Lipids in Health and Disease

Research

Epidermal anti-Inflammatory properties of 5,11,14 20:3: Effects on mouse ear edema, PGE_2 levels in cultured keratinocytes, and PPAR activation

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Published: 6 December 2002

Lipids in Health and Disease 2002, **1**:5

Received: 27 November 2002
Accepted: 6 December 2002

This article is available from: http://www.Lipidworld.com/content/1/1/5

Keywords: 5,11,14 Eicosatrienoate, Fatty acid, Non-methylene interrupted fatty acid, Peroxisome proliferated activated receptor, Tumor necrosis factor

Abstract

Background: 5,11,14 20:3 is similar to 20:4n-6 but lacks the internal Δ8 double bond essential for prostaglandin and eicosanoid synthesis. When previously fed to laboratory animals as a gymnosperm seed oil component it has shown anti-inflammatory properties.

Results: Herein, topically applied *Podocarpus nagi* methyl esters (containing 26% 5,11,14 20:3) were incorporated into mouse ear phospholipids, reduced 20:4n-6, and reduced 20:4n-6- and TPA-induced mouse ear edema. Purified 5,11,14 20:3 was taken up by cultured human skin keratinocytes, reduced 20:4n-6, and reduced PGE_2 levels dramatically. Purified 5,11,14 20:3 did not affect PPARα, PPARγ, or PPARδ transactivation.

Conclusions: Topical application of 5,11,14 20:3 to skin surfaces can thus reduce inflammatory processes, most likely by displacing 20:4n-6 from phospholipid pools and reducing downstream inflammatory products derived from 20:4n-6 such as PGE_2 and leukotrienes. It could have potential use in treating clinical skin disorders resulting from overproduction of 20:4n-6-derived eicosanoid products.

Background

Steroidal and non-steroidal anti-inflammatory drugs are known to induce various cutaneous side-effects following systemic and topical application to treat inflammatory skin diseases such as chronic eczema, psoriasis, and systemic lupus erythematosus [1–4]. Such side effects may be overcome by replacement or co-utilization with orally or topically applied anti-inflammatory lipids, such as fish oil, containing 20:5n-3 and 22:6n-3 [5–7].

A very rarely studied fatty acid (FA) with anti-inflammatory potential is the non-methylene interrupted fatty acid (NMIFA) 5,11,14 20:3 having structural similarity to 20:4n-6, but as a result of lacking the internal Δ8 double bond, is not a substrate for prostaglandin and leukotriene production, although small amounts of aborted side products may be formed [8]. Very little 5,11,14 20:3 is elongated [9] or converted to 20:4n-6 in mammals due to limited (if any) Δ8 desaturase activity [10].

Most commonly consumed vegetable oils are derived from angiosperms. In contrast, 5,11,14 20:3 is found in various gymnosperm (conifer) species [11–14] and can be synthesized in gymnosperms and animals by elongation followed by "front-end" Δ5-desaturation (*i.e.*, 9,12 18:2 → 11,14 20:2 → 5,11,14 20:3). Like fish oil-derived 20:5n-3, 5,11,14 20:3 is also incorporated into PC and PE pools resulting in reduced levels of 20:4n-6. However, 5,11,14 20:3 has more selectivity for the phosphatidylinositol (PI) and phosphatidylinositolbisphosphate (PIP_2) pools when fed to mice [15,16], and when incubated with HepG2 cells [17,18]. This could affect diacylglycerol-protein kinase C signaling [19,20]; as well as monoacylglycerol signaling, particularly via interactions with cannabinoid receptors [21]. 5,11,14 20:3 also has poorer affinity for phospholipase A_2, leading to potential accumulation in phospholipid (PL) pools at low doses [22]. Further, as compared to fish oil, NMIFA preparations have a "non-fishy" odor, and reduced oxidizability with only 2 methylene interrupted double bonds [23]. Previous studies showing anti-inflammatory potential of 5,11,14 20:3 are reviewed below.

In autoimmune mice, feeding of 5,11,14 20:3 suppressed production of pathologic anti-erythrocyte- and anti-double stranded DNA antibodies, and prolonged survival [24,25]. In mice injected with collagen-adjuvant emulsions, mortality was lowest in mice fed Juniper oil with 11% 5,11,14 20:3 as compared to fish oil and controls [26]. In mice fed Juniper oil and injected with lipopolysaccharide, PGE_2, TXB_2, 6-ketoPGF$_{1\alpha}$, IL-6, and IL-10 were decreased compared to controls, and Juniper oil was as effective as fish oil in decreasing these pro-inflammatory markers [27]. Oils containing 5,11,14 20:3 can also lower plasma cholesterol in experimental animals [28].

Herein, we explored the topical anti-inflammatory potential of 5,11,14 20:3. We set out to answer the following questions:

A) Is 5,11,14 20:3 incorporated into mouse ear PL when applied topically, can it displace 20:4n-6 from PL pools, and does 5,11,14 20:3 have potential to reduce ear edema?

B) Is 5,11,14 20:3 taken up by cultured human keratinocyte PL, can it displace 20:4n-6 from PL pools and reduce PGE_2 levels, and does it affect tumor necrosis factor α(TNFα) production?

C) Does 5,11,14 20:3 affect activation of peroxisome proliferated activated receptor (PPARs), suggested to be modulators of the inflammatory response?

Results and discussion

Incorporation of topical 5,11,14 20:3 into mouse ear combined phospholipids

In total mouse ear PL, 5,11,14 20:3 increased from 0.2–13.6 %, and 20:4n-6 decreased from 9.9–2.2 % and 22:6n-3 from 9.3–1.0 % (Table 1). It is likely 5,11,14 20:3 replaced these PUFA on the *sn*-2 position of PL. The increase in 18:2n-6 from 16.8–27.0 following *P. nagi* administration may indicate some 20:2n-6 in *P. nagi* was retroconverted to 18:2n-6 [29]. The replacement of 20:4n-6 with 5,11,14 20:3 is consistent with *P. nagi* oil having anti-inflammatory properties when applied topically to skin.

Incorporation of topical 5,11,14 20:3 into mouse ear individual phospholipids

Although the amount of ear tissue available was marginal for individual PL determination, chromatographic analysis indicated there was incorporation of 5,11,14 20:3 into all individual PL classes examined: sphingomyelin, PC, phosphatidylserine, PI, and PE. Interestingly, the greatest incorporation was in the PI pool (data not shown). Previously, feeding experiments revealed greater incorporation of 5,11,14 20:3 into mouse liver, spleen, kidney and heart PI and PIP_2, relative to other PL pools [15,16].

Incorporation of topical 5,11,14 20:3 into mouse ear neutral lipids

As in previous mouse feeding experiments [16], there was less incorporation of 5,11,14 20:3 into NL than PL pools (2.2 vs.13.6%). In both NL and PL pools, there was a small increase in 5,11 20:2, a minor *P. nagi* component (Table 1).

Effects on edema

Topical *P. nagi* ME (rich in 5,11,14 20:3) inhibited 20:4n-6-induced edema 57% (measured 6 h after 20:4n-6 addition; Figure 1). Control ME did not reduce edema and did not produce irritant effects (data not shown).

Arachidonic acid induced edema could have been reduced via the following mechanisms: 1) the incorporation of 5,11,14 20:3 into mouse ear PL reduced endogenous levels of 20:4n-6 available for release and conversion to edema-inducing eicosanoids, such as PGE_2; and leukotrienes; and 2) less exogenously added 20:4n-6 could be convert-

Table 1: Fatty acids in lipid pools following application of control and *P. nagi* ME to mouse ears

FA Percentage	Phospholipid		Neutral lipid	
	Control	*P. nagi*	Control	*P. nagi*
16:0	14.6	8.2	13.8	13.5
16:1n-7	1.6	5.9	28.8	33.6
18:0	14.8	7.6	1.8	1.5
18:1n-9	20.0	15.5	31.8	28.8
18:2n-6	16.8	27.0	12.1	8.9
18:3n-3	0.0	0.3	0.4	0.3
20:2 5,11	0.0	0.2	0.0	0.4
20:3 5,11,14	0.2	13.6	0.1	2.2
20:2n-6	0.8	4.4	0.3	1.2
20:3n-6	0.7	0.2	0.1	0.1
20:4n-6	9.9	2.2	0.7	0.5
20:5n-3	0.3	0.0	0.2	0.3
23:0	0.3	0.0	0.1	0.0
22:2n-6	0.2	0.0	0.1	0.0
22:4n-6	1.1	0.0	0.1	0.0
22:5n-3	1.0	0.0	0.1	0.0
22:6n-3	9.3	1.0	0.6	0.5
Σ 12:0, 13:0, 14:0, 15:0	1.9	2.5	4.1	4.5
Σ 20:0, 22:0, 24:0, 26:0	4.4	9.7	2.2	0.8
Σ 20:1n-9, 22:1n-9, 24:1n-9	2.1	1.7	2.8	3.0

Lipids were extracted from 4 combined ear biopsies (20 mg tissue), PL and NL separated on silica columns, methylated, and analyzed by gas chromatography. Values represent the mean of 1 pooled replicate (duplicate gas chromatographic analysis). Control ME mixture consists of safflower/sunflower/apricot (43:2:50, by vol); *P. nagi* refers to the ME mixture derived from a gymnosperm seed oil described in the text.

ed to edema-inducing eicosanoids. There is not evidence from previous experiments that 5,11,14 20:3 can inhibit conversion of free 20:4n-6 to eicosanoids, whether released from membranes or added exogenously. Exogenously added 20:4n-6 may need to first be incorporated into PL before release and conversion to eicosanoids. It could be topically added 5,11,14 20:3 impaired the incorporation of exogenously added 20:4n-6 into PL. The fact that indomethacin reduced 20:4n-6-induced edema, is also consistent with 20:4n-6 conversion to pro-inflammatory cyclooxygenase products.

In another set of experiments, relative to TPA alone, after daily application of 20 wt% *P. nagi* intact oil for 5 d, ear edema was significantly ($p < 0.05$) reduced from 6.2 ± 1.0 to 3.4 ± 0.7 (1/100 mm; mean of 5 replicates ± 1 SD), 1 h after addition of 0.01% TPA (45% reduction); and from 31.0 ± 1.8 to 22.0 ± 1.8, 24 h after addition of TPA (29% reduction). There was also a reduction in edema with a 5 wt% concentration of *P. nagi* intact oil, with edema reduced from 6.2 ± 1.0 to 1.8 ± 1.3, 1 h after addition of TPA (71% reduction; $p < 0.05$); and from 31.0 ± 1.8 to 28.6 ± 1.6, 24 h after addition of TPA (8% reduction, not significant). In the above experiment, dexamethasone (0.1%) was used as a positive control.

Relative to the same concentration of control ME mix, 20 wt% *P. nagi* ME reduced ear edema from 17.0 ± 3.3 to 3.4 ± 0.7, 1 h after TPA addition (80% reduction), but did not reduce ear edema 24 h after TPA addition. In this experiment, an irritant effect was however apparent after 1 h, since edema values for TPA alone were only 6.2 ± 1.0. Thus a lower concentration of ME was also evaluated. Relative to 10 wt% concentration control ME, *P. nagi* ME significantly reduced edema from 6.4 ± 0.7 to 3.4 ± 1.4, 1 h after TPA addition (47% reduction); no reductions were seen 24 h after TPA addition.

Some of TPA's biological actions are to enhance the release of 20:4n-6 from PL via kinase activation of phospholipase A_2 and to inhibit 20:4n-6 conversion to PGE_2 by inhibiting cyclooxygenase-2 [30,31]. It is likely that following the 5 d application of *P. nagi* lipids, less 20:4n-6 substrate was available for TPA-induced 20:4n-6 release from PL [32].

Other investigators have similarly shown TPA-induced mouse ear edema to be reduced by feeding purified 20:5n-3 and 22:6n-3, FA known to reduce 20:4n-6 in PL [6]. Additionally, in a recent study, various topically applied plant extracts used in traditional East Asian medicine against different skin disorders, were found to inhibit

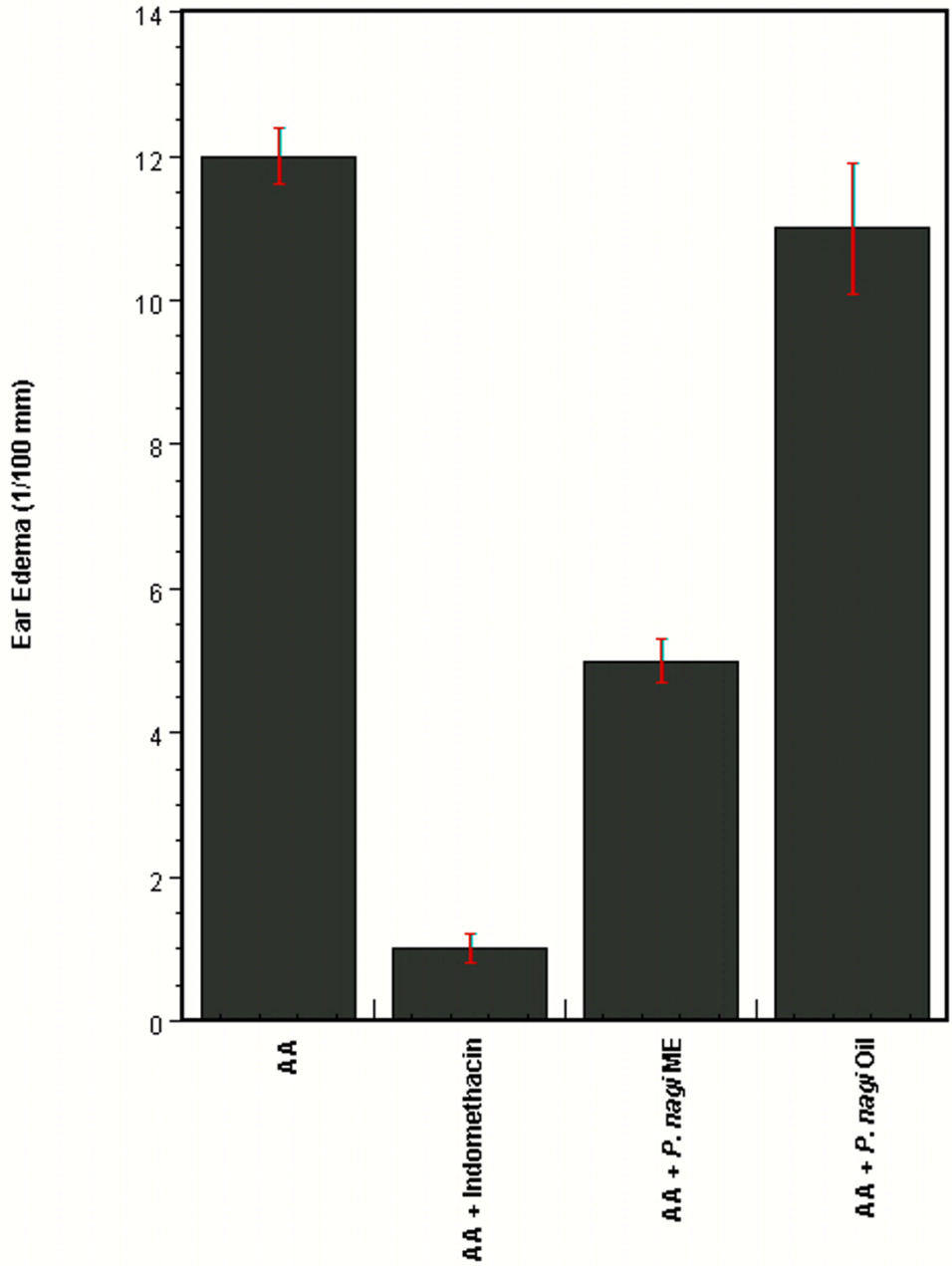

Figure 1
Reduction in mouse ear edema following addition of *P. nagi* ME *P. nagi* ME or oil (20 wt% in acetone) was applied topically once daily for 5 d, and edema measured 6 h after 20:4n-6 addition. Indomethacin was used as a positive control. A control methyl ester mix did not reduce edema (not shown in figure). Values represent mean of 5 replicates. Error bars represent 1 SD. AA, arachidonic acid.

TPA- and 20:4n-6-induced edema [33]. The content of 5,11,14 20:3 in the tested plants was not reported.

Although eicosanoids can also be generated from 20:4n-6 released from monoacylglycerols or diacylglycerols [34], it is not likely 5,11,14 20:3 influenced this process since little 5,11,14 20:3 was incorporated into NL pools (Table 1). There is also the speculative possibility topically applied 5,11,14 20:3 was converted to an *N*-5,11,14-eicosatrienoylethanolamine derivative [35] with anti-inflammatory properties [36].

In the above experiments, it is not clear why the intact *P. nagi* oil was effective in the TPA induced edema assay, but not the 20:4n-6 induced edema assay. Nor is it clear why intact *P. nagi* oil was effective 1 and 24 h after TPA addition, whereas *P. nagi* ME was only effective 1 h after TPA addition. This difference could be related to enhanced stability of the triacylglycerol form compared to the ME form. Nevertheless, there were important first indications that the active component, 5,11,14 20:3 had the potential to reduce edema following a 20:4n-6 or TPA challenge.

Incorporation of 5,11,14 20:3 into cultured keratinocytes

Cellular incubation conditions were carefully developed to possibly mimic the cellular metabolism of dietary 5,11,14 20:3, and test the anti-inflammatory potential of 5,11, 14 20:3 *in vitro*. 5,11,14 was incubated for a long period of time to allow it to be taken up by cellular PL and accumulate in PL (as a result of a supposed poor affinity for phospholipase A_2); and to eventually, displace 20:4n-6 from PL pools resulting in less production of 20:4n-6 derived anti-inflammatory products, following challenge.

Under the incubation conditions employed, 15 μM purified 5,11,14 20:3 ME was found to be incorporated into cultured human skin keratinocyte PL at 3.3% and to decrease 20:4n-6, whether 5,11,14 20:3 was added alone (Experiment 3 vs 2; Table 2), or with exogenous 20:4n-6 present in the system (Experiment 5 vs 4).

Effect of 5,11,14 20:3 on PGE_2 and TNFα secretion in TPA-treated keratinocytes

Thus far, we have shown that 5,11,14 20:3 can be incorporated into cultured keratinocytes and mouse ear PL, and in both cases, reduce 20:4n-6 pools. The mouse ear experiments indicated that the reduction in 20:4n-6 might reduce edema, following a 20:4n-6 or TPA challenge. In the next set of *in vitro* experiments, we determined whether 5,11,14 20:3 might directly reduce the pro-inflammatory eicosanoid, PGE_2, and the pro-inflammatory cytokine TNFα, since both these products are known to be at least partly modulated by cellular 20:4n-6 levels.

Relative to 6 d incubation with 20:4n-6 alone, PGE_2 was decreased with 100–200 μM 5,11,14 20:3 added during the last 4 d of incubation in combination with 20:4n-6 (Figure 2), but TNFα was slightly increased (Figure 3). PGE_2 was in fact reduced to the same levels as seen with hydrocortisone (data not shown). Although the incubation conditions were not identical to those employed for the experiments in Table 2, it is likely that a 5,11,14 20:3-mediated reduction in 20:4n-6 is responsible for the observed decrease in PGE_2. The concentration of 100–200 μM 5,11,14 20:3 may be on the high side as far as fatty acid cell culture doses are concerned, but there were no indications based on microscopic evalution of the cells, and lack of killing of the cells, that this was a cytotoxic concentration, producing detergent like effects.

The increase in TNFα may be the result of either TNFα synthesis or secretion. Mechanistically, the increase may be related to the observed decrease in PGE_2, since high levels of PGE_2 can inhibit TNFα production and transcription [37–39]. Although TNFα is a pro-inflammatory marker, we have evidence 5,11,14 20:3 reduced skin inflammation in the ear edema assay and decreased PGE_2 in keratinocytes. The kinetics of formation of TNFα and a range of eicosanoids needs to be studied to more fully understand anti-inflammatory actions of 5,11,14 20:3.

Effects of 5,11,14 20:3 on PPAR transactivation

PPARs are an extremely important class of nuclear receptors with a plethora of diverse roles. Recently, PPARs were suggested to modulate the inflammatory response [40,41]. PPARs can be activated by polyunsaturated FA [42] including FA with non-methylene interrupted double bond arrangements such as conjugated linoleic acid [43]. Thus, it was important to evaluate whether 5,11,14 20:3 and other FA could activate PPARs (Table 3). As previously reported, PPARα was induced with 22:6n-3 [44] and 9,11/10,12 18:2 (conjugated linoleic acid) [43]. The induction PPARγ and δ with 22:6n-3 may be a new finding. Importantly, 5,11,14 20:3 did not affect transactivation of human PPARα, δ and γ expressed in HeLa cells. It is thus unlikely activation or inhibition of PPARs is important for biological activity of 5,11,14 20:3. These experiments should be repeated in keratinocytes transfected with different PPAR sub-types.

Conclusions and Key findings

Previous studies showed that the 20:4n-6 analog, 5,11,14 20:3, is biologically active when fed to mice [15,16]. Herein, we showed that 5,11,14 20:3 is biologically active when applied topically in cell culture and in vivo. In cultured keratinocytes, 5,11,14 20:3 was incorporated into PL, reduced levels of 20:4n-6, reduced levels of the 20:4n-6-derived pro-inflammatory product, PGE_2, and temporally increased TNFα production. In the mouse ear-edema

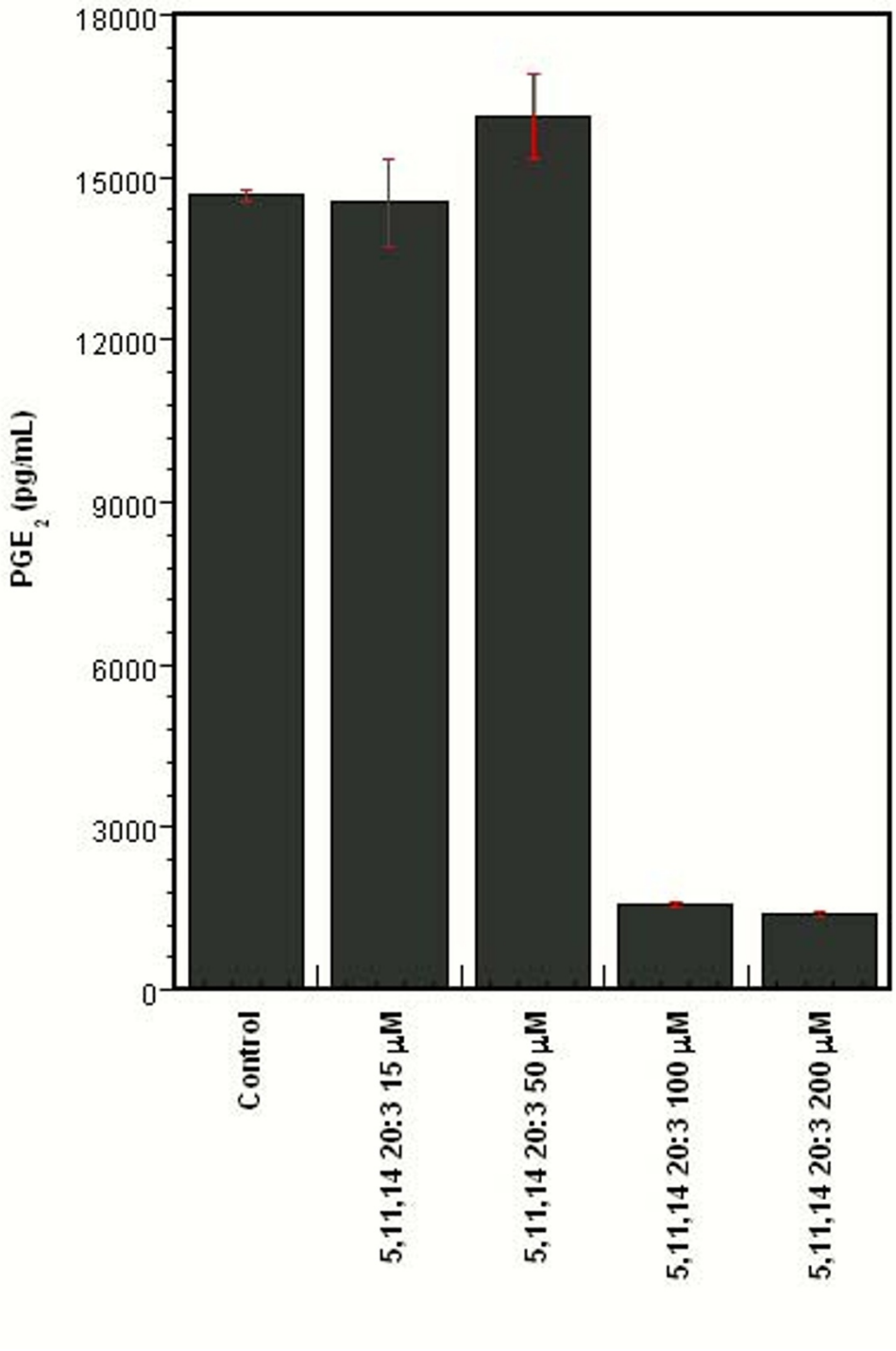

Figure 2
Reduction in PGE_2 secretion of human keratinocytes following incubation with 5,11,14 20:3 ME DK2-NR keratinocytes were incubated for 6 d with 15 μM 20:4n-6 (control) or with 2 d 15 μM 20:4n-6 plus 4 d of 15–200 μM 5,11,14 20:3. In all experiments, 10 ng/mLTPA was also added. Values represent mean of 3 replicates. Error bars represent 1 SD.

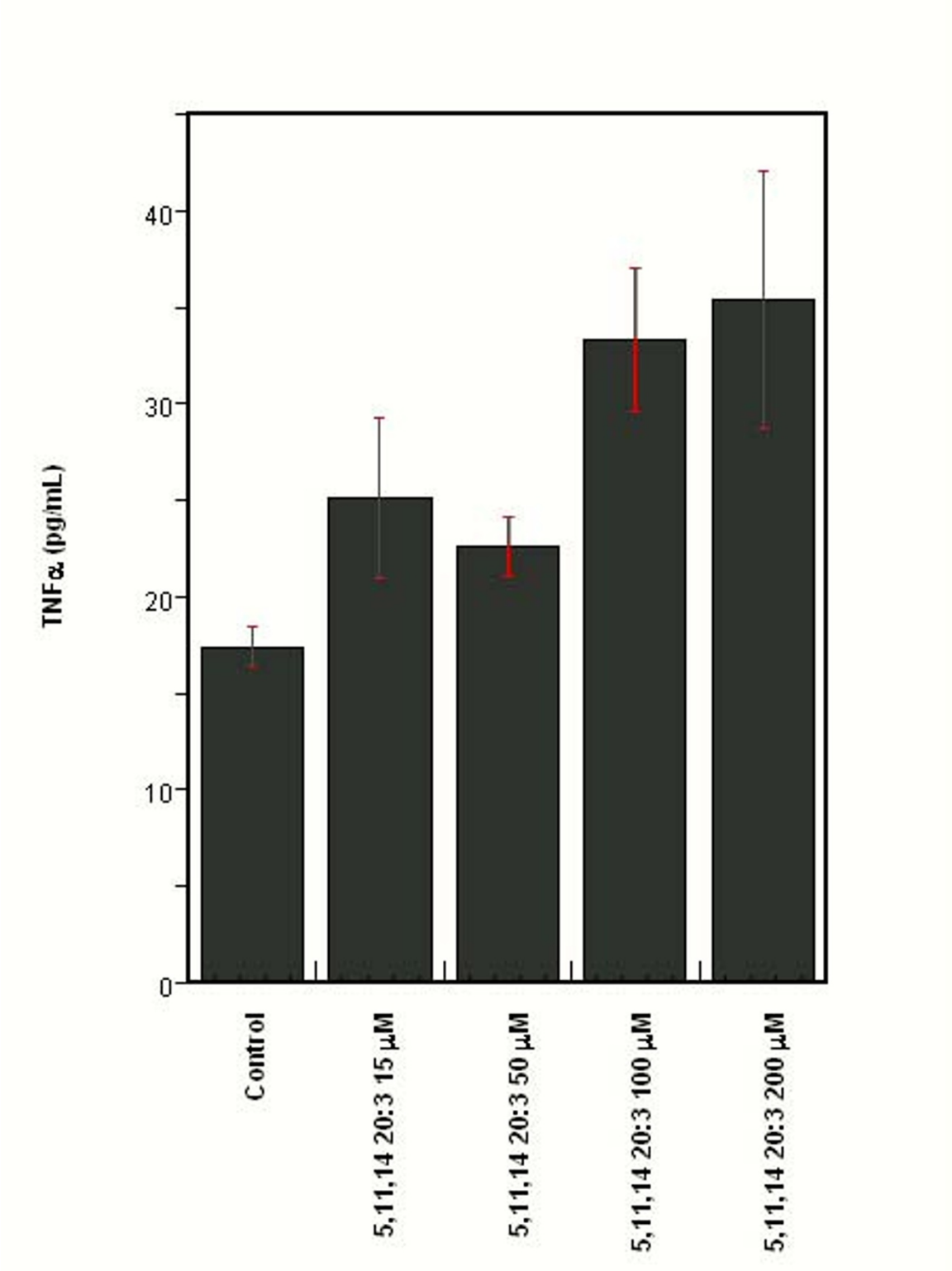

Figure 3
Increase in TNFα secretion of human keratinocytes following incubation with 5,11,14 20:3 ME. Refer to Figure 2 for experimental details. Values represent mean of 3 replicates. Error bars represent 1 SD.

Table 2: Incorporation of 5,11,14 20:3 into cultured keratinocyte phospholipids

	Treatment with fatty acids			Fatty acids incorporated into total phospholipids (%)			
Exp #	Day 0	Day 2	Day 4	5,11,14 20:3	20:3n-6	20:4n-6	20:5n-3
1	-	EtOH	EtOH	0.00	0.11	0.43	0.04
2	-	5,11,14 20:3	5,11,14 20:3	3.34	0.17	0.47	0.06
3		20:4n-6	20:4n-6	0.30	0.55	10.39	0.00
4	20:4n-6	5,11,14 20:3	5,11,14 20:3	2.12	0.38	5.30	0.03
5	20:4n-6	20:4n-6	20:4n-6	0.09	0.85	11.80	0.03

Human DK2-NR keratinocytes were incubated in NR-2 medium containing 15 μM concentrations of various FA methyl esters for up to 6 d as indicated above. Exp, Experiment. Values represent mean of 3 replicates.

Table 3: Induction of transactivation of PPARs

	PPARα		PPARγ		PPARδ	
FA	100 μM	200 μM	100 μM	200 μM	100 μM	200 μM
18:2n-6	23	43	8	12	11	13
20:3n-6	17	19	3	3	20	4
20:3n-3	19	49	18	30	53	67
5,11,14 20:3	16	35	22	27	28	56
9,11/10,12 18:2	76	98	11	14	15	32
22:6n-3	65	574	50	298	140	428

Luciferase activity in HeLa cells transfected with human PPARα, δ and γ cDNAs measured 24 h after addition of unesterified FA at indicated concentration. Values represent % of transactivation values obtained in presence of the positive control Wy14,643 at 10 μM for PPARα, and of BRL49653 at 1 μM for PPARγ and PPARδ, taken as 100%. Significant activation is considered >50% of Wy14,643 for PPARα; >75% of BRL49653 for PPARγ and >100% of BRL49653 for PPARδ. Under the assay conditions employed, inhibition values could not be determined. Values represent mean of 3 replicates in independent experiments, with 4 replicates in each experiment.

assay, 5,11,14 20:3, as a component of *P. nagi* ME and oil, was similarly incorporated into PL (and the PI pool), reduced levels of 20:4n-6, and decreased 20:4n-6- and TPA-induced edema production. PPARs are most likely not involved in the cutaneous biological activity of 5,11,14 20:3.

NMIFA preparations have the potential to reduce inflammation when applied directly to superficial tissues, and could also active when injected underneath superficial tissues. Topical application of NMIFA could be extended to inflammations of the buccal, optic, nasal, colonic, anal, and vaginal surfaces [45].

For 5,11,14 20:3 to be developed for such dermatologic and medical uses, it must not only be effective, but readily available and safe. 5,11,14 20:3 can be synthesized chemically [46,47], or purified from a gymnosperm seed oil as described herein. In addition to the *P. nagi* starting source, it is found at levels of 6–90 wt% in various other gymnosperms [14]. The fungal species *Mortierella alpina* is a current commercial source of 20:4n-6 with Generally Regarded as Safe (GRAS) status in the United States for infant formula applications (FDA GRAS Notice No. GRN 000041, May 17, 2001). By chemically inhibiting the Δ6 desaturase in this species, or by developing mutants with a poor Δ6 desaturase [48], and incubating the culture medium with the precursor 20:2n-6 [49], large amounts of 5,11,14 20:3 can be potentially produced.

With respect to safety, there is some evidence 5,11,14 20:3 is already consumed in the diet. In Japan, *T. nucifera* (11% 5,11,14 20:3) is consumed in the form of salad oil, crackers and bean paste [13]. In China and in Chinese herbal markets in the United States (and perhaps elsewhere), *Platycladus orientalis* seeds (3% 5,11,14 20:3) are consumed [12]. Of particular interest, the large tasty seeds of *Araucaria araucana* (Monkey puzzle, Chilean Pine; 90% 5,11,14 20:3) are still consumed by indigenous peoples in Argentina and Chile [14].

In summary, 5,11,14 20:3 remains a promising natural compound for reducing inflammation induced by overproduction of 20:4n-6 and might be valuable clinically for treating various skin diseases. The anti-inflammatory properties of 5,11,14 20:3 should be further evaluated in combination with nutritional components having different mechanisms of action. These include: 1) the fish oil component 20:5n-3 which reduces 20:4n-6 more from PC and PE than PI pools, and leads to 15-lipoxygenase conversion to the anti-inflammatory 15-hydroxyeicosapentaenoic acid product; 2) the fish oil component 22:6n-3 which has anti-inflammatory properties following 15-lipoxygenase conversion to 17-hydroxydocosahexaenoic acid; 3) the borage and evening primrose oil components 18:3n-6 and 20:3n-6 [50], which have anti-inflammatory properties following cyclooxygenase conversion of 20:3n-6 to PGE_1, and 15-lipoxygenase conversion of 20:3n-6 to 15-hydroxyeicosatrienoic acid; and 4) with 18:2n-6 which has antiproliferative properties following 15-lipoxygenase conversion to 13-hydroxy-9,11-octadecadienoic acid [7]. 5,11,14 20:3 may also reduce inflammation synergistically when combined with non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase-2, particularly at lower doses, where the conversion to prostaglandins, but not the release of 20:4n-6 from PL pools via phospholipase A_2, is inhibited [51].

Materials and methods

Seeds

Podocarpus nagi (Podocarpaceae) seeds were obtained from Carter Seeds (Vista, CA) and contained 14% fat (dry wt. basis), 26% 5,11,14 20:3, and trace amounts of other trienes [11].

Lipid extraction, degumming, bleaching, and methylation

Seeds (1 Kg) were extracted with isopropanol and $CHCl_3$ mixtures, evaporated under N_2 (further operations were performed under N_2 where possible), dissolved in hexane, and washed with 1% NaCl [52]. Crude oil (90 g) was degummed at 70°C for 1 min, mixed with 270 μL 85% ortho-phosphorate, heated 10 min with 1.8 mL HOH, and centrifuged at 2000 g 5 min. For bleaching, 2.4 g of active coal (Carbopal Gn-A, Chemische Fabrik, Brugg, Switzerland) and 80 g degummed oil were heated at 80°C for 20 min under vacuum, and filtered through a 50°C Buchner funnel to obtain 76 g oil [53]. For methylation, 20 g degummed/decolored oil in 50 mL $CHCl_3$ was mixed with 100 mL 2% methanolic H_2SO_4, heated 22 h at 65°C, neutralized with 200 mL 5% NaCl, redissolved in 80 mL hexane, and washed with 80 mL 2% Na bicarbonate [54]. FA composition of *P. nagi* methyl ester (ME) mix (before column purification) was: 2.4% 16:0; 1.4% 18:0; 16.4% 18:1n-9; 43.1% 18:2n-6; 0.4% 5,11 20:2; 26.3% 5,11,14 20:3; 8.6% 20:2n-6; and 1.4% 20:0/22:0/24:1n-9 combined. FA ME were analyzed on an HP 6890 gas chromatograph with SGE BPX-70 capillary column and identified by retention time and gas chromatography/mass spectroscopy [48].

Florisil column chromatography to purify 5,11,14 20:3

Argentation chromatograpy, which separates compounds based on number of double bonds, was used to obtain 5,11,14 20:3 free of other trienes. Florisil (600 g, 60–100 mesh; Fluka Chemie AG, Buchs, Switzerland) was double acid-washed with H_2SO_4, MeOH and $CHCl_3$ [55], mixed with 5% aq $AgNO_3$ (25% apparent impregnation), dried, and activated at 110°C [56]. A 300 g slurry of sorbent, 500 mL hexane and 20 g *P. nagi* ME were added to a 100 × 4 cm column with 10°C water-cooling jacket. Flow rate was adjusted to 4 L/d. Various 2 L vol ratios of hexane/diethyl ether from 100:0–0:100 (v/v) were added, and 33 1 L fractions collected over 10 d [57,58]. From 20.0 g *P. nagi* ME (containing 5.3 g 5,11,14 20:3) applied to the column, 4.0 g 5,11,14 20:3 were recovered in combined fractions; 4 fractions contained 1.3 g 5,11,14 20:3 in 100 % measurable purity. For cell culture experiments, 10 mg 5,11,14 20:3 ME was processed to reduce any contaminating silver ions [59]; for PPAR experiments, ME were converted to unesterified FA [54].

Preparation of control ME mix for ear edema experiments

A ME control oil mixture prepared from safflower/sunflower/apricot (43:2:50, by vol.) contained: 5.4% 16:0; 0.3% 16:1n-7; 1.9% 18:0; 43.9% 18:1n-9; 48.5% 18:2n-6 and 0.1% 20:5n-3. In control oil, 18:1n-9 (43.9%) content approximately equaled 18:1n-9 (16.4%) + 5,11,14 20:3 (26.3%) content in *P. nagi* oil; 18:2n-6 (48.5%) content approximately equaled 18:2n-6 (43.1%) + 20:2n-6 (8.6%) content in *P. nagi* oil. Thus, the less common FA (20:2n-6) and FA with anti-inflammatory potential (5,11,14 20:3) in *P. nagi* oil were replaced by 18:2n-6 and 18:1n-9 in control oil.

Lipid extraction and analysis of ear phospholipids

Following topical application of *P. nagi* or control ME, 4 ear biopsies per treatment were pooled to yield 20 mg ear tissue, which was ground with a teflon pestle, extracted with 0.8 mL HOH, 1 mL $CHCl_3$ and 2 mL MeOH, vortexed, centrifuged (3 min, 1000 g), and the residue re-extracted with 1 mL $CHCl_3$. Pooled supernatants were filtered through sintered glass, washed with 1 mL 0.88% KCl, vortexed, centrifuged, and the organic phase redissolved in 2 mL $CHCl_3$ and applied to pre-washed 3 mL 500 mg silica solid phase extraction columns (Supelco Inc., Buchs, Switzerland). Neutral lipid (NL) were obtained with 4 mL $CHCl_3$; and PL with 4 mL $CHCl_3$/MeOH (2:1, by vol) and 4 mL MeOH [60]. Total PL were checked for purity by high performance thin layer chromatography (HPTLC); or separated into individual classes by HPTLC,

scraped, methylated, and FA composition determined by gas chromatography [Table 1; [61]].

Ear edema assay

Right mouse ears were topically treated 1 X/d for 5 d with 20 wt% *P. nagi* ME or intact oil (4 μg in 20 μL acetone) containing 0.5 wt% α-tocopherol and 0.2 wt% palmitoylascorbate as antioxidants [62]. Left ears received 20 μL acetone. Unesterified 20:4n-6 (20 wt%) in acetone was then applied topically 1 h after the last administration of *P. nagi* ME, and left on the ears for 6 h, during which time ear edema was measured with calipers (Figure 1). Ear biopsies were taken for PL analysis 2 h after 20:4n-6 addition in some studies (Table 1). Ear edema experiments were approved by an internal animal care and use committee.

Growing conditions for keratinocytes and incorporation of 5,11,14 20:3 into PL

The newly developed SV40 T-Ag immortalized human keratinocyte line DK2-NR, having a highly conserved metabolic profile, was used to study 5,11,14 20:3 metabolism [63]. Cells were cultured in NR-2 serum-free medium (Biosource Inc., Rockville, MD) developed for immortalized keratinocytes [63]. This is an essential FA-deficient culture medium with low Ca^{2+} concentration (0.11 mM Ca^{2+}) containing bovine pituitary extract, EGF, insulin, hydrocortisone and transferrin. To improve cell adhesion, culture dishes were preincubated with a coating solution consisting of 1 L basal medium (without growth factors) supplemented with 10 mg/L human fibronectin (Becton Dickinson), 100 mg/L BSA (Biosource Inc., Rockville, MD), and 160 mg collagen/L (Vitrogen 100, Corporation, Palo Alto, CA). Cells were cultured in NR-2 medium until confluency, then shifted to high Ca^{2+} (1.5 mM Ca^{2+}). After 4 d, purified 5,11,14 20:3 ME was suspended in NR-2 medium and added to DK2-NR cell cultures (experiments 1–2, Table 2). Controls were incubated with 15 μM 20:4n-6 ME under identical conditions (experiment 3, Table 2). Medium containing fresh FA was added every 2 d in experiments exceeding 2 d incubation. Cells were also pre-incubated 2 d with 15 μM 20:4n-6 ME for 2 d and subsequently with 15 μM 5,11,14 20:3 ME, or with only 15 μM 20:4n-6 ME added every 2 d (experiments 4 and 5, Table 2). 5,11,14 20:3 ME and 20:4n-6 were warmed and sonicated in 1 mg/mL FA-free bovine serum albumin (BSA, Sigma) prior to addition. Following incubations, cells were washed in Hank's Balanced Salt solution (HBSS) containing 0.1% BSA, harvested, centrifuged in 1 mL HBSS (1000 rpm), and lipids extracted, PL separated by HPTLC, methylated, and FA ME quantified by gas chromatography (Table 2).

As compared to primary keratinocytes, we used the above cell line because it is more standardized, previously adapted to essential fatty acid deficient growing conditions, and because its FA metabolism has been previously evaluated under these conditions [63]. Cells were shifted to high calcium to induce differentiation, to better mimmic the *in vivo* situation, and because cells differentiated in this manner were known to be less sensitive to high doses of dietary FA, including 18:2n-6 and 18:3n-3 [63]. For DK2-NR cell experiments, we used ME, rather than unesterified FA, because ME are more stable to oxidation when using serum-free media with limited antioxidants; and because our cell line was previously established to posess sufficient methyl esterase activity to convert the ME to unesterified fatty acids.

Measurement of PGE_2 and TNFα in keratinocytes

Confluent DK2-NR cell cultures were incubated in NR-2 medium containing 1.5 mM $CaCl_2$. Cells were incubated for 2 d with 15 μM 20:4n-6 then for 4 d with either: 15 μM 20:4n-6; or 15 μM 20:4n-6 + 15-, 50-, 100-, or 200 μM 5,11,14 20:3 ME (Figs. 2,3; similar to Experiment 4 of Table 2 except there was co-incubation of 20:4n-6 with 5,11,14 20:3 during the final 4 d of the 6 d incubation period). In other experiments, the total time of incubation with FA was up to 10 d (data not shown). Fresh FA were added every 2 d; and 10 ng/mL of the PGE_2-inducer TPA was added on incubation d 5. On d 6, supernatant was collected for ELISA-quantification of TNFα (R&D systems Minneapolis, USA) and PGE_2 (Cayman Chemical Company, Ann Arbor, MI, USA).

PPAR Transactivation assay

Three copies of acyl-CoA oxidase PPRE (5' GATCCCCGAACGTGACCTTTGTCCTGGTCC-3') were cloned into the BglII site of pGL3 vector (Promega, France) containing SV40 promoter and luciferase gene. Human PPARα, δ and γ cDNAs were cloned in the mammalian expression vector pSG5. HeLa cells were seeded in a 150 mm diameter Petri dish in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) delipidized fetal calf serum. After 24 h, cells were co-transfected with the PPAR expression and reporter plasmids using $CaPO_4$ precipitation [64]. After 18 h, cells were trypsinized, seeded into 96 well clusters, and unesterified FA added to culture medium (Table 3). Positive controls for PPARα and PPARδ/γ activation were 10 μM Wy-14,643 and 1 μM BRL49653, respectively. Luciferase activity was determined after 24 h with a Luclite kit (Packard Instrument, France) on a Microbeta Trilux Wallac luminescence counter (EG & G Berthold, France; Table 3). Unesterified FA, as opposed to ME, were tested because the HeLa cell PPAR expression reporter assay had been validated with unesterified FA; and because there would be little oxidation risk during the 1 h short incubation period (unesterified FA being less stable than ME). The higher doses of 100–200 μM were not cytotoxic to cells under our culture conditions as assessed by trypan blue exclusion and the fact that a wide range of FA

with known activation properties behaved as reported in literature (*e.g.,* 22:6n-3, Table 3).

Abbreviations

BSA bovine serum albumin

FA fatty acid

HPTLC high performance thin layer chromatography

ME methyl ester

NL neutral lipid

NMIFA non-methylene interrupted fatty acid

PC phosphatidylcholine

PE phosphatidylethanolamine

PI phosphatidylinositol

PL phospholipid

PPAR peroxisome proliferated activated receptor

TNF tumor necrosis factor

TPA 12-*O*-tetradecanoylphorbol-13-acetate

Authors' contributions

AB wrote the majority of the manuscript and was responsible for the overall planning and development of the project. AB and IM purified the fatty acids and starting materials and developed lipid methodology. MB and CC performed all keratinocyte experiments. IS performed the PPAR experiments. AJ performed the ear edema experiments.

All authors have read and approved the final manuscript.

Acknowledgements

The authors thank Nestlé Management for their support of this research.

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