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The nuclear receptor PPAR-α mediates the antiinflammatory actions of palmitoylethanolamide

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PPAR: peroxisome proliferator-activated receptor
PEA: palmitoylethanolamide
OEA: oleoylethanolamide
FAAH: fatty acid amide hydrolase
TPA: 12-O-tetradecanoylphorbol-13-acetate

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Abstract

Palmitoylethanolamide (PEA), the naturally occurring amide of palmitic acid and ethanolamine (Kuehl et al., 1957; Bachur et al., 1965), reduces pain and inflammation (Benvenuti et al., 1968; Mazzari et al., 1996; Calignano et al., 1998; Jaggar et al., 1998) through an as-yet-uncharacterized mechanism. Here we identify the nuclear receptor PPAR-α (peroxisome proliferator-activated receptor-α) as the molecular target responsible for the antiinflammatory properties of PEA. PEA selectively activates PPAR-α *in vitro* with a half-maximal effective concentration (EC_{50}) of 3.1 ± 0.4 μM, and induces the expression of PPAR-α mRNA when applied topically to mouse skin. In two animal models, carrageenan-induced paw edema and phorbol ester-induced ear edema, PEA attenuates inflammation in wild-type mice, but has no effect in mice deficient in PPAR-α. The natural PPAR-α agonist oleoylethanolamide (OEA) and the synthetic PPAR-α agonists GW7647 and Wy-14643 mimic these effects in a PPAR-α-dependent manner. These findings indicate that PPAR-α mediates the antiinflammatory effects of PEA and suggest that this fatty-acid ethanolamide may serve, like its analog OEA (Fu et al., 2003) as an endogenous ligand of PPAR-α.

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Introduction

PEA was identified over five decades ago (Long and Martin, 1956; Bachur et al., 1965) and shown to reduce allergic reactions and inflammation in animals (Benvenuti et al., 1968; Perlik et al., 1971), along with influenza symptoms in humans (Kahlich et al., 1979). Interest in this compound faded, however, until the discovery that one of its structural analogues, anandamide (arachidonoylethanolamide), serves as an endogenous ligand for cannabinoid receptors, the molecular target of Δ^9-tetrahydrocannabinol in marijuana (Devane et al., 1992). Since this finding, PEA has been shown to inhibit peripheral inflammation (Mazzari et al., 1996; Berdyshev et al., 1998) and mast-cell degranulation (Aloe et al., 1993), as well as to exert neuroprotective (Lambert et al., 2001) and antinociceptive (Calignano et al., 1998; Jaggar et al., 1998) effects in rats and mice. These actions are accompanied by changes in nitric oxide production (Ross et al., 2000), neutrophil influx (Farquhar-Smith and Rice, 2003) and expression of proinflammatory proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Costa et al., 2002).

Despite its potential clinical significance, the receptor(s) responsible for the analgesic and antiinflammatory properties of PEA remains unknown. The structural similarity between PEA and anandamide first suggested that both lipid mediators might target cannabinoid type-2 (CB_2) receptors. In support of this view, PEA was initially reported to displace the binding of a high-affinity cannabinoid agonist from rat basophilic leukemia cells (RBL-2H3), which were also found to express CB_2 receptor mRNA (Facci et al., 1995). However, this result has been difficult to replicate in other laboratories

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(Ross et al., 1999; Sugiura et al., 2000; Lambert et al., 2002). Moreover, blockade of CB_2 receptors with the selective antagonist SR144528 was found to prevent PEA-induced antinociception (Calignano et al., 1998; Jaggar et al., 1998; Calignano et al., 2001; Farquhar-Smith and Rice, 2001), but not the prolonged antiinflammatory effects of this fatty-acid amide (Costa et al., 2002). To reconcile these contradictory observations, it was proposed that PEA may act by inhibiting anandamide hydrolysis (Jonsson et al., 2001), which is catalyzed by the enzyme fatty-acid amide hydrolase (FAAH) (Cravatt and Lichtman, 2002). According to this hypothesis, PEA may compete with anandamide for FAAH-mediated degradation, causing an increase in tissue anandamide levels and an enhanced activation of CB_2 receptors. This model does not account, however, for the observation that PEA-induced antiinflammation is not affected by the CB_2-receptor antagonist SR144528 (Conti et al., 2002).

We have recently shown that OEA, a lipid amide structurally related to PEA, elicits satiety and stimulates lipolysis in rodents by activating the nuclear receptor PPAR-α (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzman et al., 2004). The fact that activation of PPAR-α by synthetic agonists causes profound antiinflammatory effects (Chinetti et al., 2000) prompted us to ask whether PEA might also interact with this receptor to inhibit inflammation.

Materials and Methods

Chemicals.

We prepared fatty-acid ethanolamides as described (Rodríguez de Fonseca et al., 2001). SR144528 was provided by RBI (Natick, Massachusetts) as part of the Chemical

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Synthesis Program of the National Institutes of Health (NIH). Palmitic acid was purchased from Nu-Chek Prep (Elysian, Minnesota). All other chemicals were from Tocris (Avonmouth, United Kingdom) or Sigma (St. Louis, Missouri). Fresh drug solutions were prepared immediately before use in acetone (for topical drug application) or in a vehicle of 90% sterile saline/ 5% polyethylene glycol/ 5% Tween 80 (for systemic drug injections).

Animals

We purchased male C57BL6 PPAR-$\alpha^{-/-}$ (B6.129S2-*Ppara*^{tm1Gonz}N12) and wild-type C57BL6 mice (25-30g) from Taconic (Germantown, New York). PPAR-$\alpha^{-/-}$ mice were generated in SV129 mice and backcrossed for more than 10 generations to C57BL6 mice by the vendor. The mice were maintained on a 12-h/12-h light/dark cycle with free access to water and standard chow (RMH 2500, Prolab, Framingham, Massachusetts). All procedures met the NIH guidelines for the care and use of laboratory animals, and were approved by the University of California Irvine Institutional Animal Care and Use Committee.

RNA isolation and complementary DNA synthesis

We stored tissues in RNA*Later* (Ambion, Austin, Texas), extracted total RNA with TRIzol® Reagent (Invitrogen, Carlsbad, California) and quantified it by UV spectroscopy (λ = 260 nm). We synthesized cDNA using oligo(dT) primers (Ambion) and SuperscriptII RNase H-reverse transcriptase (Invitrogen).

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Polymerase chain reaction

We performed reverse transcription of total RNA (2 μg) using Oligo(dT)$_{12-18}$ primers (0.2 μg) for 50 min at 42 °C and real-time quantitative (RTQ) PCR using an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, California). We designed primer/probe sets using Primer Express software (Applied Biosystems) and gene sequences obtained from the Genbank database. Primers and fluorogenic probes were synthesized at TIB (Berlin, Germany). The primer/probe sequences for PPAR-α were: forward (F): 5'-CTTCCCAAAGCTCCTTCAAAAA-3', reverse (R): 5'-CTGCGCATGCTCCGTG-3', probe (P): 5'-TGGTGGACCTTCGGCAGCTGG-3'. The primer/probe sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: forward (F): 5'-TCACTGGCATGGCCTTCC-3', reverse (R): 5'-GGCGGCACGTCACATCC-3', probe (P): 5'-TTCCTACCCCCAATGTGTCCGTCG-3'. RNA levels were normalized by using (GAPDH) as an internal standard, and measurements conducted as described (Fu et al., 2003).

Transactivation assays

We cultured HeLa cells in Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (10%), and transfected them using Fugene 6 reagent (3 μl, Roche, Indianapolis, Indiana) with 1μg of the luciferase reporter plasmid pFR-luc, containing a hygromycin resistance gene (Stratagene, La Jolla, California). We replaced the media after 18 h with DMEM containing hygromycin (0.1 mg-ml^{-1} ,Calbiochem, San Deigo, California) to select for cells expressing the pFR-luc plasmid.

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After 4 weeks, we isolated the surviving clones and selected a cell line that showed the highest luciferase activity.

We generated plasmids containing the ligand-binding domain of human PPAR-α (nucleotides 499–1,407), PPAR-β/δ (412–1323) or PPAR-γ (610–1518) fused to the DNA-binding domain of the yeast regulatory protein GAL4 and a neomycin resistance gene, under the control of the human cytomegalovirus promoter. We transfected HeLa cells expressing the pFR-luc plasmid with our plasmid constructs, using Fugene 6 (3 µl) reagent. We selected for stably transfected cells using G418 sulfate (0.2 mg-ml^{-1}, Calbiochem). These cells were maintained in DMEM containing hygromycin and G418.

For transactivation assays, we seeded cells in six-well plates and incubated them for 7 h in DMEM, containing appropriate concentrations of test compounds. We used a dual-luciferase reporter assay system (Promega, Madison, Wisconsin) and an MIX Microtiter plate luminometer (Dynex, Chantilly, Virginia) to determine luciferase activity in cell lysates.

TPA-induced ear edema

We dissolved 12-O-tetradecanoylphorbol-13-acetate (TPA) (0.03% weight-vol^{-1}) and drugs in acetone, and applied them topically on the ear pinna (Sheu et al., 2002). Drugs were administered twice, 45 minutes and 4 hours after TPA. 18 hours after TPA application, the mice were killed and four ear punches (diameter = 2 mm) were excised and weighed for edema measurement (Sheu et al., 2002). SR144528 was dissolved in a vehicle of 90% sterile saline/ 5% polyethylene glycol/ 5% Tween 80, and administered 30 min prior to the first drug treatment by intraperitoneal (i.p.) injection.

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Carrageenan-induced paw edema.

We initiated an inflammatory response by injecting λ-carrageenan (2% weight-vol^{-1} in sterile saline, 20 μl) subcutaneously into the hind paw using a 27-gauge needle. We administered drugs by i.p. injection 30 minutes before carrageenan and measured edema with a mouse plethysmometer (Ugo Basile, Varese, Italy).

Biochemical analyses

We measured fatty-acid ethanolamide levels by high-performance liquid chromatography coupled with mass spectrometry (LC/MS) (Fu et al., 2003).

Statistical analyses.

Results are expressed as the mean ± s.e.m. of *n* experiments. The significance of differences between groups was evaluated using Student's *t* test for within group analysis or a one-way analysis of variance followed by a Tukey's multiple comparison test or a Dunnett's post-hoc test, as appropriate. A two-way analysis of variance followed by a Bonferroni's post-hoc test was used as required. Analyses were done with GraphPad Prism software (GraphPad Software, San Diego, California).

Results

We genetically modified HeLa cells to stably express a luciferase reporter gene together with the ligand-binding domain of human PPAR-α (Willson et al., 2000) and used these cells to determine whether PEA engages this receptor. PEA activated PPAR-α with a

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EC_{50} of 3.1 ± 0.4 μM (*n* = 3-7) (Fig. 1*a*). Under identical conditions, palmitic acid (a product of PEA hydrolysis) and stearoylethanolamide (a PEA analog) were ineffective (Fig. 1a). Consistent with previous reports, two synthetic agonists of PPAR-α, GW7647 and Wy-14643 (Ren et al., 1996; Brown et al., 2001), activated the receptor with EC_{50} values of 187 ± 42 nM and 1.4 ± 0.1 μM, respectively (Fig. 1*a*). Unlike its structural analogue, OEA, which activates both PPAR-α (120 ± 1 nM) and PPAR-β/δ (1.1 ± 0.1 μM)(Fu et al., 2003), PEA selectively activates PPAR-α, as the compound failed to engage PPAR-β/δ or PPAR-γ in HeLa cells expressing these receptor isoforms (Fig. 1*b*).

If PEA activates PPAR-α, this compound should also modulate the expression of PPAR-α-regulated genes (Berger and Moller, 2002; Mandard et al., 2004). To test this possibility, we assessed the effects of PEA administration on PPAR-α mRNA levels, which are known to be upregulated by PPAR-α agonists (Fu et al., 2003). We elicited an inflammatory response in mouse abdominal skin by applying the phorbol ester TPA (Sheu et al., 2002). Topical treatment with PEA (150 nmol-cm^{-2}, 45 min and 4 hour after TPA) caused a marked elevation in skin PPAR-α mRNA levels (Fig. 2*a*), which was associated with a parallel reduction in TPA-induced edema (Fig. 2*b*). The effects of PEA were mimicked by the PPAR-α agonist GW7647 (150 nmol-cm^{-2}) (Fig. 2*a* and *b*).

To determine whether topical PEA treatment provided adequate drug dosing, we measured PEA levels by LC/MS. In untreated abdominal skin, PEA levels were 8.0 ± 1.2 nmol-g^{-1}; after application of vehicle, PEA content in abdominal skin was on average 10.4 ± 1.3 nmol-g^{-1}, while after application of PEA it was 250.2 ± 22.4 nmol-g^{-1} (untreated vs. vehicle, $P > 0.05$; vehicle vs. PEA, $P < 0.001$; *n* = 5-11; one way ANOVA, followed by a Bonferroni post hoc test). The latter value exceeds the EC_{50} of PEA for

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PPAR-α (3.1 ± 0.4 μM) by over fiftyfold, which indicates that effective tissue concentrations are reached following topical PEA administration. Interestingly, PEA treatment did not enhance anandamide levels in skin, but rather decreased them, indicating that PEA does not prevent anandamide hydrolysis *in vivo* (vehicle treated anandamide levels were 1.6 ± 0.1 pmol/g and 1.1 ± 0.1 pmol/g following PEA treatment (Student's *t* test, P <0.01, *n* = 5). The results described above suggest that PEA selectively activates PPAR-α.

To examine whether this phenomenon contributes to the antiinflammatory effects of PEA we used mice deficient in PPAR-α. In a first series of experiments, we elicited paw edema by local injection of the polysaccharide carrageenan (Conti et al., 2002). Systemic treatment with PEA (10 mg-kg^{-1}, i.p., 30 min prior to carrageenan) decreased edema in wild-type, but not in PPAR-α$^{-/-}$ mice (Fig. 3). As expected from previous results (Sheu et al., 2002), the PPAR-α agonist Wy-14643 (20 mg-kg^{-1}, i.p.) exerted similar effects, which were also abolished in mutant mice (Fig. 3*b*). In these animals, PEA caused a small increase in the size of the edema, which did not reach, however, statistical significance (Fig. 3*b*).

In a second set of experiments, we initiated an inflammatory response on the ear pinna by applying the phorbol ester TPA (Sheu et al., 2002). Topical treatment with PEA (15 nmol-cm^{-2}, 45 min and 4 h after TPA) reduced edema formation in wild-type, but not in PPAR-α$^{-/-}$ mice (Fig. 4*a*). Similar responses were noted with the natural PPAR-α agonist OEA (Fig. 4*b*), as well as with the synthetic PPAR-α agonist GW7647 (Fig. 4*c*). The latter retained, however, a weak antiinflammatory activity in mutant mice (Fig. 4*c*), which might be due to its ability to interact with PPAR-β/δ or PPAR-γ (Brown et al.,

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2001). We interpret these findings to indicate that PEA modulates inflammation by engaging PPAR-α.

Finally, we asked whether the CB_2 antagonist SR144528, which blocks PEA-induced antinociception (Calignano et al., 1998; Farquhar-Smith et al., 2002) also affects the ability of PEA to modulate inflammation. Systemic administration of a maximal dose of SR144528 (2 mg kg^{-1}, i.p) failed to inhibit the antiinflammatory effects of either PEA (Fig. 5*a*) or GW7647 (Fig. 5*b*) in the TPA model, which is suggestive of a lack of involvement of CB_2 receptors in this response.

Discussion

In the present study, we have shown that the natural fatty-acid amide, PEA, activates the nuclear receptor PPAR-α with a potency comparable to that of the synthetic agonist Wy-14643, whereas it does not engage two related PPAR isoforms, PPAR-β/δ and PPAR-γ. Relatively high concentrations of PEA (high nM to low μM) are present in many animal tissues, where this substance undergoes active biosynthesis and breakdown (Schmid et al., 1996; Cadas et al., 1997; Hansen et al., 1998). This suggests that PEA might serve, like its structural analogue OEA (Fu et al., 2003), as an endogenous activator of PPAR-α. An unequivocal demonstration of such a role will require however, further experimentation.

While the signaling functions of endogenous PEA are still hypothetical, our results clearly show that this fatty-acid amide can activate PPAR-α when it is applied as a drug, and that such activation underlies its ability to inhibit the inflammatory responses induced in mice by TPA and carrageenan. We found, indeed, that the antiinflammatory

actions of PEA are absent in PPAR-$\alpha^{-/-}$ mice and are mimicked by three structurally distinct PPAR-α agonists: OEA, GW7647 and Wy-14643. Our findings are consistent with a growing body of literature, which indicates a role for PPAR-α in the modulation of inflammatory processes. For example, it has been shown that PPAR-$\alpha^{-/-}$ mice display longer inflammatory responses (Devchand et al., 1996) and that synthetic PPAR-α agonists reduce peripheral inflammation in a PPAR-α-dependent manner (Sheu et al., 2002). Our results also rule out the possibility that PEA acts by blocking anandamide hydrolysis to increase the levels of this endocannabinoid compound ('entourage effect')(Jonsson et al., 2001). We found in fact that topical PEA treatment decreases anandamide levels in skin, which is suggestive that PEA interferes with anandamide synthesis rather than anandamide degrading pathways.

Previous studies have reported that the CB_2 antagonist SR144528 prevents the antinociceptive actions of PEA, suggesting that either CB_2 receptors or an as-yet unidentified SR144528-sensitive target contributes to such actions. Here, we have confirmed previous reports showing that SR144528 does not interfere with the ability of PEA to inhibit inflammation (Costa et al., 2002). A more thorough investigation of the target selectivity of SR144528 and of the possible relationship between PPAR-α and CB_2 receptors is needed to reconcile these apparently conflicting observations.

In conclusion, our results indicate that PEA reduces inflammatory responses by engaging the nuclear receptor PPAR-α. These findings provide a framework to understand the biological functions of this naturally occurring fatty-acid ethanolamide and may help design novel PPAR-α agonists with improved potency and target selectivity.

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Legends for Figures

Fig. 1. PEA activates PPAR-α *in vitro*. a, Effects of various agents on human PPAR-α activation in HeLa cells; PEA (closed circles), GW7647 (closed squares),Wy-14643 (open squares), palmitic acid (open circles) and stearoylethanolamide (closed triangles). **b**, Effects of PEA on activation of PPAR-α (closed circles), PPAR-β/δ (open circles) and PPAR-γ (closed squares) (n = 12).

Fig. 2. PEA activates PPAR-α *in vivo*. Effects of topical PEA or GW7647 (each at 150 nmol-cm^{-2}, 45 min and 4 h after TPA) on (**a**) expression of PPAR-α mRNA (left), normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (right) and (**b*)***, TPA-induced abdominal edema in wild-type C57BL6 mice (n = 4-5). V, vehicle; UN, untreated. *, $p < 0.05$, **, $p < 0.01$.

Fig. 3. PPAR-α is required for the anti-inflammatory actions of PEA: paw edema model. Effects of vehicle (open circles), PEA (closed circles; 10 mg-kg^{-1}, i.p.) or Wy-14643 (closed squares; 20 mg-kg^{-1}, i.p.) on carrageenan-induced paw edema in (**a**) wild-type C57BL6 (+/+) or (**b**) PPAR-α (–/–) mice (n = 5-6). V, vehicle (90% sterile saline/ 5% PEG/ 5% Tween 80). *, $p < 0.05$ vs. V, **, $p < 0.01$ vs. V.

Fig. 4. PPAR-α is required for the antiinflammatory actions of PEA: ear edema model. Effects of (**a**) PEA, (**b**) OEA or (**c**) GW7647 (each at 15 nmol-cm^{-2}, 45 min and 4 h after TPA) on TPA-induced ear edema in wild-type (+/+) and PPAR-α (-/-) mice (n =

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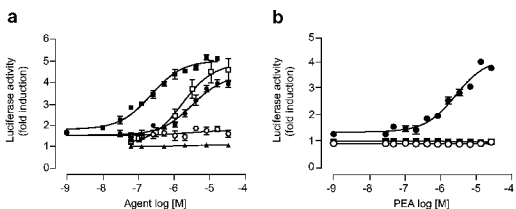
3-16). V, vehicle (acetone) *, $p < 0.05$, **, $p < 0.01$, vs. V. ††, $p < 0.01$ vs. GW7647 in wild-type mice.

Fig. 5. Effects of the CB_2 receptor antagonist SR144528 on PEA and GW7647. Effects of SR144528 (SR2, 2 mg kg^{-1}, i.p.) or vehicle (V, 90% saline/5% PEG/5% Tween 80), 30 minutes prior to (**a**) PEA or (**b**) GW7647 (each at 15 nmol-cm^2, 45 min and 4 h after TPA), following TPA-induced ear edema in wild-type mice (n = 4-13). V, vehicle *, $p < 0.05$ vs. V, **, $p < 0.01$ vs. V, ***, $p < 0.001$ vs. V †††, $p < 0.001$ vs. SR144528 alone.

Figure 1


a
b
Luciferase activity
(fold induction)
Agent log [M]
PEA log [M]
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1
2
3
4
5
6
-9
-8
-7
-6
-5
-4

Figure 2

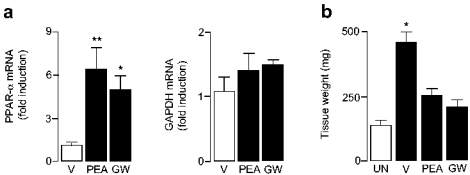
a
PPARα mRNA (fold induction)
9
6
3
0
**
*
V
PEA
GW
GAPDH mRNA (fold induction)
2
1
0
V
PEA
GW
b
Tissue weight (mg)
500
250
0
*
UN
V
PEA
GW

Figure 3

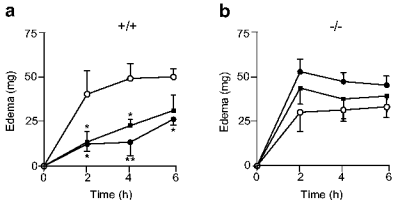

Figure 4

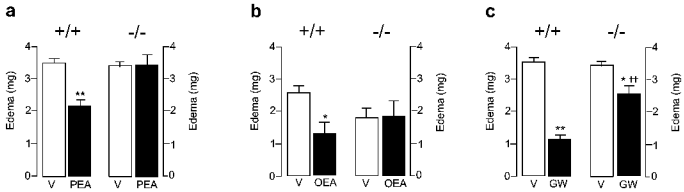

Figure 5