Inhibition of peroxisome-proliferator-activated receptor (PPAR)α by MK886

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Although MK886 was originally identified as an inhibitor of 5-lipoxygenase activating protein (FLAP), recent data demonstrate that this activity does not underlie its ability to induce apoptosis [Datta, Biswal and Kehrer (1999) Biochem. J. **340**, 371–375]. Since FLAP is a fatty-acid binding protein, it is conceivable that MK886 may affect other such proteins. A family of nuclear receptors that are activated by fatty acids and their metabolites, the peroxisome-proliferator-activated receptors (PPARs), have been implicated in apoptosis and may represent a target for MK886. The ability of MK886 to inhibit PPAR-α, -β and -γ activity was assessed using reporter assay systems (peroxisome-proliferator response element–luciferase). Using a transient transfection system in monkey kidney fibroblast CV-1 cells, mouse keratinocyte 308 cells and human lung adenocarcinoma A549 cells, 10–20 μM MK886 inhibited Wy14,643 activation of PPARα by approximately 80 %. Similar inhibition of PPARα by MK886 was observed with a stable transfection reporter system in CV-1 cells. Only minimal inhibitory effects were seen on PPARβ and PPARγ. MK886 inhibited PPARα by a non-competitive mechanism as shown by its effects on the binding of arachidonic acid to PPARα protein, and a dose–response study using a transient transfection reporter assay in COS-1 cells. An assay assessing PPAR ligand–receptor interactions showed that MK886 prevents the conformational change necessary for active-complex formation. The expression of keratin-1, a protein encoded by a PPARα-responsive gene, was reduced by MK886 in a culture of mouse primary keratinocytes, suggesting that PPAR inhibition has functional consequences in normal cells. Although Jurkat cells express all PPAR isoforms, various PPARα and PPARγ agonists were unable to prevent MK886-induced apoptosis. This is consistent with MK886 functioning as a non-competitive inhibitor of PPARα, but may also indicate that PPARα is not directly involved in MK886-induced apoptosis. Although numerous PPAR activators have been identified, the results show that MK886 can inhibit PPARα, making it the first compound identified to have such an effect.

Key words: activating protein, apoptosis, cell signalling, 5-lipoxygenase.

INTRODUCTION

Fatty acid metabolites are involved in various signalling pathways and some data suggest that those produced by lipoxygenase (LOX) enzymes play an important role in apoptosis [1]. However, there is a significant amount of conflicting information, largely because of the reliance on LOX inhibitors with unclear specificity that induce apoptosis in some systems while inhibiting it in others [2–5]. MK886, an indole compound originally identified as a potent inhibitor of leukotriene biosynthesis, with an IC_{50} of approx. 3 nM [6], has been used to induce apoptosis [3,7,8] with the assumption that its mechanism is related to its ability to inhibit 5-LOX activating protein (FLAP). This protein is an integral part of the 5-LOX pathway of arachidonic acid metabolism [6]. However, it is now evident that MK886-induced apoptosis is unrelated to 5-LOX, since this compound induces apoptosis in cells lacking this and other LOX enzymes [9], the concentration needed (10 μM) is approx. 100 times greater than that required to completely inhibit LOX activity [6], and the potency of MK886 in terms of inducing apoptosis is independent of FLAP levels [10]. Additional activities attributable to MK886 that may be involved in its ability to induce apoptosis have not been identified.

FLAP enhances 5-LOX activity by specifically binding arachidonic acid and presenting this fatty acid to the enzyme. FLAP can thus be viewed as a fatty-acid transport protein. MK886 competes for the arachidonic acid binding site of FLAP [11], but whether or not it might affect fatty-acid binding at other sites is not known. Given the ability of non-esterified fatty acids and various oxidized lipid species to cause apoptosis in some cells [12–15], the released polyunsaturated fatty acids could directly, or indirectly, generate potent apoptotic mediators. Perturbations in the control of cellular arachidonic acid levels have been shown to induce apoptosis [16], and a similar mechanism has been proposed to explain the apoptosis-inducing properties of various non-steroidal anti-inflammatory drugs (NSAIDs) [17].

A family of proteins that may be of particular importance to the activity of MK886 are the peroxisome-proliferator-activated receptors (PPARs). These proteins have been labelled as 'fatty acid receptors' [18], because they are activated by endogenous fatty acids, as well as exogenous fatty acid-like chemicals called peroxisome proliferators (PPs). Upon activation, PPARs are converted into transcriptionally active complexes ('transcription factors') that control gene expression by interacting with specific DNA response elements called PPAR-responsive elements (PPREs). Genes containing PPRE motifs include acyl-CoA

Abbreviations used: CARLA, coactivator-dependent receptor ligand assay; CMV, cytomegalovirus; DTT, dithiothreitol; EMEM, Eagle's minimal essential medium; FLAP, 5-lipoxygenase activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; LOX, lipoxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; PPs, peroxisome proliferators; PPARs, peroxisome-proliferator-activated receptors; PPREs, PPAR-responsive elements; RXR, 9-*cis* retinoic acid receptor; SRC-1, steroid receptor coactivator-1.

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oxidase, peroxisomal bifunctional enzyme, liver fatty acid-binding protein and cytochrome P450A ('CYP4A') [18]. PPARs also appear to be involved in the expression of several growth regulatory genes. Recently, the mechanism by which PPs serve as tumour promoters has been linked to apoptosis, since PPs decrease this form of cell death in hepatocytes [19,20]. A role for PPARs was further shown by overexpression of a dominant negative PPARα, thereby abolishing its activity and promoting apoptosis [21], and by studies with hepatocytes from PPARα null mice that were resistant to PP-induced suppression of apoptosis [22]. In contrast, both PPARα and PPARγ ligands can induce apoptosis in a variety of cells, including macrophages activated with tumour necrosis factor-α/interferon-γ [23], hepatoma cell lines [24] and astrocytes [25].

The purpose of the present study was to determine the effects of MK886 on PPARs, and the ability of PPs to affect the apoptosis induced by this compound. The results show that MK886 can inhibit PPARα more effectively than either PPARβ or PPARγ, making it one of the first compounds identified to have such a receptor-subtype effect. The results suggest that this inhibition is non-competitive and show that it can affect the expression of a PPARα-regulated gene. A role for the inhibition of PPARα in MK886-induced apoptosis is not yet known.

MATERIALS AND METHODS

Cell culture

A549 human lung adenocarcinoma cells, originally obtained from the A.T.C.C. (Rockville, MD, U.S.A.), were cultured in Dulbecco's minimal essential medium (pH 7.4) supplemented with 10 % (v/v) fetal calf serum, 3.7 mg/ml sodium bicarbonate and 100 μg/ml gentamicin. Monkey kidney fibroblast CV-1 cells, originally obtained from the A.T.C.C., were grown in Eagle's minimal essential medium (EMEM) (pH 7.5) supplemented with 10 % (v/v) fetal calf serum, 1 % (w/v) sodium pyruvate, 1 % (w/v) non-essential amino acids, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Monkey kidney fibroblast COS-1 cells (simian-virus-40 transformed), originally obtained from the A.T.C.C., were maintained in α-minimal essential medium (Sigma) supplemented with 8 % (v/v) fetal calf serum, 0.2 mg/ml streptomycin and 200 units/ml penicillin. Cultures were passaged at confluency (approximately every 3 days) and were removed from monolayer stock cultures with trypsin/EDTA. Cells were counted using a haemacytometer and were plated in Falcon 6-well dishes (9.6 cm^2/well). The volume of medium used was 2 ml/well.

Jurkat human acute lymphocytic leukaemia cells, originally obtained from the A.T.C.C., were cultured in RPMI 1640 medium (pH 7.4) with glutamine, supplemented with 10 % (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cultures were passaged with fresh medium every 48 h. All cells were grown at 37 °C in 5 % CO_2. Fetal calf serum was obtained from either Life Technologies (Grand Island, NY, U.S.A.) or Summit Biotechnology (Fort Collins, CO, U.S.A.).

Murine epidermal keratinocytes were harvested from newborn SENCAR (SSIN) mice (Veterinary Resources Division, M. D. Anderson Cancer Center, The University of Texas, TX, U.S.A.) by trypsinization as previously described [26], and were plated at 10^6 cells/35 mm dish in enriched Waymouth's medium (1.2 mM Ca^{2+}) containing 10 % (v/v) fetal bovine serum. The cells were allowed to attach for 2.5 h, at which time the medium was replaced with EMEM (0.05 mM Ca^{2+}) plus 8 % (v/v) chelexed serum, but without added growth factors. The cells were then grown for another 24 h prior to transfection. Cells from the keratinocyte cell line 308 [27] were grown in EMEM containing 1 % (v/v) fetal calf serum. The cells were used for transfection when they reached 70 % confluence.

Chemicals and treatments

MK886, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, and ciglitazone were obtained from Biomol Research (Plymouth Meeting, PA, U.S.A.). MK591 was a gift from Merck Frosst Canada, Inc. (Pointe Claire-Dorval, Quebec, Canada). Wy14,643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio acetic acid], > 98 % pure, was purchased from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.). Bezafibrate was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.), conjugated linoleic acid was obtained from NuCheck Prep (Elysian, MN, U.S.A.) and 8-S-HETE was purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific radioactivity of 100 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, U.S.A.). DNase I was obtained from Life Technologies. All chemicals were dissolved in DMSO or methanol, and treatments were added directly to cell culture media. The final concentration of vehicle in media was maintained at 0.1 % (v/v) and appropriate vehicle controls were used in all cases.

Western-blot analysis

PPAR-α, -β and -γ were translated *in vitro* from pSG5-PPARα, pSG5-PPARβ and pSG5-PPARγ (a gift from Dr R. M. Evans, Salk Institute, La Jolla, CA, U.S.A.) by using rabbit reticulocyte lysate from the TNT Promega transcription/translation kit (Promega, Madison, WI, U.S.A.). Western blots for PPAR proteins were performed by established procedures. Briefly, cells (5×10^6) were lysed with 150 μl of RIPA buffer (10 mM sodium phosphate, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 100 μg/ml PMSF, 30 μl/ml aprotinin and 1 mM sodium orthovanadate, pH 7.4) by repeatedly pipeting the cell suspension and incubating for 15 min at 4 °C. The lysed cells were centrifuged at 400 ***g*** for 10 min and supernatants resolved by reducing SDS/PAGE (15 % polyacrylamide gels) [loading buffer composition: 0.2 M Tris/HCl (pH 6.8), 20 % (v/v) glycerol, 4 % 2-mercaptoethanol, 4 % (w/v) SDS and 0.02 % Bromophenol Blue]. Protein was transferred on to PVDF membranes and blocked for 1 h. The membrane was then incubated with anti-PPARα (1:1000 dilution; Affinity Bioreagents, Golden, CO, U.S.A.), anti-PPARβ (1:1000 dilution; Affinity Bioreagents) or anti-PPARγ (1:600 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After membrane washing, horseradish-peroxidase conjugated anti-rabbit secondary antibodies were used (1:10000 dilution; Biosource International, Camarillo, CA, U.S.A.). Bound antibodies were detected using enhanced chemiluminescence with a kit obtained from Amersham. Protein content was determined using the method of Lowry et al. [28] using BSA as a standard.

Northern-blot analysis

Total RNA was isolated using the Tri-Reagent method (Molecular Research Center Inc., Cincinnati, OH, U.S.A.). RNA (10 μg) was fractionated through a 1 % (w/v) agarose/6 % (w/v) formaldehyde/ethidium-bromide stained gel, blotted on to a nylon membrane and hybridized with keratin-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes radiolabelled using a random primer kit (Roche Diagnostics, Indianapolis, IN, U.S.A.). The blot was washed twice for 15 min in 0.1 % SDS/2 × SSC (where 1 × SSC corresponds to 0.15 M NaCl/0.015 M sodium citrate) at room temperature, and once

for 30 min with 0.1 % SDS/0.1 × SSC at 60 °C, and was then exposed to X-ray film overnight at −80 °C. Relative band densities were determined using a scanned image and UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.).

PPAR reporter assays

The keratinocyte cell line 308 or CV-1 cells were plated in 35 mm dishes and allowed to grow to 70 % confluence. Cells were transfected with 1 ml of serum-free medium containing 1 μg/ml of the luciferase reporter construct $PPRE_3$-TK-LUC [29] (a gift from Dr R. M. Evans), 1 μg/ml pSG5-PPAR-α, -β or -γ expression vector, 0.25 μg/ml CMV-βGal (where CMV corresponds to cytomegalovirus) and 8 μl/ml LIPOFECTAMINE™ (Life Technologies) for 5 h. The transfection mix was replaced with original medium with or without Wy14,643 (10 μM), bezafibrate (50 μM) or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (10 μM) to activate PPAR-α, -β or -γ, respectively, and in the presence or absence of MK886 (10 μM). After 6 h, cells were harvested in luciferase lysis buffer (Tropix, Bedford, MA, U.S.A.). Light units from firefly luciferase activity were determined using a luminometer and were normalized to β-galactosidase activity using the β-galactosidase kit (Tropix), and to protein concentration determined using the bicinchoninic acid ('BCA') kit (Pierce, Rockford, IL, U.S.A.). For A549 cells at 70 % confluence, the transfection procedure was modified to use 8 μl/ml Fugene (Roche Diagnostics) instead of LIPOFECTAMINE™. In addition, exogenous PPARα was omitted due to the high level of endogenous expression in this cell line. Activation of PPARα was achieved with 100 μM Wy14,643 and MK886 was present at 20 μM for 6 h prior to harvesting and analyses.

The pSG5-GAL4–PPARα chimaera expression construct, containing the ligand binding domain of mouse PPARα, was kindly provided by S. A. Kliewer (Department of Molecular Endocrinology, Glaxo Wellcome Research and Development, Research Triangle Park, NC, U.S.A.) while the pFR-Luc (Gal4-UAS-Luciferase) construct was from Stratagene (La Jolla, CA, U.S.A.). A full-length PPARα–Gal4 chimaera was prepared by standard methods in the vector pM (ClonTech Laboratories, Palo Alto, CA, U.S.A.). At 75–90 % confluence, COS-1 cells were co-transfected with GAL4–PPARα, pFR-Luc and pRL (where RL is *Renilla* luciferase; Promega) as described previously [30]. The cells were co-treated with DMSO or Wy14,643 (50 μM) plus various amounts of MK886 (1–100 μM) 24 h after transfection. After 6 h of treatment, the cells were harvested and firefly and *Renilla* luciferase activities were assessed using the Dual Luciferase Assay System (Promega).

pSV-GL2-PPRE-luciferase, PPAR plasmids (in pSG5) and pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) (used for G418 selection) were transfected into CV-1 cells using calcium phosphate precipitation (ProFection, Promega), following the manufacturer's protocol. Full-length mouse PPARα cDNA and pSV-GL2-PPRE-luciferase were donated by J. Tugwood (Zeneca Central Toxicology Laboratory, Manchester, U.K.), while full-length PPARβ and PPARγ cDNAs were from P. A. Grimaldi (Centre de Biochimie, Parc Valrose, France). A 5:5:1 ratio of pSV-GL2-PPRE-luciferase/PPAR/pcDNA3 was used. Selective pressure was applied to the cells 2 days after transfection by adding 400 μg/ml G418 for 8 weeks. Surviving cells were expanded and tested for inducibility by Wy14,643 (50 μM for 24 h), bezafibrate (50 μM for 24 h) or ciglitazone (10 μM for 24 h) for PPAR-α, -β and -γ respectively. The cell lines utilized were not clonal and represented a mixed population of stably transfected cells.

PPAR ligand binding assay

Binding of MK886 to PPARs was determined using the coactivator-dependent receptor ligand assay (CARLA) [31]. A construct containing the PPARα ligand binding domain cloned in frame with glutathione S-transferase (GST) was procured from Walter Wahli (University of Lausanne, Lausanne, Switzerland). The GST–PPAR ligand binding domain fusion protein was expressed in *Escherichia coli* BL2 DE3 (pLysS). Bacterial pellets containing the fusion protein were resuspended in 10 ml of lysis buffer [PBS containing 1 % (v/v) Triton X-100 and 0.5 mM PMSF] and lysed by repeated freeze–thawing. DNA and insoluble matter were removed by centrifugation at 4500 ***g***. The fusion protein was purified using GSH–Sepharose beads at 4 °C, washed three times in lysis buffer, and equilibrated in 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40 and 1 mM dithiothreitol (DTT) supplemented with 1 % (w/v) dry milk. The amount of protein used per reaction was 1–3 μg. The beads were incubated with different concentrations of MK886 and ^{35}S-radiolabelled steroid receptor coactivator-1 (SRC-1) prepared with a pSG5 plasmid construct that can express SRC-1 (procured from Walter Wahli) *in vitro* using a coupled transcription/translation rabbit reticulocyte lysate system (Promega). Labelling was achieved by incubating for 1 h in the presence of [^{35}S]methionine (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), and the beads were recovered by centrifugation. The beads were then washed and analysed for interaction with SRC-1 using SDS/PAGE (see [32] for a scheme of this procedure). Coomassie Brilliant Blue staining of GST–ligand binding domain fusions allowed standardization between different reactions. The SRC-1 protein complex was visualized by autoradiography. Relative band densities were determined using a scanned image and UN-SCAN-IT software.

Binding of arachidonic acid to PPAR

GST–PPARα and GST–PPARγ ligand binding domain fusion proteins were expressed in *E. coli* as described above. Bacterial pellets containing the fusion proteins were resuspended in PBS (pH 7.3), supplemented with 1 mM PMSF, 10 mM DTT, 100 mM $MgCl_2$, 0.5 mg/ml lysozyme and 1.7 units/ml DNase I. The cell suspension was incubated for 1 h at 4 °C, and cell debris was removed by centrifugation at 47000 ***g*** for 20 min. The fusion protein was purified using GSH–Sepharose beads at 4 °C. The columns were washed three times with PBS and eluted with 50 mM Tris/HCl (pH 8.0) containing 20 mM GSH.

GST–PPARα or GST–PPARγ fusion proteins (10 μg), or GST alone as a control, were incubated at room temperature for 30 min in buffer containing 10 mM Tris/HCl (pH 8.0), 50 mM KCl, 10 mM DTT and [^{3}H]arachidonic acid in the presence or absence of MK886. Bound radioactivity was separated from free radioactivity by filtering through a Microcon filter device (Millipore). Bound radioactivity was quantified by liquid-scintillation counting.

Measurements of apoptosis

Phosphatidylserine externalization on the plasma membrane was measured by binding of annexin V–FITC to phosphatidylserine in the presence of Ca^{2+} (Immunotech, Miami, FL, U.S.A.). Cells were also stained with propidium iodide (100 μg/ml) to distinguish between necrotic and non-necrotic cell populations. The cells were analysed using a Coulter EPICS-XL flow cytometer equipped with an argon laser. The fluorescence for both annexin V–FITC and propidium iodide was measured on 10000 cells, in order to determine the percentage of apoptotic cells.

RESULTS

The ability of MK886 to affect PPAR-α, -β and -γ activity was assessed using transient transfection reporter assays in CV-1 and keratinocyte 308 cell lines, and a stable transfection system in CV-1 cells. In all systems examined, 10 μM MK886 was able to inhibit Wy14,643 activation of PPARα by approx. 80% (Table 1). A dose–response study in keratinocyte 308 cells showed inhibition of 30 ± 5, 65 ± 7 and 70 ± 8% at doses of 0.5, 1 and 5 μM MK886 respectively ($n = 9$). At doses between 10 and 20 μM, PPARα reporter assay activity levels were actually below the basal activity recorded in non-treated controls, suggesting inhibition of the endogenous PPARα. At doses over 20 μM, toxicity of MK886 precluded any useful measurements.

MK886 also decreased PPARα activation by fatty acids in the stable transfection system (results not shown), indicating that its effect is not specific to activation by Wy14,643. Effects of MK886 on PPARβ activated with bezafibrate were substantial (48% inhibition) using the stable transfection assay in CV-1 cells, but were not evident using the transient transfection reporter assay in CV-1 cells or keratinocytes (Table 1), perhaps because of low reporter activity. Inhibition by MK886 of PPARγ activated with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was also substantially lower than that seen with PPARα. The large standard error with PPARγ in the stable transfection system is a consequence of the very low activation achieved.

The effect of MK886 on PPARα was also investigated in A549 human lung adenocarcinoma cells. There was no enhancement in reporter activity with activators of PPARβ and PPARγ using this cell line As a result, it was not feasible to look at the effect of MK886 on PPARβ or PPARγ in this system. However, 20 μM MK886 did inhibit PPARα by 73% relative to levels seen following activation with 100 μM Wy14,643 (Table 2). Similar levels of inhibition were seen if activation was achieved with 50 μM Wy14,643 (results not shown).

MK886 (20 μM) diminished PPARα-driven reporter activity levels in A549 cells without activation by Wy14,643 or exogenously transfected PPARα by 47% relative to vehicle-treated controls (Table 2). This illustrates the high endogenous activity of PPARα in this cell line and suggests a lack of any agonist activity by MK886. In addition, in contrast with the full-length PPAR reporter systems, the fact that the PPAR–GAL4 chimaera reporter system does not require the 9-*cis* retinoic acid receptor (RXR) [33], but is still inhibited by MK886 (Figure 1), indicates that this compound acts directly on PPARs to inhibit PP activity. The effect of RXR in a similar reporter assay has not been examined. However, based on the similarity in concentration of MK886 required to decrease PPAR activity in several assays,

Table 1 Inhibition of PPAR activation by MK886

Data are expressed as the mean percentages of inhibition relative to the activity seen in control cells treated with activator alone ($\pm$S.E.M., n = three separate experiments except where noted). Wy14,643 (10 μM), bezafibrate (50 μM) or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (10 μM) were used to activate PPAR-α, -β or -γ respectively. Cells were treated with 10 μM MK886, 6 h before harvesting and luciferase measurements.

	Inhibition (%)		
Receptor subtype	CV-1 cells (stable)*	CV-1 cells (transient)†	Keratinocyte 308†
PPARα	80 ± 1	83 ± 9	76 ± 14 ($n = 9$)
PPARβ	48 ± 4	0	no effect ($n = 1$)
PPARγ	5 ± 30	22 ± 6	no effect ($n = 1$)

* PPAR activity was determined in cells stably transfected with full-length PPAR-α, -β or -γ cDNA and pSV-GL2-PPRE-luciferase as described in the Materials and methods section.

† Cells, at 70% confluence, were transiently transfected with pSG5-PPAR-α, -β or -γ expression vectors, 0.25 μg/ml CMV-βGal and $PPRE_3$-TK-LUC for 5 h as described in the Materials and methods section. The transfection mixture was replaced with original medium containing each PPAR activator $\pm$ MK886 (10 μM). After 6 h cells were harvested. Light units from luciferase activity were determined and normalized to β-galactosidase activity and to protein concentration.

Table 2 Effect of MK886 on PPARα activation in A549 cells

Data are expressed as the mean percentages of activity relative to that seen in vehicle-treated control cells ($\pm$ S.E.M., n = three separate experiments). A549 cells, at 70% confluence, were transiently transfected with 1 μg/ml $PPRE_3$-TK-LUC and 0.25 μg/ml CMV-βGal for 5 h. The transfection mixture was replaced with original medium containing 100 μM Wy14,643 $\pm$ 20 μM MK886. After 6 h cells were harvested. Light units from luciferase activity were determined and normalized to β-galactosidase activity and to protein concentration.

Treatment	Activity (%)
Control	100 ± 1
Wy14,643	198 ± 16
MK886	53 ± 9
Wy14,643 + MK886	70 ± 18

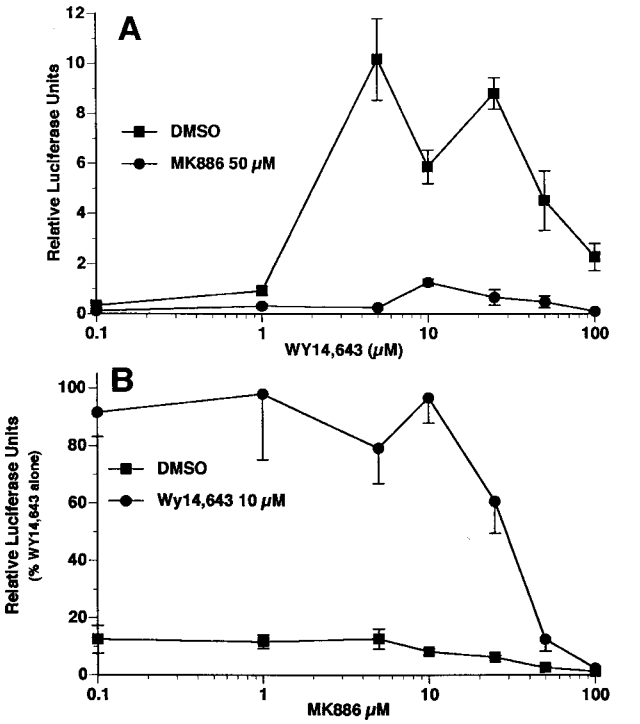

Figure 1 Effect of various doses of MK886 on PPARα activation in COS-1 cells

(**A**) Cells were co-treated with DMSO or Wy14,643 (50 μM) plus various amounts of MK886 (0.1–100 μM) 24 h after transient transfection with GAL4–PPARα, pFR-Luc and pRL as described in the Materials and methods section. After 6 h of treatment, the cells were harvested and firefly and renilla luciferase activities were assessed using the Dual Luciferase Assay System (Promega). Data are expressed as the mean percentages $\pm$ S.E.M. of the luciferase units achieved with Wy14,643 alone corrected for renilla and protein concentration (n = three to four separate experiments). (**B**) Cells were co-treated with DMSO or MK886 (25 μM) plus various amounts of Wy14,643 (0.1–100 μM) 24 h after transient transfection with GAL4–PPARα, pFR-Luc and pRL as described in the Materials and methods section. After 6 h of treatment, the cells were harvested and firefly and renilla luciferase activities were assessed using the Dual Luciferase Assay System (Promega). Data are expressed as relative luciferase units corrected for renilla and protein concentration ($\pm$S.E.M.; n = 3–4 separate experiments).

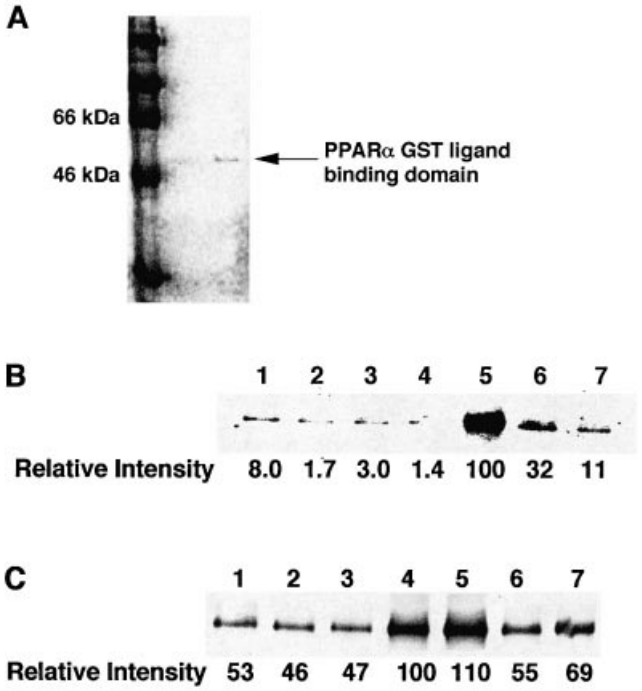

Figure 2 CARLA for PPARα

(**A**) GST–PPARα ligand binding domain fusion protein run on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue. (**B**) CARLA showing binding of SRC-1 to the PPARα ligand binding domain. Lane 1, ethanol vehicle; lane 2, DMSO vehicle; lane 3, 10 μM MK886; lane 4, 50 μM MK886; lane 5, 10 μM Wy14,643; lane 6, 10 μM Wy14,643 + 10 μM MK886; lane 7, 10 μM Wy14,643 + 50 μM MK886. Intensity values are shown relative to 10 μM Wy14,643 alone. (**C**) CARLA showing binding of SRC-1 to the PPARα ligand binding domain. Lane 1, untreated control; lane 2, DMSO vehicle; lane 3, ethanol vehicle; lane 4, 10 μM Wy14,643; lane 5: 50 μM Wy14,643; lane 6, 10 μM Wy14,643 + 10 μM MK886; lane 7, 10 μM Wy14,643 + 10 μM MK591. Intensity values are shown relative to 10 μM Wy14,643 alone.

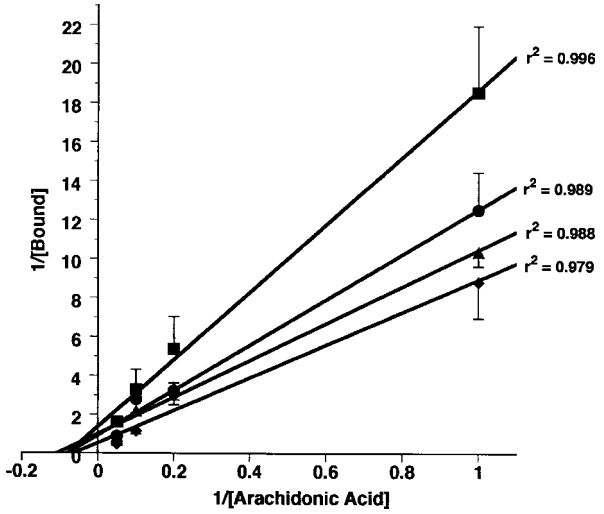

Figure 3 Double-reciprocal plot of arachidonic acid binding versus MK886 concentration

Bacterial extracts containing GST–PPARα were incubated with increasing concentrations of [^{3}H]arachidonic acid in the presence of 20 μM MK886 (■), 10 μM MK886 (●), 1 μM MK886 (▲) or vehicle (◆). Each point is the average result of binding assays performed in duplicate, and repeated three times (± S.E.M.). Lines were drawn by linear-regression analysis, and correlation coefficients are shown next to each line.

including the chimaeric receptor system, we suspect that PPAR itself and not the heterodimerization partner is the primary target.

A dose–response study examining PPARα reporter activity was performed using the full length PPARα–GAL4 chimaera transiently transfected into COS-1 cells. This reporter system was utilized because of the higher levels of Wy14,643 induction compared with PPRE-luciferase assays (20-fold compared with < 5-fold) and the fact that endogenous PPARs will not affect the results. Keeping the dose of MK886 constant at 50 μM and examining the effect of various doses of Wy14,643 at 24 h revealed complete inhibition of PPARα activation at all doses up to 100 μM (Figure 1A). Higher Wy14,643 doses were not studied, since by 100 μM some decline in activation was seen, although there was still induction over control values. These data suggest that MK886 may act in an irreversible or non-competitive manner. Maximal PPARα activation was evident between 5 and 25 μM Wy14,643 (Figure 1A). Using a fixed Wy14,643 dose of 10 μM, the data show that, in this system, 50 μM MK886 was required to completely inhibit the activation of PPARα (Figure 1B). Based on these data, an IC_{50} value of approx. 25 μM appears to exist. While this is higher than observed in the other transient reporter assay systems, these assays differ sufficiently to explain this discrepancy.

CARLA is considered semi-functional to evaluate whether a compound can bind directly to PPARs and render them functionally active [31]. In the present study, this assay was used to assess whether MK886 could directly cause this type of activation or could affect the activation induced by Wy14,643. The purity of the GST–PPARα ligand binding domain fusion protein was verified by demonstrating a single band with Coomassie Brilliant Blue staining following SDS/PAGE (Figure 2A). Because ligand binding enhances the interaction between the PPARα ligand binding domain and SRC-1, the amount of SRC-1 pulled down will be higher in the presence than in the absence of a ligand. This is evident in Figures 2(B) and 2(C) where an intense band appears following the addition of Wy14,643. No such effect was seen with up to 50 μM MK886 alone. Co-treatment with 10 μM Wy14,643 and 10 μM MK886 decreased binding, whereas the presence of 50 μM MK886 resulted in an almost complete inhibition of the PPARα–SRC-1 interaction compared with that seen with vehicle alone (Figure 2B, lanes 6 and 7 compared with lane 5). Similar results were obtained with 10 μM MK591 (Figure 2C), a more potent FLAP inhibitor [34], which is structurally analogous to MK886, containing a quinoline-based substituent at the indoyl-5 position. These data suggest that MK886 inhibits the Wy14,643-induced conformational change of PPARα, thereby inhibiting SRC-1 binding and transcriptional activity. The data also suggest that a variety of indole acetic acid derivatives have the potential to inhibit PPARs.

While CARLA clearly shows that MK886 can affect the PPARα–SRC-1 interaction, the dose–response relationships described above suggest that Wy14,643 and MK886 are not acting at the same binding site on PPARα. A non-competitive inhibitory mechanism was confirmed by examining the binding of arachidonic acid to the cloned PPARα ligand binding domain (Figure 3). The double-reciprocal binding curves meet on the *x*-axis indicating a non-competitive mechanism. The effect of MK886 on the binding of arachidonic acid to PPARγ was also examined.

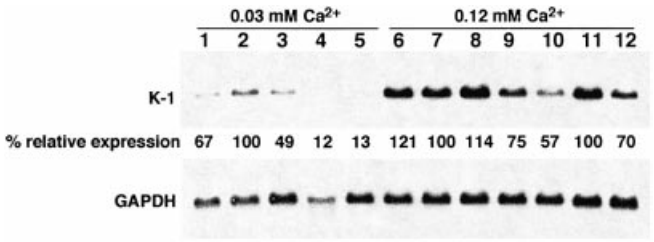

Figure 4 Keratin-1 expression in primary keratinocytes

During the experimental period, cells were incubated in the presence of either 0.03 or 0.12 mM calcium. Lanes 1 and 6, low and high calcium controls respectively; lanes 2 and 7, 0.1 μM 8-S-HETE; lanes 3 and 8, 0.1 μM 8-S-HETE + 0.5 μM MK886; lanes 4 and 9, 0.1 μM 8-S-HETE + 1 μM MK886; lanes 5 and 10, 0.1 μM 8-S-HETE + 2 μM MK886; lane 11: 0.1 μM Wy14,643; lane 12, 0.1 μM Wy14,643 + 1 μM MK886. Relative expression is expressed relative to 8-S-HETE or Wy14,643 alone and was corrected for differences in loading (determined by GAPDH mRNA levels). The blot shown is representative of four separate experiments.

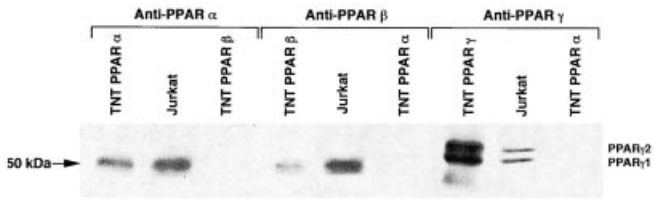

Figure 5 Protein expression of PPAR-α, -β and -γ in Jurkat cells

All three receptor types, including both γ subtypes, were evident in Jurkat cells; 50 μg of protein or 2 μl of *in vitro* translated PPARα, PPARβ or PPARγ was loaded per lane. The specificity of the antibody is shown by its strong immunoreactivity with the corresponding PPAR, but very little or no cross-reactivity with the other PPARs.

The affinity of arachidonic acid for PPARγ was only 54% of that of PPARα. As a result, 20 μM arachidonic acid was required to measure binding. At this level, 20 μM MK886 inhibited binding by approx. 40%, while lower doses had no measurable effect (results not shown). This lesser inhibition is consistent with the reporter assay results.

Functional effects caused by the inhibition of PPARα with MK886 were demonstrated by assessing the activation of keratin-1, a protein encoded by a PPARα-responsive gene, in a primary culture of mouse keratinocytes. After an initial 24 h culture period, keratinocytes were incubated with the PPARα agonists 8-S-HETE or Wy14,643, and 0.5, 1 or 2 μM MK886 for an additional 15 h in the presence of low or high levels of calcium, which causes these cells to differentiate and increases keratin-1 expression. The induction of keratin-1 was reduced to levels below those seen without any ligand treatment in the presence of 0.5–2 μM MK886 (Figure 4). Cell viability, as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide ('MTT') assay, was not affected at doses of MK886 up to 2 μM (results not shown), suggesting that the decrease in keratin-1 expression at these doses was due to down-regulation of keratin-1 expression and not to a general toxic effect of MK886.

Western-blot analyses showed that Jurkat cells expressed all three PPAR receptors, including both PPARγ subtypes (Figure 5). These cells were susceptible to MK886-induced apoptosis (Table 3). However, co-treatment with various PPAR agonists (Wy14,643 and conjugated linoleic acid for PPARα, and ciglitazone for PPARγ) did not prevent MK886-induced apoptosis (Table 3).

DISCUSSION

Nuclear hormone receptors are part of a superfamily of ligand-modulated transcription factors. These transcription factors activate various genes by binding to target DNA sequences called response elements located in the promoter regions. PPARs have been categorized into group C in subfamily 1 of the superfamily of nuclear hormone receptors [35]. PPAR binds to the DNA PPRE as a heterodimer with RXR, another member of the nuclear hormone receptor superfamily. Both phosphorylation and ligand binding then regulate receptor activity. The ligand binding induces a conformational change in the ligand binding domain allowing interaction with SRC-1 and other co-activators, further affecting transcription of various genes.

MK886 prevented PP-induced activation of PPARα in various cellular transfection systems. While this is the first report of such an effect on PPARα, the PPARγ ligand GW0072 has similar properties with that receptor, although it was described as a

Table 3 Apoptosis in Jurkat cells after treatment with MK886

Cells (10^6/ml) were treated with 10 μM MK886 and either PPARα (Wy14,643 and conjugated linoleate) or PPARγ (ciglitazone) agonists simultaneously. Apoptosis was assessed after 12 h by flow cytometry, after staining with annexin V–FITC and propidium iodide. Data are expressed as the percentages of the 10000 cells examined, that were localized to each category of viability.

	Viability			
Treatment	Live	Early apoptotic	Secondary necrotic	Necrotic
Untreated	95.8	1.1	1.8	1.3
MK886	84.6	11.1	3.8	0.6
Wy14,643 (100 μM)	94.8	2.1	2.8	0.2
MK886 + Wy14,643 (100 μM)	71.0	18.5	9.8	0.7
Wy14,643 (500 μM)	74.6	19.7	5.2	0.6
MK886 + Wy14,643 (500 μM)	78.7	8.1	12.1	1.0
Conjugated linoleate (10 μM)	96.2	1.4	1.6	0.9
MK886 + conjugated linoleate	82.9	12.3	3.7	1.1
Conjugated linoleate (50 μM)	95.2	1.9	2.0	0.9
MK886 + conjugated linoleate	82.0	13.0	4.2	0.8
Ciglitazone (10 μM)	94.6	2.0	2.5	0.8
MK886 + ciglitazone	82.0	11.4	4.4	2.2
Ciglitazone (50 μM)	90.5	4.1	3.9	1.5
MK886 + ciglitazone	75.1	14.1	8.5	2.0

partial agonist rather than as an inhibitor [36]. Furthermore, it appears that various NSAIDs can inhibit PPARβ [37].

The inhibition of PPAR activation by MK886 was quite pronounced on the α subtype, with substantially less inhibition evident with the β and γ isoforms. Given that CARLA reveals that a conformational change is not induced, it is possible that MK886 associates with components of the ligand binding site that do not induce or actually prevent conformational changes. Alternatively, MK886 may bind at a nearby site that inhibits the conformational change or prevents other ligands from accessing the conformational-sensitive site. The dose–response data from a reporter assay, as well as the lack of any agonist activity, suggested that a non-competitive mechanism was involved in the inhibition of PPARα by MK886. This suggestion was confirmed by the changes induced by MK886 in the binding curves obtained with cloned PPAR proteins and arachidonic acid.

Irrespective of the mechanism involved, it is apparent that MK886 is an effective PPARα inhibitor and can have functional effects, as demonstrated by the decreased expression of keratin-1 in keratinocytes treated with this compound. The 8-S-HETE-enhanced expression of another PPARα responsive gene, acyl-CoA oxidase, was only minimally affected in keratinocytes by MK886 (J. P. Kehrer, P. Thuillier and S. M. Fischer, unpublished work). Acyl-CoA oxidase levels are very low in keratinocytes, and the induction by 8-S-HETE was modest, suggesting that PPARα-mediated effects in this cell line are limited. It is also possible that the inability of MK886 to inhibit acyl-CoA oxidase induction reflects a redundancy in cofactor requirement for this gene that does not exist with keratin-1. This possibility is supported by the finding that acyl-CoA oxidase activity is normal in the liver of PPARα knockout mice [38].

The ligand specificity of the three PPAR isotypes is variable but, in general, includes synthetic ligands, such as the fibrate PPs and various NSAIDs, as well as natural ligands, such as polyunsaturated fatty acids and eicosanoids [35]. Interestingly, indomethacin and MK886 are structurally very similar, both being indole acetic acid derivatives. Both sulindac and indomethacin dose-dependently repress PPARβ activity in colorectal cancer cells [37]. A relationship between the repression of PPARβ and the ability of NSAIDs to inhibit tumorigenesis has been proposed [37], but alternative mechanisms remain possible.

The FLAP inhibitor MK886 is able to induce apoptosis independent of both 5-LOX [9] and FLAP [10]. Thus it is apparent that MK886 is able to activate apoptosis through actions independent of its known effects. Whether or not the inhibition of PPARα (or other PPAR subtypes) is involved in the apoptotic effect of MK886 remains unclear. Although PPARα and PPARγ agonists did not alter the apoptotic potency of MK886, a non-competitive mechanism could explain this result. Various PPAR receptor agonists can induce growth arrest and/or apoptosis in human lung cancer cells, macrophages, endothelial cells, choriocarcinoma cells, hepatoma cell lines, astrocytes and fibroblasts [23–25,39–42]. On the other hand, data are available showing that PPARα activation can prevent apoptosis [19–22], and PPARγ activation can promote tumour formation [43] in some systems. Thus while it is clear that PPARs can have effects on apoptosis, precisely how this occurs in terms of subtypes and agonist/antagonist activities remains to be determined.

FLAP is one member of a family of membrane-associated fatty-acid binding proteins, and fatty-acid transport proteins have been postulated to participate in signal transduction pathways and in fatty acid regulation of gene expression [44,45]. Some fatty-acid binding proteins also appear to be involved in cell growth and differentiation [46]. Arachidonic acid competes with a synthetic photoaffinity analogue (L-739,059) for binding to FLAP with an IC_{50} of 10–20 μM [11], interestingly close to the apoptotic dose of MK886. Higher concentrations of MK886 may displace fatty acids from FLAP and/or other membrane or cytosolic fatty-acid binding proteins. This may increase intracellular non-esterified fatty acid levels, resulting in abnormal signalling and apoptosis.

In summary, MK886 has been shown to be an effective non-competitive inhibitor of PPARα and to have functional effects on a PPAR-responsive gene. Blockage of the conformational change necessary for PPARα activation is achieved with both MK886 and the structural analogue, MK591. Given that both are indole acetic acid compounds, and that a number of NSAIDs share this structural characteristic, it is conceivable that PPAR inhibitory effects exist for additional related xenobiotics. The role of this inhibition, or effects on fatty-acid transport, in the apoptotic activity of MK886 will require further study.

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