Effect of Dietary Supplementation with n-9 Eicosatrienoic Acid on Leukotriene B_4 Synthesis in Rats: A Novel Approach to Inhibition of Eicosanoid Synthesis

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Summary

Studies were undertaken to assess the biochemical effects of dietary supplementation with n-9 eicosatrienoic acid (ETrA), an arachidonic acid analogue that is normally present in cell membranes at very low levels but is raised in the presence of essential fatty acid deficiency (EFAD). The incorporation of dietary ETrA into rat neutrophils and its effect on A23187-stimulated 5-lipoxygenase metabolism in these cells was examined; in addition, the effect of ETrA was compared with that of another arachidonic acid analogue, eicosapentaenoic acid (EPA), which is known to accumulate in cell membranes and inhibit synthesis of leukotriene B_4 (LTB$_4$), á product of the 5-lipoxygenase metabolic pathway. Rats were fed a defined diet that was sufficient in essential fatty acids and that contained EPA or ETrA (0.014% of energy) or no added fatty acid, for 3 wk. In the cells from ETrA-fed rats, LTB$_4$ synthesis was inhibited relative to control values, but synthesis of the other products of 5-lipoxygenase metabolism, 5-hydroxyeicosatetraenoic acid (5-HETE) and the all-*trans* isomers of LTB$_4$, were not inhibited. This pattern indicates inhibition of LTA hydrolase in ETrA-fed rats. In EPA-fed rats, there was inhibition of LTB$_4$ and the all-*trans* isomers of LTB$_4$, but there was no inhibition of 5-HETE. This pattern indicates inhibition of LTA synthase in EPA-fed rats. The results establish that dietary ETrA effectively inhibits synthesis of the inflammatory mediator, LTB$_4$, and suggest that ETrA may confer antiinflammatory benefits similar to those observed with EFAD or dietary fish oil (which contains EPA). Because ETrA is substantially less unsaturated than EPA, it can be expected to have greater chemical stability, which could be an important practical advantage when used as a dietary constituent or supplement.

Activation of neutrophils and monocytes stimulates the metabolism of arachidonic acid (C20:4 n-6; AA) via the sequential actions of 5-lipoxygenase/LTA synthase and LTA hydrolase to the inflammatory mediator, leukotriene B_4 (LTB$_4$) (Fig. 1). Inhibition of LTB$_4$ synthesis remains a goal of pharmacological antiinflammatory therapy. Since AA is formed from a fatty acid that is abundant in the Western diet, namely linoleic acid (C18:2 n-6; LA), a dietary strategy to reduce n-6 fatty acid incorporation and metabolism may achieve the goal of LTB$_4$ inhibition. One strategy has involved the use of dietary eicosapentaenoic acid (C20:5 n-3; EPA), an n-3 fatty acid antagonist of AA incorporation and metabolism that reduces LTB$_4$ synthesis in rats (1) and humans (2, 3) and reduces the signs and symptoms of inflammation in rheumatoid arthritis (3, 4). Another strategy might involve the use of an essential fatty acid deficient (EFAD) diet which is defined by its lack of LA. Dietary LA restriction in rats decreases the level of AA in cell membranes,

decreases LTB$_4$ synthesis (5, 6), and decreases experimental inflammation (7).

Restriction of dietary LA greatly decreases the ratio of n-6/n-9 18-carbon fatty acids available to the desaturase and elongase enzymes. The decrease in its n-6 competitor results in desaturation and elongation of oleic acid (C18:1 n-9; OA) to 8,9,11-eicosatrienoic acid (C20:3 n-9; ETrA) (8). Like AA, ETrA is also a substrate for 5-lipoxygenase/LTA synthase, the initial enzyme in the leukotriene biosynthetic pathway. ETrA, formed as a result of EFAD, is converted by 5-lipoxygenase/LTA synthase to LTA$_3$ (5) which is a poor substrate, but a potent inhibitor of LTA hydrolase (9). LTA hydrolase, in the presence of its usual n-6 substrate, LTA$_4$, catalyzes LTB$_4$ formation (Fig. 1). In EFAD rats, the decrease in LTB$_4$ synthesis (87%) was disproportionately large compared with the decrease in AA levels (34%); therefore, LTA$_3$ (formed from ETrA) was thought to contribute significantly to inhibition of LTB$_4$ synthesis (5).

In healthy human subjects, a large reduction of dietary LA by the substitution of monounsaturated oils and spreads for the usual polyunsaturated products did not cause AA reduction or ETrA formation or LTB₄ inhibition (10). Further reductions in dietary LA will be difficult to achieve. However, dietary supplementation with ETrA may accomplish LTB₄ inhibition if it can be incorporated into tissues and then metabolized to an inhibitor of LTB₄. This has not been examined. In the present study, we have fed chemically synthesized ETrA to rats and examined its incorporation into neutrophils and its effect on LTB₄ synthesis. Its effects were also compared with those resulting from EPA feeding.

Materials and Methods

Materials. 5,8,11-Eicosatrienoic acid was chemically synthesized in the Department of Organic Chemistry, Flinders University, as described (11). After purification by crystallization, this compound was hydrogenated to produce chromatographically pure 5,8,11-ETrA. EPA, free acid, was a gift from Scotia Pharmaceuticals Ltd., Guildford, Surrey, UK. A23187 (Sigma Chemical Co., St. Louis, MO) was dissolved in methanol (1 mM) and diluted in aqueous buffer immediately before use.

Animals and Diets. ETrA and EPA were dissolved (11% wt/wt) in a mixture of vegetable oils that had a polysaturated/monosaturated/saturated ratio = 0.5:1:1 and that contained added vitamin E (0.18% wt/wt). Oils containing ETrA, EPA, or neither fatty acid were mixed (5% wt/wt) with a fat-free diet, the composition of which has been described previously (12). The final dietary content of ETrA and EPA was 0.55% wt/wt (0.014 energy %). Groups of 6-wk-old, male, Hooded Wistar rats (four per group) were fed either the control diet (no ETrA or EPA) or diets containing ETrA or EPA for 3 wk.

Cell Preparation and Stimulation. Peritoneal exudates were induced by intraperitoneally peptone injection and after 4 h, peritoneal exudate cells (80–90% neutrophils) were harvested by lavage as previously described (12). After washing 2× with PBS and lysing the red blood cells with hypotonic saline, cells were resuspended in Dulbecco's PBS (DPBS) containing glucose (1 mg/ml). The cell number was adjusted to 10⁶/ml. Aliquots (1 ml) of cell suspensions were stimulated with A23187 (0.5 μM) for 5 min at 37°C. The mixtures were acidified with citric acid and, after addition of 15-hydroxyeicosatetraenoic acid (15-HETE) as an internal standard, lipids were extracted with a chloroform/methanol mixture as described (12). Quadruplicate samples were processed.

Measurement of 5-Hydroxy Fatty Acids. The lipid extract of the stimulated cells was dried and reconstituted in methanol (50 μl). LTB₄, the all-*trans* isomers of LTB₄, and 5-HETE were quantified by HPLC with UV-detection as described (12). We have established that, under the conditions of stimulation employed in this study, the ω-oxidation products of LTB₄ (20-hydroxy and 20-carboxy LTB₄) are not produced in measurable amounts (<3 ng/10⁶ cells/5 min) in rat peritoneal exudate cells.

Fatty Acids. Neutrophil phospholipids were purified on TLC. Fatty acid methyl esters were generated by methanolysis, resolved, and quantified by capillary GLC as previously described (13).

Results

Neutrophil Phospholipid Fatty Acids. Dietary EPA and ETrA were incorporated into neutrophils with the level of EPA incorporation greater than that of ETrA (Table 1). Since the

levels of EPA and ETrA and their elongation and desaturation products were low or undetectable in cells from rats on the control diet, their increased levels in the cells from rats receiving fatty acid supplemented diets suggests cellular incorporation and metabolism of these dietary fatty acids (Table 1). The sum of the levels of EPA and its elongation and desaturation products (20:5 n-3 + 22:5 n-3 + 22:6 n-3) is greater than the sum of ETrA and its elongation product (20:3 n-9 + 22:3 n-9), indicating that the neutrophil level of EPA is greater than that of ETrA as a result of relatively greater incorporation rather than relatively less metabolism of EPA by the relevant elongase and desaturase enzymes.

The incorporation of EPA into neutrophil phospholipids was associated with decreased AA and increased LA levels (Table 1). By contrast, ETrA incorporation was not associated with changes in AA or LA levels (Table 1).

Neutrophil 5-Lipoxygenase Metabolites. Both EPA- and ETrA-feeding inhibited LTB₄ synthesis and increased (slightly) 5-HETE synthesis (Table 2). However, these fatty acids had different effects on the synthesis of the all-*trans* isomers of LTB₄. EPA feeding resulted in decreased formation of the LTB₄ isomers to an extent similar to the decrease in LTB₄ formation, whereas ETrA feeding had no apparent effect on the isomers (Table 2). Decreased synthesis of LTB₄ without decreased synthesis of the all-*trans* isomers of LTB₄ indicates inhibition of LTA hydrolase since LTA₄ is the common precursor for the all-*trans* isomers of LTB₄ (formed nonenzymatically) and for LTB₄ (via LTA hydrolase) (Fig. 1).

Discussion

EFAD is associated with decreased inflammation in experimental glomerulonephritis in rats (7) and with decreased nephritis and increased survival in a murine model of systemic lupus erythematosus (14). Several actions relevant to the antiinflammatory effect of EFAD have been observed, including decreased numbers of resident tissue macrophages and decreased macrophage migration to an inflammatory focus (7), decreased macrophage spreading and adherence (15), and decreased neutrophil LTB₄ synthesis (5, 16). The latter alteration may be especially important because LTB₄ acts with vasodilator prostaglandins to induce extravasation of fluid (17) and it is a neutrophil chemotaxin and degranulating agent (18). LTB₄ may be responsible for neutrophil migration to an inflammatory focus in the acute phase of tissue inflammation although it has been argued that it is not responsible for the later phase of monocyte migration, at least in rats (7).

The mechanism for decreased LTB₄ synthesis in EFAD may partly involve decreased availability of the substrate, AA. However, neutrophil LTB₄ inhibition is disproportionately large compared with AA decrease and there is evidence of LTA hydrolase inhibition in EFAD (5). The combination of these observations suggests that ETrA and its product LTA₃ are mainly responsible for the inhibition of neutrophil LTB₄ synthesis in EFAD rats (5). However, it is apparent that not all of the depression of leukocyte function in EFAD is mediated by eicosanoids, as there is evidence that the impaired

Table 1. *Effect of Dietary EPA and ETrA on Rat Neutrophil Fatty Acid Composition*

	Neutrophil Fatty Acids (Percent total fatty acids)		
	Control	EPA-Fed	ETrA-Fed
Total Sats	40.1 ± 0.2 a	42.3 ± 0.6 b	38.9 ± 0.2 c
Total Monos.	21.4 ± 0.4 a	19.9 ± 0.1 b	19.6 ± 0.2 b
20:3 n-9 (ETrA)	0.5 ± 0.04 a	N.D.	4.8 ± 0.1 b
22:3 n-9	N.D.	N.D.	1.4 ± 0.2
18:2 n-6	6.5 ± 0.3 a	10.1 ± 0.3 b	6.7 ± 0.2 a
20:3 n-6	1.3 ± 0.1 a	1.0 ± 0.1 ab	0.9 ± 0.1 b
20:4 n-6 (AA)	26.1 ± 0.5 a	18.7 ± 1.2 b	24.9 ± 0.9 a
22:4 n-6	2.2 ± 0.1 a	0.4 ± 0.1 b	1.3 ± 0.2 c
22:5 n-6	0.7 ± 0.1 a	N.D.	0.5 ± 0.01 b
Total n-6	36.8	30.2	34.3
20:5 n-3 (EPA)	0.1 ± 0.03 a	7.0 ± 0.5 b	N.D.
22:5 n-3	N.D.	2.5 ± 0.1	N.D.
22:6 n-3	0.5 ± 0.01 a	2.5 ± 0.7 b	0.4 ± 0.02 a
Total n-3	0.6	12.0	0.4

Values represent the mean ± SD of determinations from four rats. In each row, values with different letters are significantly different from each other (Newman-Keuls Multiple Comparisons analysis, $p < 0.05$). N.D., not detectable.

macrophage spreading and adherence is mediated by decreased AA alone and not by an eicosanoid metabolite of AA (15).

From the preceding results, it is evident that cellular AA depletion is likely to be an important, but not the only factor responsible for the antiinflammatory effect of EFAD, which could be exploited for the treatment of human diseases. Theoretically, lowering cellular AA should reduce n-6 eicosanoid synthesis in inflammatory lesions. Reduction of cellular AA through dietary avoidance of its n-6 precursor, LA, requires dietary stringencies that can be achieved in rats (dietary LA <0.1 en%) (19), but which are not feasible for free-living human subjects. An alternative strategy of using dietary ETrA

supplements may confer some of the antiinflammatory benefits of EFAD if cellular ETrA is increased to an extent that alters leukocyte eicosanoid synthesis (and perhaps other properties). The general approach of using unsaturated 20-carbon fatty acids that compete directly with AA for incorporation into cell membranes and/or compete with AA for eicosanoid synthesis forms the basis for using fish oil or EPA to inhibit n-6 eicosanoid synthesis. However, the current study is the first attempt (to our knowledge) to evaluate this strategy with n-9 fatty acids and the results indicate that dietary ETrA is incorporated into leukocyte membranes. While no decrease in AA level was seen, LTB4 synthesis was inhibited and as discussed below, the ratio of AA to 20-carbon fatty acid competitors (ETrA and EPA) may be a principal determinant of the effect of these competitors on eicosanoid synthesis.

We have previously shown a correlation between EPA/AA ratios in rat neutrophils and LTB5/LTB4 synthesis (1, 12, 13) in rats fed diets of varying n-3 and n-6 fatty acid content. Using data from the present study and published studies that used rat peritoneal exudate cells, it is possible to examine the relationship between ETrA/AA ratios in rat neutrophils and LTB4 synthesis. In the present study, ETrA feeding at a di-

Table 2. *Effect of Dietary EPA and ETrA on the Synthesis of 5-Lipoxygenase Products by Rat Neutrophils*

	LTB4	LTB4 Isomers	5-HETE
		ng/10⁶ cells/5 min	
Control	23.9 ± 1.6 a	15.2 ± 1.2 a	21.7 ± 1.1 a
EPA-fed	10.3 ± 0.7 b	4.5 ± 0.3 b	26.6 ± 1.6 b
ETrA-fed	15.2 ± 1.1 c	16.6 ± 1.1 a	27.8 ± 2.5 b

Values represent the mean ± SD of determinations (in quadruplicate) from four rats. In each column, values with different letters are significantly different from each other (Newman-Keuls Multiple Comparisons analysis, $p < 0.05$).
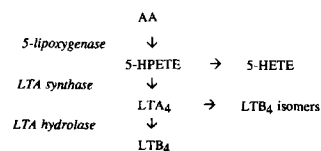

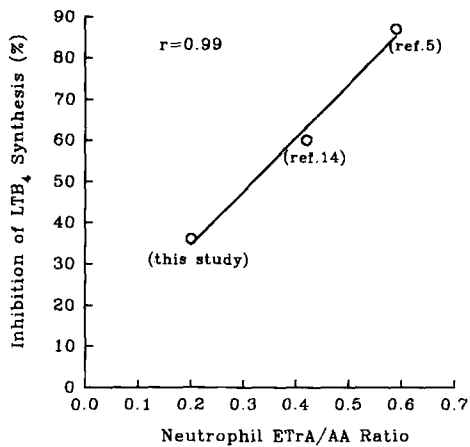**Figure 1.** Metabolism of arachidonic acid to leukotriene B4.

Figure 2. Relationship between the neutrophil ETrA/AA ratio and the extent of inhibition of leukotriene B$_4$ synthesis. The source of the data is shown beneath each symbol.

etary level of 0.014 energy % resulted in a neutrophil ETrA/AA ratio of 0.20 and a 36% decrease in neutrophil LTB$_4$ synthesis. In addition, it has been reported that rats maintained on an EFAD diet for 1 yr had a neutrophil ETrA/AA ratio of 0.59 and 87% depression of LTB$_4$ synthesis (5) and rats maintained on an EFAD diet for several generations had a neutrophil ETrA/AA ratio of 0.42 and 60% depression of LTB$_4$ synthesis (16). Using these data from the present study and the published studies (5, 16), all of which employed peritoneal elicited cells as the source of neutrophils, a strong correlation was observed between the neutrophil ETrA/AA ratio and depression of LTB$_4$ synthesis ($r = 0.99$, $p = 0.005$) (Fig. 2).

Thus, the effect of cellular ETrA on LTB$_4$ synthesis is similar for exogenous (dietary) ETrA and endogenous (formed during EFAD) ETrA. Another similarity in the effects of dietary ETrA and EFAD is the inhibition of LTA hydrolase as manifested by decreased LTB$_4$ synthesis with no decrease in LTB$_4$ isomer formation. In human neutrophils, inhibition of LTA hydrolase is likely to be more effective than inhibition of 5-lipoxygenase/LTA synthase for decreasing LTB$_4$ syn-

thesis since LTA hydrolase is the rate-limiting enzyme and is saturated with substrate (LTA$_4$), at least during A23187 stimulation (20). However, it is not known whether LTA hydrolase is rate-limiting for LTB$_4$ synthesis in A23187-stimulated rat neutrophils and thus, inhibition of rat neutrophil LTA hydrolase may be no more effective than inhibition of either the 5-lipoxygenase or LTA synthase reactions for inhibition of LTB$_4$ synthesis.

In contrast to the ETrA-induced inhibition of LTA hydrolase, dietary EPA appeared to inhibit LTB$_4$ synthesis at the LTA synthase step, i.e., at a reaction after the production of 5-hydroperoxy eicosatetraenoic acid (5-HPETE), which is reduced to 5-HETE and before the formation of LTA$_4$, which is the immediate precursor for both LTB$_4$ and all-*trans* isomers of LTB$_4$, both of which were decreased after EPA feeding. Despite the apparent inhibition of different enzymes by ETrA and EPA, it can be argued that their potencies for inhibition of LTB$_4$ synthesis are similar. In the present study, EPA dietary supplementation resulted in a neutrophil EPA/AA ratio of 0.37. If EPA/AA and ETrA/AA have similar relationships with the extent of inhibition of LTB$_4$ synthesis, the predicted inhibition of LTB$_4$ synthesis for a ratio of 0.37 is 56% (see Fig. 2). The observed inhibition was 57%, suggesting that the inhibitory potency of ETrA and EPA is similar under these experimental conditions in rat neutrophils.

Since dietary EPA and ETrA inhibited LTB$_4$ synthesis at different reactions in the LTB$_4$ biosynthetic pathway, combinations of the two fatty acids may give additive or even synergistic inhibition of LTB$_4$ synthesis. Also, dietary ETrA may have additive or synergistic effects with oral pharmacological inhibitors of 5-lipoxygenase for inhibition of LTB$_4$ synthesis. Insufficient synthetic ETrA was available to test these propositions.

We have shown that dietary ETrA supplementation can modify LTB$_4$ production in the presence of an essential fatty acid sufficient diet. This approach is analogous to dietary fish oil or EPA supplementation but has the advantage that ETrA is odorless and is more stable (because it is less unsaturated) than EPA, both features that would allow its use as a food additive rather than a pharmacological supplement in situations in which reduced eicosanoid production is desirable.

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