

Vol. 63, No. 5
2185/1060073
Printed in U.S.A.

Activation and Binding of Peroxisome Proliferator-Activated Receptor γ by Synthetic Cannabinoid Ajulemic Acid

JILIN LIU, HUI LI, SUMNER H. BURSTEIN, ROBERT B. ZURIER, and J. DON CHEN[1]

Departments of Biochemistry and Molecular Pharmacology (J.L., H.L., S.H.B., J.D.C.) and Medicine (R.B.Z.), University of Massachusetts Medical School, Worcester, Massachusetts

Received September 30, 2002; accepted February 5, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Ajulemic acid (AJA) is a synthetic analog of the tetrahydrocannabinol (THC) metabolite THC-11-oic acid; THC is a major active ingredient of the drug marijuana derived from the plant *cannabis*. AJA has potent analgesic and anti-inflammatory activity without the psychotropic action of THC. Unlike the nonsteroidal anti-inflammatory drugs, AJA is not ulcerogenic at therapeutic doses, making it a promising anti-inflammatory drug. However, the mechanism of AJA action remains unknown. Here we report that AJA binds directly and specifically to the peroxisome proliferator-activated receptor γ (PPARγ), a pharmacologically important member of the nuclear receptor superfamily. Functional assay indicates that AJA activates the transcriptional activity of both human and mouse PPARγ at pharmacological concentrations. Activation of PPARγ by AJA requires the AF-2 helix of the receptor, suggesting that AJA activates PPARγ through the ligand-dependent AF-2 function. AJA binding consistently enables PPARγ to recruit nuclear receptor coactivators. In addition, we show that AJA inhibits interleukin-8 promoter activity in a PPARγ-dependent manner, suggesting a link between the anti-inflammatory action of AJA and the activation of PPARγ. Finally, we find that AJA treatment induces differentiation of 3T3 L1 fibroblasts into adipocytes, a process mediated by PPARγ. Together, these data indicate that PPARγ may be a molecular target for AJA, providing a potential mechanism for the anti-inflammatory action of AJA, and possibly other cannabinoids. These studies also implicate other potential therapeutic actions of AJA through PPARγ activation in multiple signaling pathways.

The mood-altering drug marijuana derived from the hemp plant *Cannabis sativa* contains a group of biosynthetically related substances known collectively as cannabinoids. Tetrahydrocannabinol (THC), one of the major cannabinoids in marijuana, has potent analgesic and anti-inflammatory activities, but it also exhibits psychotropic effects, which limit its clinical application. Considerable effort has been expended toward the goal of creating nonpsychotropic cannabinoid derivatives that retain therapeutic actions but are free of psychotropic activity. A useful template for this search is the THC metabolite THC-11-oic acid (Fig. 1A), because it does not induce changes in mood in human or behavior in animal models. Unfortunately, THC-11-oic acid has only modest analgesic and anti-inflammatory activities (Burstein et al., 1992). By adding two carbons to the side chain of THC-11-oic acid, and introducing two methyl groups, the dimethylheptyl analog of THC-11-oic acid, ajulemic acid (AJA; Fig. 1B), has been developed (Burstein et al., 1992). AJA has potent and prolonged analgesic and anti-inflammatory activities that are comparable with those of morphine (Burstein et al., 1992, 1998; Dajani et al., 1999). Also, in contrast to the nonsteroidal anti-inflammatory drugs (NSAIDs), AJA is not ulcerogenic at therapeutic doses, and does not exhibit tolerance or cause mutagenesis (Dajani et al., 1999; Burstein, 2000). Furthermore, AJA does not have psychotropic actions in animal models (Burstein et al., 1992; Zurier et al., 1998). AJA also suppresses both acute inflammation induced by injection of interleukin (IL)-1β and tumor necrosis factor α in the murine subcutaneous air pouch model, and joint tissue damage in the adjuvant-induced polyarthritis model in rats (Zurier et al., 1998). Based on these findings, AJA has been approved recently for a phase II

J.D.C. is a Research Scholar funded by the Leukemia and Lymphoma Society. This work was made possible by grants DK52542 and DK52888 (to J.D.C.), DA09439 and DA09017 (to S.H.B.), and AR38501 (to R.B.Z.) from the National Institutes of Health.

[1] Present Address: Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey.

ABBREVIATIONS: THC, tetrahydrocannabinol; AJA, ajulemic acid; NSAID, nonsteroidal anti-inflammatory drug; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; LBD, ligand binding domain; RXR, retinoid X receptor; PCR, polymerase chain reaction; TK, thymidine kinase; PPRE, peroxisome proliferator-responsive element; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcriptase; DMSO, dimethyl sulfoxide; RA, retinoic acid; RAC3, receptor-associated coactivator-3; DRIP, vitamin D receptor interacting protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

clinical trial for reduction of pain in humans. However, the mechanism by which AJA exerts its therapeutic activity remains largely unknown, because it does not bind efficiently to either of the cannabinoid-specific cell surface receptors CB1 and CB2 (Pertwee, 1997).

Peroxisome proliferator-activated receptor γ (PPARγ) is a pharmacologically important member of the nuclear receptor superfamily (Houseknecht et al., 2002). It plays important roles in a diverse array of biological processes, including lipid metabolism, glucose homeostasis, and adipocyte differentiation. The crystal structure of the PPARγ ligand-binding domain reveals a large hydrophobic cavity for ligand binding (Uppenberg et al., 1998; Xu et al., 2001). Indeed, PPARγ binds to a wide range of synthetic and naturally occurring substances, including the antidiabetic drugs thiazolidinediones (Lehmann et al., 1995; Willson et al., 1996), the synthetic tyrosine analog GW347845 (Cobb et al., 1998), polyunsaturated fatty acids (Kliewer et al., 1997), metabolites of arachidonic acid, including 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (Forman et al., 1995; Kliewer et al., 1995), NSAIDs (Lehmann et al., 1997), and compounds of oxidized low-density lipoprotein, such as 13-hydroxyoctadecadienoic acid and 15-hydroxyeicosatraenoic acid (Nagy et al., 1998). Several of these PPARγ ligands exhibit anti-inflammatory activity in vivo (Kawahito et al., 2000; Naito et al., 2001), and activation of PPARγ is directly linked to anti-inflammatory (Jiang et al., 1998) and antitumor (Patel et al., 2001) processes. Accordingly, activation of PPARγ inhibits the expression of cytokines such as IL-1β, tumor necrosis factor α, and nitric oxide at both transcription and translation levels (Jiang et al., 1998; Ricote et al., 1998). PPARγ is expressed in adipose tissue, skeletal muscle, adrenal gland, colonic epithelium, heart, pancreas, and liver (Mukherjee et al., 1997; Sarraf et al., 1998). It is also expressed in immune system-related cells such as splenocytes (Kliewer et al., 1994; Clark et al., 2000), synoviocytes (Kawahito et al., 2000; Ji et al., 2001; Simonin et al., 2002), helper T cells (Clark et al., 2000), and activated monocytes and macrophages (Jiang et al., 1998; Ricote et al., 1998; Kawahito et al., 2000), suggesting that PPARγ has a direct role in modulating inflammation in addition to its role in lipid metabolism and glucose homeostasis.

The functional similarity between the anti-inflammatory activity of AJA and known PPARγ ligands and the promiscuity of ligand binding by PPARγ led us to consider AJA as a potential ligand for PPARγ. In line with this speculation, some NSAIDs also bind to PPARγ (Lehmann et al., 1997). In this study, we demonstrate that AJA binds specifically and selectively to PPARγ but not PPARα or PPARδ. Binding of AJA activates PPARγ's transcriptional activity and enables recruitment of transcriptional coactivators to the receptor. We also show that AJA inhibits the induction of interleukin-8 promoter activity in a PPARγ-dependent manner. Furthermore, we demonstrate that addition of AJA to 3T3 L1 cells in vitro induces differentiation of these cells into adipocytes, a process mediated by PPARγ. Thus far, an intracellular receptor for AJA has not been reported; our data suggest that PPARγ may serve this function.

Materials and Methods

Reagents. The PPARγ-specific ligand GW347845, PPARα-specific ligand GW32, and PPARδ-specific ligand GW36 were kindly provided by Dr. Timothy M. Willson (GlaxoSmithKline, Research Triangle Park, NC). Ajulemic acid was synthesized by Organix, Inc. (Woburn, MA). All other chemicals were purchased from commercial sources.

Plasmids. The pGEX-DRIP205 (527–970) was a gift from Dr. Leonard P. Freedman (Merck, Inc., West Point, PA). The Gal4-hPPAR ligand-binding domain (LBD) constructs were provided by

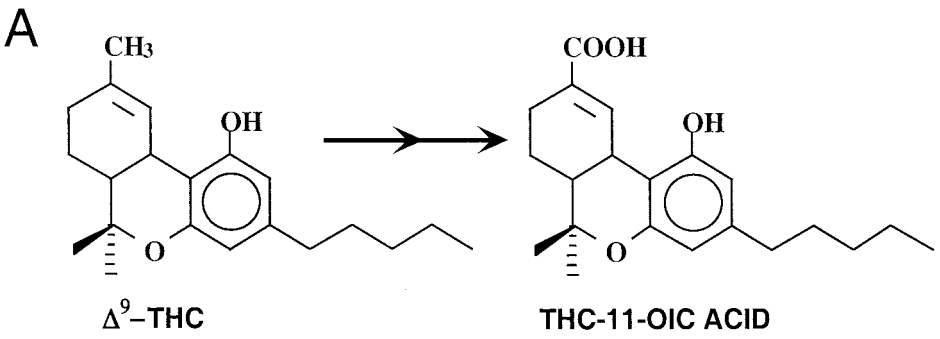
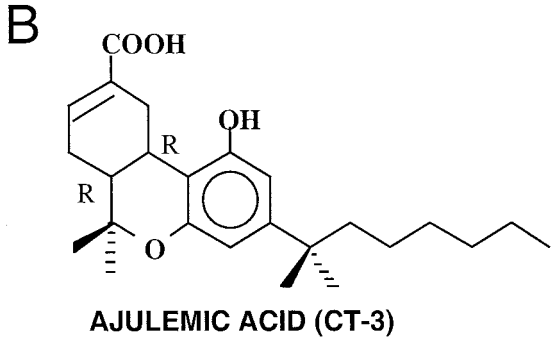


Fig. 1. Chemical structures of THC, THC-11-oic acid, and ajulemic acid



Dr. Steven A. Kliewer (Southwestern Medical Center, Dallas, TX). The IL8-luciferase reporter was provided by Dr. Bernd Stein (Celgene, San Diego, CA). The plasmids mPPARα, mPPARγ1, mPPARδ, and hRXRα were subcloned in the pCMX vector (Umesono et al., 1991). The pCMX-Gal4-mPPARγ1 was constructed by fusing the mPPARγ1 coding sequence to the yeast GAL4 DNA binding domain (amino acids 1–147) in the pCMX vector. The AF-2 helix-truncated mutant of mPPARγ1 (PPARγ ΔAF2) was generated by polymerase chain reaction (PCR) amplification to introduce a stop codon and an *Nhe*I restriction site after amino acid 489, and then subcloned into the pCMX vector. The PPRE-TK-LUC, MH100-TK-LUC, and DR1-TK-LUC reporters have been described previously (Umesono et al., 1991; Forman et al., 1995).

Partial Protease Digestion Assay. Partial protease digestion was carried out as described previously (Leng et al., 1993). PPAR proteins were made by in vitro transcription/translation reactions in reticulocyte lysate according to manufacture's instructions (Promega, Madison, WI). AJA, GW compounds, or vehicle alone was incubated with the ^{35}S-labeled PPARs at room temperature for 1 hour before trypsin digestion. Reactions were stopped by boiling in SDS-containing sample buffer, and lysates were subjected to SDS-PAGE and autoradiography.

Expression and Purification of GST Fusion Protein. The glutathione *S*-transferase (GST)-DRIP205 (amino acids 527–970) and GST-RAC3 receptor interaction domain (amino acids 613–752) fusion proteins were expressed in the *Escherichia coli* BL21 cells by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside at room temperature for 4 h. The bacterial pellet was resuspended in buffer containing 150 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA, with 5 mM dithiothreitol, 1 mM phenylmethlsulfonyl fluoride, and 1.5% Sarkosyl. The cell suspension was sonicated and centrifuged at 7400 rpm at 4°C for 30 min. The supernatant was isolated and 0.02% Triton-X 100 was added. The mixture was then incubated with 1 ml of 50% slurry of glutathione-Sepharose beads on a nutator at 4°C for 30 min. The beads were spun down at 3000 rpm for 10 min, the supernatant was removed, and the beads were suspended in 1 ml of cold phosphate-buffered saline (PBS).

GST Pull-Down Assay. Approximately 5 μg of GST-DRIP205 or GST-RAC3 bound on glutathione-Sepharose beads and 4 μl of [^{35}S]methionine-labeled mPPARγ1 were incubated with the indicated concentrations of AJA, GW347845, or vehicle alone in H buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Nonidet P40, 0.1 mM methionine, and 1 mM dithiothreitol) containing 1 mg/ml bovine serum albumin on a nutator at 4°C overnight. After three washes with cold PBS, the bound proteins were eluted in SDS sample buffer and boiled for 10 min before SDS-PAGE and autoradiography. To ensure that equal amounts of GST fusion proteins were recovered in the pull-down assay, the gel was stained with Coomassie blue before autoradiography.

Cell Culture and Transient Transfection. For PPARγ activation assay, the HEK293 cells were plated in 12-well cell culture plates and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Cells were changed to phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum 3 h before transfection by a standard calcium-phosphate precipitation method. Twelve hours after transfection, the cells were washed with PBS and fed again with fresh medium containing the indicated concentrations of specified compounds. After 36 h, cells were harvested for β-galactosidase and luciferase activities as described previously (Li et al., 1997). The average normalized luciferase activity was determined in triplicate experiments. For IL-8 promoter assay, HeLa cells were maintained and transfected as described above. After transfection, cells were recovered for 4 h before treatment with AJA, GW347845, or solvent. After 3 to 4 h, cells were treated with or without 25 nM of phorbol 12-myristate 13-acetate (PMA) for 24 h.

Adipocyte Differentiation Assay and RT-PCR. The adipocyte differentiation assay was performed as described by Mukherjee et al. (2000). The 3T3 L1 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% calf serum. Two days after reaching confluence, cells were treated with AJA, GW347845, or vehicle (0.1% DMSO) in the presence of 10 μg/ml insulin every other day. After 10 days of treatment with AJA or 7 days with GW347845 at confluence, cells were fixed and stained with Oil Red O (Sigma, St. Louis, MO). For RT-PCR, total cellular RNAs were isolated by TRIzol (Invitrogen, Carlsbad, CA). Reverse transcriptase (RT)-PCR was performed using the superscript first-strand synthesis kit (Invitrogen). After first-strand cDNA was synthesized by use of oligo(dT), cDNA was amplified by PCR. The forward and reverse primers used in the amplifications were 5′-GCT GTT ATG GGT GAA ACT CTG GGA G-3′, and 5′-CTT CAT GAG GCC TGT TGT AGA GC-3′, respectively, for PPARγ2, 5′-GAG CAA ATG GAG TTC CCA GAT G-3′ and 5′-GCA AAC AAT GGG AAT AGT TCA CAG TAG-3′, respectively, for aP2, and 5′-GAC CAC AGT CCA TGC CAT CAC-3′ and 5′-CAT ACC AGG AAA TGA GCT GAC-3′, respectively, for GAPDH. PCR amplifications were performed in 50 μl volume with *Taq*DNA polymerase for 30 cycles.

Results

Binding of Ajulemic Acid to PPARγ. AJA is a synthetic dimethylheptyl analog of the THC metabolite THC-11-oic acid (Fig. 1). To test whether AJA binds to PPARs, we conducted partial proteinase digestion assays as described previously (Leng et al., 1993). Ligand binding is known to cause conformational changes of nuclear receptors, a crucial process for transcriptional activation by the receptors (Allan et al., 1992). Such conformational change can be detected by limited proteinase digestion based on alteration of the accessibility of proteolytic sites on the surface of the receptor. In vitro translated ^{35}S-labeled PPAR proteins were incubated with AJA for 60 min before being subjected to partial trypsin digestion. The proteolytic patterns of PPAR proteins were then analyzed by SDS-PAGE and autoradiography and compared with vehicle-treated control samples.

In the experiment illustrated in Fig. 2A, we observed clear differences in the proteolytic profiles between AJA and vehicle-treated PPARγ proteins. Two prominent trypsin-resistant fragments of 30 and 24 kDa were detected after incubation with 20 μM AJA when compared with the control sample, in which the 30-kDa band disappeared completely after a 60-min digestion and two smaller fragments emerged after 30 min of digestion. As a positive control, the potent PPARγ agonist GW347845 (Cobb et al., 1998; Suh et al., 1999) also produced a proteinase digestion pattern similar to the one seen in AJA-treated sample. These findings suggest that AJA treatment can cause a conformational change in PPARγ, suggesting that AJA binds directly to PPARγ.

The AJA concentrations required to protect PPARγ from trypsin digestion were measured. The two proteinase-resistant PPARγ fragments were observed at 2, 20, and 100 μM AJA concentrations, with a slight dose-dependent increase between 2 and 20 μM (Fig. 2B). In contrast, AJA at all tested concentrations did not affect the proteinase sensitivity of PPARα (Fig. 2C) or PPARδ (Fig. 2D), although PPARα- and PPARδ-specific ligands protected these proteins against protease digestion. These data indicate that AJA binds selectively and specifically to PPARγ.

Transcriptional Activation by PPARγ upon Ajulemic Acid Treatment. The above observation indicates that AJA induces a conformational change in PPARγ in a manner similar to a known PPARγ agonist. This led us to speculate

that AJA might be a PPARγ agonist capable of activating PPARγ. Therefore, transient transfection assay was performed using the PPRE-TK-LUC reporter (Kliewer et al., 1994) cotransfected with PPARγ expression vector into the human kidney HEK293 cells followed by AJA treatment of the transfected cells. In this assay, 1 μM GW347845 stimulated the transcriptional activity of PPARγ by 4-fold. Intriguingly, AJA also stimulated PPARγ transcriptional activity 2- to 3-fold at 1 to 10 μM concentrations in a dose-dependent manner (Fig. 3A). These results demonstrate that AJA can indeed activate transcriptional activity of PPARγ, consistent with its binding to PPARγ and induction of an active conformation.

In an effort to confirm that AJA activates PPARγ selectively, we analyzed the ability of AJA to activate PPARα and PPARδ in a similar transient transfection assay. PPARα had a 3-fold higher basal transcriptional activity than PPARγ in the absence of ligand; however, no further activation was observed after treatment with 1 to 20 μM AJA, whereas a PPARα-specific ligand activated PPARα strongly (Fig. 3B; data not shown). Similarly, AJA did not activate PPARδ in this assay, whereas a PPARδ-specific ligand activated PPARδ strongly (Fig. 3C). These data indicate that AJA selectively activates PPARγ in cultured human cells, consistent with its ligand-binding specificity in vitro.

PPARγ forms a permissive heterodimeric complex with the retinoid X receptor (RXR). Ligands for either PPARγ or RXR have both been shown to activate the receptor heterodimer (Leblanc and Stunnenberg, 1995). To rule out the possibility that the observed activation by AJA in cells might act through endogenous RXR, we analyzed the ability of AJA to activate RXRα on a DR1-LUC reporter, where RXR forms a homodimer that is activated by RXR-specific ligands. In this experiment, we found that the RXR-specific ligand 9-*cis*-RA indeed activated the reporter 3-fold, but AJA failed to activate the reporter even at saturating concentrations (Fig. 3D), suggesting that AJA does not bind or activate RXRα. These data indicate that transcriptional activation of PPARγ by AJA in vivo is not mediated through its heterodimeric partner RXRα.

PPARγ contains two transcriptional activation domains—a constitutive N-terminal AF-1 domain and a ligand-dependent C-terminal AF-2 domain. The AF-2 function depends on the presence of an AF-2 helix (helix 12) located at the extreme C terminus of the LBD. To examine whether activation of PPARγ by AJA is mediated through the ligand-dependent AF-2 function, we deleted the AF-2 helix of PPARγ to create a PPARγ ΔAF2 mutant, and tested whether AJA could still activate the PPRE-TK-LUC reporter through the ΔAF2 mutant. AJA and GW347845 both failed to activate expression of

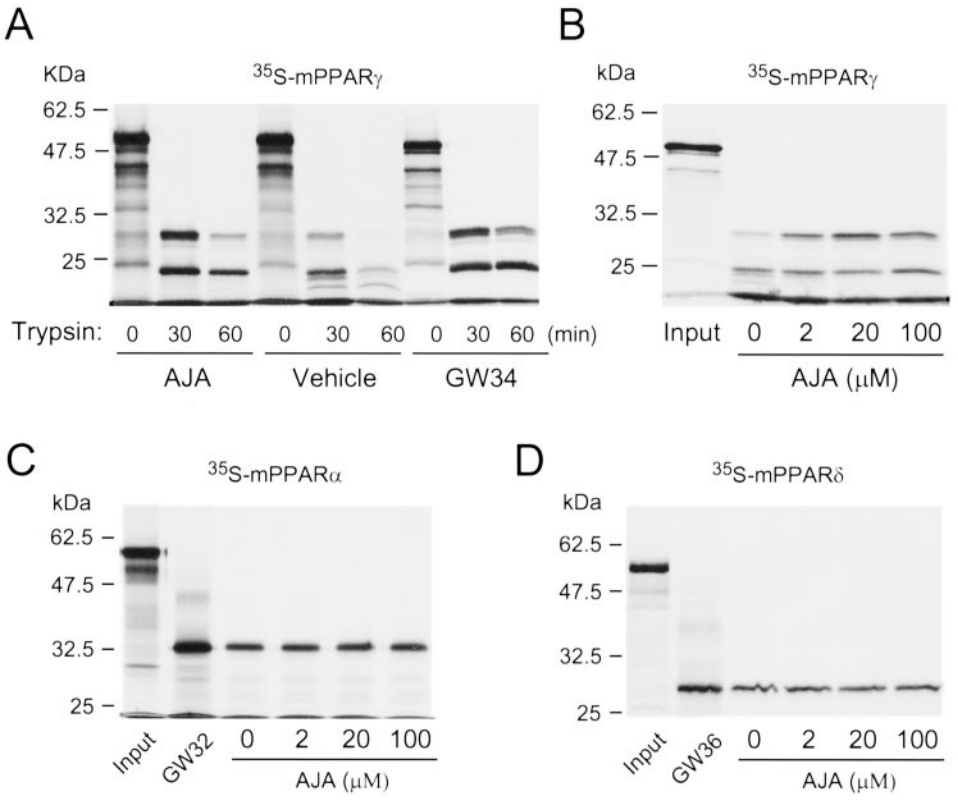


Fig. 2. Binding of AJA to PPARγ. The PPAR proteins were synthesized and labeled with [^{35}S]methionine in reticulocyte lysate and incubated with AJA, GW compounds, or vehicle alone at room temperature for one hour before trypsin digestion. The reactions were terminated by boiling in SDS-containing protein sample buffer. The PPAR proteolytic patterns were analyzed by SDS-PAGE and autoradiography. A, AJA treatment protects mPPARγ from trypsin digestion. The final concentrations of ligands were 20 μM AJA and 1 μM GW347845 in 0.2% DMSO (vehicle). Partial proteinase digestions were conducted with 30 μg/ml of trypsin for 30 or 60 min as indicated. Note that the two trypsin resistant fragments (30 and 24 kDa) were better protected from trypsin digestion in the presence of AJA or GW347845. Two additional smaller proteolytic fragments were observed only in the vehicle control. B, AJA concentration-dependent protection of mPPARγ against trypsin digestion. The PPARγ treated with the indicated concentrations of AJA was digested with 20 μg/ml of trypsin for 30 min. A clear protection of PPARγ could be observed starting at 2 μM AJA. C and D, trypsin sensitivity of PPARα and PPARδ in the absence or increasing concentrations of AJA. In contrast to PPARγ, PPARα, and PPARδ were not protected from trypsin digestion in the presence of 2 to 100 μM AJA, whereas the PPARα-specific ligand GW32 and the PPARδ-specific ligand GW36 partially protected PPARα and PPARδ from proteinase digestion, respectively.

the reporter gene (Fig. 3E), suggesting that activation of PPARγ by AJA is mediated through the AF-2 function. This is consistent with the hypothesis that AJA is an activating ligand for PPARγ.

To further confirm the activation of PPARγ by AJA, and to determine species specificity, we assessed the ability of AJA to activate the chimeric Gal4-DBD/PPAR fusion proteins on a Gal4-dependent MH100-Luc reporter (Fig. 4A). In this system, the activation of reporter is mediated through the chimeric exogenous Gal4-DBD fusion protein, thus eliminating potential interference from any endogenous receptor. In this assay, both AJA and GW347845 activated the reporter gene expression significantly (Fig. 4B). The activation of Gal4-DBD/mPPARγ by AJA was concentration dependent, with an estimated EC_{50} of approximately 13 μM (Fig. 4C). We also tested the efficacy of AJA to activate human PPARγ in this assay and confirmed that AJA could also activate human PPARγ as efficiently as its ability to activate mouse PPARγ (Fig. 4D). AJA consistently did not activate human PPARα or PPARδ. Taken together, these data strongly indicate that AJA activates both mouse and human PPARγ specifically at pharmacologically relevant concentrations.

AJA Stimulates Coactivator Binding to PPARγ. Nuclear receptor coactivators are known to interact with ligand-activated receptors to enhance transcriptional activation by recruiting chromatin modifying enzymes and RNA polymerase. The receptor-associated coactivator-3 (RAC3) of the p160/SRC family (Leo and Chen, 2000) and the DRIP205 subunit of the DRIP coactivator complex (Yang et al., 2000), are known PPARγ coactivators. To provide insight into the mechanism whereby AJA influences PPARγ transcriptional activity, we examined the ability of PPARγ to interact with DRIP205 and RAC3 in response to AJA treatment by GST-pull down assay. In this experiment, the [^{35}S]methionine-labeled PPARγ showed negligible binding to GST alone, GST-DRIP205, or GST-RAC3 in the absence of ligand (Fig. 5, A

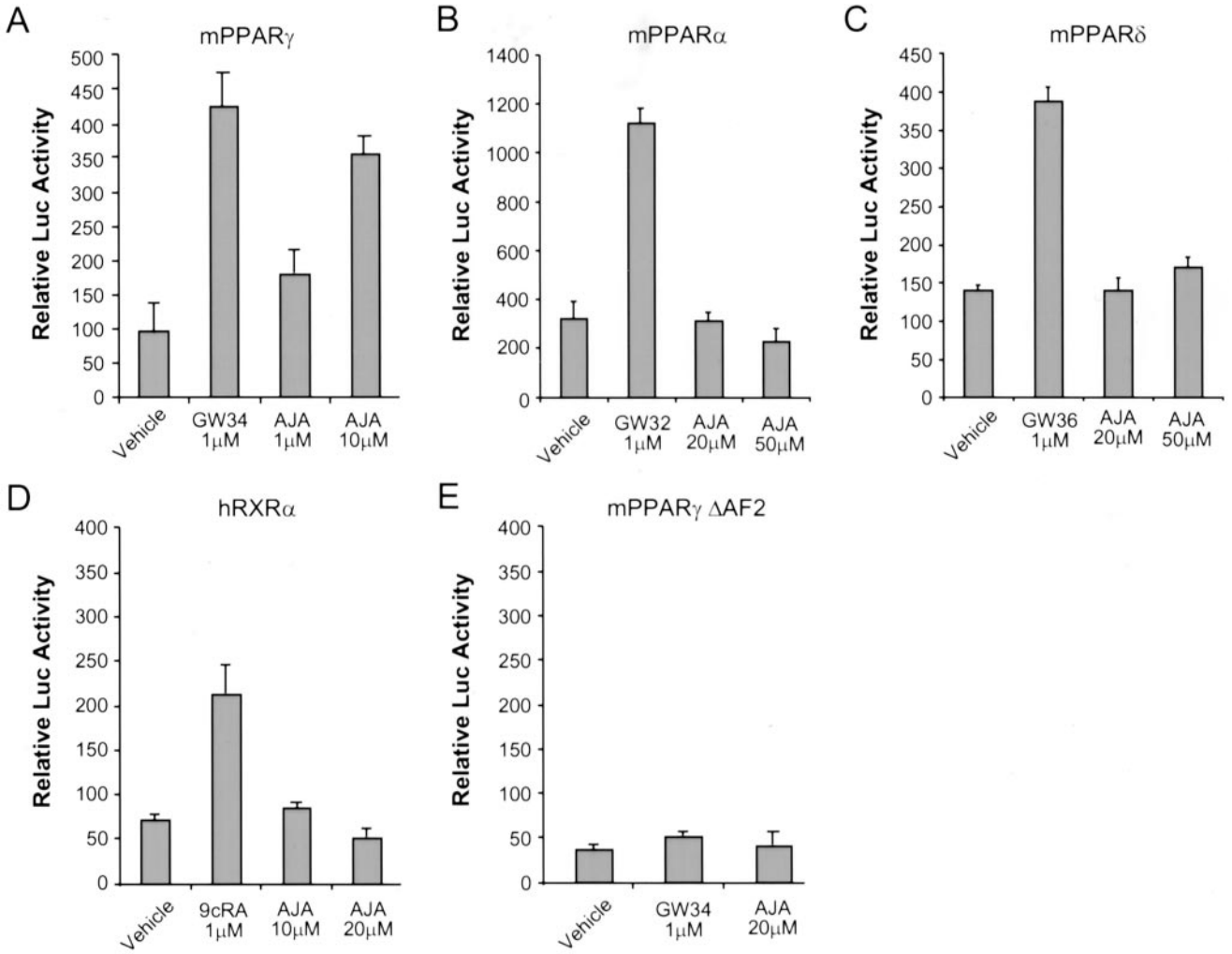


Fig. 3. Transcriptional activation of PPARγ by AJA. The reporter gene assay was performed in HEK293 cells by transient transfection with the indicated PPAR expression vectors and the PPRE-TK-LUC reporter. The average luciferase activities were normalized with the cotransfected β-galactosidase in triplicate experiments. After transfections, cells were treated with indicated concentrations of ligands or vehicle alone for 36 h before harvesting for β-galactosidase and luciferase assays. A, the mouse PPARγ is activated by GW347845 and AJA in a concentration-dependent manner. B to C, the mouse PPARα and PPARδ were not activated by AJA in the reporter gene assay, whereas they were activated by GW32 and GW36, respectively. D, AJA does not activate RXR. The hRXRα and a DR1-TK-LUC reporter were cotransfected into HEK293 cells, followed by 9-*cis*-RA or AJA treatments. Unlike the 9-*cis*-RA, AJA does not activate hRXRα homodimer on the DR1 element. E, activation of PPARγ by AJA requires AF-2 helix of the receptor. The AF-2 helix truncated PPARγ ΔAF2 mutant could not be activated by AJA or GW347845, suggesting that AJA and GW347845 activate the ligand-dependent AF-2 function of the receptor.

and B). In contrast, AJA and GW347845 pretreatment caused significantly increased binding of PPARγ to GST-DRIP205 and GST-RAC3. The interaction between PPARγ and DRIP205 seemed to be stronger than the interaction between PPARγ and RAC3, consistent with the finding that DRIP205 is a more potent coactivator than RAC3 for PPARγ (Zhu et al., 1997; Yang et al., 2000). These data indicate that AJA treatment promotes the interaction of PPARγ with nuclear receptor coactivators, further corroborating the hypothesis that AJA binds directly to PPARγ and activates its transcriptional activity.

Effect of PPARγ Activation by AJA on IL-8 Promoter Activity. In an effort to link activation of PPARγ by AJA to the anti-inflammatory activity of AJA, we determined the effect of AJA on IL-8 promoter activity and the role of PPARγ in this process. Transient transfection assays were conducted using an IL-8 promoter-regulated luciferase reporter cotransfected with wild-type or PPARγ ΔAF2 mutant in mammalian cells. IL-8 is a biomarker for inflammation, and reduction of IL-8 levels correlates with a decrease in inflammation. The involvement of PPARγ in regulating IL-8 promoter activity was determined by comparing the IL-8 promoter activity in the presence of wild-type PPARγ or its ΔAF2 mutant. In the experiment illustrated in Fig. 6A, PMA stimulated IL-8 promoter activity 3-fold, which was set as 100% promoter activity. AJA treatment reduced the IL-8 promoter activity by about 40% at 20 μM concentration in cells transfected with the wild-type PPARγ. This effect seems concentration-dependent, and the reduction of IL-8 promoter activity is statistically significant at 10 and 20 μM AJA concentrations. AJA had no effect on IL-8 promoter activity in cells transfected with the PPARγ ΔAF2 mutant (Fig. 6B), suggesting that the reduction of IL-8 promoter activity is mediated through activation of PPARγ by AJA. As a control, GW347845 dramatically reduced IL-8 promoter activity in cells cotransfected with the wild-type PPARγ but not with the ΔAF2 mutant

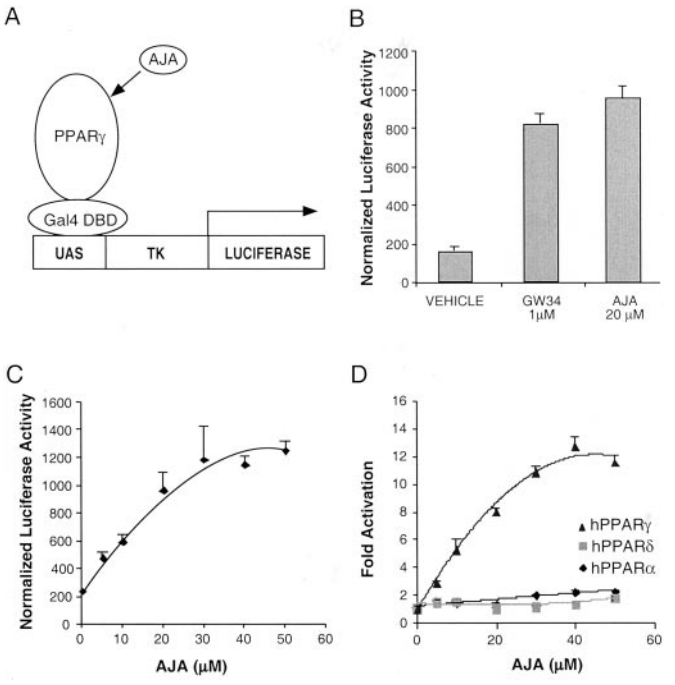


Fig. 4. Transcriptional activation by Gal4 DBD/PPARγ fusion protein in response to AJA treatment. A, schematic of the Gal4 based reporter system. The PPARs were expressed as Gal4 DBD fusion proteins, which bind to the UAS promoter containing four copies of a synthetic Gal4 binding site upstream of the minimal thymidine kinase (TK) promoter. Transient transfection was conducted in HEK293 cells with the mPPARγ expression vector and the MH100-tk-Luc reporter. The Gal4 DBD/PPAR fusion protein activates the reporter gene expression in response to agonist binding. B, PPARγ was significantly activated by 20 μM AJA or 1 μM GW347845 in the Gal4 reporter system. C, AJA concentration-dependent activation of mPPARγ. Transfected cells were treated with increasing concentrations of AJA from 10 to 60 μM, and the luciferase activities were determined in triplicated experiments. The estimated EC_{50} for activation of PPARγ by AJA is 13 μM in this assay. D, activation of human PPARγ by AJA. The Gal4 DBD/hPPARγ fusion protein activates the reporter gene expression in a concentration-dependent manner in the Gal4 DBD system. In contrast, AJA failed to activate hPPARα and hPPARδ. Normalized luciferase activity was plotted as fold activation relative to vehicle control.

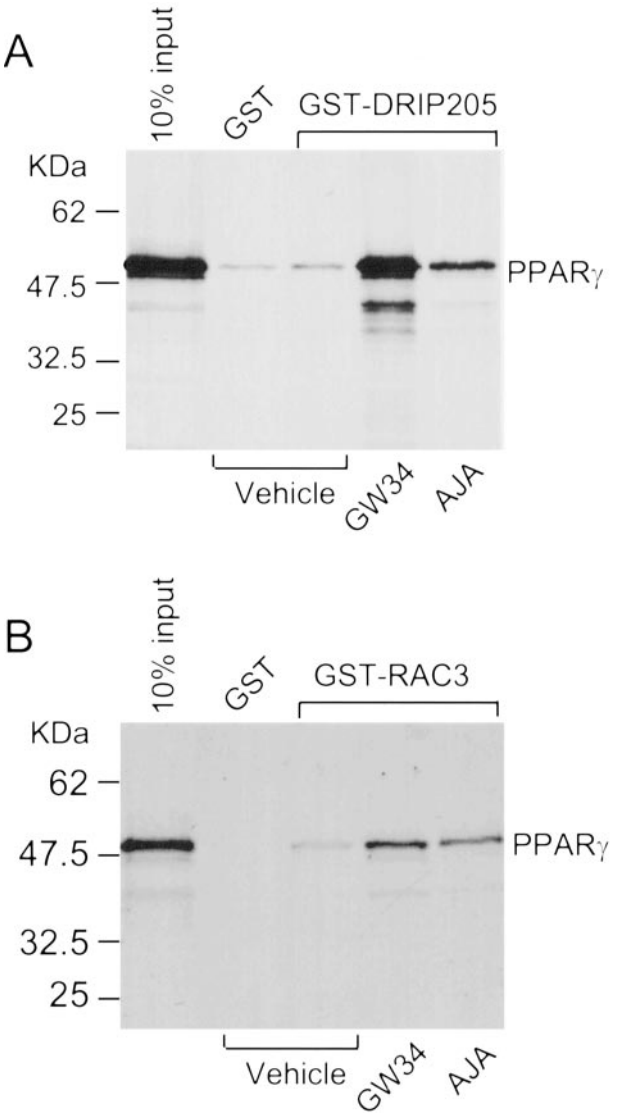


Fig. 5. Binding of AJA enables coactivator recruitment to PPARγ. GST pull-down assay shows interactions of PPARγ with the coactivators DRIP205 and RAC3. The GST-DRIP205 (amino acids 527–970) and the GST-RAC3 (amino acids 613–752) fusion proteins were purified and used in binding reactions containing in vitro translated ^{35}S-labeled PPARγ in the presence of vehicle (0.1% DMSO), GW347845 (GW34, 1 μM), or AJA (20 μM). GST alone was used as a negative control in the pull-down reaction. A, the PPARγ interacted with the GST-DRIP205 in the presence of GW347845 or AJA. B, the PPARγ also interacted with GST-RAC3 in the presence of GW347845 or AJA but to a lesser degree than the interactions with GST-DRIP205.

(Figs. 6, C and D). These data indicate that activation of PPARγ by AJA is involved in the inhibition of IL-8 promoter activity, suggesting a potential mechanism for the anti-inflammatory action of AJA.

Induction of Adipocyte Differentiation by Ajulemic Acid. Activation of PPARγ by its ligands is an essential process for the onset of adipocyte differentiation, which is characterized by morphological changes, droplet formation, and induction of adipocyte-specific genes such as PPARγ2 and aP2 (Tontonoz et al., 1994; Barak et al., 1999; Rosen et al., 2002). PPARγ ligands can substitute for the adipogenic hormones during differentiation of preadipocytes into adipocytes (Chawla and Lazar, 1994), and ectopically expressed PPARγ is able to transdifferentiate myoblasts into adipocytes (Hu et al., 1995). To provide biological evidence that AJA is a *bona fide* activator for PPARγ, we assessed the ability of AJA to induce differentiation of 3T3 L1 fibroblasts into adipocytes. The cells were treated for 10 days with AJA, or 7 days with GW347845, and lipid accumulation in cells was assessed by Oil Red O staining. A dramatic increase in lipid droplet staining in the cytoplasm was observed after treatment with AJA or GW347845 (Fig. 7A), suggesting that both AJA and GW347845 can induce differentiation of 3T3 L1 fibroblasts into adipocytes.

To confirm that AJA and GW347845 induce 3T3 L1 cell differentiation into adipocytes, we measured expression levels of the adipocyte-specific genes PPARγ2 and aP2 by RT-PCR. Total RNA was isolated after AJA or GW347845 treatment and compared with vehicle control. Reverse transcription was conducted and the relative amounts of PPARγ2 and aP2 transcripts were measured by PCR reactions using primer sets specific to each gene. Both AJA and GW347845 enhanced PPARγ2 and aP2 gene expression significantly in comparison with vehicle control treatment (Fig. 7B). As a control for the induction specificity and the PCR amplification reaction, the expression levels of GAPDH were measured in all samples and found to be unaffected by any treatment. These data indicate that AJA can induce differentiation of 3T3 L1 fibroblasts into adipocytes, further demonstrating that AJA is an activating ligand for PPARγ.

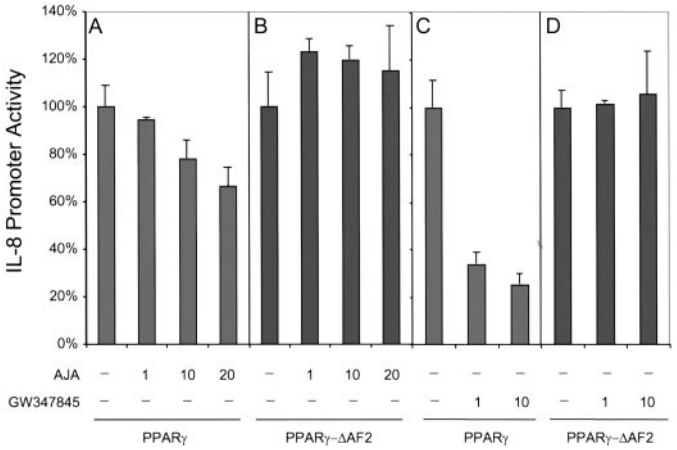


Fig. 6. Down-regulation of IL-8 promoter activity by AJA. The IL-8 luciferase reporter controlled by the human IL-8 promoter (−97/−69) was cotransfected with mPPARγ or mPPARγ ΔAF2 mutant into HeLa cells. After transfection, cells were treated with AJA, GW347845, or solvent after PMA treatment for 24 h. PMA was able to induce IL-8 promoter activity 3-fold above untreated cells, which was set as 100% IL-8 promoter activity. A, in PPARγ -transfected cells, AJA reduces the PMA-activated IL-8 promoter activity in a concentration (micromolar)-dependent manner. B, AJA had no effect on IL-8 promoter activity in the PPARγ ΔAF2-transfected cells, suggesting that the inhibition of IL-8 promoter activity by AJA depends on the transcriptional activity of PPARγ. C, GW347845 reduces the IL-8 promoter activity in cells transfected with wild-type PPARγ. D, in cells transfected with the PPARγ ΔAF2 mutant, GW347845 had no effect on IL-8 promotor activity.

Discussion

In the present study, we investigated the molecular basis for the therapeutic action of AJA, a promising agent for relieving pain and inflammation. We find that AJA binds selectively to PPARγ in vitro and AJA activates the transcriptional activity of PPARγ in vivo. Activation of PPARγ by AJA depends on the presence of the AF-2 helix in the receptor. AJA binding enables PPARγ to recruit nuclear receptor coactivators. In addition, AJA inhibits IL-8 promoter activity in a PPARγ-dependent manner and induces differentiation of 3T3 L1 fibroblasts into adipocytes. Our data suggest that AJA may exert its therapeutic actions at least in part through transcriptional activation of PPARγ.

The structural and functional similarity between AJA and several known PPARγ ligands led us to consider AJA as a potential PPARγ ligand. Indeed, in a partial proteinase digestion assay, AJA effectively protects PPARγ from proteinase digestion (Fig. 2), reflecting direct binding of AJA to PPARγ. The two trypsin resistant fragments probably contain the LBD of PPARγ, because ligand binding induces a compact conformation of the LBD (Xu et al., 2001), which is expected to be more resistant to proteinase digestion compared with the unliganded receptor. Because AJA causes proteinase resistance only to PPARγ, but not PPARα or PPARδ, it is clear that AJA binds selectively to PPARγ. Indeed, the three PPARs have distinct ligand binding specificities (Xu et al., 2001) and physiological functions (Berger and Moller, 2002). The selective binding of AJA to PPARγ suggests that PPARγ may mediate the therapeutic activity of AJA.

Consistent with the binding of AJA to PPARγ, the transient reporter gene assay further demonstrates that AJA activates the target promoter through PPARγ but not PPARα or PPARδ (Fig. 3). These data indicate that AJA is a PPARγ-specific agonist, a conclusion that is further supported by the inability of AJA to activate the PPARγ ΔAF2 mutant, which is defective in ligand-dependent transcriptional activation (Fig. 3E). The observation that PPARγ activation by AJA requires the AF-2 function is consistent with the hypothesis that AJA binds to PPARγ LBD and activates its ligand-dependent transcriptional function. By using the Gal4-DBD fusion protein system, the EC_{50} of AJA for PPARγ activation was measured at 13 μM (Fig. 4), a concentration that is within the pharmacologically effective doses of AJA (Burstein, 2000). Furthermore, AJA also activates human PPARγ equally well compared with mouse PPARγ, implying that the PPARγ-dependent therapeutic activity of AJA observed in mice might be applicable to humans.

In line with the hypothesis that AJA is an agonist for PPARγ, binding of AJA enables PPARγ to recruit nuclear receptor coactivators (Fig. 5). Nuclear receptor coactivators are known to interact with liganded receptors to facilitate transcriptional activation of target promoters by recruiting histone acetyltransferase activity to the receptor (Leo and

Chen, 2000). The interaction of PPARγ with DRIP205 in response to AJA treatment seems to be more prominent than the interaction with RAC3. This is consistent with a report showing that DRIP205 is a potent coactivator for PPARγ (Yang et al., 2000). AJA may induce formation of a coactivator-binding surface to allow docking of the coactivator LXXLL motif. Furthermore, addition of AJA causes differentiation of 3T3 L1 fibroblasts into adipocytes (Fig. 7), a process that is mediated by PPARγ. Together, these data provide strong evidence to suggest that AJA is an agonist of PPARγ. These data also implicate a potential involvement of PPARγ in the signaling of this cannabinoid class of analgesic and anti-inflammatory drugs.

It is likely that activation of PPARγ is at least partly responsible for the anti-inflammatory action of AJA and perhaps other cannabinoids as well. In fact, several natural cannabinoids, such as THC and cannabidiol, also activate PPARγ (J. Liu and J. D. Chen, unpublished data). The involvement of PPARγ in AJA-mediated anti-inflammatory activity is further supported by the observation that AJA inhibits IL-8 promoter activity in a PPARγ-dependent manner (Fig. 6). This inhibition occurs only in the presence of wild-type PPARγ, not the ΔAF-2 mutant, suggesting that transcriptional activation by PPARγ is required for the repression of IL-8 promoter activity by AJA. It is not clear whether inhibition of cytokine promoter activity by PPARγ is caused by direct binding of PPARγ to the promoter. Because there is no evidence for direct binding of PPARγ to the cytokine promoter, this inhibition may be indirect. Treatment of peritoneal macrophages with several PPARγ ligands, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 also suppresses expression of the inducible nitric-oxide synthase, gelatinase B, and scavenger receptor A in response to phorbol ester stimulation. The promoters of these genes were found to possess binding sites for activator protein-1, nuclear factor κB, and the signal transducer and activator of transcription. Furthermore, the inhibition of inflammatory response by PPARγ ligands in macrophages was produced in part by antagonizing the activities of these transcription factors (Ricote et al., 1998). Although 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was shown in some studies to be able to mediate an anti-inflammatory action in a PPARγ-independent manner (Straus et al., 2000; Tsubouchi et al., 2001), the inhibition of inflammatory responses by activation of PPARγ was confirmed by several recent studies both in vitro (Ji et al., 2001) and in vivo (Dubuquoy et al., 2000; Kawahito et al., 2000; Naito et al., 2001). It is interesting to note that, like the activation of PPARγ, the immunosuppressive functions of some cannabinoids were also reported to be exerted by the inhibition of activator protein-1, nuclear factor κB, and the signal transducer and activator of transcription (Jeon et al., 1996; Zheng and Specter, 1996; Faubert and Kaminski, 2000).

The complete mechanism of the anti-inflammatory action of AJA is likely to involve a complicated signaling network. PPARγ is highly expressed in several immune cells, such as macrophages and monocytes. Therefore, AJA may activate PPARγ in these cells to modulate immune inflammatory responses. Further studies are needed to investigate whether

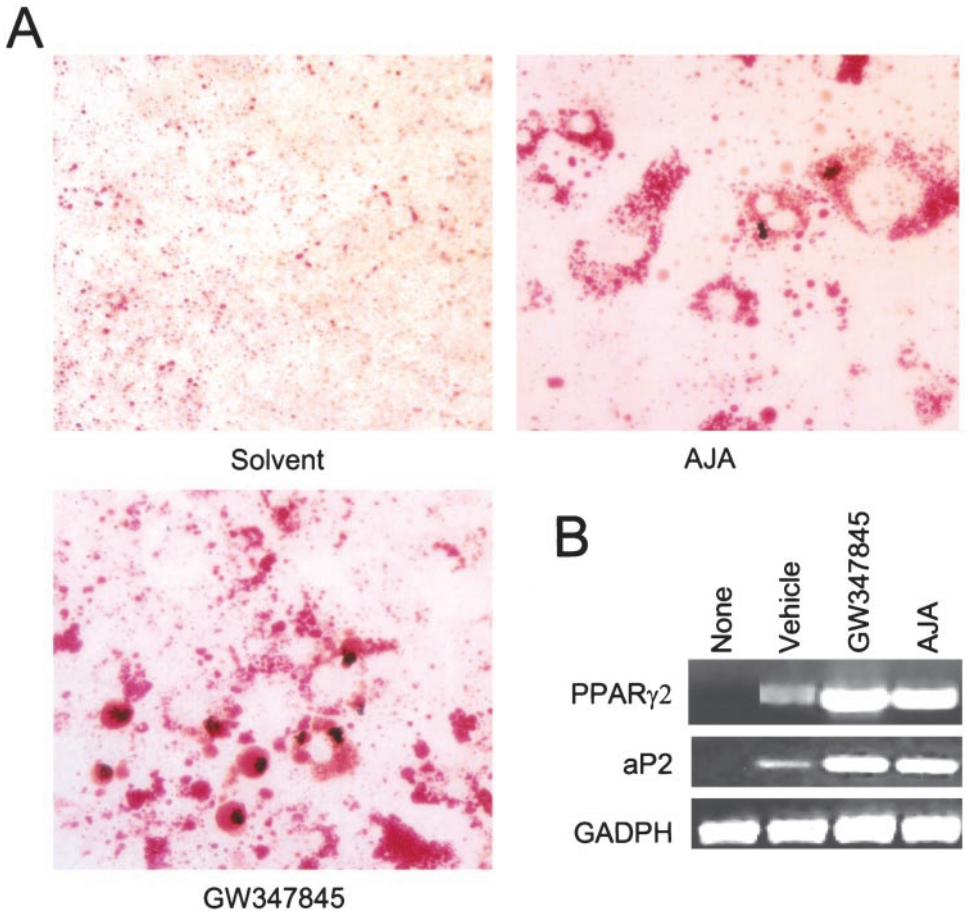


Fig. 7. Induction of adipocyte differentiation by AJA. 3T3 L1 fibroblasts were cultured in DMEM supplemented with 10% calf serum. Two days after confluence, cells were treated with 0.1% DMSO, 20 μM AJA, or 1 μM GW347845 in the presence of 10 μg/ml of insulin. A, Oil Red O staining for detection of adipocyte differentiation. After 7 days of treatment with GW347845 and 10 days of treatment with AJA, cells were fixed and stained with Oil Red O. Red staining indicates lipid droplets in the cytoplasm. B, RT-PCR analysis of adipocyte specific genes. PCR products were analyzed on 1% agarose gel and stained with ethidium bromide. PPARγ2 and aP2 were induced significantly after treatment with 1 μM GW347845 or 20 μM AJA compared with vehicle-treated cells. The housekeeping gene GAPDH expressed equally in all samples.

other cannabinoids are capable of activating PPARγ and whether other cytokines or chemokines are inhibited by AJA. Because PPARγ is involved in several physiological processes including lipid metabolism, glucose homeostasis, and adipocyte differentiation, the discovery that AJA is an active ligand for PPARγ suggests that AJA may have a broader range of pharmacological activities than previously expected. Activation of PPARγ by several synthetic and natural compounds is known to correlate well with the ability of these compounds to control glucose concentrations. Therefore, it may be interesting to test in the future whether AJA is a useful agent for treatment of diabetes mellitus.

Acknowledgments

We are very grateful to Timothy Willson and Steve Kliewer for providing the PPAR ligands and expression vectors. We thank Amy (Hong-Bing) Chen for technical support and the other Chen Laboratory members for helpful discussion during the course of this work.

References

Allan GF, Leng X, Tsai SY, Weigel NL, Edwards DP, Tsai MJ, and O'Malley BW (1992) Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation. *J Biol Chem* **267:**19513–19520.

Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, and Evans RM (1999) PPAR gamma is required for placental, cardiac and adipose tissue development. *Mol Cell* **4:**585–595.

Berger J and Moller DE (2002) The mechanisms of action of PPARs. *Annu Rev Med* **53:**409–435.

Burstein SH (2000) Ajulemic acid (CT3): a potent analog of the acid metabolites of THC. *Curr Pharm Des* **6:**1339–1345.

Burstein SH, Audette CA, Breuer A, Devane WA, Colodner S, Doyle SA, and Mechoulam R (1992) Synthetic nonpsychotropic cannabinoids with potent anti-inflammatory, analgesic and leukocyte antiadhesion activities. *J Med Chem* **35:** 3135–3141.

Burstein SH, Friderichs E, Kogel B, Schneider J, and Selve N (1998) Analgesic effects of 1′,1′ dimethylheptyl-delta8-THC-11-oic acid (CT3) in mice. *Life Sci* **63:**161–168.

Chawla A and Lazar MA (1994) Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. *Proc Natl Acad Sci USA* **91:**1786–1790.

Clark RB, Bishop-Bailey D, Estrada-Hernandez T, Hla T, Puddington L, and Padula SJ (2000) The nuclear receptor PPAR gamma and immunoregulation: PPAR gamma mediates inhibition of helper T cell responses. *J Immunol* **164:**1364–1371.

Cobb JE, Blanchard SG, Boswell EG, Brown KK, Charifson PS, Cooper JP, Collins JL, Dezube M, Henke BR, Hull-Ryde EA, et al. (1998) *N*-(2-Benzoylphenyl)-L-tyrosine PPARgamma agonists. 3. Structure-activity relationship and optimization of the *N*-aryl substituent. *J Med Chem* **41:**5055–5069.

Dajani EZ, Larsen KR, Taylor J, Dajani NE, Shahwan TG, Neeleman SD, Taylor MS, Dayton MT, and Mir GN (1999) 1′,1′-Dimethylheptyl-Δ-8-tetrahydrocannabinol-11-oic acid: a novel, orally effective cannabinoid with analgesic and anti-inflammatory properties. *J Pharmacol Exp Ther* **291:**31–38.

Dubuquoy L, Bourdon C, Peuchmaur M, Leibowitz MD, Nutten S, Colombel JF, Auwerx J, and Desreumaux P (2000) Peroxisome proliferator-activated receptor (PPAR) gamma: a new target for the treatment of inflammatory bowel disease. *Gastroenterol Clin Biol* **24:**719–724.

Faubert BL and Kaminski NE (2000) AP-1 activity is negatively regulated by cannabinol through inhibition of its protein components, c-fos and c-jun. *J Leukoc Biol* **67:**259–266.

Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, and Evans RM (1995) 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* **83:**803–812.

Houseknecht KL, Cole BM, and Steele PJ (2002) Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: a review. *Domest Anim Endocrinol* **22:**1–23.

Hu E, Tontonoz P, and Spiegelman BM (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc Natl Acad Sci USA* **92:**9856–9860.

Jeon YJ, Yang KH, Pulaski JT, and Kaminski NE (1996) Attenuation of inducible nitric oxide synthase gene expression by Δ^9-tetrahydrocannabinol is mediated through the inhibition of nuclear factor-κB/Rel activation. *Mol Pharmacol* **50:**334–41.

Ji JD, Cheon H, Jun JB, Choi SJ, Kim YR, Lee YH, Kim TH, Chae IJ, Song GG, Yoo DH, et al. (2001) Effects of peroxisome proliferator-activated receptor-gamma (PPAR-gamma) on the expression of inflammatory cytokines and apoptosis induction in rheumatoid synovial fibroblasts and monocytes. *J Autoimmun* **17:**215–221.

Jiang C, Ting AT, and Seed B (1998) PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature (Lond)* **391:**82–86.

Kawahito Y, Kondo M, Tsubouchi Y, Hashiramoto A, Bishop-Bailey D, Inoue K, Kohno M, Yamada R, Hla T, and Sano H (2000) 15-deoxy-$\Delta^{12,14}$-PGJ_2 induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. *J Clin Investig* **106:**189–197.

Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, and Evans RM (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA* **91:**7355–7359.

Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, and Lehmann JM (1995) A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* **83:**813–819.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, et al. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. *Proc Natl Acad Sci USA* **94:**4318–4323.

Leblanc BP and Stunnenberg HG (1995) 9-cis retinoic acid signaling: changing partners causes some excitement. *Genes Dev* **9:**1811–1816.

Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, and Kliewer SA (1997) Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* **272:**3406–3410.

Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, and Kliewer SA (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *J Biol Chem* **270:**12953–12956.

Leng X, Tsai SY, O'Malley BW, and Tsai MJ (1993) Ligand-dependent conformational changes in thyroid hormone and retinoic acid receptors are potentially enhanced by heterodimerization with retinoic X receptor. *J Steroid Biochem Mol Biol* **46:**643–661.

Leo C and Chen JD (2000) The SRC family of nuclear receptor coactivators. *Gene* **245:**1–11.

Li H, Gomes PJ, and Chen JD (1997) RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci USA* **94:**8479–8484.

Mukherjee R, Jow L, Croston GE, and Paterniti JR Jr (1997) Identification, characterization and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARγ2 versus PPARγ1 and activation with retinoid X receptor agonists and antagonists. *J Biol Chem* **272:**8071–8076.

Mukherjee R, Hoener PA, Jow L, Bilakovics J, Klausing K, Mais DE, Faulkner A, Croston GE, and Paterniti JR Jr (2000) A selective peroxisome proliferator-activated receptor-gamma (PPARgamma) modulator blocks adipocyte differentiation but stimulates glucose uptake in 3T3–L1 adipocytes. *Mol Endocrinol* **14:**1425–1433.

Nagy L, Tontonoz P, Alvarez JG, Chen H, and Evans RM (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* **93:**229–240.

Naito Y, Takagi T, Matsuyama K, Yoshida N, and Yoshikawa T (2001) Pioglitazone, a specific PPAR-gamma ligand, inhibits aspirin-induced gastric mucosal injury in rats. *Aliment Pharmacol Ther* **15:**865–873.

Patel L, Pass I, Coxon P, Downes CP, Smith SA, and Macphee CH (2001) Tumor suppressor and anti-inflammatory actions of PPARgamma agonists are mediated via upregulation of PTEN. *Curr Biol* **11:**764–768.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74:**129–180.

Ricote M, Li AC, Willson TM, Kelly CJ, and Glass CK (1998) The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature (Lond)* **391:**79–82.

Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, and Spiegelman BM (2002) C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev* **16:**22–26.

Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, et al. (1998) Differentiation and reversal of malignant changes in colon cancer through PPARgamma. *Nat Med* **4:**1046–1052.

Simonin MA, Bordji K, Boyault S, Bianchi A, Gouze E, Becuwe P, Dauca M, Netter P, and Terlain B (2002) PPAR-gamma ligands modulate effects of LPS in stimulated rat synovial fibroblasts. *Am J Physiol* **282:**C125–C133.

Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, and Glass CK (2000) 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proc Natl Acad Sci USA* **97:**4844–4849.

Suh N, Wang Y, Williams CR, Risingsong R, Gilmer T, Willson TM, and Sporn MB (1999) A new ligand for the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), GW7845, inhibits rat mammary carcinogenesis. *Cancer Res* **59:** 5671–5673.

Tontonoz P, Hu E, and Spiegelman BM (1994) Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* **79:**1147–1156.

Tsubouchi Y, Kawahito Y, Kohno M, Inoue K, Hla T, and Sano H (2001) Feedback control of the arachidonate cascade in rheumatoid synoviocytes by 15-deoxy-Delta^{12,14}-prostaglandin J2. *Biochem Biophys Res Commun* **283:**750–755.

Umesono K, Murakami KK, Thompson CC, and Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone induced gene expression through a common responsive element. *Cell* **65:**1255–1266.

Uppenberg J, Svensson C, Jaki M, Bertilsson G, Jendeberg L, and Berkenstam A (1998) Crystal structure of the ligand binding domain of the human nuclear receptor PPARgamma. *J Biol Chem* **273:**31108–31112.

Willson TM, Cobb JE, Cowan DJ, Wiethe RW, Correa ID, Prakash SR, Beck KD, Moore LB, Kliewer SA, and Lehmann JM (1996) The structure-activity relationship between peroxisome proliferator-activated receptor gamma agonism and the antihyperglycemic activity of thiazolidinediones. *J Med Chem* **39:**665–668.

Xu HE, Lambert MH, Montana VG, Plunket KD, Moore LB, Collins JL, Oplinger JA, Kliewer SA, Gampe RT Jr, McKee DD, et al. (2001) Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA* **98:**13919–13924.

Yang W, Rachez C, and Freedman LP (2000) Discrete roles for peroxisome proliferator-activated receptor gamma and retinoid X receptor in recruiting nuclear receptor coactivators. *Mol Cell Biol* **20:**8008–8017.
Zheng ZM and Specter S (1996) Delta-9-tetrahydrocannabinol: an inhibitor of STAT1 alpha protein tyrosine phosphorylation. *Biochem Pharmacol* **51:**967–73.
Zhu Y, Qi C, Jain S, Rao MS, and Reddy JK (1997) Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. *J Biol Chem* **272:**25500–25506.
Zurier RB, Rossetti RG, Lane JH, Goldberg JM, Hunter SA, and Burstein SH (1998) Dimethylheptyl-THC-11 oic acid: a nonpsychoactive anti-inflammatory agent with a cannabinoid template structure. *Arthritis Rheum* **41:**163–170.

Address correspondence to: J. Don Chen, Ph.D., Department of Pharmacology, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, 675 Hoes Lane, Room 414, Piscataway, NJ 08854. E-mail: chenjd@umdnj.edu