Research

**Epidermal anti-Inflammatory properties of 5,11,14 20:3: Effects on mouse ear edema, PGE2 levels in cultured keratinocytes, and PPAR activation**

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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 PGE2 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 PGE2 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 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 (PIP2) 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 A2, 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, PGE2, TXB2, 6-ketoPGF1α, 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 PGE2 levels, and does it affect tumor necrosis factor α (TNFa) 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, phosphatidylinerine, 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 PL and PIP2, 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 eedema-inducing eicosanoids, such as PGE2 and leukotrienes; and 2) less exogenously added 20:4n-6 could be convert-
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 A2 and to inhibit 20:4n-6 conversion to PGE2 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

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**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.
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-eicosatrienoyl ethanolamine 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 A2); 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 PGE2 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, PGE2, 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, PGE2 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). PGE2 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 PGE2. 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 evaluation 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 PGE2, since high levels of PGE2 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 PGE2 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, PGE2, 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.
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].

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

| Exp # | Treatment with fatty acids | 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            | 3.34  | 0.17  | 0.47  | 0.06        |        |        |        |
| 3     | 20:4n-6 20:4n-6           | 0.30  | 0.55  | 10.39 | 0.00        |        |        |        |
| 4     | 5,11,14 20:3              | 2.12  | 0.38  | 5.30  | 0.03        |        |        |        |
| 5     | 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

| FA              | 100 μM PPARα | 200 μM PPARα | 100 μM PPARγ | 200 μM PPARγ | 100 μM PPARδ | 200 μM PPARδ |
|-----------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 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, 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.
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-hydroxyicosapentaenoic acid product; 2) the fish oil component 22:6n-3 which has anti-inflammatory properties following 15-lipoxygenase conversion to 15-hydroxydocosahexaenoic acid; 3) the borago 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 PGE1, 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 A2, 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 CHCl3 mixtures, evaporated under N2 (further operations were performed under N2 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-phosphoric, heated 10 min with 1.8 mL HOH, and centrifuged at 2000 g 5 min. For bleaching, 2.4 g of active coal (Carbopals G-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 CHCl3 was mixed with 100 mL 2% methanolic H2SO4, 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 spectrometry [48].

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

Argentation chromatography, which separates compounds based on number of double bonds, was used to obtain 5,11,14 20:3 free of other trienes. Floresil (600 g, 60–100 mesh; Fluka Chemie AG, Buchs, Switzerland) was double acid-washed with H2SO4, MeOH and CHCl3 [55], mixed with 5% aq AgNO3 (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 18:2n-6 (43.1%) + 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 1 mL CHCl3. Pooled supernatants were centrifuged (3 min, 1000 g), and the residue re-extracted with 4 mL CHCl3; and PL with 4 mL CHCl3/MeOH (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].
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% palmitoyl-lascorbate 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 Ca2+ concentration (0.11 mM Ca2+) 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 with 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 mg/mL of the PGE2-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 PGE2 (Cayman Chemical Company, Ann Arbor, MI, USA).

**PPAR Transactivation assay**

Three copies of acyl-CoA oxidase PPRE (5′ GATCCCGAAACGTGACCITTTGGCTCGTCC-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 CaPO4 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.

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