TETRAENE AND PENTAENE LEUKOTRIENES: SELECTIVE PRODUCTION FROM MURINE MASTOCYTOMA CELLS AFTER DIETARY MANIPULATION

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ABSTRACT

A neoplastic mast cell tumor was grown in mice which had been raised since birth on a diet enriched with eicosapentaenoic acid. Intact harvested mastocytoma cells were stimulated with calcium ionophore A23187 to produce lipoxygenase products from the polyunsaturated fatty acids liberated from the cellular membranes. Leukotriene B_4, B_5, C_4 and C_5 were isolated and characterized by HPLC retention time, ultraviolet absorption spectrometry and mass spectrometry. The arachidonic acid content of the mast cell tumor lipids was altered from 9.2 to 3.9 mole % while eicosapentaenoic acid increased from 0.5 to 4.5 mole % in response to the fish oil-supplemented diet.

The relative amounts of arachidonic and eicosapentaenoic acids (3.9 and 4.5 mole % respectively) were associated with similar amounts of LTB_4 and LTB_5 synthesized by the cells. These results suggest that the epoxide leukotriene (LTA) derivative can be made efficiently from either arachidonic or eicosapentaenoic acids when both are present in cellular lipids. In contrast, the ratio of LTC_4 to LTC_5 (10 to 1) indicates that the reaction of LTA with glutathione may be critically dependent upon the structure of the unsaturated fatty acid with the ratio of LTC_4/LTB_4 (2.0) more than 10 times greater than that (0.16) for LTC_5/LTB_5.

INTRODUCTION

The two major types of polyunsaturated fatty acids are derived from linoleic acid (18:2(n-6)) and linolenic acid (18:3(n-3)) respectively. Recent studies have indicated that dietary polyunsaturated fatty acids of the linolenic type (designated(n-3)) may have a significant impact in reducing thrombosis and vascular hemostasis (1,2). Particular emphasis has been placed on the effects of eicosapentaenoic acid (20:5(n-3)) in regulating the biosynthesis of prostaglandins and thromboxane (3). Analytical results comparing the abundance of this fatty acid in the diet with its abundance in the resultant serum lipids and platelet phospholipids have been correlated to decreased tendencies in platelet aggregation (4,5). Increased abundance of eicosapentaenoic acid relative to arachidonic acid in the tissue lipids of subjects

consuming appreciable quantities of maritime products may exert an effect by competitive interactions with cycloxygenase in two different ways. Eicosapentaenoic acid may form trienoic prostaglandin derivatives which may have either greatly diminished or even antagonistic activities on thrombogenesis and vasospasm compared to the corresponding products from arachidonic acid (6). On the other hand, the inability of the n-3 fatty acid derivatives to be oxygenated by cyclooxygenase under conditions of low peroxide tone (7,8) allows this acid to serve as a competitive inhibitor of dienoic prostaglandin biosynthesis under the physiologic conditions within the cells (8,9).

Leukotrienes are now recognized as important metabolic derivatives of arachidonic acid (10,11) which arise from the action of lipoxygenase rather than cyclooxygenase activity. Leukotriene C and D are found in the preparations of the slow reacting substance of anaphylaxis from animals (12,13) and humans (14). In addition, leukotriene B is a potent chemotactic factor comparable to C_{5a} (15) as a chemokinetic factor and inducer of neutrophil aggregation (15) as well as being able to stimulate accumulation of PMNs and macrophages and affect capillary permeability (16). A recent report indicated that leukotriene C_5 (LTC_5) can be synthesized when eicosapentaenoic acid is incubated with murine mastocytoma cells and that the product has less biological activity than LTC_4 in inducing contraction of guinea pig lieum (17). Because of the marked differences in reactivity noted for the n-3 and n-6 fatty acids with cylooxygenase under physiologic conditions (8), we felt it would be of value to determine the relative capacity of these two acids to be converted into leukotriene by the lipoxygenase enzyme system. The model selected for study was the murine mastocytoma cell carried in mice that had been maintained on a diet rich in eicosapentaenoic acid. The mastocytoma cell containing elevated levels of eicosapentaenoic acid harvested, stimulated with calcium ionophore (A23187), and the resultant leukotrienes were isolated. This report describes the selective conversion of two different acids, 20:4(n-6) and 20:5(n-3), into leukotriene derivatives.

MATERIALS AND METHODS

Feeding protocol

A rodent fat free test diet was obtained from ICN-Nutritional Biochemicals (Cleveland, OH) and was suplemented with 5% (w/w) menhaden fish oil (Zapata Haynie; Reedville, VA) and 0.05% butylated hydroxytoluene. The diet was prepared fresh every two days with pellets always available to the mice. Control mice were fed Purina lab chow. Pregnant CB6F mice were obtained from Jackson Laboratories (Bar Harbor, ME) and as soon as litters were delivered, the mothers were provided the fish oil supplemented diet. After weaning, the offspring were also provided the fish oil supplemented diet. After 6 weeks, the mice were injected in the peritoneal cavity with $3x10^6$ CXBG ABMCT-1 tumor cells which was originally

obtained from Litton Bionetics (Kensington, MD). The cells were carried as an ascites tumor for two weeks, after which time approximately $1x10^8$ cells per mouse were harvested.

Incubations

The tumor cells from 12 mice ($7.1x10^8$ cells) were suspended in buffer (150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 35 mM KH_2PO_4, 15.6 mM dextrose, pH7.3) to a concentration 10^7 cells/ml. Then after warming to 37° the incubation was made 20 μM in ionophore A23187 (Calbiochem, San Diego, CA). The suspension was made 1.8 mM in Ca^{2+} to stimulate the cell and incubated for 20 min. after which time 4 volumes of ethanol was added to terminate the incubation. The ethanolic suspension was cooled at 4° for 2 hours, centrifuged, and the pellet was washed with 0.5 volume ethanol. The combined ethanol solutions were taken to dryness by rotary evaporation at 30° and the residue stored at -20 .

Isolation

The ethanol-soluble residue was dissolved in 100 ml water and added to 100 ml bed volume XAD-7 column (Mallinckrodt, St. Louis, MO). The column was washed with 100 ml water followed by 200 ml ethanol. This later fraction was taken to dryness and dissolved in 5 ml 30% methanol in water. Two ml aliquots were injected onto a Polygosil 60D reverse phase column (10x230 mm) and chromatographed using methanol: water: acetic acid (69/31/0.02) adjusted to pH 5.7 with 1M NH_4OH. The HPLC effluent was monitored at 280 nM using an ultraviolet monitor (Model III Laboratory Data Control, Riviera Beach, FL). Fractions were collected and rechromatographed on a Nucleosil C-18 column (4.6x250 mm) using a mobile phase of methaonl:water:acetic acid (75/25/.01) for leukotriene B and isomers and methanol: water: acetic acid (65:35:0.01) for leukotriene C. Authentic leukotriene C_4 and leukotriene B_4 were obtained following previously published procedures from mastocytoma cells (10) and human PMNs (18) and used as chromatographic standards.

Fatty Acid Analysis

The lipids from milligram amounts of organs, cells, or fish oil were extracted essentially by the method of Bligh and Dyer (19). The organic layer extract was treated with methanolic HCl (5 ml, 5 N HCl) and heated 2 hr. at 70° to effect transesterification. Hexane (15 ml, twice) was used to extract the methyl esters prior to gas chromatography using 3 OV-17 (6 ft x 0.25 cm, 100/200 mesh Supelcoport) and temperature programming from 170-250° at 6°/min. The fatty acid methyl esters were quantitated by measurement of peak height and verified by gas chromatography-mass spectrometry. Mass spectrometry was carried out with a model 3200 Finnigan quadrupole mass spectrometer (Sunnyvale, CA) under electron impact conditions, 70eV. Mass spectra were acquired under computer control, using model 6100 data system.

Bioassay

The isolated guinea pig ileum was suspended in Tyrodes buffer containing atropine sulfate (10^{-6}M) and pyrilamine maleate (10^{-6}M), and SRS activity was determined as previously described (10).

RESULTS

The fatty acid content in the lipids of several different tissues of the mice was appreciably altered when the diet was supplemented with 5 menhaden oil as shown in Table 1. The tumor cells and other major organs within the peritoneal cavity showed significantly increased abundances of eicosapentaenoic acid and markedly decreased levels of linoleic acid. The tumor cells also showed appreciable increases in the other n-3 fatty acids and a significant decrease in the content of arachidonic acid. In contrast to the decreased mole per cent of arachidonic acid observed in liver and tumor cells, the kidney and heart tissues showed somewhat elevated contents of arachidonic acid. The most abundant polyunsaturated fatty acid present in tissue lipids following the fish oil-supplementation was docosahexaenoic acid. A slight contribution of 24:2 was detected by mass spectrometry.

Table 1

Mole percent composition of n-6 and n-3 acids.*

Tissue	Diet	18:2(n-6)	20:4(n-6)	20:5(n-3)	22:5(n-3)	22:6(n-3)
CxBG Cells	normal	21. ±1.	9.2±0.3	0.5±0.2	0.8±0.3	5.6±1.
	+ oil	1.7±0.3	3.9±0.2	4.5±0.6	6.1±0.3	9.9±0.9
Liver	normal	23. ±2.	11.8±0.9	0.5±0.1	0.4±0.1	8. ±1.
	+ oil	1.0±0.2	2.0±0.4	4.2±0.6	1.3±0.2	12.6±0.7
Heart	normal	17. ±1.	5.3±0.4	0.2±0.1	1.1±0.2	20. ±2.
	+ oil	0.8±0.1	6.2±0.6	6.3±0.4	1.4±0.3	18.5±0.9
Kidney	normal	32.0±0.6	3.4±0.2	0.2±0.1	0.3±0.2	3.2±0.1
	+ oil	1.0±0.3	6.4±0.6	5.4±0.3	1.7±0.2	19.2±0.8
Menhaden oil		1.4	0.3	11.0	1.2	5.3

*Fatty acids such as 16:1, 16:2, 20:1, 20:2, 20:3, 24:1 (or 22:3) are not reported and typically were less than 1% each. Saturated fatty acids 14.0, 16:0 and 18:0 constituted about 45-50%. Oleate (18:1(n-9)) was 10-15 mole % with normal diets and was doubled by the oil-supplemented diet.

The leukotrienes produced from the mastocytoma cells following stimulation with A23187 were separated by high pressure liquid chromatography as illustrated in Fig. 1 Selected fractions were combined when appropriate as indicated by their absorbance at 280 nm and examined further. Material eluting in the peak labeled LTC_4 displayed the characteristic UV absorbance spectrum for conjugated trienes (10) with a maximum absorbance at 280 nm. The spectrum for materials eluted in the LTC_5 fraction had considerable interference by materials eluted with the solvent front with significant absorption below 250 nm. Nevertheless, there was definite indication of a triplet absorbance band centered at 280 nm. All HPLC fractions were subjected to the guinea pig ileum bioassay to estimate slow reacting substance activity. Only material

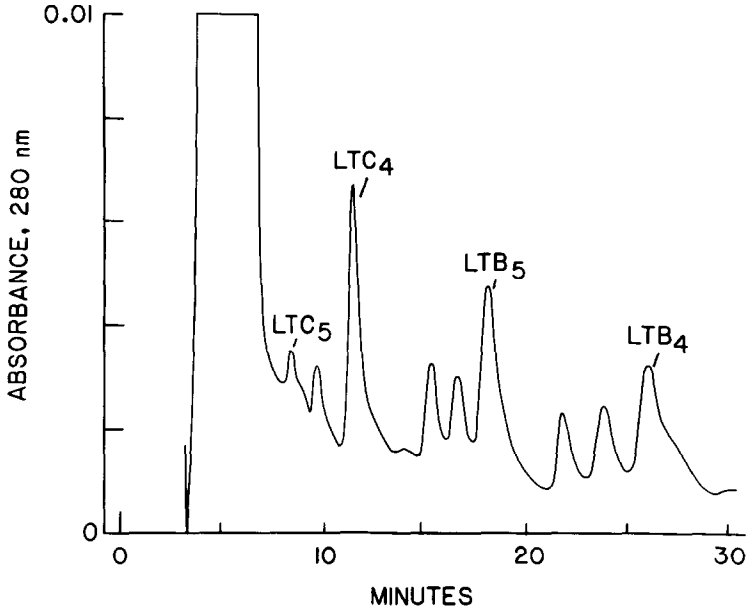

Figure 1. Preparative HPLC separation monitored at 280 nm of crude extract from A23187 stimulation of mastocytoma cells grown in Menhaden fish oil fed mice. The isocratic mobile phase at 4 ml/min was: methanol: water: acetic acid (69:31:0.02, pH 5.7).

from the peaks labeled LTC_5 and LTC_4 provided a dose dependent slow contraction of the ileum which could be inhibited by FPL55712. Rechromatography of the material in peak LTC_4 on an analytical reversed phase column yielded material with a retention time identical to authentic LTC_4. Similar chromatography of the LTC_5 fraction resulted in a peak with an earlier retention time that was comparable to that reported previously for LTC_5 (17). The peak appearing between LTC_5 and LTC_4 (Fig. 1) did not have the characteristic leukotriene absorption spectrum and was not biologically active on the guinea pig ileum.

The fractions eluting between 14 and 30 minutes (Fig. 1) were pooled and rechromatographed on the analytical Nucleosil C-18 column shown in Figure 2. The peak labeled LTB_4 had the UV absorbance, HPLC retention, gas chromatographic and mass spectrometric behavior of LTB_4 generated from human polymorphonuclear leukocytes by the method of Borgeat and Samuelsson (18), as well as to the physico chemical behavior published by other investigators (16). The two peaks eluting at 9.1 and 8.2 minutes were identical in the above parameters to the all-trans isomers of 5,12-dihydroxyeicosa-pentaenoic acid also described by Borgeat and Samuelsson (18).

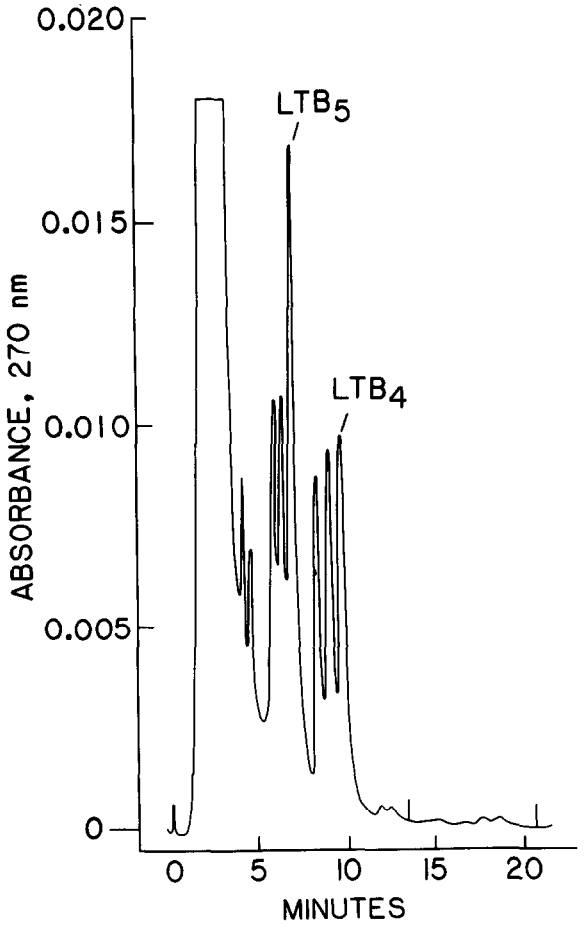

Figure 2. Analytical HPLC separation monitored at 280 nm of the pooled eluate from 14 to 30 min of the HPLC in figure 1. An isocratic mobile phase, methanol: water: acetic acid (75:25: 0.1, pH 4.2) at 1 ml/min was used.

The component labeled LTB_5 possessed the UV absorption triplet characteristic of conjugated triene species with a maximum at 270 nm and shoulders at 260 and 290 nm. The mass spectrum of 200 ng of the methyl ester trimethylsilyl ether derivative of this component is shown in figure 3. There is an apparent molecular ion at m/z 492 and ions at m/z 477, 461 and 402 corresponding to the loss of CH_3, OCH_3 and trimethylsilanol from the molecular ion. The abundant ions at m/z 203 and 383, and the weak but significant ion at 391, are consistent with cleavage adjacent to trimethylsilyl ether moiety with charge retention on the oxygen-containing fragment. Other abundant ions, such as m/z 217, 229, 267,

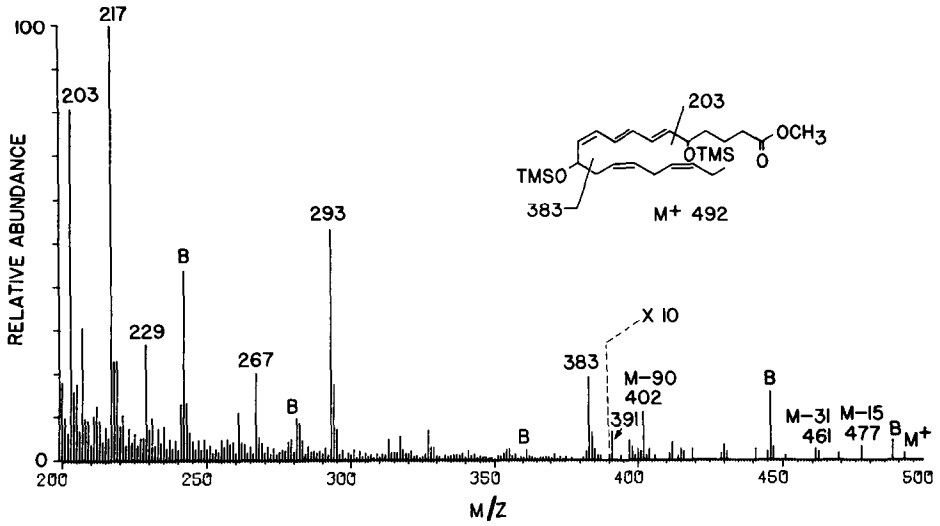

Figure 3. Mass spectrum of the HPLC purified mastocytoma metabolite eluting at 7.0 min in figure 2 following conversion to the methy ester, trimethylsilyl ether derivative. The ions indicated with "B" were prominent background ions in the gas chromatograph - mass spectrometer system. The structure indicated may not indicate the absolute double bond configuration.

and 293 are identical to those in the mass spectrum of the 5,12-dihydroxyeicosanoic acid derivative. The collected data for this component therefore suggests the 5,12-dihydroxy-6, 8, 10, 14, 16-eicosapentaenoic acid structure. The designation of the peak as LTB_5 is made by analogy to the HPLC behavior of the isomeric 5,12-dihydroxyeicosatetraenoic acid species (20). The two components eluting prior to LTB_5 (Fig. 2) had UV and mass spectrometric behavior identical to the LTB_5. Since they were separable by gas chromatography, they are most likely isomeric to LTB_5.

The yield of HPLC-purified leukotrienes following stimulation of the tumor cells was estimated from UV absorbance at 280 nm and 270 nm for LTC and LTB respectively and is indicated in table 2. There was an approximate 12-fold reduction in the yield of leukotriene C (C_5 and C_4) and 17-fold reduction of leukotriene B (B_5 and B_4) compared to tumor cells from mice on a control diet.

Table 2

Production of leukotrienes from mastocytoma cells carried in normal and fish oil fed mice following stimulation with A23187 (10 ng/ml) under standard conditions.

	pmoles/10^7 cells			
	LTC_4	LTC_5	LTB_4	LTB_5
CxBG normal	200±40[a]	-	270±30[b]	-
CxBG MFO	15	1.5	6.3	9.6

(a) ± S.E., n=5; (b) ± S.E., n=3

DISCUSSION

The feeding protocol employed in this study produced nearly equal amounts of eicosapentaenoic and arachidonic acid in the fatty acids of the mastocytoma cells within the peritoneal cavity. Following stimulation with A23187, the total abundance of the tetraenoic leukotrienes (from 20:4(n-6)) was slightly greater than that of pentaenoic derivatives (from 20:5(n-3)). This difference could be attributed either to greater release of the arachidonic acid from cellular lipids or to a slight preference of the lipoxygenase enzyme for arachidonic acid. Clearly both LTA_4 and LTA_5 had been produced following ionophore challenge of these cells, and there is little evidence for a selective requirement of peroxide that is demonstrated by the cyclooxygenase enzyme with different reactivity for these two fatty acids (8). The relative abundances of LTB_5 and LTB_4 do, in fact, reflect the relative abundances of their precusor fatty acid in the mastocytoma lipids. Thus, we expect that more detailed studies will show similar rates of oxygenation by the mastocytoma lipoxygenase (and hydration by LTA hydrase) for the eicosapentaenoic and eicosatetraenoic acids. On the other hand, a dramatic selectivity appears to occur with the LTC synthase in catalyzing the reaction of glutathione with the LTA intermediate. The pentaenoic derivative forms much less of the LTC_5 than the LTB_5, indicating a relative slow reactivity of the LTC synthase with the pentaenoic intermediate, LTA_5. In contrast, LTC_4 was more abundant than LTB_4 indicating a preferred utilization of the LTA_4 intermediate for LTC synthesis. The ratio

of LTC_4 to LTB_4 (2.0) was more than 10 times greater than that of LTC_5 to LTB_5 (0.16).

The selectivity observed in the biosynthesis of LTC derivatives open a new important area of inquiry in the manner by which fish products may have beneficial effects on human health. Emphasis on the inhibition of thromboxane biosynthesis is compatible with the recognized diminution in platelet function observed epidemiologically in Greenland Eskimos (21) and also in subjects fed fish products (5). This emphasis on platelet function provides important mechanisms in relating human nutrition to the integrity of cardiovascular and cerebrovascular systems (22,23). Our current results allow us to extend the interpretations of a previous report on the effect of dietary fatty acids on cerebral vascular integrity (23). When stroke was induced by ligation of the middle cerebral artery, the infarcted area was significantly less in animals that were previously fed with n-3 polyunsaturated fatty acids. In this model, the infarction was not induced by the formation of a thrombus in the large vessel, but rather may have depended more on the factors which regulate the integrity of the microvasculature of the cerebrum. These factors, which include edema and microvascular pressure, are identical to pharmacologic properties already described for the leukotrienes. The beneficial effects of fish oil supplements in the diet described in that report combined with our evidence for a lower rate of LTC_5 biosynthesis in the present report leads us to suggest that leukotriene synthesis may be a major regulatory factor in causing edema and related changes that produce infarction in ischaemic tissues. The protective effect of dietary n-3 fatty acid observed in the coronary thrombotic model with dogs (24) was not clearly correlated with the size of thrombus in the major vessel. Further studies may indicate that a selective reduction in LTC synthase activity in the presence of the pentaenoic intermediates may be a significant feature in the protection of organs from ischaemic damage and infarction.

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