Inhibition of peroxisome proliferator-activated receptor (PPAR)-mediated keratinocyte differentiation by lipoxygenase inhibitors

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Lipoxygenase (LOX) metabolites from arachidonic acid and linoleic acid have been implicated in atherosclerosis, inflammation, keratinocyte differentiation and tumour progression. We previously showed that peroxisome proliferator-activated receptors (PPARs) play a role in keratinocyte differentiation and that the PPARα ligand 8*S*-hydroxyeicosatetraenoic acid is important in this process. We hypothesized that blocking LOX activity would block PPAR-mediated keratinocyte differentiation. Three LOX inhibitors, nordihydroguaiaretic acid, quercetin and morin, were studied for their effects on primary keratinocyte differentiation and PPAR activity. All three LOX inhibitors blocked calcium-induced expression of the differentiation marker keratin 1. In addition, activity of a PPAR-responsive element was inhibited in the presence of all three inhibitors, and this effect was mediated primarily through PPARα and PPARγ. LOX inhibitors decreased the activity of a chimaeric PPAR-Gal4-ligand-binding domain reporter system and this effect was reversed by addition of PPAR ligands. Ligand-binding studies revealed that the LOX inhibitors bind directly to PPARs and demonstrate a novel mechanism for these inhibitors in altering PPAR-mediated gene expression.

Key words: eicosanoid, flavonoid, nuclear receptor, skin.

INTRODUCTION

Arachidonic acid is a fatty acid and component of lipid membranes and a major substrate for lipoxygenase (LOX) enzymes. LOXs are enzymes that convert primarily arachidonic acid into hydroxyeicosatetraenoic acids (HETEs) or leukotrienes [1,2]. Increasing evidence suggests that LOX-catalysed metabolites play a role in inflammation (including allergic rhinitis, colitis, inflammatory bowel disease, bronchial asthma, rheumatoid arthritis, glomerulonephritis and psoriasis) [3] and have a profound influence on the development and progression of cancers [1,3], including skin cancers [4]. Some of the better-characterized biological activities of LOX products include neoplastic cell growth [5], growth factor [6] and transcription factor [7] activation, oncogene induction [8], stimulation of cell adhesion [9] and regulation of apoptotic cell death [10]. LOX metabolites are also involved in proliferation, differentiation and inflammation in mouse skin [11–13]. These effects appear to be mediated primarily by leukotrienes and HETEs although their mechanism of action remains unclear.

HETEs have recently been shown to be ligands for a family of nuclear receptors, the peroxisome proliferator-activated receptors (PPARs) [14–16]. PPARs regulate adipocyte differentiation and lipid homoeostasis [17]. PPARs have also been implicated in cancer, in some cases promoting progression, as in colon cancer [18], and in others preventing it, as in breast cancer [19]. Recent reports suggest a role for PPARs in inflammation, proliferation and differentiation [17]. The mechanisms by which PPARs modulate these processes require ligand activation, heterodimerization with the retinoic X receptor α (RXRα) and binding to a specific DNA response element (PPAR-responsive element, PPRE). Despite the availability of many pharmacological PPAR ligands, very few endogenous ligands have been shown to activate PPARs *in vivo*. Some of the most promising natural PPAR ligands are the LOX products derived from arachidonic acid and linoleic acid. Those metabolites that have been best studied include leukotriene B4, 15Δprostaglandin J2, and 5-, 8-, 12- and 15*S*-HETE [14,20–22].

We recently demonstrated [23] that 8*S*-HETE induced keratinocyte differentiation by activation of PPARα. Other PPARα ligands had the same effect on murine keratinocytes and similar results were reported with human keratinocytes, further supporting a role for PPARα in keratinocyte differentiation [23,24]. In addition, 8-LOX transgenic mice have an increase in expression of the differentiation marker keratin 1 (K1) and display significant hyperkeratosis [23]. These studies demonstrated that LOX products not only induced keratinocyte differentiation, but also this effect was mediated through PPARα. In addition, the effect of 8*S*-HETE and PPARα on keratinocyte differentiation can be blocked by the five lipoxygenase-activating protein (FLAP) inhibitor MK886, a novel PPARα inhibitor [25]. Although the properties of LOX inhibitors have been well characterized and range from antioxidant to chemopreventive, there is no information regarding their effect on primary keratinocyte differentiation. Hence, we hypothesized that blocking LOX activity would abrogate PPAR-mediated differentiation of keratinocytes.

Abbreviations used: FLAP, five lipoxygenase-activated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HSD, honestly significant difference; K1, keratin 1; LBD, ligand-binding domain; LOX, lipoxygenase; NDGA, nordihydroguaiaretic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-responsive element; RXR, retinoic X receptor; SPA, scintillation proximity assay; Wy14,643, 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid.

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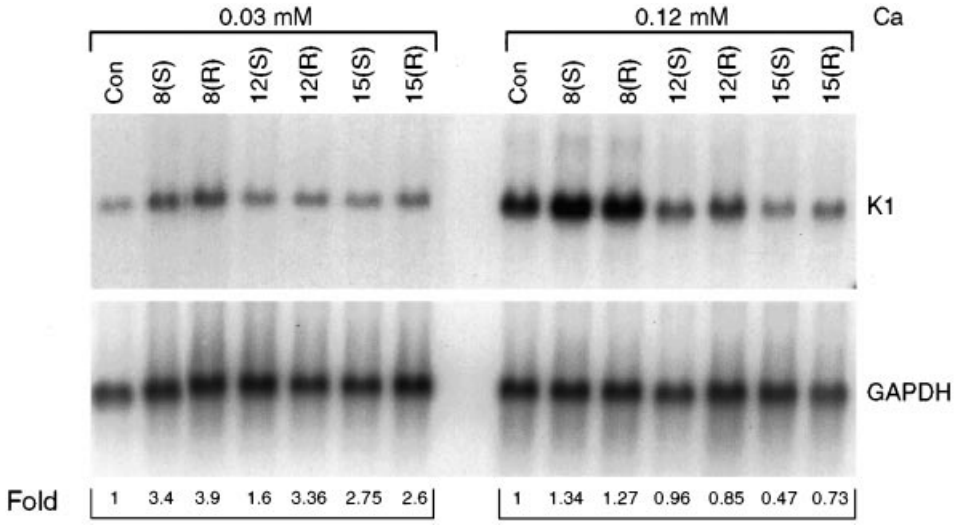


Figure 1 Effect of HETEs on keratinocyte differentiation

Primary keratinocytes were treated with 1 μM of one of the six HETEs (8*S*, 8*R*, 12*S*, 12*R*, 15*S* or 15*R*) in low- (0.03 mM) or high- (0.12 mM) calcium media for 18 h. Total RNA was harvested and fractionated on 1% agarose/formaldehyde gel and blotted on to a nitrocellulose membrane. Membranes were hybridized with ^{32}P-radiolabelled probe specific for K1 or GAPDH. The Northern blot shown is a representative from three different experiments. The numbers below show the relative fold induction of K1 expression corrected for GAPDH and compared with untreated cells (Con).

In the present study, we chose three natural compounds for their demonstrated ability to block LOX activity. Nordihydroguaiaretic acid (NDGA), which occurs in the resinous exudates of many plants, is used as an antioxidant in fats and oils [26]. Two flavonoids, quercetin and morin, have also been widely used as LOX inhibitors [27]. Our results suggest that these LOX inhibitors can inhibit keratinocyte differentiation and that this effect is mediated through PPARα and PPARγ more effectively than PPARδ. Surprisingly, we found that these inhibitors can interact directly with PPARs and appear to compete with ligands for binding. These results also suggest that this inhibition can affect the expression of PPAR-regulated genes. This study demonstrates a novel role for LOX inhibitors as direct effectors of PPAR-mediated gene regulation.

MATERIALS AND METHODS

Cell culture

Murine epidermal keratinocytes were harvested from newborn SENCAR (SSIN) mice by trypsinization as described previously [28] and plated at 10^6 cells per 35 mm dish in an enriched Waymouth's media (1.2 mM calcium) 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 keratinocyte growth medium ('KGM') (0.05 mM calcium) medium (ClonTech, Palo Alto, CA, U.S.A.). The cells were then grown for another 24 h prior to treatment or transfection. The cells were used for transfection when they reached 70% confluence.

Western blots

Total protein was isolated from primary keratinocytes as described previously [33]. Protein immobilized on PVDF membranes (Pierce, Rockford, IL, U.S.A.) was detected by chemiluminescence following incubation with anti-PPARα, anti-PPARδ (both 1:500; Affinity Bioreagents, Golden, CO, U.S.A.), anti-PPARγ (1:2000) or anti-RXRα (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibodies.

Chemicals and treatments

MK886 was obtained from Biomol Research (Plymouth Meeting, PA, U.S.A.). NDGA, morin and quercetin were purchased from Sigma (St. Louis, MO, U.S.A.). Troglitazone was a gift from Warner–Lambert (Ann Arbor, MI, U.S.A.). Wy14,643 [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid], > 98% pure, was purchased from Chemsyn Science Laboratories (Lenexa, KS, U.S.A.). 8*S*-HETE was obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.), and 8*S*-, 8*R*-, 12*S*-, 12*R*-, 15*S*- and 15*R*-HETE were purified as described previously [29]. All chemicals were dissolved in DMSO or methanol and treatments were added directly to cell culture media. The final concentration of vehicle in the media was 0.1% (v/v) and appropriate vehicle controls were used in all cases.

Northern blots

Total RNA was isolated from primary keratinocytes using the Tri-Reagent method (MRC, Cincinnati, OH, U.S.A.). RNA (10 μg) was fractionated through a 1% agarose/6% formaldehyde/ethidium bromide-stained gel, blotted on to a nylon membrane and hybridized with K1 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 is 0.15 M NaCl/15 mM sodium citrate) at room temperature and once for 30 min with 0.1% SDS/0.1 × SSC at 60 °C and exposed to X-ray film overnight at −80 °C.

PPAR reporter assays

Primary keratinocytes were plated in 35 mm dishes at 1.5×10^6 cells/dish. To test for PPRE activity, cells were trans-

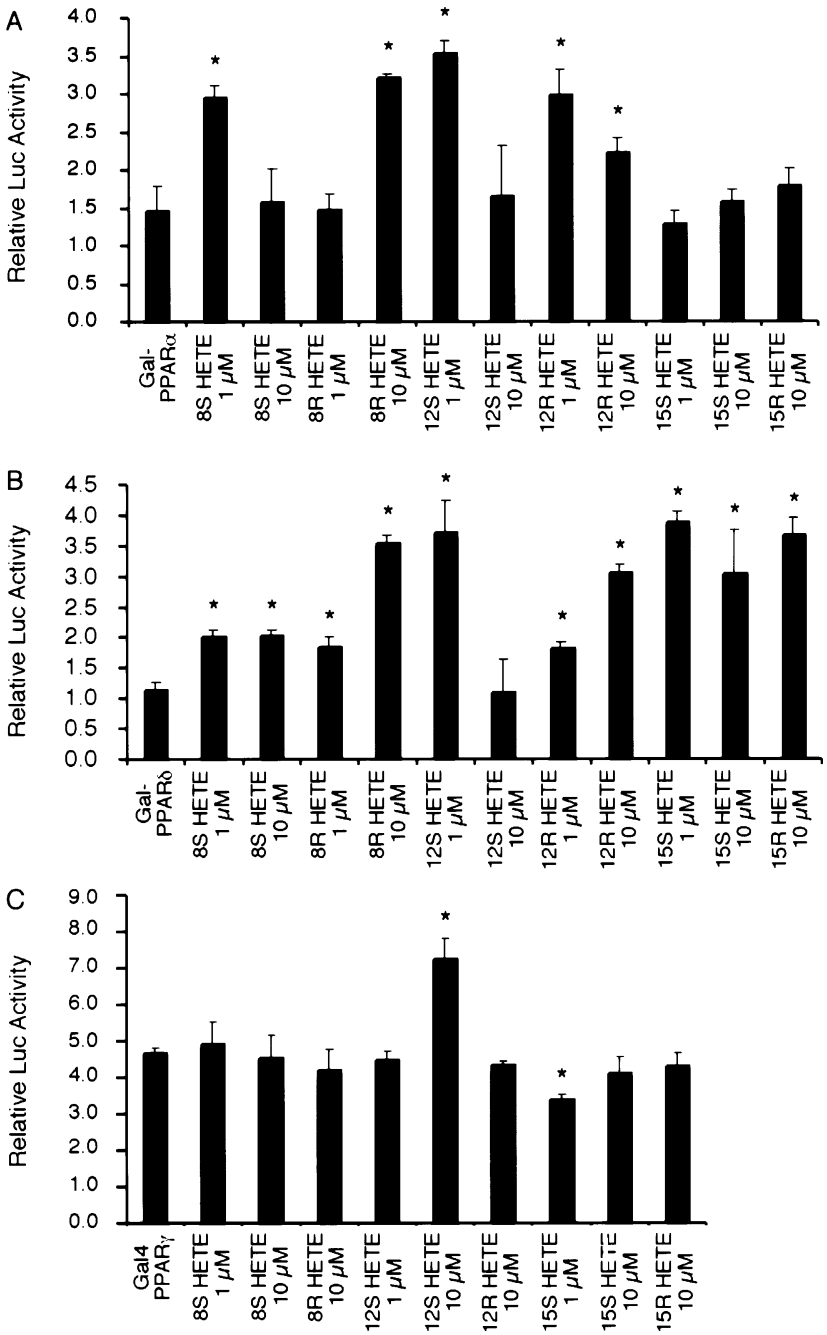


Figure 2 Affinity of 8-, 12- and 15-HETE for the LBD of PPAR-α, δ and γ

Primary keratinocytes were transfected with 0.5 μg of a MH-100-TK-Luc reporter plasmid, 0.125 μg of a β-galactosidase plasmid and with 0.5 μg of one of the three plasmids Gal4-PPARα-LBD (**A**), Gal4-PPARδ-LBD (**B**) or Gal4-PPARγ-LBD (**C**), and treated for 18 h with one of the six HETEs (8*S*, 8*R*, 12*S*, 12*R*, 15*S* or 15*R*). Relative luciferase (Luc) activity was determined and corrected for efficiency with β-galactosidase activity. *Data were analysed by ANOVA (Tukey's HSD) with $P < 0.05$ for all treatment groups from 3–5 individual experiments.

fected with 1 μg/dish of the luciferase reporter construct $PPRE_3$-TK-LUC [22] (generously given by Dr R. M. Evans, The Salk Institute, San Diego, CA, U.S.A.), and 0.125 μg/dish CMV-βGal and 3 μl of Fugene 6/2 μg of DNA (Roche Diagnostics) for 5 h. To test for specific PPAR ligand-binding activity, cells were transfected with 1 μg/dish of the luciferase reporter construct MH-TK-LUC [22], in the presence of Gal4-PPARα-LBD, Gal4-PPARδ-LBD or Gal4-PPARγ-LBD (where LBD is ligand-binding domain; generously given by Dr R. M. Evans) and 0.125 μg/dish CMV-βGal and 3 μl of Fugene 6/2 μg of DNA for 5 h. Wy14,643 (10 μM) or 8*S*-HETE (1 μM), bezafibrate (25 μM) or troglitazone (10 μM) were then added directly to each dish to activate PPARα, δ or γ respectively, in the presence or absence of NDGA, quercetin or morin. After 6 h, cells were harvested in luciferase lysis buffer (Tropix, Bedford, MA, U.S.A.). Relative light units from firefly luciferase activity were determined using a luminometer and normalized to the relative β-galactosidase activity using the β-galactosidase kit (Tropix) and to the relative

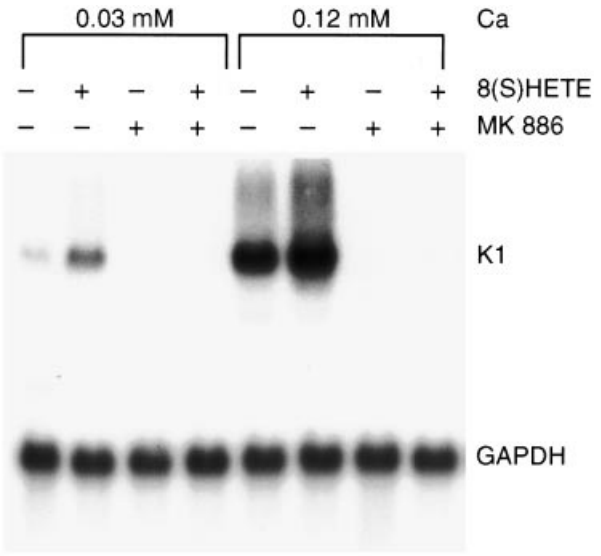


Figure 3 The PPAR inhibitor MK886 blocks 8*S*-HETE induction of keratinocyte differentiation

Primary keratinocytes were treated with 1 μM 8*S*-HETE in either low-calcium (0.03 mM) or high-calcium (0.12 mM) media with or without 1 μM MK886 for 18 h. Total RNA was harvested and fractionated on 1% agarose/formaldehyde gel and blotted on to a nitrocellulose membrane. Membranes were hybridized with ^{32}P-radiolabelled probe specific for K1 or GAPDH. The Northern blot shown is a representative from three different experiments.

protein concentration determined using the bicinchoninic acid ('BCA') kit (Pierce). Activation of PPARα was achieved with 1 μM Wy14,643 and LOX inhibitors were present for 6 h and up to 24 h prior to harvesting and analyses. Data were analysed by one-way ANOVA with Tukey's honestly significant difference (HSD) *post-hoc* test to compare means (SPSS Mac V.10; SPSS, Chicago, IL, U.S.A.).

Binding assay

Scintillation proximity assays (SPAs) were performed as described previously for PPARγ [31]. In brief, the PPARα, δ and γ LBDs were expressed in *Escherichia coli* as polyhistidine-tagged fusion proteins. The proteins were purified, biotinylated and immobilized on streptavidin-modified SPA beads. [^{3}H]-GW362433X was used as radioligand for PPARα and δ, and [^{3}H]rosiglitazone was used as radioligand for determination of binding to PPARγ. The buffer for all assays was 50 mM Hepes (pH 7), 50 mM KCl, 5 mM CHAPS, 0.1 mg/mg BSA and 10 mM dithiothreitol. Samples were incubated with increasing concentrations of NDGA, quercetin or morin for at least 1 h at room temperature, and bound radioactivity for each well was determined in a Wallac 1450 Microbeta counter (Turku, Finland) and expressed as a percentage of the control (radioligand only).

Measurement of cell viability by MTT cell proliferation assay

Primary keratinocytes were treated with increasing doses of NDGA, quercetin or morin for 6 h or 24 h or with vehicle alone. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl Blue; Sigma] was added to each well to a final concentration of 0.5 mg/ml and cells were incubated for another 3 h at culture conditions described above. After removing the media, cells were incubated for 10 min in 200 μl of DMSO and

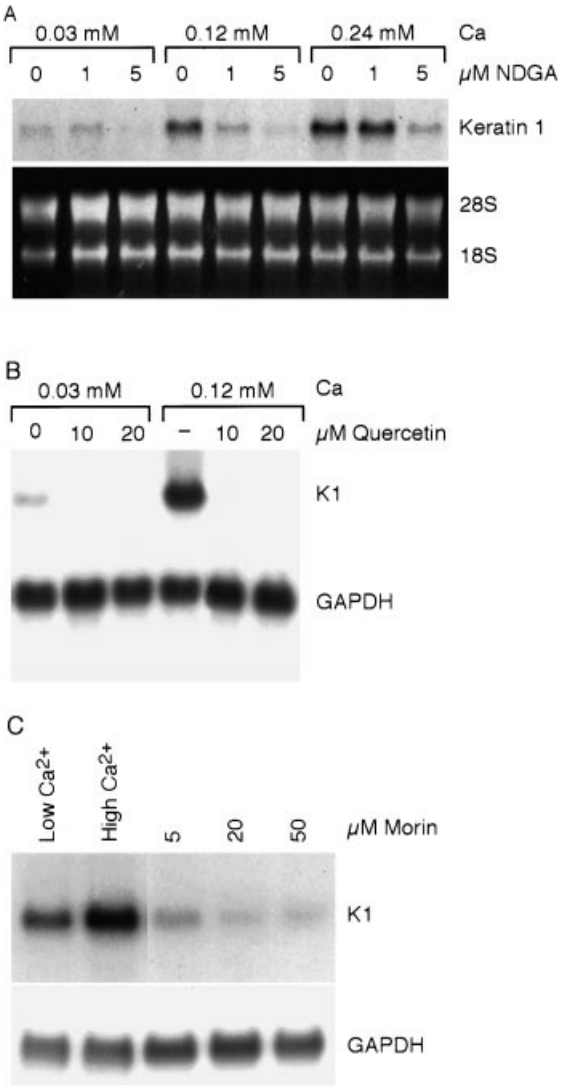


Figure 4 LOX inhibitors block K1 expression

Primary keratinocytes were treated with either low-calcium (0.03 mM) or high-calcium (0.12 or 0.24 mM) media and with 0, 1 or 5 μM NDGA (**A**), 0, 10 or 20 μM quercetin (**B**) or 0, 5, 20 or 50 μM morin (**C**) for 18 h. Total RNA was harvested and fractionated on 1% agarose/formaldehyde gel and blotted on to a nitrocellulose membrane. Membranes were hybridized with ^{32}P-radiolabelled probe specific for K1 or GAPDH. The Northern blot shown is a representative from three different experiments.

subsequently solubilized by trituration. Absorbance of the solution was determined on a microplate reader at 570 nm after subtracting for background, according to the manufacturer's instructions.

Inhibition of LOX activity

Primary keratinocytes (5×10^6 cells/sample) were trypsinized, centrifuged, washed in PBS and resuspended in 500 μl of PBS containing 1 mM $CaCl_2$. Cells were incubated for 2 min at 37 °C and another 5 min with or without inhibitors. Cells were incubated for another 10 min after addition of 2.5 μl of 1 mM A23187 (Sigma) and for a further 10 min following addition of 7.5 μl of 10 mM arachidonic acid in ethanol (Sigma). Samples were placed on ice and the pH was reduced to 3 by addition of 1 M citric

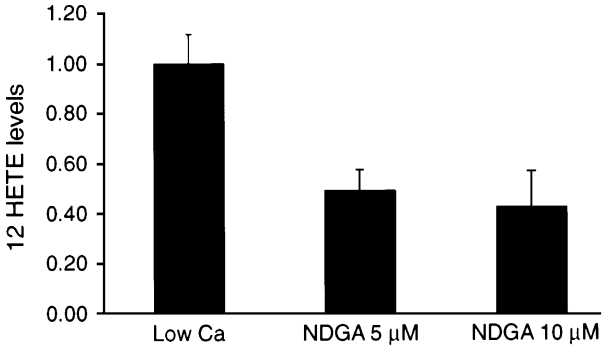


Figure 5 Effect of NDGA on LOX activity

Primary keratinocytes in low-calcium media (Low Ca) were treated with vehicle or with 5 or 10 μM NDGA. Total production of 12*S*-HETE was measured as described in the Materials and methods section and expressed as ng/5 $\times$ 10^6 cells. Data were analysed by ANOVA (Tukey's HSD) with $P < 0.01$ for both NDGA groups from different experiments.

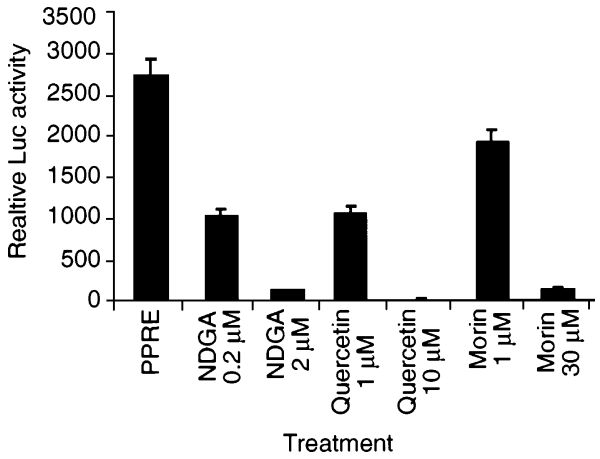


Figure 6 LOX inhibitors block PPRE

Primary keratinocytes were transfected with 0.5 μg of a PPRE-Luc reporter plasmid and 0.125 μg of a β-galactosidase plasmid and treated for 18 h with 0, 0.2 or 2 μM NDGA, 1 or 10 μM quercetin or 1 or 30 μM morin. Relative luciferase (Luc) activity was determined and corrected for efficiency with β-galactosidase activity. Data were analysed by ANOVA (Tukey's HSD) with $P < 0.01$ for all treatment groups from three individual experiments.

acid (cell suspensions at 80 μl/ml of media) and 10 % butylated hydroxytoluene at 10 μl/ml of media were added. 12*S*-HETE-d8 (20 μl; 100 ng/ml; Cayman Chemicals) was then added (as an internal standard) and cells were extracted three times with 2 ml of 1:1 hexane/ethyl acetate. Samples were dried under nitrogen gas and reconstituted in 200 μl of methanol/10 mM ammonium acetate, pH 8.5 (70:30, v/v). The levels of eicosanoids were determined by liquid chromatography-tandem MS using the modified method of Kempen et al. [32]. Briefly, instrumentation consisted of a Quattro Ultima tandem mass spectrometer (Micromass, Beverly, MA, U.S.A.) equipped with an Agilent HP1100 binary pump HPLC inlet. Eicosanoid metabolites were separated using a Luna phenyl-hexyl 2.0 mm $\times$ 150 mm analytical column (3 μm; Phenomenex, Torrance, CA, U.S.A.). Mobile phase consisted of 10 mM ammonium acetate, pH 8.5 (buffer A) and methanol (buffer B). Flow rate was 300 μl/min and sample injection volume was 25 μl. The mass spectrometer was operated in electrospray negative-ion mode with a cone voltage of 100 V, source temperature was 120 °C and collision cell pressure was 2.1×10^{-3} Torr using argon as collision gas. The collision energy was 19 V.

RESULTS

We determined the effect of the most naturally abundant HETEs in murine skin [4] on primary keratinocyte differentiation by measuring the levels of expression of the early marker of keratinocyte differentiation, K1. Under low-calcium conditions (0.03 mM), K1 was expressed at very low levels and was induced by increasing calcium levels in the media (Figure 1). Addition of 8*S*-HETE to the cells resulted in a 3–4-fold and approx. 1.5-fold increase in K1 mRNA levels in low- and high-calcium-treated cells respectively. The same effect was seen with 8*R*-HETE as well as with 12*S*-, 12*R*-, 15*S*- and 15*R*-HETE in low-calcium medium. In contrast, 12*S*-, 12*R*-, 15*S*- and 15*R*-HETE did not enhance differentiation in high-calcium media. Also, 12- and 15-HETE decreased K1 expression at 0.12 mM calcium, through unknown mechanisms.

We reported previously [23] that 8*S*-HETE activated a PPRE reporter construct in primary murine keratinocytes. Since all three PPAR isoforms are expressed in primary murine keratinocytes [33] and can activate PPREs on target genes, we determined the ability of each HETE to individually activate PPARα, δ or γ. The chimaeric Gal4-DBD-PPAR-LBD system was used to evaluate the ability of each HETE to activate each PPAR isoform. In primary keratinocytes, 8*S*-HETE activated primarily PPARα and δ and had apparently no affinity for PPARγ (Figure 2). In contrast, 15*S*-HETE had no effect on PPARα activity but increased PPARδ activity. 12*S*-HETE was the only HETE tested to activate all three PPAR isoforms. The lowest concentrations, 1 μM 8*S*- and 12*S*-HETE, were more efficacious than 10 μM for PPARα (Figure 2A) and 1 μM 12*S*-HETE was more efficacious than 10 μM for PPARδ (Figure 2B). Interestingly only the highest concentrations of 12*S*-HETE activated PPARγ, whereas other HETEs tested had no effect or caused inhibition. This suggests that in primary keratinocytes endogenous HETEs synthesized by LOX might preferentially bind and exert their effect through PPARα and PPARδ.

We previously showed [23] that overexpression of PPARα induced keratinocyte differentiation and that 8*S*-HETE further amplified this effect. To further show that the effect of 8*S*-HETE on keratinocyte differentiation depended on PPARα we used MK886, a novel PPAR inhibitor that preferentially blocks PPARα [25]. Treatment of primary keratinocytes with 1 μM MK886 completely blocked 8*S*-HETE induction of differentiation, both in low and high calcium (Figure 3). Since blocking PPARs was sufficient to inhibit keratinocyte differentiation, as measured by K1 expression, and some of the LOX products induce keratinocyte differentiation through PPARs, we hypothesized that blocking LOX activities would inhibit PPAR-mediated differentiation in keratinocytes. Primary keratinocytes induced to differentiate with high calcium were treated with increasing amounts of several LOX inhibitors. This resulted in a significant reduction of K1 mRNA expression with 1 μM NDGA and almost complete abrogation of K1 with 5 μM NDGA (Figure 4A). Similar results were obtained with quercetin and morin. At doses smaller than 10 μM quercetin, K1 expression was not significantly reduced (results not shown). Higher doses of morin and quercetin were needed to achieve the same levels

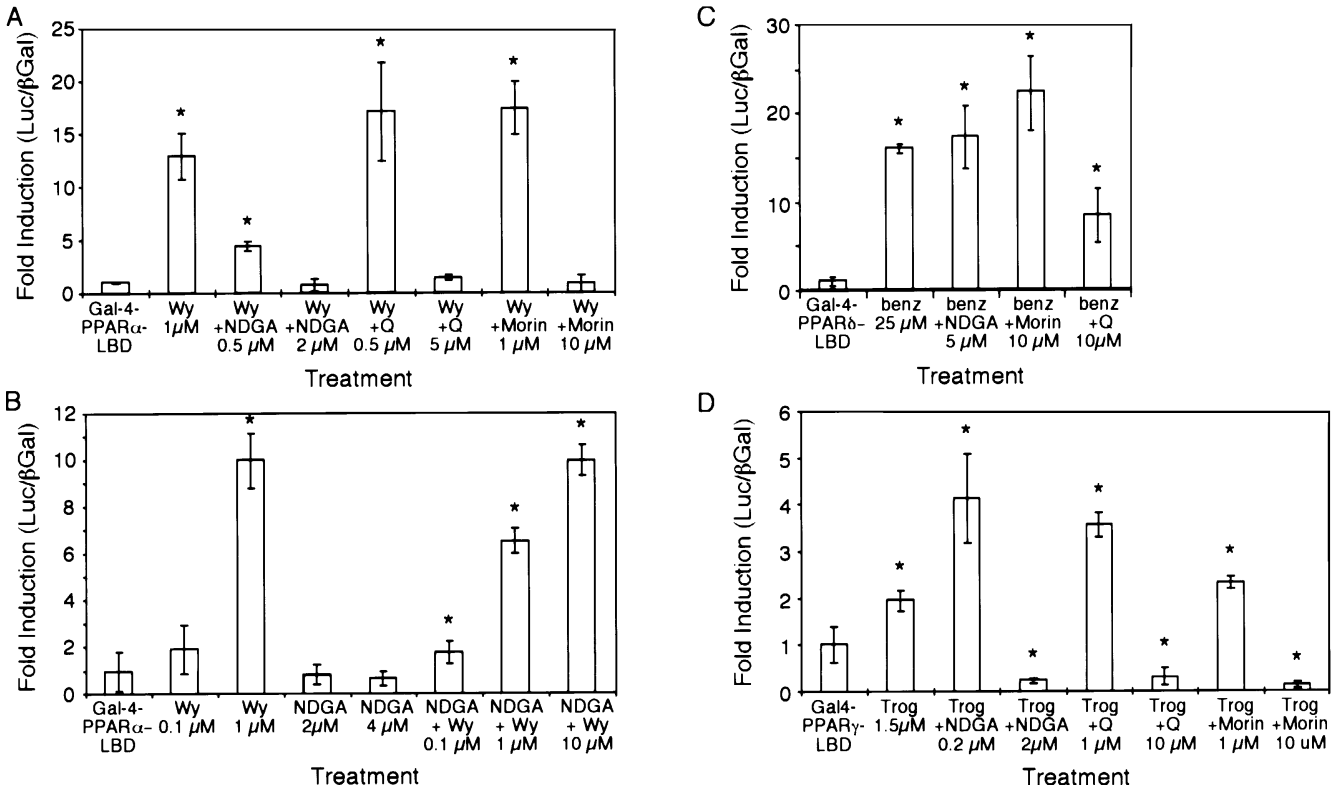


Figure 7 Effect of LOX inhibitors on the LBD of PPARα, δ and γ

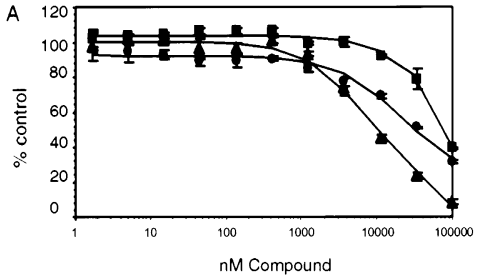
Primary keratinocytes were transfected with 0.5 μg of a TK-MH-Luc reporter plasmid, 0.125 μg of a β-galactosidase plasmid and 0.5 μg of Gal4-PPARα-LBD, and treated for 18 h with or without 1 μM Wy14,643 4(Wy) and increasing amounts of NDGA, quercetin (Q) or morin (**A**), or Gal4-PPARα-LBD and treated for 18 h with or without 0.1, 1 or 10 μM Wy14,643 and 2 μM or 4 μM NDGA (**B**), or Gal4-PPARδ-LBD and treated for 18 h with or without 25 μM bezafibrate (benz) and increasing amounts of NDGA, quercetin or morin (**C**) or Gal4-PPARγ-LBD and treated for 18 h with or without 1 μM troglitazone (Trog) and increasing amounts of NDGA, quercetin or morin (**D**) as indicated. Relative luciferase (Luc) activity was determined and corrected for efficiency with β-galactosidase (βGal) activity. *Data were analysed by ANOVA (Tukey's HSD) with $P < 0.05$ for all treatment groups from 3–5 individual experiments.

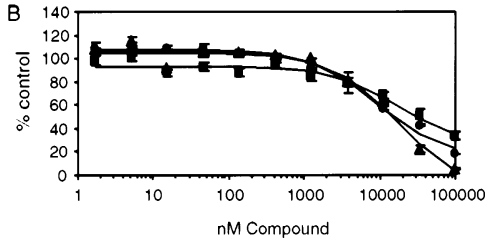
of K1 inhibition (Figures 4B and 4C) observed with NDGA (Figure 4A). None of the doses used affected cell viability. However, higher doses of NDGA (10 μM) and quercetin (30 μM) reduced cell growth (results not shown). To demonstrate that the doses of the LOX inhibitors were sufficient to inhibit LOX activity, keratinocytes were treated with 5 or 10 μM NDGA and HETE synthesis was quantified. As shown in Figure 5, 5 μM NDGA was sufficient to inhibit HETE synthesis; a higher concentration of NDGA did not seem to further reduce LOX activity.

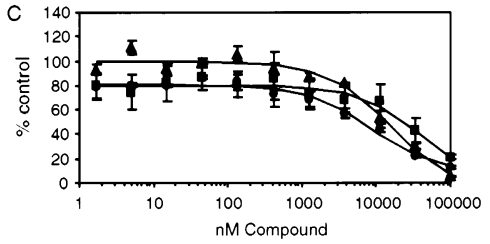
Decreasing HETE levels in the cells should have an effect on endogenous PPAR activities if these metabolites truly activate PPARs. In addition, if the mechanism by which LOX inhibitors block K1 expression involves PPAR, then we would predict that they would also affect the ability of PPARs to activate target genes through their PPRE. Hence, we determined the effect of LOX inhibitors on the PPRE activity in primary keratinocytes. As little as 0.2 μM NDGA inhibited endogenous PPRE activity in primary keratinocytes and this effect was dose-dependent (Figure 6). Similar results were obtained with morin and quercetin, although higher doses were needed to reach levels of inhibition comparable with those seen with NDGA. Since LOX inhibitors inhibited PPRE activity, we speculated that the PPAR isoforms that would be the most affected by these inhibitors would be PPARα and PPARδ, since they appear to have the highest affinity for the HETEs that were tested (Figure 2). Since NDGA reduced production of endogenous PPAR ligands (Figure 5), we tested its ability to prevent PPARα activation. To do so, primary keratinocytes were transfected with the Gal4-PPARα-LBD plasmid system and the cells treated with increasing amounts of inhibitor. As seen in Figure 7(B), NDGA inhibited endogenous PPARα activity and adding back the PPARα ligand Wy14,643 restored PPARα activity. However, the levels of activation were lower when both NDGA and Wy14,643 were present than when Wy14,643 alone was added to the cells, suggesting a possible direct effect of NDGA on PPARα ligand-binding activity. To explore this possibility, we treated primary keratinocytes transfected with Gal4-PPAR constructs with known PPAR ligands and challenged them with increasing concentrations of inhibitors. Surprisingly, all three inhibitors blocked PPARα and PPARγ ligand-binding activity (Figures 7A and 7D), whereas only quercetin had a modest effect on PPARδ (Figure 7C). Wy14,643 (1 μM) increased PPARα activity by 12-fold and this effect was completely blocked with 2 μM NDGA, 5 μM quercetin or 10 μM morin (Figure 7A). PPARγ activation by troglitazone was also blocked with 2 μM NDGA and 10 μM morin, but required 10 μM quercetin (Figure 7D), suggesting a higher affinity of quercetin for PPARα. In contrast, 10 μM quercetin partially inhibited PPARδ activity, whereas morin or NDGA had no effect (Figure 7C).

Because the Gal4-PPAR-LBD reporter assay assesses the ability of a ligand to bind to PPAR, we concluded that the effect observed with the LOX inhibitors corresponded to a direct interaction between the inhibitors and PPARs and that this inhibition

was reversible. However, ligands could also activate the system through effects on co-regulators or covalent modifications of the receptor. To address this issue we used an SPA and determined the ability of each LOX inhibitor to bind to the LBD of the human PPARα, δ or γ. Consistent with our data with the murine LBD reporter assay (Figure 7), NDGA was more efficient at blocking PPARα and PPARγ than PPARδ. The apparent K_i was approx. 20 and 8.9 μM for PPARα and PPARγ, whereas it required higher concentrations of NDGA to displace the PPARδ ligand GW362433X (Figure 8A), and this effect was negligible. Quercetin was more selective for PPARα with a K_i of 9 μM, whereas K_i values for PPARγ and δ were 17 and 18 μM respectively (Figure 8B). Morin was also more effective in blocking ligand binding to PPARα than to PPARγ and had a more modest effect on PPARδ (Figure 8C). Clearly, PPARα and PPARγ were affected to the greatest extent at low doses of the inhibitors, whereas it took higher doses to have an effect on PPARδ (Figure 8D).







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Ki μM	NDGA	Quercetin	Morin
PPARα	20.9	9.0	8.6
PPARγ	8.7	17.0	16.0
PPARδ	61.0	18.0	43.0

Figure 8 PPAR-binding assay

SPAs of (**A**) NDGA, (**B**) quercetin and (**C**) morin on PPARα (●), γ (▲) or δ (■) LBDs as described in the Materials and methods section. IC_{50} was determined from the graphs. The graphs shown are representative of two separate experiments.

Finally, we determined whether LOX inhibitors had any effect on relative PPAR levels in primary keratinocytes. We previously showed [23] that PPARα levels increased with differentiation, whereas PPARδ and γ remained unchanged. Treatment with LOX inhibitors had no effect on either PPARα or δ (results not shown) or RXRα, but NDGA and quercetin decreased PPARγ levels by 80 and 70 % respectively, in high-calcium differentiating medium (Figures 9A and 9B, sixth lanes). Morin had no effect on the level of expression of PPARγ (Figure 9C).

DISCUSSION

Agents that block LOX activity have been used to treat inflammatory diseases, such as asthma, arthritis, ulcerative colitis and psoriasis [27]. Since LOX products play an important role in growth-related signals and proliferation [10], the use of LOX inhibitors as potential tools for chemoprevention of cancer has generated great interest. Although the mode of action includes antioxidant activity and inhibition of LOX and in some case cyclo-oxygenases, the full mechanism of action of these compounds remains unclear. In the present study, we showed that LOX inhibitors block keratinocyte differentiation and unravelled a novel mechanism of action for these LOX inhibitors by showing that they directly inhibit the nuclear receptors PPARs.

We previously demonstrated [23] that HETEs and activated PPARs induce keratinocyte differentiation. Since some of the HETEs are PPAR ligands [21], we further proposed that LOX inhibitors would inhibit PPAR-mediated differentiation of keratinocytes. We first evaluated three of the most abundant HETEs (8*S*, 12*S* and 15*S*) [34] in murine skin for their potency in inducing keratinocyte differentiation. We found that 8*S*-HETE

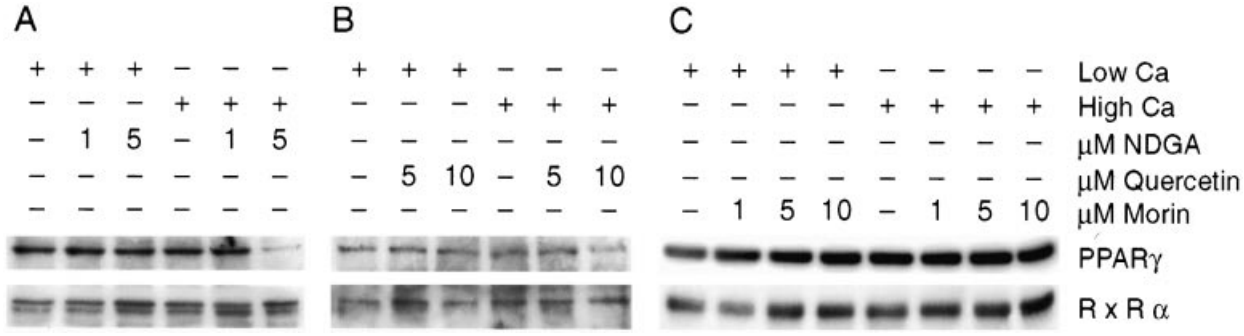

Figure 9 Effect of LOX inhibitors on PPARγ expression levels

Primary keratinocytes were treated with increasing amounts of NDGA, morin or quercetin in low- (**A** and **B**, lanes 1–3; **C**, lanes 1–4) or high- (**A** and **B**, lanes 4–6; **C**, lanes 5–8) calcium media as indicated. Proteins (40 μg) were analysed by Western blot with specific antibodies against PPARγ or RXRα. The blots shown are representative of four different experiments.

was the most potent activator of differentiation. Interestingly, 10–100 nM doses of 8- or 15-HETE were sufficient to trigger keratinocyte differentiation in low-calcium media (results not shown), further suggesting the high specificity of action of these compounds. In addition, levels of these HETEs in primary keratinocytes are in the nanomolar range and have been detected at levels 50–60-fold higher in tumours [4]. This suggests that the concentrations used to stimulate keratinocyte differentiation are within the physiological range for these metabolites in skin. The higher doses are pharmacological and the fact that they are less effective suggests that they may have other effects. We have observed a similar effect on K1 expression, where high doses (10 μM 8*S*-HETE) were less effective in inducing K1 compared with lower doses (10 nM to 1 μM; P. Thuillier, unpublished work). The nature of the effects seen at higher doses requires further investigation and would be highly speculative at this point. 8*S*-HETE was also the most effective activator of PPRE activity and was a better ligand for PPARα and δ than for PPARγ. This is consistent with the previous characterization of 8*S*-HETE as a PPARα ligand [14,21].

Although many reports clearly demonstrate a role for PPARs in keratinocyte differentiation [33,35], the relative contribution of each isoform and the mechanism by which they operate remains unclear. In human keratinocytes, PPARα ligands also induce keratinocyte differentiation [35], and more recently farnesol appeared to also play a role in differentiation through activation of the farnesoid X-activated factor ('FXR') receptor [24]. However, PPARα-knockout mice have no skin phenotype [36], suggesting that PPARα is not necessary for proper homoeostasis of the skin. Alternatively, the lack of phenotype in skin of PPAR$\alpha^{-/-}$ mice could suggest a compensatory mechanism from the other PPAR isoform(s). In support of this idea, PPARγ ligands can also induce keratinocyte differentiation. We observed that 13*S*-hydroxyoctadecadienoic acid (13*S*-HODE) and 9*S*-HODE (results not shown), both PPARγ ligands, induced keratinocyte differentiation. In fact, a role for PPARγ in skin is supported by several studies. Westergaard et al. [37] showed a direct correlation between PPARγ expression and keratinocyte differentiation, and PPARγ ligands improve psoriasis in human skin [38].

The support for 12-HETE, 15-HETE and 13-HODE as endogenous PPARγ ligands was recently greatly strengthened by studies in macrophages showing the generation of these endogenous ligands for PPARγ by 12/15-LOX [39]. Hence, the modest effect of 15-HETE on K1 expression and PPRE activity (results not shown) suggests a smaller contribution of endogenous PPARγ on keratinocyte differentiation. We tested other PPARγ ligands and they were also able to induce keratinocyte differentiation, but were never as potent as 8*S*-HETE (P. Thuillier, unpublished work). Hence, it would appear that, other than calcium, 8*S*-HETE and PPARα might be the most effective stimulator of keratinocyte differentiation found so far. This is supported by the fact that keratinocyte differentiation is blocked by MK886. MK886 was originally characterized as a FLAP inhibitor [40], we recently showed [25] that it was a non-competitive PPARα inhibitor. Interestingly, primary keratinocytes have almost undetectable levels of 5*S*-HETE, suggesting they may not express 5-LOX [41]. Hence, the effect observed with MK886 is most likely FLAP-independent and further substantiates the important role that PPARα plays in keratinocyte differentiation.

Surprisingly, PPARγ levels were decreased 4–5-fold (Figures 9A and 9B) in the presence of NDGA or quercetin but only in high-calcium medium, suggesting a calcium-dependent mechanism for this process. In support of our observations, genistein, another flavonoid, was recently shown to inhibit PPARγ expression in 3T3-L1 cells [42]. In addition, several other flavonoids bound directly to PPARγ and affected cyclo-oxygenase and nitric oxide synthase expression in mouse macrophages [43]. Interestingly, quercetin was recently shown to downregulate expression of both the vitamin D receptor and RXRα in human keratinocytes [44]. Hence, a common specific mechanism might exist to selectively down-regulate these factors.

Although it is expressed at much higher levels in the skin than the other two isoforms, much less is known about PPARδ, presumably because of the lack of identification of target genes. However, the recent generation of PPAR$\delta^{-/-}$ mice has provided new insights into the role of PPARδ and suggests a function in epidermal cell proliferation induced by PMA [45] and in the control of mouse proliferation during wound healing [46]. The increasing availability of specific PPARδ ligands should help in better understanding its function.

While the respective contributions of each PPAR isoform and their ligands to skin differentiation are being unravelled, the potential endogenous PPAR effectors are the LOX metabolites from arachidonic and linoleic acid. Our data further magnify the importance of eicosanoids and PPARs in keratinocyte differentiation. LOX inhibitors decreased HETE synthesis and inhibited both keratinocyte differentiation and PPRE activity. Although we expected that LOX inhibitors would impair endogenous PPAR activities due to depletion of endogenous ligands, we did not anticipate that these inhibitors would interact directly with PPARs. The discovery that these LOX inhibitors block not only the production of eicosanoids, but also directly block their nuclear receptor targets adds a new level of complexity regarding their mechanism of action.

It is interesting that we were able to block PPAR activities with doses lower or within the range of doses usually used in chemoprevention studies [47]. Studies in skin suggest that the possible mechanism(s) of the anti-mutagenic and anti-tumourigenic activities of NDGA [26], quercetin and morin may be due to their multiple effects as inhibitors of carcinogen metabolism and DNA-adduct formation, scavengers of carcinogen free radicals and inhibitors of phorbol ester-induced ornithine decarboxylase activity. Hence, our results suggest that the chemopreventive effect of LOX inhibitors could result not only from blocking the pro-tumourigenic effect of eicosanoids, but also from blocking the activation of specific genes through PPAR inhibition. Although these target genes remain to be identified, PPARs play a role in cancer, and recent studies suggest an involvement in many types of cancers, including, but not limited to, colon (for a review see [48]) and skin [33]. However, the effects of PPAR activation on cancer appear to enhance carcinogenesis in some cases [49] and inhibit it in others [33]. Similarly the effect of LOX inhibitors on PPARs might be cell- and tissue-specific, as suggested by a recent report [50] in which NDGA mediated apoptosis in human breast cancer lines and up-regulated PPARγ. The finding that LOX inhibitors compete with HETEs for binding to PPARs is not unreasonable. This may occur because the inhibitors 'fit' the active site of LOX, which would be expected to be spatially similar to the LBD of PPARs. This idea is supported by X-ray crystallography studies. Recent resolution of the crystal structure of the PPAR LBDs [50] suggest the presence of a larger ligand-binding pocket size than other nuclear receptors. The size of the pocket can accommodate a large variety of natural and synthetic ligands and explains the relative promiscuity of PPARs for their ligands. The implications of PPAR inhibition by LOX inhibitors both for the differentiation programme and for tumour progression remain to be understood and warrant further studies.

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REFERENCES

1 Funk, C. D. (1996) The molecular biology of mammalian lipoxygenases and the quest for eicosanoid functions using lipoxygenase-deficient mice. Biochim. Biophys. Acta **1304**, 65–84

2 Brash, A. R. (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. **274**, 23679–23682

3 Steele, V. E., Holmes, C. A., Hawk, E. T., Kopelovich, L., Lubet, R. A., Crowell, J. A., Sigman, C. C. and Kelloff, G. J. (2000) Potential use of lipoxygenase inhibitors for cancer chemoprevention. Expert Opin. Invest. Drugs **9**, 2121–2138

4 Krieg, P., Kinzig, A., Ress-Loschke, M., Vogel, S., Vanlandingham, B., Stephan, M., Lehmann, W. D., Marks, F. and Furstenberger, G. (1995) 12-Lipoxygenase isoenzymes in mouse skin tumor development. Mol. Carcinog. **14**, 118–129

5 Chen, B. K., Liu, Y. W., Yamamoto, S. and Chang, W. C. (1997) Overexpression of Ha-ras enhances the transcription of human arachidonate 12-lipoxygenase promoter in A431 cells. Biochim. Biophys. Acta **1344**, 270–277

6 Liu, Y. W., Chen, B. K., Chen, C. J., Arakawa, T., Yoshimoto, T., Yamamoto, S. and Chang, W. C. (1997) Epidermal growth factor enhances transcription of human arachidonate 12-lipoxygenase in A431 cells. Biochim. Biophys. Acta **1344**, 38–46

7 Timar, J., Raso, E., Fazakas, Z. S., Silletti, S., Raz, A. and Honn, K. V. (1996) Multiple use of a signal transduction pathway in tumor cell invasion. Anticancer Res. **16**, 3299–3306

8 Amstad, P., Crawford, D., Muehlematter, D., Zbinden, I., Larsson, R. and Cerutti, P. (1990) Oxidants stress induces the proto-oncogenes, C-fos and C-myc in mouse epidermal cells. Bull. Cancer **77**, 501–502

9 Liu, B., Timar, J., Howlett, J., Diglio, C. A. and Honn, K. V. (1991) Lipoxygenase metabolites of arachidonic and linoleic acids modulate the adhesion of tumor cells to endothelium via regulation of protein kinase C. Cell Regul. **2**, 1045–1055

10 Tang, D. G., Chen, Y. Q. and Honn, K. V. (1996) Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc. Natl. Acad. Sci. U.S.A. **93**, 5241–5246

11 Ziboh, V. A. (1996). The significance of polyunsaturated fatty acids in cutaneous biology. Lipids **31** (suppl.), S249–S253

12 Hagerman, R. A., Fischer, S. M. and Locniskar, M. F. (1997) Effect of 12-O-tetradecanoylphorbol-13-acetate on inhibition of expression of keratin 1 mRNA in mouse keratinocytes mimicked by 12(*S*)-hydroxyeicosatetraenoic acid. Mol. Carcinog. **19**, 157–164

13 Fischer, S. M. (1997) Prostaglandins and cancer. Front. Biosci. **2**, d482–d500

14 Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M. and Lazar, M. A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem. **270**, 23975–23983

15 Mangelsdorf, D. J. and Evans, R. M. (1995) The RXR heterodimers and orphan receptors. Cell **83**, 841–850

16 Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. and Evans, R. M. (1995) 15-Deoxy-Δ 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR γ. Cell **83**, 803–812

17 Kliewer, S. A., Xu, H. E., Lambert, M. H. and Willson, T. M. (2001) Peroxisome proliferator-activated receptors: from genes to physiology. Recent Progr. Horm. Res. **56**, 239–263

18 Park, B. H., Vogelstein, B. and Kinzler, K. W. (2001) Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cells. Proc. Natl. Acad. Sci. U.S.A. **98**, 2598–2603

19 Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S. and Spiegelman, B. M. (1998) Terminal differentiation of human breast cancer through PPAR γ. Mol. Cell **1**, 465–470

20 Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J. and Wahli, W. (1996) The PPARα-leukotriene B4 pathway to inflammation control. Nature (London) **384**, 39–43

21 Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M. and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc. Natl. Acad. Sci. U.S.A. **94**, 4318–4323

22 Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H. and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. Cell **93**, 229–240

23 Muga, S. J., Thuillier, P., Pavone, A., Rundhaug, J. E., Boeglin, W. E., Jisaka, M., Brash, A. R. and Fischer, S. M. (2000) 8*S*-lipoxygenase products activate peroxisome proliferator-activated receptor α and induce differentiation in murine keratinocytes. Cell Growth Differ. **11**, 447–454

24 Hanley, K., Komuves, L. G., Ng, D. C., Schoonjans, K., He, S. S., Lau, P., Bikle, D. D., Williams, M. L., Elias, P. M., Auwerx, J. and Feingold, K. R. (2000) Farnesol stimulates differentiation in epidermal keratinocytes via PPARα. J. Biol. Chem. **275**, 11484–11491

25 Kehrer, J. P., Biswal, S. S., La, E., Thuillier, P., Datta, K., Fischer, S. M. and Vanden Heuvel, J. P. (2001) Inhibition of peroxisome-proliferator-activated receptor (PPAR)α by MK886. Biochem J. **356**, 899–906

26 Wang, Z. Y., Agarwal, R., Zhou, Z. C., Bickers, D. R. and Mukhtar, H. (1991) Antimutagenic and antitumorigenic activities of nordihydroguaiaretic acid. Mutat. Res. **261**, 153–162

27 Steele, V. E., Holmes, C. A., Hawk, E. T., Kopelovich, L., Lubet, R. A., Crowell, J. A., Sigman, C. C. and Kelloff, G. J. (1999) Lipoxygenase inhibitors as potential cancer chemopreventives. Cancer Epidemiol. Biomarkers Prev. **8**, 467–483

28 Kulesz-Martin, M., Kilkenny, A. E., Holbrook, K. A., Digernes, V. and Yuspa, S. H. (1983) Properties of carcinogen altered mouse epidermal cells resistant to calcium-induced terminal differentiation. Carcinogenesis **4**, 1367–1377

29 Schneider, C., Boeglin, W. E. and Brash, A. R. (2000) Enantiomeric separation of hydroxy eicosanoids by chiral column chromatography: effect of the alcohol modifier. Anal. Biochem. **287**, 186–189

30 Reference deleted

31 Nichols, J. S., Parks, D. J., Consler, T. G. and Blanchard, S. G. (1998) Development of a scintillation proximity assay for peroxisome proliferator-activated receptor γ ligand binding domain. Anal. Biochem. **257**, 112–119

32 Kempen, E. C., Yang, P., Felix, E., Madden, T. and Newman, R. A. (2001) Simultaneous quantification of arachidonic acid metabolites in cultured tumor cells using high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. Anal. Biochem. **297**, 183–190

33 Thuillier, P., Anchiraico, G. J., Nickel, K. P., Maldve, R. E., Gimenez-Conti, I., Muga, S. J., Liu, K. L., Fischer, S. M. and Belury, M. A. (2000) Activators of peroxisome proliferator-activated receptor-α partially inhibit mouse skin tumor promotion. Mol. Carcinog. **29**, 134–142

34 Cameron, G. S., Baldwin, J. K., Jasheway, D. W., Patrick, K. E. and Fischer, S. M. (1990) Arachidonic acid metabolism varies with the state of differentiation in density gradient-separated mouse epidermal cells. J. Invest. Dermatol. **94**, 292–296

35 Komuves, L. G., Hanley, K., Lefebvre, A. M., Man, M. Q., Ng, D. C., Bikle, D. D., Williams, M. L., Elias, P. M., Auwerx, J. and Feingold, K. R. (2000) Stimulation of PPARα promotes epidermal keratinocyte differentiation *in vivo*. J. Invest. Dermatol. **115**, 353–360

36 Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez-Salguero, P. M., Westphal, H. and Gonzalez, F. J. (1995) Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. **15**, 3012–3022

37 Westergaard, M., Henningsen, J., Svendsen, M. L., Johansen, C., Jensen, U. B., Schroder, H. D., Kratchmarova, I., Berge, R. K., Iversen, L., Bolund, L. et al. (2001) Modulation of keratinocyte gene expression and differentiation by PPAR-selective ligands and tetradecylthioacetic acid. J. Invest. Dermatol. **116**, 702–712

38 Ellis, C. N., Varani, J., Fisher, G. J., Zeigler, M. E., Pershadsingh, H. A., Benson, S. C., Chi, Y. and Kurtz, T. W. (2000) Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-γ inhibit keratinocyte proliferation. Arch. Dermatol. **136**, 609–616

39 Huang, J. T., Welch, J. S., Ricote, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D. and Glass, C. K. (1999) Interleukin-4-dependent production of PPAR-γ ligands in macrophages by 12/15-lipoxygenase. Nature (London) **400**, 378–382

40 Ford-Hutchinson, A. W., Gresser, M. and Young, R. N. (1994) 5-Lipoxygenase. Annu. Rev. Biochem. **63**, 383–417

41 Fischer, S. M., Baldwin, J. K., Jasheway, D. W., Patrick, K. E. and Cameron, G. S. (1988) Phorbol ester induction of 8-lipoxygenase in inbred SENCAR (SSIN) but not C57BL/6J mice correlated with hyperplasia, edema, and oxidant generation but not ornithine decarboxylase induction. Cancer Res. **48**, 658–664

42 Harmon, A. W. and Harp, J. B. (2001) Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am. J. Physiol. Cell. Physiol. **280**, C807–C813

43 Liang, Y. C., Tsai, S. H., Tsai, D. C., Lin-Shiau, S. Y. and Lin, J. K. (2001) Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-γ by flavonoids in mouse macrophages. FEBS Lett. **496**, 12–18

44 Segaert, S., Courtois, S., Garmyn, M., Degreef, H. and Bouillon, R. (2000) The flavonoid apigenin suppresses vitamin D receptor expression and vitamin D responsiveness in normal human keratinocytes. Biochem. Biophys. Res. Commun. **268**, 237–241

45 Peters, J. M., Lee, S. S., Li, W., Ward, J. M., Gavrilova, O., Everett, C., Reitman, M. L., Hudson, L. D. and Gonzalez, F. J. (2000) Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor β (δ). Mol. Cell. Biol. **20**, 5119–5128
46 Michalik, L., Desvergne, B., Tan, N. S., Basu-Modak, S., Escher, P., Rieusset, J., Peters, J. M., Kaya, G., Gonzalez, F. J., Zakany, J. et al. (2001) Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)α and PPARβ mutant mice. J. Cell Biol. **154**, 799–814
47 Lamson, D. W. and Brignall, M. S. (2000) Antioxidants and cancer, part 3: quercetin. Altern. Med. Rev. **5**, 196–208
48 Park, B. H., Breyer, B. and He, T. C. (2001) Peroxisome proliferator-activated receptors: roles in tumorigenesis and chemoprevention in human cancer. Curr. Opin. Oncol. **13**, 78–83
49 He, T. C., Chan, T. A., Vogelstein, B. and Kinzler, K. W. (1999) PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell **99**, 335–345
50 Avis, I., Hong, S. H., Martinez, A., Moody, T., Choi, Y. H., Trepel, J., Das, R., Jett, M. and Mulshine, J. L. (2001) Five-lipoxygenase inhibitors can mediate apoptosis in human breast cancer cell lines through complex eicosanoid interactions. FASEB J. **15**, 2007–2009

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