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Inhibition of Neutrophil Leukotriene B₄ Production by a Novel Synthetic N-3 Polyunsaturated Fatty Acid Analogue, β-Oxa 21:3n-3

Brenton S. Robinson,* Deborah A. Rathjen,²§ Neil A. Trout,*# Christopher J. Easton,¶ and Antonio Ferrante³#†

We recently reported the synthesis and anti-inflammatory properties of a novel long chain polyunsaturated fatty acid (PUFA) with an oxygen atom in the β-position, β-oxa-21:3 n-3 (Z,Z,Z)-(octadeca-9,12,15-trienyloxy) acetic acid. Our data, from studies aimed at elucidating the mechanism of its action, show that pretreatment of human neutrophils with the β-oxa-PUFA substantially depresses the production of leukotriene B₄ (LTB₄) in response to calcium ionophore, A23187, comparable to standard leukotriene inhibitors such as zileuton and nordihydroguaiaretic acid. Interestingly, the n-6 equivalent, β-oxa-21:3 n-6, is also a strong inhibitor of LTB₄ production. In contrast, naturally occurring PUFA only slightly reduce, for eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, or increase, for arachidonic acid (20:4n-6), the formation of LTB₄. The parent β-oxa-21:3:3-molecule, rather than its derivatives (methyl ester, saturated, monohydroperoxy, or monohydroxy forms), is exclusively responsible for attenuation of LTB₄ formation. β-Oxa-21:3n-3 inhibits the conversion of [³H]20:4n-6 to [³H]5-hydroxyeicosatetraenoic acid and [³H]LTB₄ by neutrophils in the presence of calcium ionophore and also suppresses the activity of purified 5-lipoxygenase, but not cyclooxygenase 1 and 2. β-Oxa-21:3n-3 is taken up by neutrophils and incorporated into phospholipids and neutral lipids. In the presence of calcium ionophore, the leukocytes convert a marginal amount of β-oxa-21:3n-3 to a 16-monoxygenated-β-oxa-21:3n-3 derivative. After administration to rodents by gavage or i.p. injection, β-oxa-21:3n-3 is bound to be incorporated into the lipids of various tissues. Thus, β-oxa-21:3n-3 has the potential to be used in the treatment of inflammatory diseases, which are mediated by products of the lipoxigenase pathway. The Journal of Immunology, 2003, 171: 4773–4779.

Polyunsaturated fatty acids (PUFA) are ubiquitous components of mammalian cell membranes, where they are generally esterified in phospholipids and neutral lipids. In addition to membranes, another PUFA-bearing domain in cells can be lipid bodies, which are lipid-rich cytoplasmic inclusions (2–4). Most cellular PUFA are derived from the essential dietary fatty acids, octadecadienoic acid (linoleic acid, 18:2n-6) and octadecatrienoic acid (α-linoleic acid, 18:3n-3) by the process of chain elongation and desaturation (5) and are mainly degraded by β-oxidation (6). Apart from their important role as structural components and as a source of energy, some of these fatty acids, in particular eicosapentaenoic acid (arachidonic acid, 20:4n-6) and eicosapentaenoic acid (20:5n-3), act as precursors of physiologically active oxygenated metabolites, collectively termed eicosanoids. These include prostaglandins, thromboxanes, prostacyclins (cyclooxygenase products), hydroxylated fatty acids, leukotrienes, lipoxins (lipoxygenase products), and epoxyenated fatty acids (epoxyenase products) (7–11).

When leukocytes such as neutrophils are challenged with certain agonists, the formation of leukotrienes is initiated by the release of 20:4n-6 from phospholipids by the activation of phospholipase A₂. The unesterified 20:4n-6 then undergoes 5-lipoxygenation to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is further converted to an unstable epoxide leukotriene A₄ (LTA₄). This intermediate can be enzymatically hydrolyzed to LTB₄. Alternatively, LTA₄ is transformed, by the addition of glutathione at C-6, into leukotriene C₄ (LTC₄). Stepwise enzymatic elimination of glutamic acid and glycine in the peptide side chain leads to formation of LTD₄ and LTE₄, respectively (9–11). The presumed asthma mediator slow-reacting substance of anaphylaxis has now been identified as a biological activity composed of LTC₄, LTD₄, and LTE₄ (cysteinyl-leukotrienes), which are all potent bronchoconstrictors (12–14). In addition, these leukotrienes have been implicated in hypoxic vasoconstriction and the pathophysiology of respiratory distress syndromes (15). The closely related compound, LTB₁, is a potent mediator of leukocyte-dependent inflammation (16, 17) and hence is important for the inflammatory component of asthma and other pulmonary affictions.

Our recent studies have shown that PUFA also have the ability to directly stimulate leukocytes (neutrophils and macrophages) to...
release superoxide, degranulate, adhere, and display increased expression of CR3 receptors (18–20). We have observed that the modulation of biological activity induced by PUFA is related to neutrophils are clearly proinflammatory and therefore would exacerbate the asthmatic state.

20:5n-3 and docosahexaenoic acid (22:6n-3), which are prominent in diets enriched with fish oil, competitively inhibit the conversion of 20:4n-6 to eicosanoids. With respect to leukotriene products of the 5-lipoxygenase pathway in leukocytes, LTB₄, the dihydroxy derivative of 20:5n-3 formed from LTA₄, is a weak and partial agonist compared with LTB₄ in eliciting inflammatory responses (11, 22, 23). The pentaenoic cysteinyl-leukotrienes are equipotent with their tetraenoic counterparts (11, 24, 25). Dietary strategies involving fish oils rich in 20:5n-3 and 22:6n-3 for increasing the amount of LTB₄ production at the expense of LTB₄ production have attracted interest because of their potential use in the management of a range of human diseases, including asthma, rheumatoid arthritis, systemic lupus erythematosus, and cardiovascular diseases (26). However, to date the results of such studies have essentially remained controversial and disappointing with respect to their use in the treatment of inflammatory diseases (27). This is probably due to 20:5n-3 and 22:6n-3 having direct proinflammatory effects, such as stimulation of leukocyte respiratory burst, degranulation, and adherence.

Recently we described a chemically synthesized novel PUFA, with an oxygen atom in the β-position, β-oxa-21:3n-3 (28), that has immunosuppressive properties, but, unlike its natural PUFA counterpart, was a weak stimulator of neutrophil functions (29). In the present study we have assessed whether such chemically engineered PUFA inhibit calcium ionophore-induced production of LTB₄ by human neutrophils.

Materials and Methods

Fatty acids
β-Oxa-PUFA, β-oxa-21:3n-3, and β-oxa-21:3n-6, were synthesized as described by Pitt et al. (28). β-Oxa-21:3n-3 methyl ester was formed by treatment of β-oxa-21:3n-3 with diazomethane in diethyl ether, and β-oxa-21:0 was prepared by hydrogenation of β-oxa-21:3n-3 in the presence of platinum oxide (30). 16-Monohydroperoxy-β-oxa-21:3n-3 was prepared by incubation of β-oxa-21:3n-3 with soybean lipoxidase (31), and 16-monoxygenase-β-oxa-21:3n-3 was obtained by reduction of the 16-monohydroperoxy product with sodium borohydride (31). 20:4n-6, docosahexaenoic acid (22:6n-3), eicosapentaenoic acid (20:5n-3), and nonadecanoic acid (nonadecyl acid, 19:0) methyl ester were obtained from Sigma-Aldrich (St. Louis, MO).

Biochemical and inhibitors

The calcium ionophore A23187, nordihydroguaiaretic acid (NDGA), dihydroxy derivative of 20:5n-3 on purification by HPLC, was a weak stimulator of neutrophil functions (29). In the majority of cases the preparation of neutrophils was of >99% purity and >99% viability, as judged by morphological examination of cytospin preparations and the ability of viable cells to exclude trypan blue stain. The neutrophils were washed three times, resuspended in HBSS (5 × 10⁶ cells/ml), and used within 30 min of preparation. Trypan blue exclusion and lack of lactate dehydrogenase release indicated that the cells remained viable throughout subsequent incubations.

Measurement of LTB₄ production

Neutrophils (5 × 10⁶) were preincubated with or without fatty acids or derivatives (2.5–40 μM), Zileuton (10 μM, 5-lipoxygenase inhibitor) or NDGA (10 μM, 5-, 12-, and 15-lipoxygenase inhibitor) in 0.5 ml of HBSS at 37°C for up to 60 min. The cells were subsequently incubated in the presence or the absence of the calcium ionophore A23187 (5 μM) in 1 ml final volume of HBSS at 37°C for 30 min. The incubates were immediately centrifuged at 14,000 × g at 4°C for 5 min, and the supernatants were stored at −70°C until analysis. The amount of LTB₄ in the medium samples was measured using commercial enzyme immunoassay kits.

Determination of [3H]-5-hydroxyeicosatetraenoic acid ([3H]-5-HETE) and [3H]LTB₄ production from [3H]20:4n-6

The production of [3H]-5-HETE and [3H]LTB₄ by [3H]20:4n-6-prelabeled neutrophils challenged with the calcium ionophore A23187 was assessed as follows. Neutrophils (6 × 10⁶) were incubated with [3H]20:4n-6 (10 μCi; 24 nM) in 2 ml of HBSS at 37°C for 75 min. The cells were washed three times with 10 ml of HBSS containing BSA (0.001%, w/v), once with 10 ml of HBSS, and finally resuspended in HBSS. Approximately 70% of the radiolabeled fatty acid substrate was taken up by the cells, of which >98% was esterified in lipids. [3H]20:4n-6-labeled neutrophils (2 × 10⁶) were preincubated with or without β-oxa-21:3n-3 (20 μM) in 2 ml of HBSS at 37°C for 15 min. The cells were then incubated in a 4-ml final volume of HBSS containing the calcium ionophore A23187 (5 μM) at 37°C for up to 30 min. Lipids were extracted by the addition of 15 ml of chloroform/methanol/acetic acid (1/2/0.2, v/v/v) (33). The mixture was left at room temperature for 1 h and subsequently partitioned by the addition of 5 ml of chloroform and 5 ml of water (34). Concentrated lipid extracts were applied to silica gel 60 TLC plates and developed in diethyl ether/hexane/acetate (60/40/1, v/v/v) or the organic phase of ethyl acetate/isopropanol/acetic acid/water (11/5/2/10, v/v/v/v) to isolate [3H]-5-HETE and [3H]LTB₄ (35, 36). Identification of the lipids was based on a comparison of their TLC mobility with that of authentic unlabeled standards. The 5-HETE and LTB₄ zones were located with I₂ vapor or by comparison of their TLC mobility with that of authentic unlabeled standards. The 5-HETE and LTB₄ zones were located with I₂ vapor or by comparison of their TLC mobility with that of authentic unlabeled standards.

Presentation of fatty acids and agonists

Fatty acids and derivatives, Zileuton, NDGA, and calcium ionophore A23187 (concentrated stocks in ethanol stored at −20°C) were diluted with HBSS immediately before use. Ethanol alone appropriately diluted in HBSS was used as a control. The final concentration of ethanol in neutrophil incubations was <0.2% (v/v). In some cases fatty acids were also solubilized by preparing mixed DPC/fatty acid micelles (4/1, w/v) in HBSS by sonication (19). Controls contained micelles of DPC alone similarly diluted. TLC and gas-liquid chromatography (GLC)-mass spectrometry (MS) indicated that the lipids were of high purity. Agonists and media were shown to be free of endotoxin contamination using the Limulus amebocyte lysate assay. In the TLC analysis the oxo fatty acid is UV active, such that when the TLC plate is sprayed with dichlorofluorescein and viewed under UV light, it quenches the fluorescence of the dichlorofluorescein to show up as a dark purple spot on a green background.

Isolation of neutrophils

Human neutrophils were isolated from the peripheral blood of healthy volunteers by the rapid single-step method described by Ferrante and Thong (32) in which the blood was layered onto Ficoll-Hypaque medium (density, 1.114 g/l) and centrifuged at 600 × g for 30–40 min at room temperature. The leukocytes resolved into two distinct bands, with the lower containing neutrophils. In the majority of cases the preparation of neutrophils was of >99% purity and >99% viability, as judged by morphological examination of cytospin preparations and the ability of viable cells to exclude trypan blue stain. The neutrophils were washed three times, resuspended in HBSS (5 × 10⁶ cells/ml), and used within 30 min of preparation. Trypan blue exclusion and lack of lactate dehydrogenase release indicated that the cells remained viable throughout subsequent incubations.

Assessment of 5-lipoxygenase, LTA₄ hydrolase, and cyclooxygenase activity

The effects of β-oxa-21:3n-3 on purified 5-lipoxygenase, cyclooxygenase-1 and 2, and LTA₄ hydrolase were determined by PanLabs (Bothwell, WA). Cyclooxygenase 1 activity was measured using the enzyme purified
from ram seminal vesicle using radiolabeled 20:4n-6 substrate at 37°C for 20 min (37). Cyclooxygenase 2 activity was similarly measured using the enzyme purified from sheep placenta (38). LTA₄ hydrolyase activity was determined by incubating the enzyme purified from guinea pig lungs and radiolabeled LTA₄ substrate incubated at 25°C for 4 min (39). The effects on cyclooxygenase activity was determined using the enzyme purified from rat RBL-1 cells and radiolabeled 20:4n-6 as substrate incubated at 25°C for 8 min (40).

These studies were conducted under standard operating procedures developed by Panlabs. Concurrent testing of standard reference compounds (indomethacin at 1.7 μM for cyclooxygenase 1; 2.4 μM for cyclooxygenase 2; 49 μM esbenol for LTA₄ hydrolyase; 0.25 μM NDGA) was performed with each protocol.

**Assessment of β-oxa-21:3n-3 incorporation into cellular lipids**

Neutrophils (4 × 10⁶) were incubated with or without 20 μM β-oxa-21:3n-3 in 4 ml of HBSS at 37°C for up to 60 min. The incubate was centrifuged at 800 × g for 5 min at room temperature, and the medium was discarded. The cell pellet was resuspended in 2 ml of water, and lipids were extracted by the addition of 7.5 ml of chloroform/methanol/acetic acid (1/2/0.02, v/v/v) (33). The mixture was left at 4°C overnight and then partitioned by the addition of 2.5 ml of chloroform and 2.5 ml of water (34).

The amount of β-oxa-21:3n-3 associated with total cellular lipid was measured as follows. Total lipid extracts were evaporated to dryness under N₂ and transesterified with 5 ml of 1.5% (v/v) H₂SO₄ in methanol at 75°C for 4 h. The resulting fatty acid methyl esters were extracted with 1 ml of water and 2 ml of hexane and then purified on silica gel 60 TLC plates, which were developed in dichloromethane. Authentic standards were cochromatographed to assist identification of the methyl ester species. The β-oxa-fatty acid methyl ester zone was located under UV light after spraying the plate with dichloro fluorescein and viewed under UV light, it quenches the fluorescence of the dichloro fluorescein and is UV active, so on a TLC plate sprayed with the dichloro fluorescein to show up as a dark spot on a green background. The samples were cochromatographed to assist identification of the fatty acid methyl esters, and the liberated fatty acid methyl esters were cochromatographed to assist identiﬁcation of the fatty acid species.

The lipid zones were located under UV light after spraying the plates with 0.1% (w/v) dichloro fluorescein and viewed under UV light, it quenches the fluorescence of the dichloro fluorescein and is UV active, so on a TLC plate sprayed with the dichloro fluorescein to show up as a dark spot on a green background. The samples were cochromatographed to assist identiﬁcation of the fatty acid methyl esters, and the liberated fatty acid methyl esters were cochromatographed to assist identiﬁcation of the fatty acid species.

**Results**

**Inhibition of 5-HETE and LTB₄ production**

Pretreatment of human neutrophils with engineered β-oxa-PUFA (β-oxa-21:3n-3, β-oxa-21:2n-6) markedly inhibited the generation of LTD₄ when the cells were stimulated with the calcium ionophore A23187 (Fig. 1). This effect was comparable to that observed with the commercially available leukotriene inhibitors, Zileuton and NDGA. In contrast, pretreatment with naturally occurring PUFAs only slightly suppressed, in the case of 20:5n-3 and 22:5n-3, or increased, in the case of 20:4n-6, the ionophore-induced LTD₄ production (Fig. 1). The fatty acids were presented to the leukocytes with ethanol as diluent (0.1%, v/v, final concentration); however, similar results were observed using mixed fatty acid-DPC micelles. Trypan blue exclusion and lack of lactate dehydrogenase release showed that the cells remained viable under our experimental conditions (data not presented). The inhibitory effect of β-oxa-21:3n-3 on LTD₄ production was independent of pretreatment time (Fig. 2A) and was significant with a concentration of ≥5 μM (Fig. 2B).

Further studies were conducted to determine whether the generation of [³H]5-HETE was affected by β-oxa-21:3n-3. Pretreatment of [³H]20:4n-6 prelabelled neutrophils with β-oxa-21:3n-3 significantly inhibited calcium ionophore-enhanced production of [³H]5-HETE (Fig. 3A) and [³H]LTB₄ (Fig. 3B). The high 5-HETE and LTD₄ seen at time zero is probably a result of some neutrophil recovered oxygenated fatty acid derivatives were taken up in methanol and characterized by electrospray MS according to the method described by Pitt et al. (43).

**Statistical analyses**

Results are expressed as the mean ± SEM. Statistical analyses were performed by one-way ANOVA, followed by Dunnett’s test for multiple comparisons or by two-tailed Student’s t test for unpaired data. A value of p < 0.05 was considered significant.
activation during the 75 min in preincubation with \([1^H]20:4n-6\). The level of 5-HETE and LTB₄ remained constant during 10- to 30-min incubation, instead of the expected decrease if LTB₄ was \(\omega\)-oxidized. This is either due to an excess amount being produced or the fact that the LTB₄ measured included the two-\(\omega\)-trans isozymes of LTB₄, which are not as well \(\omega\)-oxidized.

Interestingly, pretreatment with \(\beta\)-oxa-21:3n-3 suppressed the production of \([1^H]5\)-HETE and \([1^H]LTB₄\) by leukocytes challenged simultaneously with \([1^H]20:4n-6\) and the calcium ionophore (Fig. 3C). This suggested that the \(\beta\)-oxa-fatty acid could be directly inhibiting the 5-lipoxygenase enzymes.

**Inhibition of 5-lipoxygenase activity by \(\beta\)-oxa-21:3n-3**

Fig. 4 shows that the \(\beta\)-oxa-PUFA substantially inhibited the activity of purified 5-lipoxygenase (IC₅₀ = 26 ± 2 \(\mu\)M; mean ± SEM of three analyses). Conversely, \(\beta\)-oxa-21:3n-3 had no significant effect on the activity of purified LTA₄ hydrodase, cyclooxygenase-1, or cyclooxygenase-2 (results not shown). It remains to be determined whether \(\beta\)-oxa-21:3n-3 can inhibit the activity of LTA₄ synthase in the neutrophil LTB₄ pathway. Overall, the data show that \(\beta\)-oxa-21:3n-3 is a direct inhibitor of 5-lipoxygenase in human neutrophils, leading to reduced formation of 5-HETE and, consequently, LTB₄.

**Incorporation into phospholipids and neutral lipids**

A substantial amount of \(\beta\)-oxa-21:3n-3 was taken up by neutrophils during the first 15 min of incubation and thereafter plateaued up to 60 min. The \(\beta\)-oxa-PUFA taken up by the leukocytes after 60 min was mainly present in the unesterified form, with smaller amounts incorporated in neutral lipids (predominantly cholesterol...
Metabolism of β-oxa-21:3n-3 in stimulated neutrophils

After neutrophils were pretreated with β-oxa-21:3n-3 for 15 min, followed by stimulation with A23187 for 30 min, a small amount of a single oxygenated fatty acid product was observed. The total ion chromatogram produced by electrospray MS analysis of the product showed a molecular ion at m/z 337 (M+−1), which was expected for a monohydroxylated analog of β-oxa-21:3n-3. A daughter ion was found at m/z 238, corresponding to the loss of a C16H11O fragment resulting from C15-C16 bond cleavage. This fragment unambiguously confirms the identification of the oxygenated product with a monohydroxyl group at carbon atom 16. Pretreatment of the leukocytes with NDGA (lipoxigenase inhibitor) markedly suppressed the formation of the oxygenated product, whereas indomethacin (a cyclooxygenase inhibitor) had no effect. In the absence of calcium ionophore stimulation, a negligible amount of the product was recovered. Together these results provide evidence that neutrophils convert β-oxa-21:3n-3 to a 16-monohydroxylated derivative (16-OH-β-oxa-21:3n-3) by the lipoxigenase enzyme pathway. Derivatization of β-oxa-21:3n-3 to methylated, saturated, 16-monohydroperoxy or 16-monohydroxy forms resulted in the loss of its effect (Fig. 5), indicating that the structure of the parent molecule is mandatory for the inhibitory effect.

Discussion

Data from investigations examining the effects of addition of different types of fatty acids to neutrophils on LTB4 production demonstrated that 20:4n-6 significantly increased production compared with the n-3 fatty acids, 20:5n-3 and 22:6n-3, which significantly decreased production. This is consistent with reports in the literature that the lipoxygenase form different and less proinflammatory metabolites from these n-3 fatty acids (44). Presumably this

![Image](https://example.com/image.jpg)

**FIGURE 4.** Effect of β-oxa-21:3n-3 on the activity of purified 5-lipoxygenase. The influence of β-oxa-21:3n-3 (0–100 μM) on purified 5-lipoxygenase activity (using 30 μM 20:4n-6 as substrate) was determined as described in Materials and Methods. Each point represents the mean ± SEM for three separate experiments performed in triplicate. *, p < 0.05; **, p < 0.01 (for significant differences between pretreatment with β-oxa-21:3n-3 and controls, by one-way ANOVA, followed by Dunnett’s test for multiple comparisons).

![Image](https://example.com/image.jpg)

**FIGURE 5.** Effect of β-oxa-21:3n-3 derivatives on LTβ4 production by neutrophils stimulated with the calcium ionophore A23187. Neutrophils (5 × 10⁶) were pretreated with β-oxa-21:3n-3 (20 μM), β-oxa-21:3n-3 derivatives (20 μM), or diluent (control) in 0.5 ml of HBSS at 37°C for 15 min. The cells were then incubated in a 1-ml final volume of HBSS containing the calcium ionophore A23187 (5 μM) at 37°C for 30 min. The mass of LTβ4 in the medium was determined as described in Materials and Methods. Each bar represents the mean ± SEM for three determinations. This experiment was performed three times with similar results. *, p < 0.01 (for significant differences between pretreatment with compounds and controls, by one-way ANOVA, followed by Dunnett’s test for multiple comparisons). The basal production of LTβ4 by the cells was negligible and was not significantly affected by agonist pretreatment. β-oxa-21:3n-3ME, β-oxa-21:3n-3 methyl ester; β-oxa-21:0, saturated β-oxa-21:3n-3; β-oxa-21:3n-3OOH, 16-monohydroperoxy-β-oxa-21:3n-3; β-oxa-21:3n-3OH, 16-monohydroxy-β-oxa-21:3n-3.

### Table 1. Incorporation of β-oxa-21:3n-3 into phospholipids and neutral lipids of neutrophils

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Recovered β-Oxa-21:3n-3 (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>6.6 ± 0.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>12.3 ± 1.3</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>13.3 ± 1.8</td>
</tr>
<tr>
<td>18:3</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>20:4</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>20:5</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>22:6</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

*Neutrophils (4 × 10⁷) were incubated with β-oxa-21:3n-3 (20 μM) or diluent (control) in 4 ml of HBSS at 37°C for 60 min. The amount of β-oxa-21:3n-3 associated with phospholipids and neutral lipids of the cells was determined as described in Materials and Methods. The results represent the mean ± SEM of three separate experiments performed in triplicate and are expressed as a percentage of the total recovered β-oxa-21:3n-3.

After β-oxa-21:3n-3 was administered to mice and rats by gastric or ip injection (daily doses of 100 mg/kg body weight for 3 days), it was found to be incorporated into the lipids of various tissues, including liver, lungs, kidneys, heart, spleen, brain, adipose tissue, and blood (data not presented). The animals given β-oxa-21:3n-3 maintained normal body weight and retained normal liver and kidney function (29).
were found to only slightly reduce LTB₄ production. It is evident its inhibitory action on 5-lipoxygenase, since these derivatives /H9252 LTB 4 production. While 22:6n-3 and the novel n-3 fatty acid also correlate with our previous findings that the latter is a stronger anti-inflammatory agent than the former (29). It is therefore tempting to speculate that the ω-3 fatty acid is likely to be more useful than the fish oil fatty acids for treating inflammatory diseases.

The role of the structure of ω-3 21:3n-3 in the inhibition of LTB₄ production was evaluated, and it was evident that the unsaturation was important, since a ω-3 21:0 fatty acid was not active. However, the position of the double bonds made only a slight difference in activity, where ω-3 21:3n-6 was almost as active as the n-3 equivalent. In contrast, methylation of the carboxyl group, hydroxylation, and formation of the hydroperoxy all resulted in the total loss of activity of the molecule. This demonstrates that the unoxidised parent ω-3 21:3n-3 is the inhibitory agent, which is consistent with our finding that most of the added fatty acid was found as a free fatty acid in stimulated neutrophils.

The inhibition of production of LTB₄ was upstream of LTB₄, since production of 5-HETE was similarly inhibited by ω-3 21:3n-3. Since this fatty acid also dramatically inhibited the oxidation of radiolabeled 20:4n-6 to the above metabolites in neutrophils when both the labeled fatty acid and calcium ionophore were added together, it implies a direct effect on the lipoxygenase enzymes. Using the purified enzyme, this fatty acid was shown to directly inhibit the 5-lipoxygenase enzyme activity. Indeed, it showed selectivity for this enzyme, since it did not inhibit purified LTA₄ hydrolyase, cyclooxygenase-1, and cyclooxygenase-2. The ω-3 21:3n-3 was rapidly taken up by neutrophils, and its inhibitory effects were also very rapid. Thus, >80% inhibition of LTB₄ production occurred even when the fatty acid and the agonist were added simultaneously to the cells. Most of the fatty acid added was taken up by neutrophils and went into the various lipid pools. Importantly, 38% was present as the free fatty acid, presumably due to the inability to be oxidized via ω-3 oxidation. It is highly probable that this was responsible for the potent inhibition of LTB₄ production.

Isomeric forms of monohydroxylated 20:4n-6 (containing the hydroxy group at carbon atoms 5, 8, 9, 11, 12, or 15) are synthesized from 20:4n-6 by activated neutrophils and certain other cells via the actions of stereospecific lipoxygenase enzymes (9, 10). In human neutrophils 5-lipoxygenase is the most active, which is responsible for the relatively high formation of 5-HETE and LTB₄ in response to a stimulus (9, 11). The lipoxygenase positional isomer specificity is governed by the carbon chain length from the methyl end of the fatty acid substrate (34). Since ω-3 21:3n-3 has an additional carbon atom in its chain compared with 20:4n-6, it is probable that the 16-monohydroxylated 21:3n-3 derivative is formed by the 15-lipoxygenase in neutrophils. The conversion of ω-3 21:3n-3 to 16-monohydroperoxy-ω-3 21:3n-3 and 16-monohydroxy-ω-3 21:3n-3 by neutrophils is unlikely to mediate its inhibitory action on 5-lipoxygenase, since these derivatives were found to only slightly reduce LTB₄ production. It is evident that unmodified ω-3 21:3n-3 is primarily responsible for 5-lipoxygenase modulation, and its relatively low cellular metabolism enhances this effect.

LTB₄ controls many neutrophil functions, including chemotaxis, respiratory burst, and degranulation (21). It is therefore highly likely that the observation that the ω-3 21:3n-3 inhibition of LTB₄ production is responsible for the previously observed effects of this fatty acid on acute inflammation induced by carrageenan (29), in which neutrophils play a dominant role (45, 46). Our data together with our previous findings that ω-3 21:3n-3 inhibited carrageenan-induced paw inflammation (29) suggest an important role for the 5-lipoxygenase pathway in this inflammatory reaction (47). ω-3 21:3n-3 was also shown (29) to inhibit the delayed-type hypersensitivity response and in vitro inhibition of T lymphocyte function. However, this appeared to be due to a different mechanism, involving inhibition of protein kinase C and the mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2, particularly as evidence suggests that T lymphocytes lack lipoxygenase activity (29).

Our observations suggest that ω-3 21:3n-3 and other ω-3 PUFA are potent, direct inhibitors of 5-lipoxygenase activity in human leukocytes, resulting in reduced production of 5-HETE and LTB₄. Thus, these ω-3 PUFA will provide new avenues to develop improved therapeutic strategies for inflammatory diseases, such as asthma, rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis. The feasibility of using this fatty acid to treat these diseases is supported by our findings of the lack of toxicity (29) and ability to incorporate into lipids of tissues following oral administration.

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References