

Interleukin-2 Suppression by 2-Arachidonyl Glycerol Is Mediated through Peroxisome Proliferator-Activated Receptor γ Independently of Cannabinoid Receptors 1 and 2

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ABSTRACT

2-Arachidonyl glycerol (2-AG) is an endogenous arachidonic acid derivative that binds cannabinoid receptors CB1 and CB2 and is hence termed an endocannabinoid. 2-AG also modulates a variety of immunological responses, including expression of the autocrine/paracrine T cell growth factor interleukin (IL)-2. The objective of the present studies was to determine the mechanism responsible for IL-2 suppression by 2-AG. Because of the labile properties of 2-AG, 2-AG ether, a nonhydrolyzable analog of 2-AG, was also used. Both 2-AG and 2-AG ether suppressed IL-2 expression independently of CB1 and CB2, as demonstrated in leukocytes derived from CB1/CB2-null mice. Moreover, we demonstrated that both 2-AG and 2-AG ether treatment activated peroxisome proliferator-activated receptor γ (PPAR γ), as evidenced by forced differentiation of 3T3-L1 cells into adipocytes, induction of aP2 mRNA levels, and activation of a PPAR γ -specific luciferase reporter in transiently transfected 3T3-L1 cells. Consequently, the putative role of PPAR γ in IL-2 suppression by 2-AG and 2-AG ether was examined in Jurkat T cells. Concordant with PPAR γ involvement, the PPAR γ -specific antagonist 2-chloro-5-nitro-*N*-(4-pyridyl)-benzamide (T0070907) blocked 2-AG- and 2-AG ether-mediated IL-2 suppression. Likewise, 2-AG suppressed the transcriptional activity of two transcription factors crucial for IL-2 expression, nuclear factor of activated T cells and nuclear factor κ B, in the absence but not in the presence of T0070907. 2-AG treatment also induced PPAR γ binding to a PPAR response element in activated Jurkat T cells. Together, the aforementioned studies identify PPAR γ as a novel intracellular target of 2-AG, which mediates the suppression of IL-2 by 2-AG in a manner that is independent of CB1 and/or CB2.

2-Arachidonyl glycerol (2-AG) is an endogenous arachidonic acid derivative that binds cannabinoid receptors CB1 and CB2 and is hence termed an endocannabinoid. 2-AG also modulates a variety of immunological responses, including expression of the autocrine/paracrine T cell growth factor interleukin (IL)-2. The objective of the present studies was to determine the mechanism responsible for IL-2 suppression by 2-AG. Because of the labile properties of 2-AG, 2-AG ether, a nonhydrolyzable analog of 2-AG, was also used. Both 2-AG and 2-AG ether suppressed IL-2 expression independently of CB1 and CB2, as demonstrated in leukocytes derived from CB1/CB2-null mice. Moreover, we demonstrated that both 2-AG and 2-AG ether treatment activated peroxisome proliferator-activated receptor γ (PPAR γ), as evidenced by forced differentiation of 3T3-L1 cells into adipocytes, induction of aP2 mRNA levels, and activation of a PPAR γ -specific luciferase reporter in transiently transfected 3T3-L1 cells. Consequently, the putative role of PPAR γ in IL-2 suppression by 2-AG and 2-AG ether was examined in Jurkat T cells. Concordant with PPAR γ involvement, the PPAR γ -specific antagonist 2-chloro-5-nitro-*N*-(4-pyridyl)-benzamide (T0070907) blocked 2-AG- and 2-AG ether-mediated IL-2 suppression. Likewise, 2-AG suppressed the transcriptional activity of two transcription factors crucial for IL-2 expression, nuclear factor of activated T cells and nuclear factor κ B, in the absence but not in the presence of T0070907. 2-AG treatment also induced PPAR γ binding to a PPAR response element in activated Jurkat T cells. Together, the aforementioned studies identify PPAR γ as a novel intracellular target of 2-AG, which mediates the suppression of IL-2 by 2-AG in a manner that is independent of CB1 and/or CB2.

The cannabinoid receptors CB1 and CB2 belong to the G-protein-coupled receptor superfamily (Matsuda et al., 1990; Munro et al., 1993). Although both receptor subtypes are expressed in a variety of different tissues, the levels of expression of CB1 and CB2 can vary greatly between cell types. In immune cells, CB2 expression is markedly higher than that of CB1, as determined by mRNA levels (Kaminski et al., 1992; Munro et

al., 1993; Berdyshev, 2000). The cannabinoid receptors, in particular CB2, have been implicated in the modulation of a number of different immunological responses, including inhibition of anti-tumor immunity in mice, enhancement of B cell proliferation, and inhibition of antigen processing by macrophages (Berdyshev, 2000; Zhu et al., 2000).

The endogenous arachidonic acid derivative 2-AG is a high-affinity agonist for CB1 and CB2, hence termed an endocannabinoid (Mechoulam et al., 1995). In addition to a variety of other tissues, 2-AG has been detected in a number of immune cell types, including macrophages, and lymphocytes (Berdyshev et al., 2001; Maccarrone et al., 2002). 2-AG ether, which

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ABBREVIATIONS: 2-AG, 2-arachidonyl glycerol; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPARE, peroxisome proliferator-activated receptor response element; NFAT, nuclear factor of activated T cells; AP-1, activator protein 1; NF κ B, nuclear factor κ B; T0070907, 2-chloro-5-nitro-*N*-(4-pyridinyl)-benzamide.; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*H*-pyrazole-3-carboxamidehydrochloride; SR144528, *N*-[(1*S*)-endo-1,3,3-trimethyl-bicyclo[2,2,1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; 15-deoxy-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; BCS, bovine calf serum; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; aP2, adipocyte fatty acid-binding protein; IFN, interferon; PCR, polymerase chain reaction; VH, vehicle; PMA, phorbol 12-myristate 13-acetate; COX, cyclooxygenase; DMSO, dimethyl sulfoxide.

is nonhydrolyzable and therefore metabolically more stable than 2-AG, was originally synthesized as an ether-linked analog of 2-AG (Fig. 1). Although 2-AG has relatively equal affinity for both CB1 and CB2, 2-AG ether binds to CB1 with a much higher affinity than to CB2 (Hanus et al., 2001).

Within the immune system, 2-AG has been reported to have a number of effects, including calcium influx in HL-60 cells, enhancement of antibody formation in murine splenocytes, and induction of the migration of human peripheral blood monocytes and HL-60 cells (Lee et al., 1995; Sugiura et al., 2000; Kishimoto et al., 2003). 2-AG has also been shown to inhibit cytokine production, including tumor necrosis factor α release from both lipopolysaccharide-treated rat microglial cells as well as murine macrophages, IL-6 production in J774 macrophages, and IL-2 secretion in activated murine splenocytes (Ouyang et al., 1998; Gallily et al., 2000; Chang et al., 2001; Facchinetti et al., 2003). Although CB2 has been implicated in many 2-AG-mediated immune effects, the role of CB2 and/or CB1 in the immunosuppressive effects of 2-AG upon cytokine release has yet to be conclusively determined.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors, to which the steroid hormone receptors also belong. Like many other nuclear receptors, PPAR exists as a heterodimer with the retinoid X receptor (RXR). In the resting state, PPAR/RXR associates with corepressors. Upon ligand activation, the corepressors dissociate from PPAR/RXR and the heterodimer then binds to PPAR response elements (PPREs), which are located in the regulatory regions of various target genes (Daynes and Jones, 2002). The expression of the target genes is thus regulated by the PPAR/RXR complex, either alone or in conjunction with other transcription factors. In addition to transactivation, PPARs have also been found to produce transrepression through physical association with, and the subsequent inhibition of, transcription factors, such as nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), and nuclear factor of the κ -enhancer in B cells (NF κ B) (Ricote et al., 1998; Yang et al., 2000). Although all three PPAR subtypes, α , δ , and γ , have been detected in a number of different immune cell types, PPAR γ in particular has been reported to produce a variety of different immune effects. In T cells, PPAR γ has been found to inhibit cytokine production, suppress proliferation, and, in some instances, to either induce or prevent apoptosis (Ricote et al., 1998; Clark et al., 2000; Wang et al., 2002).

The overall objective of the present studies was to rigorously examine the mechanism for IL-2 suppression by 2-AG in activated T cells. IL-2 is an autocrine/paracrine factor secreted by activated T cells and is important for T cell survival, proliferation, and in some cases, differentiation. As such, IL-2 is critically involved in the development of an adaptive immune response. IL-2 is also a hallmark of T cell activation in that it exhibits virtually no basal level expression, but is rapidly secreted upon T cell activation. The current studies rule out the involvement of CB1 and CB2 in the

suppression of IL-2 by 2-AG but also suggest an obligatory role by PPAR γ . In addition, these studies demonstrate that 2-AG and 2-AG ether treatment impairs the transcriptional activity of two transcription factors crucial for IL-2 expression, NFAT and NF κ B, in the absence but not in the presence of the PPAR γ antagonist T0070907. The current findings are significant in that they identify a novel pathway by which 2-AG can exert biological activity independent of CB1 and/or CB2 involvement.

Materials and Methods

Reagents. 2-AG, SR141716A, and SR144528 were provided by the National Institute on Drug Abuse. 2-AG ether, ciglitazone, 15-deoxy-PGJ₂, and T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Animals and Cell Culture. CB1/CB2 receptor double knockout (CB1^{-/-}/CB2^{-/-}) mice were developed in C57BL/6J mice as described previously (Jarai et al., 1999). Female C57BL/6J and B6C3F1 mice, 6 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). Spleens were isolated aseptically and made into single-cell suspensions (1×10^6 c/ml). Cells were cultured in RPMI 1640 medium supplemented with 100 units penicillin/ml, 100 units streptomycin/ml, 50 μ M 2-mercaptoethanol, and 2% bovine calf serum (BCS). Jurkat E6-1 and 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA). Jurkat T cells were cultured in RPMI 1640 medium supplemented with 100 units/ml streptomycin, 100 units/ml penicillin, 10 mM nonessential amino acids, 100 mM sodium pyruvate, and 10% bovine calf serum. 3T3-L1 murine fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 100 units/ml streptomycin, 100 units/ml penicillin, 10 mM nonessential amino acids, 100 mM sodium pyruvate, and 10% bovine calf serum.

IL-2 ELISA. Splenocytes were cultured in triplicate (1×10^6 c/ml) in 48-well culture plates (800 μ l/well). The supernatants were collected 24 h after stimulation, and IL-2 protein was quantified by a sandwich ELISA method as described previously (Faubert Kaplan et al., 2003). The IL-2 standard (mouse or human recombinant IL-2), purified rat anti-mouse or mouse anti-human IL-2 antibody, and biotinylated anti-mouse or anti-human IL-2 antibody were purchased from BD Pharmingen (San Diego, CA).

3T3-L1 Differentiation Assay and Oil Red O Staining. 3T3-L1 cells, grown to confluence, were cultured in media alone or in the presence of either 2-AG, 2-AG ether, ciglitazone, or vehicle (0.1% ethanol) for 7 days. The cells were then washed in PBS, fixed in 10% formalin, stained with Oil Red O (0.2% in 60% isopropanol) for 10 min, rinsed in 60% isopropanol, washed in tap water for 1 to 2 min, counterstained with 6% hematoxylin for 30 s, and washed in PBS. The number of differentiated cells was quantified by enumerating the total number of differentiated cells per well.

Real-Time PCR. Total RNA was isolated from 3T3-L1 cells or splenocytes using TRI reagent (Sigma, St. Louis, MO), according to the manufacturer's protocol. The relative expression levels of aP2, IL-4, and IFN γ , were determined by TaqMan one-step real-time multiplex PCR (Applied Biosystems, Foster City, CA). Relative mRNA expression for aP2, IL-4, and IFN γ was calculated as described previously and normalized to the endogenous reference, 18S ribosomal RNA (Farraj et al., 2004). Primers and probe for aP2 were

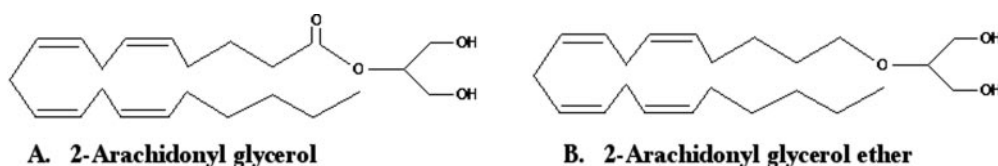


Fig. 1. The chemical structures of 2-AG and 2-AG ether.

designed to exclude the detection of genomic DNA using PrimerExpress software (Applied Biosystems), were synthesized by Applied Biosystems, and are as follows: forward primer, 5'-AAGTGGGAGTGGGCTTTGC-3'; reverse primer, 5'-TCCCCATTTACGCTGATGATC-3'; and probe, 5'-CAGGCATGGCCAAGCCCACC-3'. The probe was labeled with 6-carboxyfluorescein dye on the 5' end and 5-carboxytetramethylrhodamine quencher on the 3' end. TaqMan predeveloped primers and probe were used for the detection of IL-4, IFN γ , and 18S ribosomal RNA.

Transient Transfection Assay. 3T3-L1 cells (1.38×10^6) were cultured in growth medium for 16 to 20 h. The cells were then incubated with the transfection reagents [37 μ g of plasmid and 115 μ l of Lipofectamine 2000 (Invitrogen, Carlsbad, CA)] for 5 h in serum-free media. The transfected cells were then trypsinized, washed, resuspended in growth media, and plated (3×10^4 cells per well in a 24-well plate). After another 5-h incubation, the cells were cultured in the presence of either 2-AG, 2-AG ether, ciglitazone, or vehicle (0.1% ethanol). Treatments were performed in triplicate.

Jurkat T cells (5×10^5 c/ml) were incubated with transfection reagents (1.5 μ g of plasmid and 3 μ l of Lipofectamine 2000 for every 5×10^5 cells) for 12 h in RPMI 1640 medium with 2% BCS. The transfected cells were washed, resuspended in RPMI 1640 medium with 2% BCS, and treated with 2-AG or vehicle (VH) in the presence or absence of T0070907.

After a 16-h (3T3-L1 cells) or 12-h (Jurkat cells) incubation, luciferase activity was assayed using a commercially available kit according to the manufacturer's protocol (Promega, Madison, WI).

Plasmids. The trans-acting PPAR γ reporter gene plasmid was constructed by fusing the ligand-binding domain of mouse PPAR γ to the DNA binding domain of the yeast transcription factor, Gal4, under the control of the simian virus 40 promoter as described previously (Bility et al., 2004). The plasmid also encoded the UAS-firefly luciferase reporter under the control of the Gal4 DNA response element.

NFAT-luc, NF κ B-luc, AP1-luc, and pTA-luc reporter gene plasmids are commercially available from Clontech (Mountain View, CA). The pTA-luc plasmid served as a negative control and is identical to the NFAT-luc, NF κ B-luc, and AP1-luc plasmids, except for the absence of the NFAT, NF κ B, and AP-1 response elements.

Nuclear Protein Isolation. Jurkat T cells were cultured in triplicate (5×10^5 c/ml) in 60-mm culture dishes (10 ml/plate). The cells were treated with 2-AG or VH (0.1% ethanol), for 30 min before treatment with 40 nM PMA and 0.5 μ M ionomycin. The cells were then incubated for 2.5 h at 37°C. The nuclear proteins were extracted using a commercially available kit (Panomics, Redwood City, CA), according to the manufacturer's protocol. Protein concentrations were determined using the method of Bradford (1976).

ELISA-Based DNA/Protein Binding Assay. PPAR γ /PPRE binding was quantified using the Transbinding PPAR γ assay kit from Panomics according to the manufacturer's protocol. The nonradioactive probe (provided by the manufacturer) is an unlabeled PPAR consensus oligonucleotide that competes with the biotinylated PPAR probe to confirm specificity. The recombinant PPAR γ protein served as a positive control for the assay.

Calculation of IC₅₀ Values. IC₅₀ values were calculated using Prism (ver. 4.03 for Windows; GraphPad Software, San Diego, CA).

Statistical Analysis. The mean \pm S.E. was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by one- or two-way parametric analysis of variance. Dunnett's two-tailed *t* test was used to compare treatment groups to the VH control when significant differences were observed, using SigmaStat software from SPSS Inc. (Chicago, IL).

Results

2-AG and 2-AG Ether Suppress IL-2 Secretion in Activated Splenocytes Independently of CB1 and CB2. We have shown previously that 2-AG suppresses the produc-

tion of IL-2 by T cells in a concentration-dependent manner (Ouyang et al., 1998). To ascertain whether IL-2 suppression is mediated by 2-AG or a hydrolysis product of 2-AG, a nonhydrolyzable form of 2-AG, 2-AG ether, was employed. 2-AG ether treatment markedly inhibited IL-2 secretion in a concentration-dependent manner as observed with 2-AG, demonstrating that 2-AG hydrolysis is not required for IL-2 suppression (Figs. 2 and 3). The involvement of CB1 and/or CB2 in IL-2 suppression by 2-AG and 2-AG ether was initially assessed through the use of the CB1 and CB2 antagonists SR141716A and SR144528. Pretreatment of splenocytes with SR141716A and SR144528, in combination, did not attenuate the suppression of PMA plus ionomycin-induced IL-2 production by 2-AG, suggesting the absence of CB1 and CB2 involvement (Fig. 2). At the highest concentration at which SR141716A and SR144528 were used (5 μ M), antagonist treatment alone induced suppression of IL-2. Concordant with the above cannabinoid receptor antagonist studies, both 2-AG and 2-AG ether also induced suppression of IL-2 in splenocytes from CB1/CB2-null mice thus confirming the absence of a role by CB1 and/or CB2 (IC₅₀ values: 2-AG, 6.9 and 7.3 μ M, and 2-AG ether, 7.7 and 6.2 μ M, for wild-type and CB1/CB2-null splenocytes, respectively) (Fig. 3).

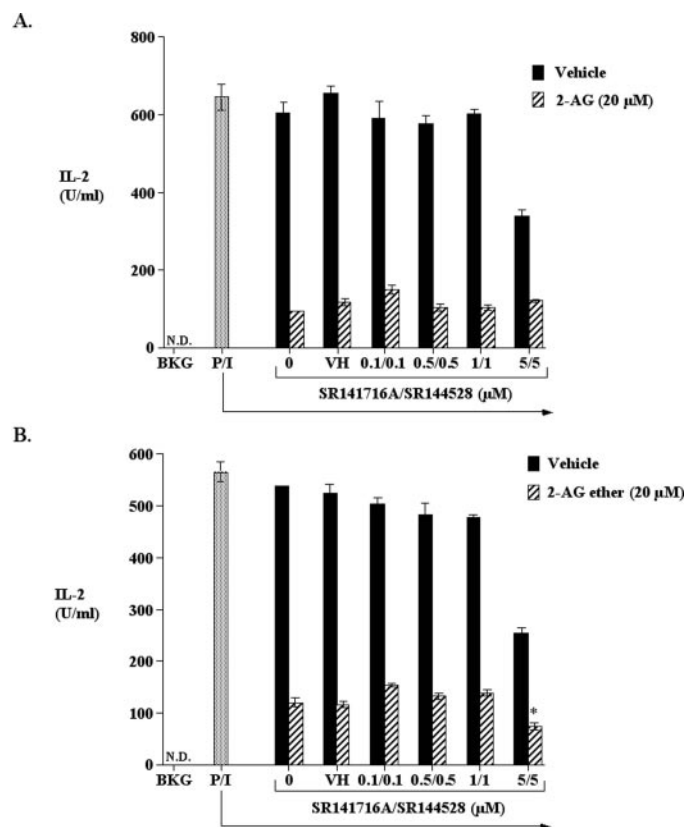


Fig. 2. Effect of cannabinoid receptor antagonists on inhibition of interleukin-2 secretion by 2-AG and 2-AG ether. Splenocytes (1×10^6 cells/ml) were pretreated with both SR141716A and SR144528 or VH (0.1% DMSO) for 30 min followed by treatment with either 2-AG (20 μ M; A) or 2-AG ether (20 μ M; B) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μ M ionomycin for 24 h. The supernatants were harvested, and IL-2 production was measured by ELISA analysis. Cellular viability was $\geq 85\%$ for all treatment groups as assessed by trypan blue exclusion. The results are the mean \pm standard error of triplicate cultures. *, *p* < 0.05 compared with VH + 2-AG ether group. None of the 2-AG-treated groups was significantly different from the VH + 2-AG group.

2-AG-Induced Differentiation of 3T3-L1 Fibroblasts into Adipocytes. The absence of CB1 and/or CB2 involvement in 2-AG and 2-AG ether-mediated IL-2 suppression spurred exploration of other putative cellular targets. PPAR γ was among those considered because of the structural similarity between known ligands of PPAR γ , such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) and eicosatetraynoic acid, to 2-AG and 2-AG ether. In addition, PPAR γ expression has recently been established in T cells, and its activation correlated with IL-2 suppression (Clark et al., 2000). Based on the above, we examined the effect of 2-AG and 2-AG ether treatment upon PPAR γ activation using multiple and well established PPAR γ -mediated biological responses, including PPAR γ -dependent adipogenesis in 3T3-L1 cells. 3T3-L1 cell differentiation was assessed morphologically by the rela-

tively round shape of the adipocytes and by the presence of prominent lipid vacuoles, which are readily stained by Oil Red O. 2-AG and 2-AG ether each induced adipogenesis in 3T3-L1 cells (Fig. 4B) that was morphologically indistinguishable from that induced by the other or that induced by the positive control, ciglitazone (Fig. 4A) (C. Rockwell and N. Kaminski, unpublished observations). However, 2-AG ether induced a greater magnitude of adipogenesis than 2-AG as quantified by enumeration of the total number of differentiated cells per well. These studies demonstrate PPAR γ activation by 2-AG/2-AG ether treatment. In addition, it was also observed that because 3T3-L1 fibroblast adipogenesis typically requires approximately 7 days coupled with the efficient processing of lipids by 3T3-L1 cells, higher concentrations of 2-AG and 2-AG ether were required than in other short-term

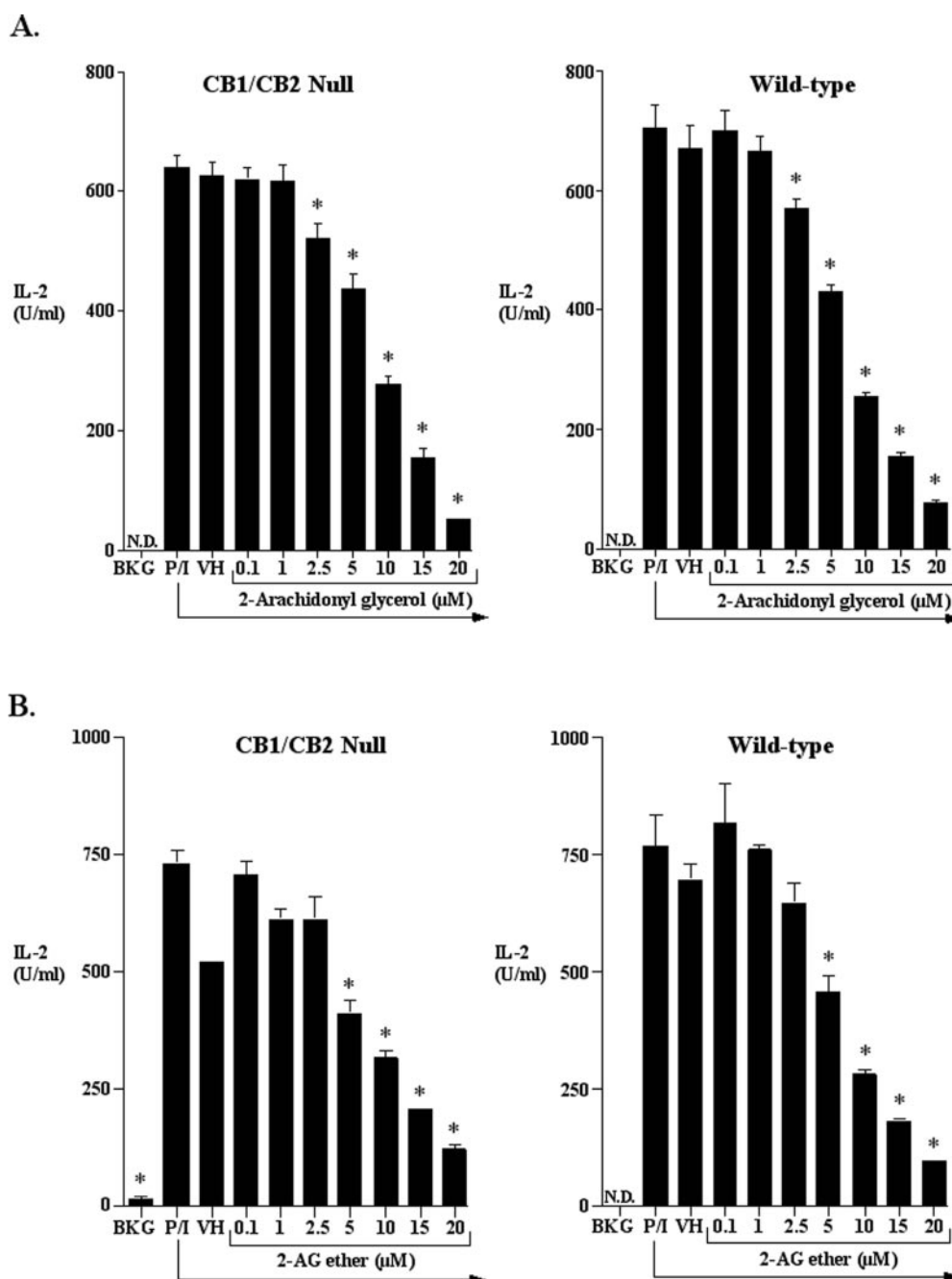


Fig. 3. Effect of 2-AG and 2-AG ether upon PMA/ionomycin-stimulated IL-2 production in splenocytes derived from CB1/CB2-null and wild-type mice. Splenocytes (1×10^6 cells/ml) were treated with 0.1 to 20 μ M concentrations of either 2-AG (A) or 2-AG ether (B) for 30 min followed by activation of the cells with PMA/ionomycin (40 nM/0.5 μ M). Cells were harvested 24 h later, and the supernatants were analyzed for interleukin-2 protein by ELISA. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with VH group.

assays using other models (i.e., IL-2 secretion in primary splenocytes).

Induction of aP2 mRNA Levels by 2-AG and 2-AG Ether Treatment. Because mRNA expression is a more precisely quantifiable response than adipogenesis, the effect of 2-AG and 2-AG ether upon mRNA levels of aP2, a gene regulated by PPAR γ , was also determined. Consistent with other laboratories, we detected a robust increase in aP2 expression 4 to 7 days after PPAR γ activation by ciglitazone, with modest induction detectable at earlier timepoints (Fig. 5) (C. Rockwell and N. Kaminski, unpublished observations) (Chawla and Lazar 1993; Tontonoz et al., 1994). The slow induction of aP2 is probably due to an initial up-regulation of PPAR γ expression upon activation, which occurs before transactivation of PPAR γ -regulated genes. Compared with 2-AG, lower concentrations of 2-AG ether were required to induce aP2, suggesting hydrolysis and inactivation of 2-AG in assays requiring cell culture for extended time periods.

Induction of PPAR γ Activation by 2-AG/2-AG Ether Treatment as Determined by PPAR γ -Specific Luciferase Activity and PPRE Binding. To further evaluate the specificity of 2-AG and 2-AG ether in the activation of PPAR γ , transient transfection experiments in 3T3-L1 cells were conducted using the PPAR γ -LBD/Gal4-DBD Gal 4 lu-

ciferase reporter, which is activated by ligands for PPAR γ but not PPAR α or PPAR δ . It is noteworthy that 2-AG, 2-AG ether, and the positive control, ciglitazone, all exhibited comparable potency in the induction of luciferase activity, demonstrating that 2-AG and 2-AG ether treatment activates PPAR γ specifically (Fig. 6A). Consistent with observations made by other researchers using similar plasmids, the positive control, ciglitazone, induced a 2-fold increase in luciferase activity over background levels (Kozak et al., 2002). Although the PPAR γ -specific luciferase induction by ciglitazone, 2-AG, and 2-AG ether may not seem to correlate with the magnitude of IL-2 suppression observed with the same compounds, this is probably due to significant differences between the two assays. The inhibition of IL-2 secretion by

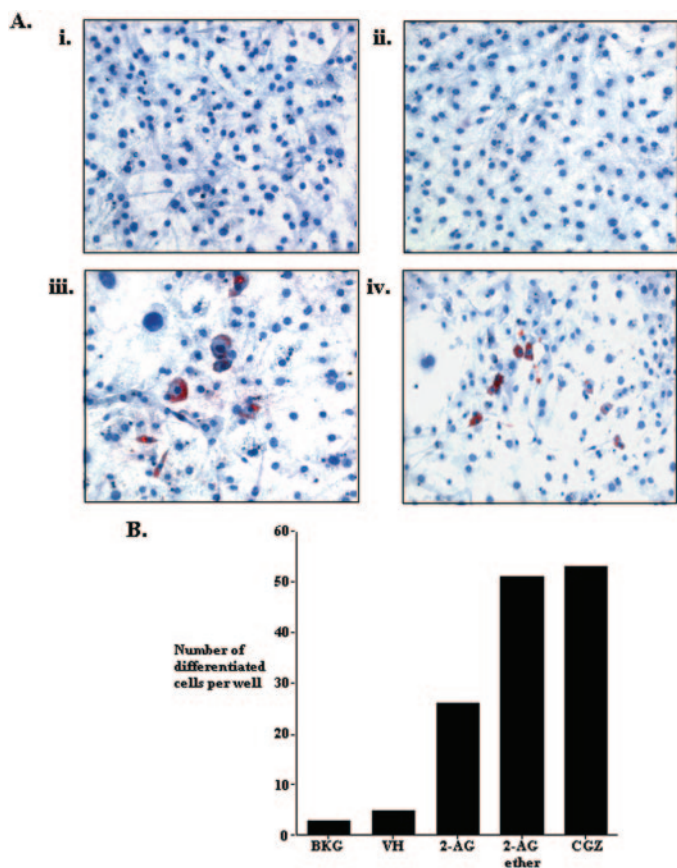


Fig. 4. Effect of 2-AG upon 3T3-L1 cellular differentiation. A, 3T3-L1 preadipocytes were cultured in plastic 2-well culture slides. Upon reaching confluence, the cells were either not treated (NA) (i) or treated with vehicle (0.1% ethanol; ii), 2-AG (50 μ M; iii), or ciglitazone (10 μ M; iv). The cells were then cultured for 7 days, after which the media was removed and the cells were washed with PBS before staining with Oil Red O. B, cellular differentiation was quantified by counting the total number of differentiated cells per well. Cellular viability was $\geq 85\%$ for all treatment groups.

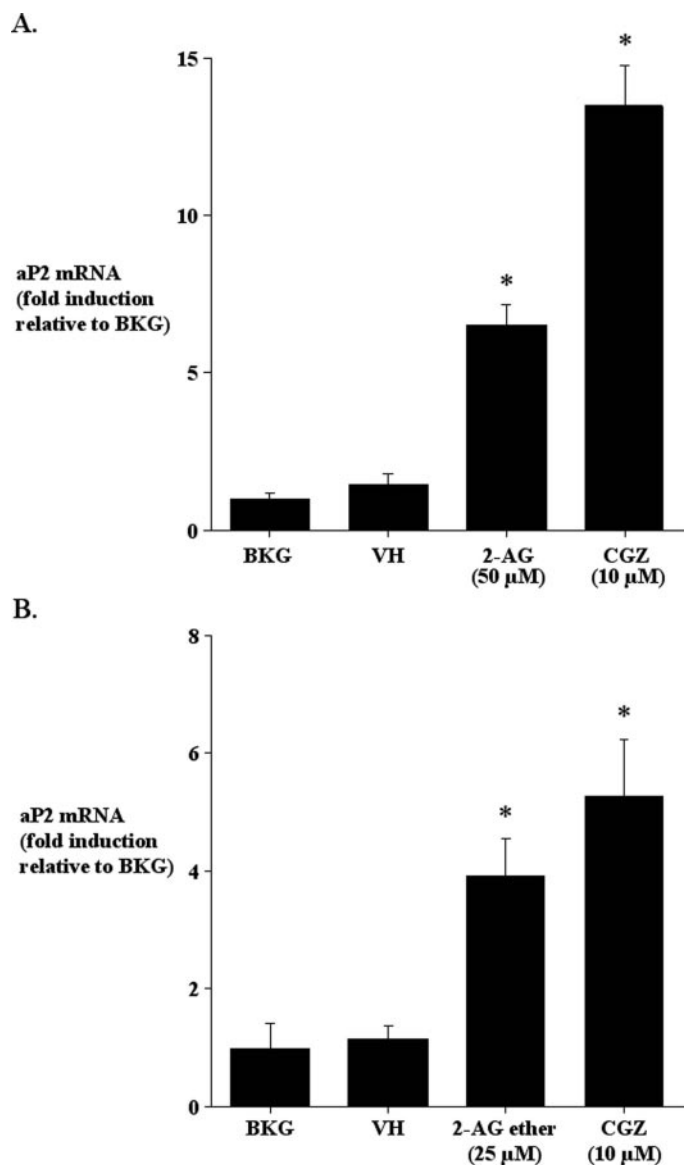


Fig. 5. Effect of 2-AG and 2-AG ether upon the induction of aP2. 3T3-L1 cells were cultured in 60-mm culture plates and allowed to grow to confluence. The cells were then either left untreated (BKG) or treated with VH (0.1% ethanol), ciglitazone (10 μ M), and either 2-AG (50 μ M; A) or 2-AG ether (25 μ M; B). Cells were cultured for 4 days after treatment at which time total RNA was isolated. aP2 mRNA was determined by real-time PCR using Taqman primers and probe. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of quadruplicate cultures. *, $p < 0.05$ compared with VH group.

PPAR γ agonists is dependent upon endogenous PPAR γ and RXR levels, whereas luciferase induction is dependent upon the expression of the PPAR γ /Gal 4 fusion protein from the exogenous plasmid in the transfected cells. These studies demonstrate that 2-AG and 2-AG ether treatment of 3T3-L1 cells specifically induces PPAR γ activation.

Likewise, to determine whether 2-AG treatment also specifically activates PPAR γ in Jurkat T cells as observed in 3T3-L1 cells, the effect of 2-AG treatment upon PPAR γ /PPRE binding was investigated. Concordant with the luciferase activity in 3T3-L1 cells, we observed that 2-AG treatment

also produced a 2-fold increase in PPAR γ /PPRE binding over the vehicle control in nuclear protein from activated Jurkat T cells as determined by ELISA-based DNA/protein binding assay (Fig. 6B). The increased PPAR γ /PPRE binding of nuclear protein from 2-AG-treated cells was abrogated in the presence of the unlabeled probe, thus demonstrating the specificity of the assay.

The PPAR γ Agonists Ciglitazone and 15d-PGJ₂ Suppress IL-2 Secretion by Activated Splenocytes. To further characterize the role of PPAR γ activation in IL-2 suppression, a series of direct addition studies were conducted using two known PPAR γ agonists, ciglitazone and 15d-PGJ₂. Both ligands suppressed IL-2 secretion by activated splenocytes in a concentration-dependent manner (Fig. 7). Also observed was that 15d-PGJ₂ (IC₅₀, 2.7 μ M) exhibited greater potency than ciglitazone (IC₅₀, 19.7 μ M), which has been previously demonstrated in other T cell activation models (Clark et al., 2000).

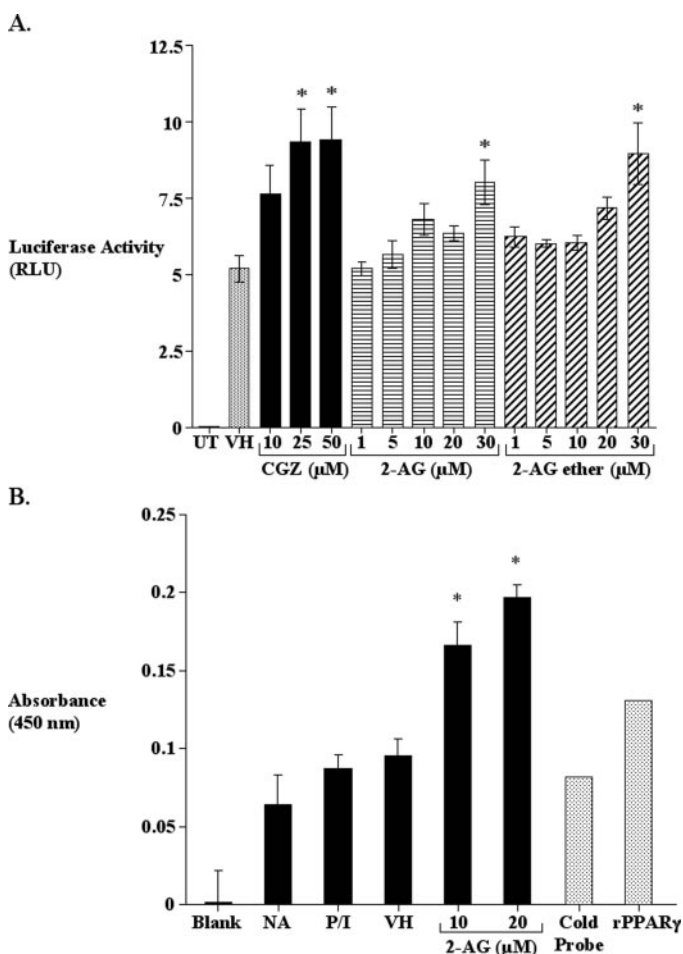


Fig. 6. Effect of 2-AG treatment upon PPAR γ transcriptional activity and PPAR γ /PPRE binding. A, 3T3-L1 cells were either transiently transfected with the PPAR γ -LBD/Gal4-DBD Gal4 luciferase reporter plasmid or treated with transfection reagents in the absence of plasmid (UT). After transfection, the cells were trypsinized, washed, resuspended in Dulbecco's modified Eagle's medium with 2% BCS, and pooled together. The cells were then cultured in 24-well plates for 5 h before treatment. Cells were then treated with 0.1% ethanol (VH), 10 to 50 μ M ciglitazone (CGZ), 1 to 30 μ M 2-AG, or 1 to 30 μ M 2-AG ether. Cells were then incubated for 16 to 20 h. The luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. B, Jurkat T cells (5×10^5 c/ml) were treated with 2-AG or VH (0.1% ethanol) for 30 min before treatment with PMA (40 nM) and ionomycin (0.5 μ M). After a 2.5-h incubation, the cells were harvested and the nuclear proteins were extracted. PPAR γ /PPRE binding was determined by light absorbance at 450 nm using an ELISA-based assay. The blank was loaded with buffer alone in the absence of nuclear protein. The nonradioactive probe was run with unlabeled oligonucleotide added to the binding buffer in addition to the biotinylated PPAR probe to confirm the specificity of the assay. The recombinant PPAR γ protein was used as a positive control for the assay. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with VH group.

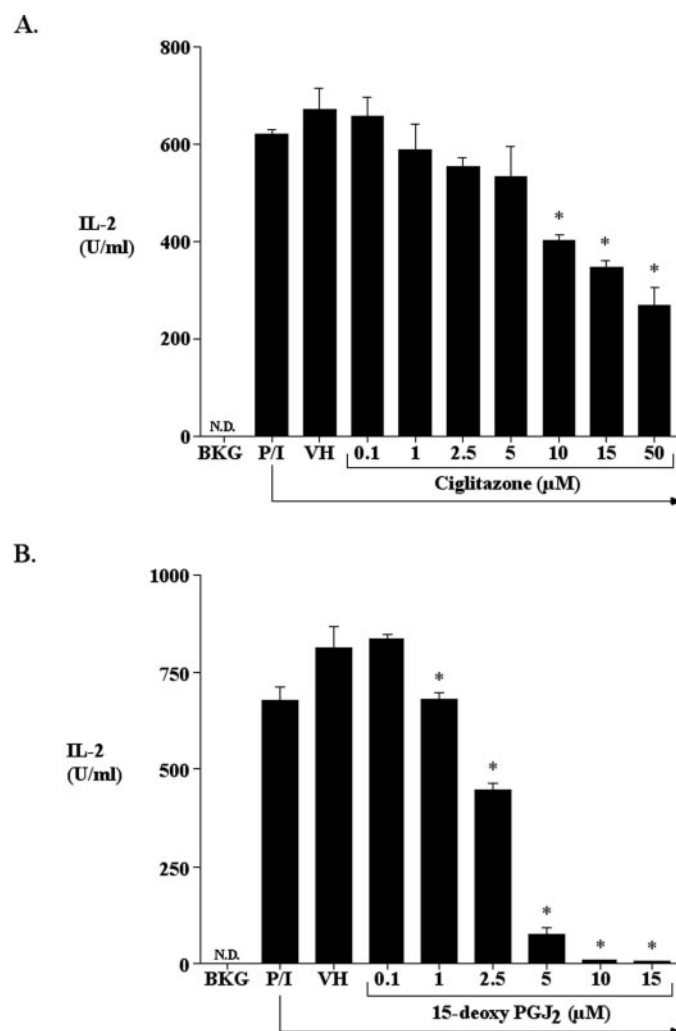


Fig. 7. Effect of ciglitazone and 15-deoxy PGJ₂ upon IL-2 secretion by activated splenocytes. Splenocytes (1×10^6 cells/ml) were treated with either ciglitazone (0.1–50 μ M; A) or 15-deoxy PGJ₂ (0.1–15 μ M; B) for 30 min. Cells were then stimulated with 40 nM PMA/0.5 μ M ionomycin for 24 h. The supernatants were harvested, and IL-2 production was measured by ELISA. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with VH.

T0070907, a Potent, Synthetic PPAR γ -Specific Antagonist, Attenuates IL-2 Suppression by 2-AG and 2-AG Ether. To establish a causal relationship between PPAR γ activation and suppression of IL-2, studies were conducted to ascertain whether the PPAR γ -specific antagonist T0070907 attenuates 2-AG and/or 2-AG ether-mediated IL-2 suppression. Pretreatment of freshly isolated splenocytes with increasing concentrations of T0070907 attenuated 2-AG-mediated IL-2 suppression in a concentration-respon-

sive manner, which is consistent with previous observations with structurally related anandamide (Fig. 8A) (Rockwell and Kaminski, 2004). It is noteworthy that T0070907 treatment alone, at the highest concentration used (10 μ M), produced a marked suppression of IL-2 in the absence of 2-AG but only in murine splenocytes. Identical experiments were performed in the human Jurkat T cell line. These studies showed that Jurkat cells were more refractory to the suppressive effects of T0070907 but almost completely abrogated 2-AG and 2-AG ether-mediated IL-2 suppression (Fig. 8, B and C).

2-AG Markedly Inhibited the Transcriptional Activity of NFAT and NF κ B in the Absence, but Not in the Presence, of T0070907 But Only Modestly Inhibited AP-1 Activity. Published studies from other researchers have demonstrated that suppression of IL-2 by activated PPAR γ probably involves the transrepression of the transcription factor NFAT (Yang et al., 2000). In addition to NFAT, there is evidence to suggest that PPAR γ transrepresses NF κ B and AP-1 as well (Ricote et al., 1998). 2-AG treatment causes a concentration-dependent decrease in NFAT and NF κ B transcriptional activity and produces only modest inhibition of AP-1 activity (Fig. 9). T0070907 abrogated the inhibitory effects of 2-AG upon NFAT and NF κ B transcriptional activity, suggesting that these effects are mediated by PPAR γ (Fig. 10). Because treatment of transfected Jurkat cells with T0070907 alone resulted in a modest decrease in AP-1 activity comparable with that of 2-AG, the role of PPAR γ in the modest suppression of AP-1 by 2-AG could not be assessed (data not shown). In addition, it was also observed that the inhibition of NFAT and NF κ B transcriptional activity by 2-AG in human Jurkat cells is markedly greater than that previously demonstrated in murine EL4 T cells, suggesting that the effect of 2-AG upon NFAT and NF κ B is dependent upon the model and/or the species used (Ouyang et al., 1998).

IFN γ and IL-4, Cytokines Also Regulated by NFAT and NF κ B, Are Suppressed by 2-AG. Because 2-AG inhibits NFAT and NF κ B transcriptional activity, we investigated the effect of 2-AG upon the transcription of other cytokines that are also regulated by NFAT and NF κ B and secreted by activated T cells. 2-AG markedly suppressed mRNA levels for both IFN γ and IL-4 in a concentration-dependent manner (Fig. 11).

Discussion

Although it has been demonstrated by a number of researchers that 2-AG inhibits cytokine production in a variety of different model systems, the role of CB1 and CB2 in these effects has been unclear (Ouyang et al., 1998; Gallily et al., 2000; Chang et al., 2001; Facchinetti et al., 2003). As such, a major objective of the current studies was to rigorously examine the role of CB1 and CB2 in 2-AG-mediated suppression of IL-2, a cytokine critical for T cell growth and development. The suppression of IL-2 by 2-AG in splenocytes derived from CB1/CB2-null mice coupled with the failure of the CB1/CB2 antagonists to block inhibition of IL-2 by 2-AG demonstrates the absence of cannabinoid receptor involvement. Furthermore, the present studies are also the first to demonstrate that 2-AG treatment inhibits NFAT and NF κ B activity and consequently suppresses IL-2 production in the

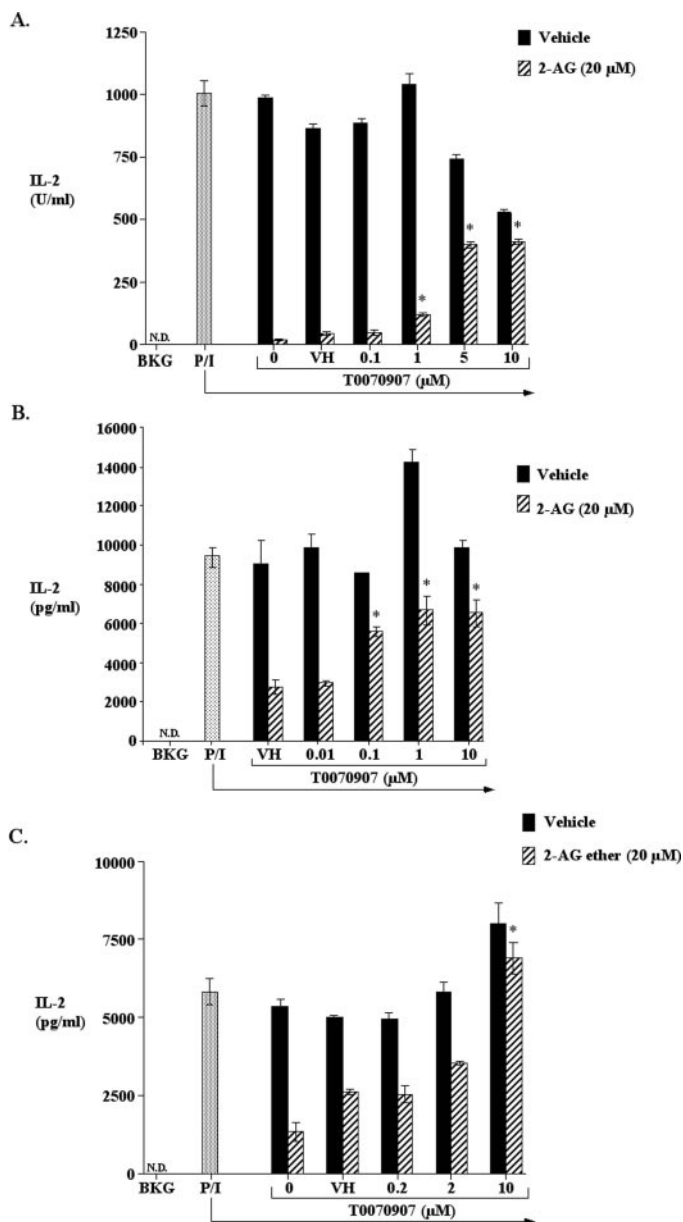


Fig. 8. Effect of T0070907 upon inhibition of IL-2 secretion by 2-AG and 2-AG ether. A, splenocytes (1×10^6 cells/ml) were treated with T0070907 (0.1–10 μ M) or vehicle (0.02% DMSO) for 30 min before treatment with 2-AG (20 μ M). B and C, Jurkat cells (5×10^5 cells/ml) were treated with T0070907 (0.01–10 μ M) or vehicle (0.02% DMSO) for 30 min before treatment with either 2-AG (20 μ M; B) or 2-AG ether (20 μ M; C). After a 30-min incubation, the cells were then stimulated with 40 nM PMA/0.5 μ M ionomycin for 24 h. The supernatants were harvested, and IL-2 production was measured by ELISA analysis. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with either VH + 2-AG group (A and B) or VH + 2-AG ether group (C).

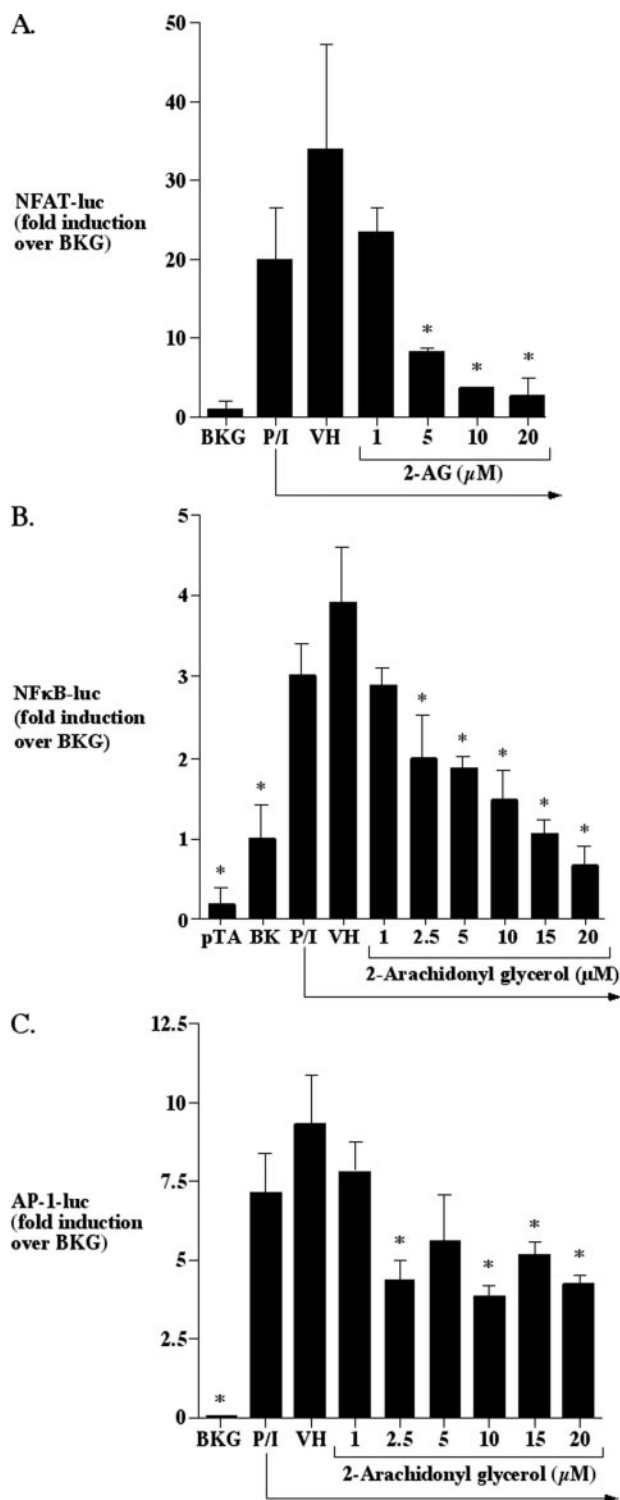


Fig. 9. Effect of 2-AG upon the transcriptional activity of NFAT, NFκB, and AP-1. Jurkat T cells were transiently transfected with either NFAT (A), NFκB (B), or AP-1 (C) luciferase reporter plasmid. After transfection, the cells were washed, resuspended in fresh media, and pooled together. The cells were then either left untreated (BKG) or treated with 2-AG or VH (0.04% ethanol) for 30 min followed by activation of the cells with PMA/ionomycin (80 nM/1 μM). Cells were then incubated for 12 h. The luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. Cellular viability was ≥85% for all treatment groups. The pTA group served as a negative control and represents cells that were transfected with the pTA-luc plasmid and later treated with PMA/Io. The results are the mean ± S.E. of triplicate or quadruplicate cultures. *, $p < 0.05$ compared with VH group.

absence, but not in the presence, of a PPARγ-specific antagonist, suggesting that the decrease in IL-2 transcription is the result of transrepression of NFAT and NFκB by PPARγ. The aforementioned results are corroborated by the activation of PPARγ in 2-AG-treated Jurkat T cells, as evidenced by increased PPARγ/PPRE binding. Likewise, inhibition of IL-2 secretion in T cells treated with 2-AG ether is also abrogated by the PPARγ antagonist, suggesting that, like 2-AG, the effects of 2-AG ether treatment upon IL-2 production occur through PPARγ activation.

In activated splenocytes, suppression of IL-2 by 2-AG is induced at concentrations as low as 2.5 μM, which is within an order of magnitude of the calculated endogenous 2-AG levels detected in human plasma (Motobe et al., 2004). It is noteworthy that the intracellular concentrations of structurally related congeners, such as anandamide, have been reported to exceed extracellular concentrations by as much as 3 orders of magnitude (Hillard and Jarrahian, 2003). Although the intracellular concentrations of 2-AG have not yet been

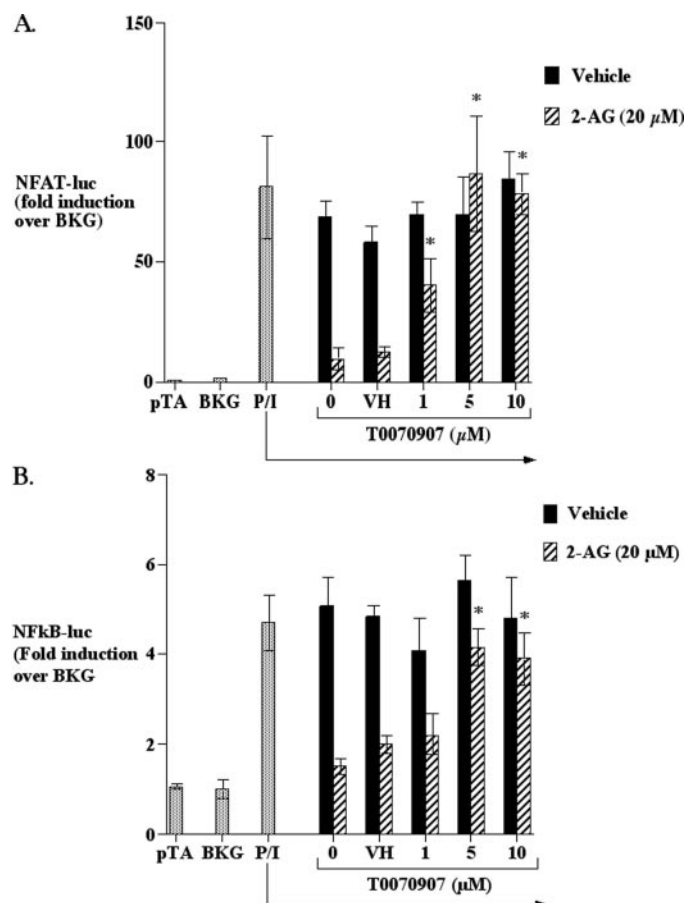


Fig. 10. Effect of T0070907 upon inhibition of NFAT and NFκB transcriptional activity by 2-AG. Jurkat T cells were transiently transfected with either NFAT (A) or NFκB (B) luciferase reporter plasmid. After transfection, the cells were washed, resuspended in fresh media, and pooled together. The cells were then treated with 2-AG or VH (0.04% ethanol) in the presence or absence of T0070907 for 30 min followed by activation of the cells with PMA/ionomycin (80 nM/1 μM). Cells were then incubated for 12 h. The luciferase activity was quantified in relative light units (RLU) by chemiluminescence assay. Cellular viability was ≥85% for all treatment groups. The pTA group served as a negative control and represents cells that were transfected with the pTA-luc plasmid and later treated with PMA/Io. The results are the mean ± S.E. of quadruplicate cultures. *, $p < 0.05$ compared with VH + 2-AG group.

reported, they are likely to greatly exceed extracellular levels because 2-AG has been found to accumulate intracellularly by mechanisms similar to or the same as those of anandamide (Beltramo and Piomelli, 2000).

Because 2-AG ether is nonhydrolyzable and produces a level of IL-2 suppression similar to produced by 2-AG, it is unlikely that the activity of 2-AG is mediated through a hydrolysis product. This notion is further supported by the inability of methyl arachidonyl fluorophosphonate, an inhibitor of fatty acid amide hydrolase and monoacylglycerol lipase (the two enzymes responsible for the hydrolysis of 2-AG), to block IL-2 suppression by 2-AG (data not shown). Nonetheless, the metabolism of 2-AG probably plays an important role in its observed activity in fibroblasts and T cells. It has been shown, for instance, that a 15-lipoxygenase metabolite of 2-AG is a PPAR α agonist (Kozak et al., 2002a). Furthermore, numerous studies have demonstrated that 2-AG is efficiently metabolized by cyclooxygenase-2 (COX-2) as well as multiple lipoxygenases (Kozak et al., 2002a,b).

Consistent with the aforementioned findings, we have found that suppression of IL-2 by 2-AG in activated Jurkat T cells is abrogated by pretreatment with NS398, a COX-2-specific inhibitor, suggesting that the decrease in IL-2 by 2-AG is mediated by a COX-2 metabolite rather than the parent molecule of 2-AG (data not shown).

A number of oxygenated arachidonate metabolites have also been found to activate PPAR γ , including the COX product, 15d-PGJ $_2$ (Kliwer et al., 1995). In immune cells, the activation of PPAR γ by 15d-PGJ $_2$ results in transrepression of transcription factors, such as NFAT, which is ultimately responsible for the suppression of cytokine secretion as well as other immunosuppressive effects (Ricote et al., 1998; Yang et al., 2000). Despite the variety of immune effects produced by 15d-PGJ $_2$, there has been considerable controversy concerning whether it is a biologically relevant PPAR γ activator as a result of the low levels at which it is detected in vivo (low picomolar range) and because 15d-PGJ $_2$ levels are not elevated under conditions in which PPAR γ is activated (Bell-Parikh et al., 2003). Whereas many studies have focused upon the role of free fatty acids and their metabolites as PPAR γ activators, our studies suggest that a 2-AG metabolite also activates PPAR γ and may be a biologically relevant ligand in cell types known to release 2-AG, such as leukocytes.

Similar to arachidonate, 2-AG is released upon the activation of a number of immune cell types (Berdyshev et al., 2001; Maccarrone et al., 2002). 2-AG is unique from arachidonate, however, in that it is a COX-2-specific substrate (Kozak et al., 2002a). Metabolism of 2-AG by COX-2 produces a variety of prostaglandin-like compounds that differ from arachidonate-derived products only in the addition of a glyceryl ester group. The inability of 2-AG to be metabolized by COX-1, thromboxane synthase, 5-lipoxygenase, as well as other oxidative enzymes, may produce a unique set of eicosanoids tailored to mediate a specific response (Kozak et al., 2002a,b). In addition, like synthetic COX-2 specific inhibitors, 2-AG would probably have minimal effects upon gastric mucosa and the coagulation system. The prostaglandin glyceryl esters are also distinguished from the free acid prostaglandins in that they are more stable, such that prostaglandin glyceryl esters may induce responses over a longer duration (Kozak et al., 2002a).

Although anandamide is also a COX-2 specific substrate, it is generally found at much lower levels than 2-AG in most cell types and is metabolized less efficiently than 2-AG by COX-2 (Kozak et al., 2002a). The release of 2-AG in a number of immune cell types by a variety of different stimuli suggests that it may play a major role in immune regulation, whereas anandamide may be more specialized for particular immune responses (Berdyshev, 2001). The high levels of anandamide found in the uterus, for example, suggest that anandamide may function to regulate maternal immune responses near the embryo during gestation (Das et al., 1995).

Early studies examining the role of PPAR γ in immune cells indicated that PPAR γ agonists can modulate a variety of immune responses, which in the majority of cases results in down-regulation of leukocyte activity (Daynes and Jones, 2002). Because a recent report demonstrated that rosiglitazone and 15d-PGJ $_2$ have anti-inflammatory effects in macrophages that lack PPAR γ , the role of PPAR γ in many of the observed inhibitory immune effects by PPAR γ agonists has

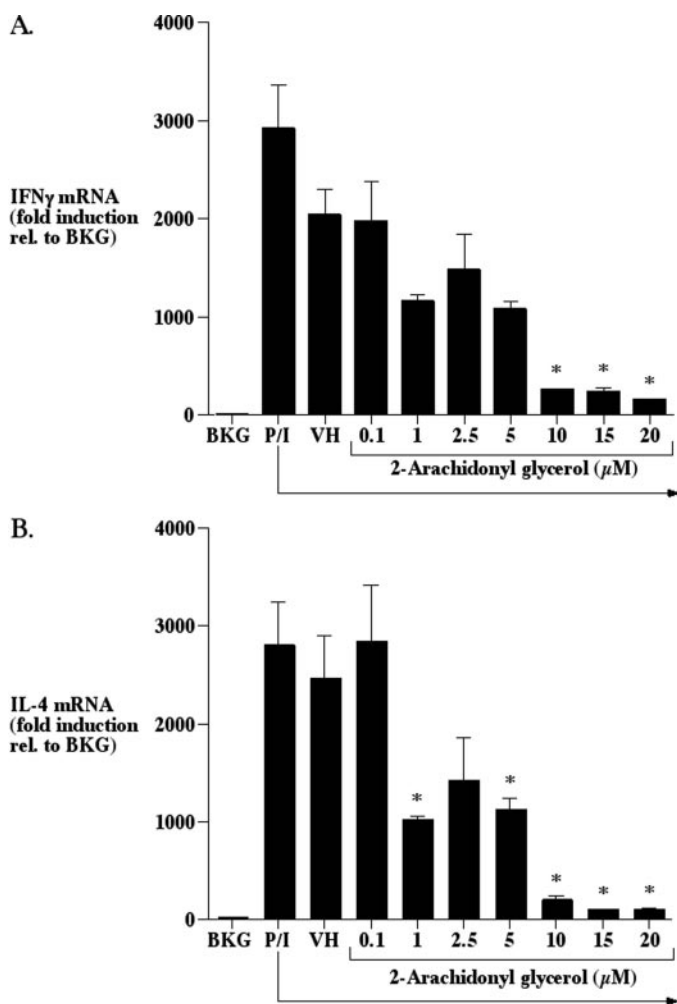


Fig. 11. Effect of 2-AG upon the transcription of other cytokines regulated by NFAT and NF κ B. Splenocytes (1×10^6 cells/ml) were treated with 0.1 to 20 μ M 2-AG for 30 min followed by activation of the cells with PMA/ionomycin (40 nM/0.5 μ M). Cells were incubated for 6 h after treatment, at which time total RNA was isolated. IFN γ and IL-4 mRNA levels were determined by real-time PCR using Taqman primers and probe. Cellular viability was $\geq 85\%$ for all treatment groups. The results are the mean \pm S.E. of triplicate cultures. *, $p < 0.05$ compared with VH group.

been questioned and may need to be confirmed (Chawla et al., 2001). As such, the current studies are relevant because a number of different means were used to carefully assess activation of PPAR γ upon 2-AG treatment as well as the subsequent downstream effects upon fibroblasts and activated T cells, including a PPAR γ -specific reporter assay, the demonstration of PPAR γ binding to PPAR response element in Jurkat cells treated with 2-AG, and an antagonist that is highly specific for PPAR γ . Together, these findings strongly suggest that the observed effects of 2-AG and 2-AG ether are in fact mediated by PPAR γ .

Although initial studies of PPAR γ centered upon lipid metabolism and glucose homeostasis, the breadth of knowledge concerning the role of PPAR γ in immune regulation is rapidly increasing. It has been reported that activation of PPAR γ can ameliorate autoimmune disease in a number of different animal models, including experimental allergic encephalitis, a model of multiple sclerosis (Natarajan and Bright, 2002). Furthermore, PPAR γ activation causes anti-inflammatory effects in T cells derived from patients with multiple sclerosis (Schmidt et al., 2004). Likewise, the modulation of a variety of immune responses by 2-AG, including cytokine secretion, suggests that 2-AG may also play an important role in immune regulation. An immunoregulatory role by 2-AG is further supported by the marked increase in 2-AG levels upon activation of various immune cell types (Berdyshev et al., 2001). In addition, 2-AG levels are much higher in the sera of patients suffering from lipopolysaccharide-induced shock compared with healthy control subjects, suggesting that 2-AG may also play a regulatory role in sepsis (Wang et al., 2001). Together, the above findings suggest that PPAR γ may execute an important function in the control of exaggerated or inappropriate immune responses, and that activation of PPAR γ by 2-AG may be important for the maintenance of immune homeostasis.

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