Dietary (n-9) Eicosatrienoic Acid from a Cultured Fungus Inhibits Leukotriene B_4 Synthesis in Rats and the Effect Is Modified by Dietary Linoleic Acid[1,2]

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ABSTRACT Eicosatrienoic acid (ETrA) is the (n-9) homologue of (n-6) arachidonic acid (AA) and (n-3) eicosapentaenoic acid (EPA). ETrA can be synthesized endogeneously, but tissue levels are normally undetectable except in essential fatty acid (EFA) deficiency. An ETrA-rich oil extracted from a cultured fungus was used to prepare diets which had varying levels of ETrA (0–8 g/kg diet) in combination with one of two levels of linoleic acid (LA, 2.2 or 9.5 g/kg diet). All diets were sufficient in essential fatty acids. Groups of rats were fed these diets for 4 wk after which leucocyte fatty acid content and leukotriene B_4 (LTB_4) synthesis were measured. The influence of dietary LA on ETrA accumulation in cells was studied and correlations with LTB_4 synthesis determined. ETrA was efficiently incorporated into peritoneal exudate cell (PEC) phospholipids with no evident saturation being observed with levels up to 10 mol/100 mol total fatty acids in peritoneal exudate cells. Cellular ETrA levels were lower ($P < 0.001$) in rats fed the higher level of LA. ETrA accumulation in peritoneal exudate cells correlated ($r^2 = 0.63$, $P < 0.05$) with reduced LTB_4 synthesis which was attributable to LTA hydrolase inhibition. Thus, dietary ETrA from a biological source can accumulate in leucocytes and suppress inflammatory eicosanoid synthesis. The findings justify further studies into the biochemical and anti-inflammatory effects of dietary ETrA, which could be incorporated into palatable food additives. J. Nutr. 126: 1534–1540, 1996.

INDEXING KEY WORDS:

- *(n-9) eicosatrienoic acid* • *linoleic acid*
- *neutrophils* • *leukotriene B_4* • *rats*

When (n-6) essential fatty acids (EFA)[4] are abundant in the diet, (n-9) eicosatrienoic acid, [ETrA, 20:3(n-9)], is a minor constituent of plasma and tissue fatty acids in adult mammals. There are exceptions, e.g., immature hyaline cartilage, where ETrA can be present normally in greater amounts within the setting of diets rich in (n-6) fat (Adkisson et al. 1990, Cleland et al. 1995). With EFA-sufficient diets in which (n-9) monounsaturated fatty acids dominate relative to (n-6) fatty acids, plasma ETrA levels increase modestly. Only in the presence of severe EFA-deficiency do ETrA levels in plasma and cells approach those of its (n-6) homologue, arachidonic acid [AA, 20:4(n-6)] (Holman 1960).

We have shown that when chemically synthesized ETrA was given as a dietary supplement to rats within an EFA-sufficient diet, it was incorporated into the phospholipids of peritoneal exudate cells (PEC), and in vitro production of leukotriene B_4 (LTB_4) by these cells was reduced (James et al. 1993). Thus, it appears that ETrA may be similar to (n-3) eicosapentaenoic acid [EPA, 20:5(n-3)] with regard to its dietary effects on LTB_4 synthesis. However, information is scant because the lack of adequate quantities of ETrA has inhibited further dietary studies.

Therefore, the present study was undertaken to as-

[1] Supported by a project grant from the National Health and Medical Research Council of Australia.

[2] The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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[4] Abbreviations used: AA, arachidonic acid; EDA, eicosadienoic acid; EFA, essential fatty acids; EPA, eicosapentaenoic acid, ETrA, eicosatrienoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; LA, linoleic acid; LT, leukotriene; PEC, peritoneal exudate cells; PG, prostaglandin; TX, thromboxane.

Manuscript received 26 September 1995. Initial review completed 15 November 1995. Revision accepted 8 February 1996.

TABLE 1

Percentage of various fatty acids in the diet

Fatty acid	Diet number								
	1	2	3	4	5	6	7	8	9
	g/100 g								
16:0	8.44	7.21	8.13	6.05	7.10	5.12	5.92	4.03	4.87
18:0	5.67	5.18	5.09	4.65	4.70	4.27	4.19	3.78	3.72
22:0	2.14	1.97	1.78	1.69	1.50	1.39	1.24	1.09	0.95
24:0	5.00	4.23	4.05	2.98	2.79	1.68	1.64	0.35	0.32
ΣSaturated	22.63	19.83	20.28	16.42	17.19	13.30	13.87	10.04	10.66
Σ*Trans*	0.16	0.10	0.08	0.25	0.11	0.29	0.26	0.37	0.30
18:1(n-9)	34.44	47.11	31.50	59.59	43.62	71.75	56.31	84.00	68.61
18:2(n-9)	13.72	10.12	10.31	6.68	6.99	3.39	3.50	0.00	0.15
20:2(n-9)	2.52	1.87	1.86	1.26	1.24	0.64	0.64	0.00	0.00
20:3(n-9)	16.46	12.16	12.22	8.09	8.07	3.99	3.99	0.00	0.00
22:1(n-9)	0.23	0.18	0.18	0.12	0.13	0.07	0.07	0.00	0.00
22:3(n-9)	0.17	0.13	0.15	0.09	0.10	0.00	0.00	0.00	0.00
24:1(n-9)	0.55	0.44	0.44	0.29	0.30	0.15	0.17	0.00	0.00
ΣMonounsaturated	38.43	50.41	34.85	62.15	46.25	73.58	58.16	85.06	69.72
Σ(n-9)	68.42	72.25	56.90	76.29	60.61	80.08	64.76	84.00	68.75
18:2(n-6)	4.57	4.39	19.17	4.38	19.27	4.42	19.08	4.35	18.89
20:4(n-6)	0.42	0.30	0.30	0.20	0.21	0.11	0.11	0.00	0.00
Σ(n-6)	5.23	4.87	19.66	4.71	19.61	4.54	19.18	4.35	18.89
18:3(n-3)	0.60	0.47	0.62	0.39	0.50	0.27	0.38	0.19	0.28
22:6(n-3)	0.17	0.12	0.07	0.05	0.05	0.00	0.00	0.00	0.00
Σ(n-3)	0.85	0.64	0.74	0.44	0.54	0.27	0.38	0.19	0.28

sess the biochemical effects in rats of diets containing an oil from a substrain of the fungus *Mortierella alpina*. The oil is rich in ETrA and will be referred to as Mut48 oil. The fatty acid composition of phospholipids in PEC was analyzed. Ex vivo production of LTB_4 and other 5-lipoxygenase metabolites by PEC was also evaluated, along with thromboxane A_2 (TXA_2) release into serum. Because we have shown previously that dietary linoleic acid [LA, 18:2(n-6)] inhibits cellular incorporation of the (n-3) fatty acid, EPA, from the diet in humans and rats (Cleland et al. 1992, James et al. 1991), this study was designed to discern the possible effects of dietary LA on cellular incorporation of dietary ETrA.

MATERIALS AND METHODS

M. alpina (Mut48) oil was obtained from Suntory, Osaka, Japan. Sunflower oil and Sunola oil™ were obtained from Meadow Lea Foods, Sydney, Australia. Sunola oil is obtained from a sunflower strain which yields an oil with a low LA concentration (4.35 g/100 g of total fatty acids) compared with regular sunflower oil which contains ~70% LA.

Diets. Mut48 oil was mixed with varying amounts of the LA-rich sunflower oil and Sunola oil to create a panel of oil mixtures containing ETrA in proportions of 12, 8 and 4 g/100 g total fatty acids in the presence of LA at levels of 4.4 and 19 g/100 g total fatty acids. Additional preparations included Mut48 oil blended with a small amount of sunflower oil (25:1) to raise the proportion of LA to 4.4 g/100 g total fatty acids, thereby ensuring that dietary EFA requirements would be met. The remaining groups were Sunola only and a blend of Sunola and sunflower oil which contained LA at a level of 18 g/100 g total fatty acids. The oil blends were mixed with a fat-free diet, which was based on the AIN-76 diet. The details of the diet have been published previously (James et al. 1991). The total fat concentration of the final diet was 5 g/100 g, and at this level of fat, the energy level of the diet is 66.1 kJ/g. The proportions of fatty acids in the nine test diets are detailed in **Table 1.** Linoleic acid was present at two levels in the nine diets, ~4.4 and 19 g/100 g total fatty acids which is equivalent to 2.2 and 9.5 g/kg diet, respectively. ETrA was present at five levels in the nine diets, ~0, 4, 8, 12, 16 g/100 g total fatty acids which is equivalent to 0, 2, 4, 6, 8 g/kg diet, respectively.

Rats. Female Dark Agouti rats were supplied by the Institute of Medical and Veterinary Science, Veterinary Services Division, Gilles Plains, South Australia. Groups of four rats were fed the test diets from 5 wk of age for 4 wk. Food intake was not monitored. Peritoneal exudates were induced by injection of peptone (3%, 10 mL) into the peritoneal cavity 4 h prior to killing of rats by cervical dislocation under halothane anaesthesia. At the time of killing, blood samples were taken and PEC were harvested by lavage as described previously (James et al. 1991). The study was approved by the Animal

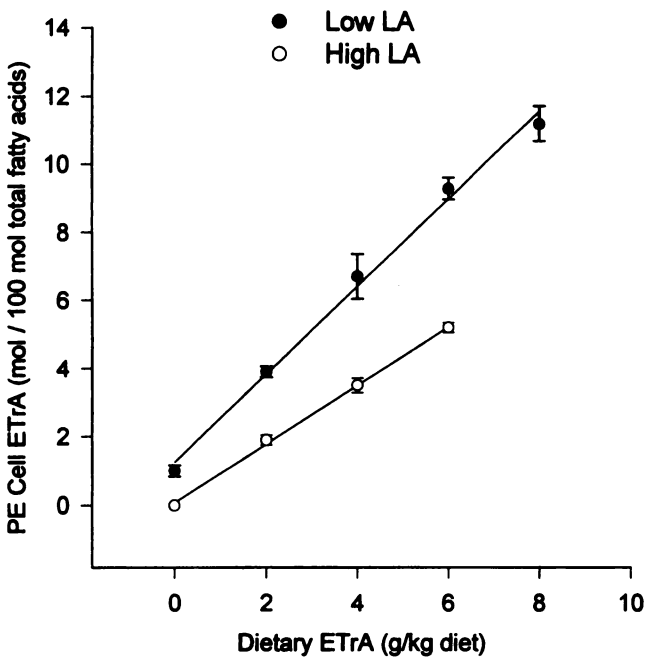

FIGURE 1 Effect of dietary eicosatrienoic acid (ETrA) on the ETrA content of peritoneal exudate (PE) cells of rats fed two levels of dietary linoleic acid (LA): low LA, 2.2 g/kg diet; high LA, 9.5 g/kg diet. Points represent means ±SD, $n = 4$ rats. Correlations: low LA, $r^2 = 0.99$, $P < 0.001$; high LA, $r^2 = 0.99$, $P < 0.001$.

Ethics Committee of the Institute of Medical and Veterinary Science/Royal Adelaide Hospital.

Fatty acid analyses. Total lipids were extracted from PEC with chloroform:methanol (7:3), and extracts were evaporated to dryness under nitrogen. The residues were dissolved in chloroform:methanol (9:1) and stored at −70°C prior to fatty acid analysis. Phospholipids were separated by thin layer chromatography as described previously (Cleland et al. 1992). The samples were then hydrolyzed and methylated in 0.102 mol/L H_2SO_4 in methanol at 70°C for 3 h. The resulting methyl esters were separated and quantified by gas liquid chromatography as described previously (Makrides et al. 1994). All organic solvents contained 0.005% butylated hydroxyanisole.

Cell preparation and stimulation. Peritoneal exudate cells were washed with PBS, and the erythrocytes were lysed with hypotonic saline. The remaining cells (80–90% neutrophils) were resuspended in Dulbecco's PBS containing 1 g/L glucose. The cell number was adjusted to 10^9/L. Aliquots (1 mL) of cell suspensions were stimulated with A23187 (0.5 μmol/L) for 5 min at 37°C. The mixtures were acidified with citric acid, and 15-hydroxyeicosatetraenoic acid (15-HETE) was added as internal standard, prior to extraction with a chloroform/methanol mixture as described (James et al. 1991). Quadruplicate samples were processed for each rat.

Measurement of 5-hydroxy fatty acids. Lipid extracts of the stimulated cells were dried and reconstituted in methanol (50 μL). Leukotriene B_4, the all-*trans* isomers of LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE) were quantified by HPLC with UV detection, using 15-HETE as internal standard, as described (James et al. 1991). We have established that under the conditions of stimulation and incubation employed, the omega oxidation products of LTB_4 (20-hydroxy and 20-carboxy LTB_4) are not produced in measurable amounts [<3 ng/(10^6 cells · 5 min)] by rat PEC.

Measurement of thromboxane synthesis. Blood was allowed to clot at 37°C for 1 h. Thromboxane B_2 (TXB_2) was measured in aliquots of serum using RIA. The thromboxane antiserum, prepared by inoculating a rabbit with TXB_2 conjugated with thyroglobulin, has been used in previous studies (James and Walsh 1988). Cross-reactivities were 0.06% for prostaglandin E_2 (PGE_2), 0.05% for 6-keto-$PGF_{1\alpha}$ and <0.05% for $PGF_{2\alpha}$.

Statistics. Correlations between fatty acid composition of PEC and dietary treatments or PEC mediator production were assessed by linear regression analysis. Differences in PEC ETrA values at fixed levels of dietary LA were assessed by multiple t tests. The effect of the diets on TXB_2 production was examined by ANOVA (Kwikstat version 3.3, Texasoft, Cedar Hill, TX).

RESULTS

Incorporation of ETrA into cellular phospholipids. ETrA was incorporated into PEC phospholipids in direct proportion to the amount of ETrA present in the

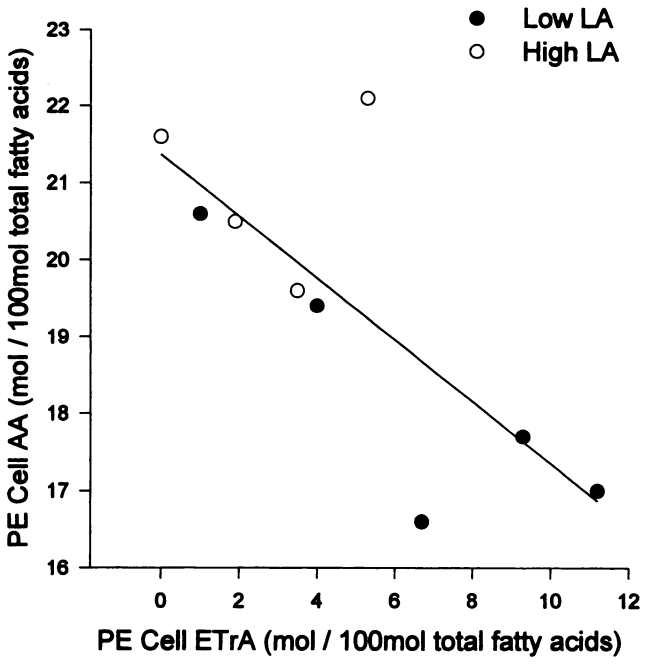

FIGURE 2 Effect of peritoneal exudate (PE) cell eicosatrienoic acid (ETrA) content on PE cell arachidonic acid (AA) content of rats fed two levels of dietary linoleic acid (LA): low LA, 2.2 g/kg diet; high LA, 9.5 g/kg diet. Correlation: $r^2 = 0.58$, $P < 0.05$.

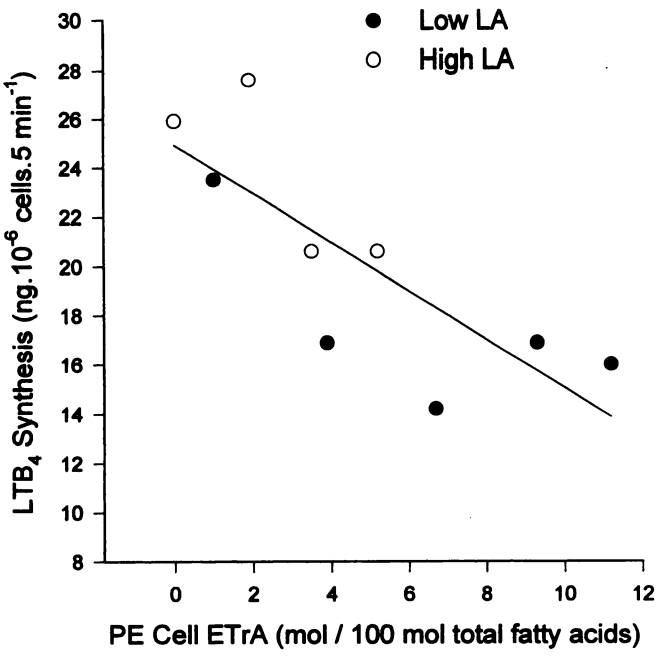

FIGURE 3 Relationship between peritoneal exudate (PE) cell eicosatrienoic acid (ETrA) levels and PE cell leukotriene B_4 (LTB_4) synthesis in rats fed two levels of dietary linoleic acid (LA): low LA, 2.2 g/kg diet; high LA, 9.5 g/kg diet. Points represent the mean values of 4 rats. Correlation: $r^2 = 0.63$, $P < 0.05$.

diet. The correlation between dietary ETrA and PEC ETrA was significant regardless of the LA concentration of the diet **(Fig. 1)**. However, dietary LA had a significant negative effect on the efficiency with which ETrA accumulated in PEC phospholipids (Fig. 1). The presence of 9.5 g/kg dietary LA significantly decreased ($P < 0.001$) the levels of ETrA in PEC by ~30%, compared with PEC ETrA levels achieved in rats fed diets containing 2.2 g/kg LA. Within the range of ETrA provided in the diets, evidence for saturation of ETrA incorporation into PEC phospholipids was not seen, even though ETrA levels reached 10 mol/100 mol total fatty acids. Incorporation was efficient, particularly in rats fed the lower LA diets.

Arachidonic acid levels in PEC decreased with increasing levels of cellular ETrA **(Fig. 2)**. However, displacement of AA accounted only partially for the increase in cellular ETrA, with displacement of oleic acid and saturated fatty acids accounting for the remainder of the ETrA increase. Linoleic acid levels in PEC were unaffected by dietary ETrA (results not shown).

Effect on 5-lipoxygenase pathway. An inverse correlation was seen between cell phospholipid ETrA and LTB_4 synthesis by stimulated PEC ($P < 0.01$) **(Fig. 3)**. No correlation was seen between ETrA in PEC phospholipids and the synthesis of 5-HETE or total 5-lipoxygenase metabolites (i.e., 5-HETE + LTB_4 + LTB_4 isomers) in response to stimulation in vitro (data not shown). However, a strong negative association was found between ETrA in PEC and a measure of LTA hydrolase activity which was the proportion of subtrate LTA_4 (assessed as the sum of LTB_4 and the nonenzymatic LTB_4 isomers) that was converted to LTB_4 (Cleland et al. 1994) **(Fig. 4)**.

Effect on thromboxane synthesis. Dietary ETrA had no discernible effect on TXA_2 release into serum, as measured by TXB_2 concentrations (ANOVA) **(Table 2)**.

DISCUSSION

The anti-inflammatory effects of EFA-deprivation have been established in laboratory rodents fed EFA-deficient diets. The potency of this effect is highlighted by observations with NZB/NZW mice. These mice develop spontaneously an illness resembling systemic lupus erythematosus when fed conventional (EFA-sufficient) nonpurified diet. Mice fed an EFA-deficient diet had less severe disease and improved survival relative to their counterparts fed an EFA-sufficient diet (Hurd and Gilliam 1981). EFA-deficient diets also reduce proteinuria and glomerular leukocyte infiltration in experimental glomerulo-nephritis in rats (Schreiner et al. 1989).

Tissue accumulation of ETrA is a feature of EFA-deficiency. As a 20-carbon fatty acid, ETrA (and its 5-hydroxy metabolites) may compete with arachidonic acid for access to eicosanoid-forming enzymes (Evans

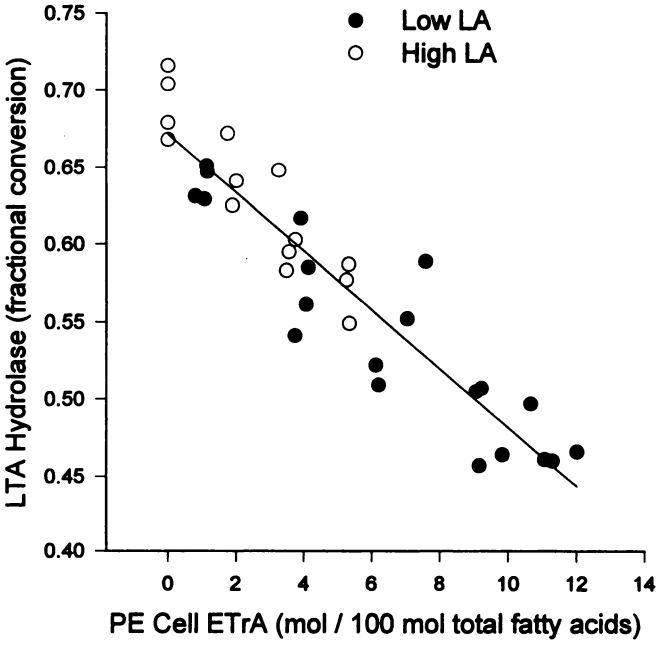

FIGURE 4 Relationship between peritoneal exudate (PE) cell eicosatrienoic acid (ETrA) levels and a measure of PE cell leukotriene A (LTA) hydrolase activity in rats fed two levels of dietary linoleic acid (LA): low LA, 2.2 g/kg diet; high LA, 9.5 g/kg diet. The LTA hydrolase activity is expressed as the proportion of substrate converted to product (fractional conversion) and is described in the text. Points represent data from individual rats. Correlation: $r^2 = 0.87$, $P < 0.001$.

TABLE 2

Thromboxane B_2 concentrations in serum of rats fed diets containing eicosatrienoic acid and two levels of linoleic acid[1]

Diet number[2]								
1	2	3	4	5	6	7	8	9
				μg/L				
465 ± 213	695 ± 110	654 ± 259	610 ± 146	679 ± 105	595 ± 41	740 ± 139	579 ± 85	505 ± 96

[1] Values represent mean ± SD, n = 4 rats.
[2] See Table 1.

et al. 1985, Lefkowith et al. 1987, Stenson et al. 1984). Because arachidonic acid is strongly conserved in EFA deficiency, even in the presence of severe depletion of its precursor LA (Cleland et al. 1994), the anti-inflammatory effects of EFA deficiency could, in part, reflect the effects of ETrA accumulation on inflammatory eicosanoid production.

The biochemical effects of dietary supplementation of rats with chemically synthesized ETrA (5.5 g/kg diet) within the context of an EFA-sufficient diet (ratio of polyunsaturated/monounsaturated/saturated fatty acids = 0.5:1:1) have been studied previously (James et al. 1993). ETrA was incorporated into the phospholipids of induced peritoneal exudate cells, and there was reduced LTB_4 synthesis relative to a comparison group receiving the background diet alone. In a further comparison group, made up of rats given EPA in amounts equivalent to the ETrA supplement, EPA incorporation and reduced LTB_4 production were also seen, as expected from the findings of earlier studies (Cleland et al. 1990, James et al. 1991). These studies indicate that ETrA, like EPA, can be given as a dietary supplement without evident toxicity and with a modulating effect on inflammatory mediator production. However, the cost of chemically synthesized ETrA precluded studies of its anti-inflammatory effects and studies of its interactions with other dietary fatty acids.

A preliminary report indicated that carrageenan-induced paw swelling was reduced in rats fed an EFA-sufficient diet containing an ETrA-rich oil (Nakamura et al. 1993). This oil was obtained from cultures of a mutant of the fungus *M. alpina* (unpublished data). The present investigations involve a more extensive analysis of the biochemical effects of an oil from this source when given within the context of defined diets which vary in LA concentration.

The results indicate that incorporation of dietary ETrA into cells occurs efficiently and that levels of at least 10 mol/100 mol total fatty acids can be achieved in PEC without evident saturation. However, the extent of incorporation was influenced negatively by the amount of LA co-administered in the diet. While this inhibitory effect of dietary LA on ETrA accumulation in cells was significant, ETrA as a proportion of phospholipid fatty acids in PEC was 5.3 mol/100 mol when ETrA was given at 6 g/kg diet in the presence of LA at 9.5 g/kg diet. The negative effect of dietary LA on ETrA incorporation is analogous to the effect of dietary LA on EPA incorporation into cells as shown in our previous studies in rats and humans (Cleland et al. 1992, James et al. 1991).

In the present study, we have shown a modulating effect of dietary ETrA on LTB_4 synthesis by PEC. Analyses of metabolites of the 5-lipoxygenase pathway indicated inhibition of LTA hydrolase, the terminal enzyme in the LTB_4 synthetic pathway. This finding is consistent with the effects of ETrA on subcellular fractions of LTA hydrolase (Evans et al. 1985, Jakschik et al. 1983, Stenson et al. 1984), the effects of exogenous ETrA on leucocytes in vitro (Cleland et al. 1994), and our earlier studies on dietary supplementation with chemically synthesized ETrA in rats (James et al. 1993). This effect of ETrA mirrors the effect of EPA on LTB_4 synthesis, although in the case of ETrA, measurable amounts of the (n-9) homologue of LTB_4 (i.e. LTB_3) are not found because LTA_3 inhibits LTA hydrolase (Evans et al. 1985). By contrast, the (n-3) homologue, LTB_5, can be detected after EPA treatment.

The (n-9) fatty acid, eicosadienoic acid [EDA, 20:2(n-9)] was a minor constituent of the diets (up to 1.25 g/kg) and accumulated to some extent in PEC (maximum 2.2 mol/100 mol phospholipid fatty acids). As a 20-carbon fatty acid, EDA could theoretically influence AA metabolism in a similar manner to that of ETrA and EPA. However, inclusion of this fatty acid with ETrA in correlations with LTA hydrolase activity (data not shown) did not increase the significance of the observed inhibitory effect. While the amounts of EDA may have been insufficient to discern an effect, the greater disparity in the number of double bonds between AA and EDA than between AA and ETrA or EPA, may render EDA a less potent competitor for AA metabolizing enzymes.

Release of TXA_2 into serum was not reduced by dietary ETrA as assessed by RIA for the stable TXA_2 metabolite, TXB_2. Immunoassays may not distinguish between homologous (n-6) and (n-3) metabolites, which, as in the case of TXA_2 and TXA_3, can have markedly different activities. However, because EPA was not detected in PEC, very little if any TXA_3 could have

formed. Because ETrA lacks the (n-6) bond necessary for cyclooxygenase action, formation of a potentially confounding (n-9) homologue of TXA_2 is precluded on structural grounds. Thus, the TXB_2 RIA can be considered to reliably reflect TXA_2 synthesis in the present context with no effect of ETrA on TXA_2 synthesis being observed.

A key issue is the relevance of these studies of ETrA supplementation in rats to dietary strategies designed to modulate risk for, or expression of, unwanted inflammation in human subjects. It is encouraging that in our studies of ETrA feeding in rats, no evident toxicity has been seen in spite of careful monitoring of the appearance and behavior of rats and a histological survey of major organs.

Although the tolerance of human subjects to dietary enrichment with ETrA has not been tested, ETrA is a normal constituent of fetal tissues with levels declining during infancy (Adkisson et al. 1990, Cleland et al. 1995). Also, ETrA accumulation was well tolerated by an adult female who became severely EFA deficient while self-administering long-term total parenteral nutrition (TPN) which she adapted due to intolerance of the prescribed fat component (Cleland et al. 1994). In her peripheral blood neutrophils, phospholipid AA was well conserved (AA, 8.6 mol/100 mol total fatty acids; reference range 10–16 mol/100 mol) in spite of a dramatic reduction of LA and consequent marked reduction of total (n-6) fatty acids. ETrA comprised more than 5 mol/100 mol total fatty acids in her neutrophil phospholipids (reference range <0.1 mol/100 mol). This study suggested a benign effect of ETrA accumulation.

A related issue is the potential anti-inflammatory effect of ETrA in humans. In the case described above, the peripheral blood neutrophils displayed markedly reduced LTB_4 synthesis when stimulated ex vivo, with inhibition occurring at the LTA hydrolase reaction (as seen with PEC in rats fed ETrA). Because the extent of inhibition of LTB_4 was disproportionate to the modest reduction in cellular AA and because ETrA has been shown to inhibit LTA hydrolase (Evans et al. 1985, Jakschik et al. 1983, Stenson et al. 1984), it was inferred that inhibition of neutrophil LTB_4 synthesis in this woman was due to the effects of accumulation of ETrA (Cleland et al. 1994). In human neutrophils in vitro, addition of exogenous ETrA or EPA resulted in dose-dependent inhibition of LTB_4 synthesis (Cleland et al. 1994). EPA is a constituent of fish oils, which have anti-inflammatory effects in several human inflammatory diseases.

While the above studies provide impetus for the further evaluation of dietary ETrA supplementation as a component of regimens designed to reduce unwanted inflammation, the evaluation of its anti-inflammatory effects to date has been confined to a single preliminary study of carrageenan-induced edema and to in vitro studies of mediator production using cells from normal rats fed ETrA. Investigations should be extended further to include studies of more complex models of inflammation that more closely mirror human inflammatory disorders. Studies of dietary treatments of animals predisposed genetically to spontaneous inflammatory disorders would be of particular interest. Dietary interventions, started when these animals are healthy, should provide a guide to the potential prophylactic role that ETrA, as a palatable food additive, may play in reducing the emergence of human inflammatory diseases in the community or in those identified as being at high risk.

ACKNOWLEDGMENT

The expert technical assistance of Cindy Hall is gratefully acknowledged.

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