Leukotriene B₅ is formed in human neutrophils after dietary supplementation with icosapentaenoic acid

(Thromocytes/n – 3 fatty acids/n – 6 fatty acids/fish oil/icosanoid formation)

THOMAS STRASSER, SVEN FISCHER, AND PETER C. WEBER

Medizinische Klinik Innenstadt der Universität München, Ziemssenstraße 1, 8000 München 2, Federal Republic of Germany

Communicated by K. M. Brinkhous, October 25, 1984

ABSTRACT Incorporation and conversion of icosapentaenoic acid (20:5, n – 3) by human polymorphonuclear leukocytes were studied in volunteers (n = 6) ingesting a normal Western diet supplemented with icosapentaenoic acid (∼4 g daily). Ingestion of icosapentaenoic acid leads to formation of biologically less active leukotriene B₅ (LTB₅) from polymorphonuclear leukocytes (PMNL) stimulated with ionophore A23187. LTB₅ was identified on HPLC by UV absorption and by GC/MS and showed a behavior identical to that of in vitro synthesized LTB₅ produced by incubation of human PMNL with icosapentaenoic acid. The ratio of icosapentaenoic acid/arachidonic acid (20:4, n – 6) in cellular phospholipids increased from 0.045 during control to 0.28 after the supplemented period. LTB₅ increased from undetectable values to 70.2 ± 18.7 pmol of LTB₅ per 10⁹ PMNL during the experimental period. Synthesis of LTB₅ did not change significantly (control, 218.8 ± 89.1; icosapentaenoic acid–enriched diet, 253.6 ± 18.7 pmol per 10⁹ PMNL). The ratio of LTB₅/LTB₄ corresponded to the ratio of arachidonic acid/icosapentaenoic acid in PMNL phospholipids. Our findings prove that LTB₅, which is 10 to 30 times less potent than LTB₄ to cause aggregation, chemotaxis, and degranulation of PMNL, can be formed in vivo in man after dietary icosapentaenoic acid. This may modify the contribution of leukotrienes in processes in which these metabolites are of pathogenetic relevance.

Human polymorphonuclear leukocytes (PMNL) convert arachidonic acid (20:4, n – 6) after release from cellular phospholipids to leukotriene B₄ (LTB₄) via the 5-lipoxygenase pathway (1, 2). LTB₄ is a potent stimulator of PMNL chemotaxis and, therefore, an important component of the cellular response in inflammatory and immune reactions (3). Under our Western dietary conditions, arachidonic acid is by far the dominant precursor fatty acid of biologically highly active icosanoids.

Icosapentaenoic acid (20:5, n – 3) predominates over arachidonic acid in marine diets and gives rise to trienoic icosanoids, which differ in biological activity from the dienoic icosanoids derived from arachidonic acid (4). Epidemiological and experimental studies suggest a therapeutic potential of icosapentaenoic acid–enriched diets in atherothrombotic and inflammatory disorders (5–9), and formation of icosapentaenoic acid–derived icosanoids has been implicated in those beneficial effects (10, 11). Indeed, in vitro synthesized LTB₅ is at least 1 order of magnitude less potent in stimulating PMNL chemotaxis and aggregation (12, 13). Our study provides the first evidence that LTB₅ is formed from cellular icosapentaenoic acid of PMNL in subjects that have supplemented their Western diet with cod liver oil, which is rich in icosapentaenoic acid.

MATERIAL AND METHODS

Materials. Percoll was purchased from Pharmacia (Freiburg, F.R.G.); bovine serum albumin (essentially fatty acid free), ionophore A23187, and icosapentaenoic acid (99% pure) were from Sigma (Munich, F.R.G.); Hank’s balanced salt solution (HBSS) and prostaglandin B₂ (PGB₂) were from Serva (Heidelberg, F.R.G.); [³H]LTB₄ (specific activity, 59.0 Ci/mmol) was purchased from New England Nuclear. Synthetic LTB₄ was a gift from J. Pike (Upjohn, Kalamazoo, MI). Cod liver oil was from Molter (Oslo, Norway).

Volunteer Study. After informed consent was obtained, six healthy male volunteers of age 26–37 yr and weight 67–90 kg supplemented their otherwise unchanged Western diet with cod liver oil (40 ml/day; ∼4 g of icosapentaenoic acid per day) for 4 wk. The study complied with the guiding principles as set forth in the Declaration of Helsinki. Subjects with abnormalities of prestudy laboratory data (including complete blood count, clinical chemistry, and kidney and liver parameters) were excluded. For preparation of PMNL, volunteers fasted for at least 10 hr.

Preparation of PMNL. Heparinized venous blood (10 units/ml) was taken and centrifuged at 150 × g for 15 min. The platelet-rich plasma was discarded, and the blood was diluted with platelet-poor plasma (10% vol/vol), layered on a Percoll gradient, and centrifuged as described (14). Contaminating cells were removed by hypotonic lysis and by washing the PMNL twice in bovine serum albumin (15 mg/dl). The PMNL fraction, resuspended in phosphate-buffered saline, was 98% pure with a viability of 97% and a leukocyte-to-platelet ratio greater than 80:1.

Incubation Conditions. PMNL (1 × 10⁷) were preincubated at 37°C for 10 min in 200 μl of phosphate-buffered saline. Then, 10 μM ionophore A23187, dissolved in HBSS containing Ca²⁺, was added to give a final volume of 0.5 ml and a Ca²⁺ concentration of 0.8 mM. The incubation was terminated after 10 min by adding 1.5 vol of ice-cold ethanol.

Extraction, Purification, and Analysis of LTB₄ and LTB₅. The ethanolic solution was centrifuged, purified, and extracted using SEP-PAK C₁₈ cartridges as described (15). Reversed-phase (RP)-HPLC was carried out with a Nucleosil 5 C₁₈ column using MeOH/H₂O/acetic acid, 70:30:0.01 (vol/vol; pH adjusted to 5.7 with NH₄OH) as the mobile phase at 1 ml/min. For quantification of biosynthesized LTB₄ and LTB₅, PGB₂ (100 ng) or [³H]LTB₄ (2 × 10⁶ cpm) were added to the sample before extraction to account for recovery. Absorbance was monitored at 280 nm; radioactivity was measured by using a radioactivity monitor.

LTB₄ and LTB₅ and the corresponding 6-trans isomers of

Abbreviations: PMNL, polymorphonuclear leukocytes; RP-HPLC, reversed-phase HPLC; LTB₄ and LTB₅, leukotrienes B₄ and B₅, respectively; HBSS, Hank’s balanced salt solution; PGB₂, prostaglandin B₂.

*To whom reprint requests should be addressed.
each compound were separated, and the Me₃Si-derivatives were further analyzed by GC/MS. The GC/MS system was a Finnigan MAT 44s, equipped with a 50-m fused silica WCOT capillary column (Carbowax CP 51; Chrompack) capable of separating LTB₄ and LTB₅. Retention times were 13.1 min for LTB₄ and 14.5 min for LTB₅. Operation conditions of the GC/MS system were: injection port, 280°C; interface, 270°C; ion source, 200°C; electron impact energy, 80 eV; current of emission, 0.9 mA; electron multiplier voltage, 1.8 kV.

Fatty Acid Analyses. Phospholipid fatty acids were analyzed by GC as described (16). Briefly, a Packard gas chromatograph was used (model 419), equipped with a 6-foot glass column, containing 10% SP-2340 on 100–120 mesh (Supelco, Munich, F.R.G.). Carrier gas had a flow rate of 25 ml/min, oven temperature was 150–200°C, the rise in temperature was 2°C/min, and the injection port was at 270°C.

**RESULTS**

Phospholipid Fatty Acid Analyses. During the control period, the icosapentaenoic acid content was low in PMNL phospholipids and contributed only 0.6 ± 0.2% of total fatty acids. At the end of the icosapentaenoic acid-supplemented diet, the polyunsaturated fatty acids in PMNL phospholipids indicated a characteristic change as compared to the control diet (Table 1). Icosapentaenoic acid increased by a factor of about 6, and arachidonic acid and linoleic acid (18:2, n–6) decreased slightly. The ratio of arachidonic acid to icosapentaenoic acid was 22.2:1 before and 3.5:1 at the end of the icosapentaenoic acid-supplemented diet.

Metabolism of Endogenous Icosapentaenoic Acid by Human PMNL. During the control period, PMNL stimulated with the ionophore A23187 generated LTB₄ and its two 6-trans-stereoisomers from cellular arachidonic acid (Fig. 1 Top). Peaks appearing on RP-HPLC with retention times of 17.5 min (compound I) and 18.8 min (compound II) for the two stereoisomers and of 20.8 min for the biologically active LTB₄ comprise the characteristic triplet of 5-lipoxygenase products of arachidonic acid. PMNL prepared after the icosapentaenoic acid-enriched diet synthesized a new triplet of lipoxygenase compounds when stimulated with the ionophore A23187 (Fig. 1 Middle). This triplet was detectable only during the icosapentaenoic acid-supplemented diet and was absent under control conditions. The three new peaks had retention times on RP-HPLC of 11.4 min (compound IV), 12.9 min (compound V), and 14.7 min (LTB₅). LTB₄ and its stereoisomers also were formed after icosapentaenoic acid-enriched diet in concentrations similar to those found in the control period (Fig. 1 Top and Middle).

Characterization of LTB₅. For identification of the new compounds as 5,12-dihydroxy analogues, including LTB₅ formed from cellular icosapentaenoic acid, control PMNL were incubated with exogenous icosapentaenoic acid and stimulated with ionophore A23187. A characteristic triplet was formed (Fig. 1 Bottom). In incubations with exogenous

| Table 1. Fatty acid composition in phospholipids of human PMNL before and after dietary icosapentaenoic acid for 4 wk in healthy male volunteers |
|-----------------|-----------------|-----------------|
| PMNL phospholipid fatty acid | Control diet | Icosapentaenoic acid-enriched diet |
| 18:2, n–6 | 13.6 ± 0.9 | 9.2 ± 2.1 |
| 20:4, n–6 | 13.3 ± 5.2 | 11.4 ± 1.9 |
| 20:5, n–3 | 0.6 ± 0.2 | 3.3 ± 0.3* |

Cod liver oil at 40 ml/day (~4 g of icosapentaenoic acid per day) was added to the diet. Results are expressed as the percentage of total fatty acid content. Values are means ± SD from six volunteers. *P < 0.01.

Fig. 1. Formation of LTB₄ and LTB₅ and their corresponding stereoisomers in human PMNL (1 × 10⁷ cells) from cellular precursor fatty acids arachidonic acid and icosapentaenoic acid before (Top) and after (Middle) a 4-wk period of cod liver oil (40 ml/day; ~4 g of icosapentaenoic acid) added to an otherwise unchanged Western diet. Formation of LTB₅ and its stereoisomers after incubation of human control PMNL with exogenous 20:5, n–3 fatty acid (Bottom); 4 × 10⁷ PMNL were used for analysis of LTB₄ and LTB₅ after the 20.5, n–3 fatty acid-enriched diet (Middle). The dotted line in the bottom panel indicates the elution profile of [³H]LTB₄ added to this sample.

icosapentaenoic acid (50–100 μM), the formation of LTB₄ and its stereoisomers from cellular arachidonic acid was suppressed (Fig. 1 Bottom). In analogy to LTB₄ and its stereo-
formed from phospholipids common identical with LTB4, and 6,8,10,14,17-icosapentaenoic acid, endogenous icosapentaenoic acid, 6,8,10,14,17-icosapentaenoic acid (Fig. 2). Specific fragments at m/z 402 (M+ - 90), 461 (M+ - 31), and 477 (M+ - 15) indicate a molecular weight of M+ = 492 and one additional double bond as compared to LTB4 (M+ = 494). LTB3 formed from endogenous cellularicosapentaenoic acid after cod liver oil supplementation showed fragments appearing with identical intensities on GC/MS: m/z 477 (M+ - 15) and 461 (M+ - 31), both specific for LTB3, and m/z 383 and 293, both common to LTB3 and LTB4, suggesting an identical structure from C16 to C18 for both compounds. Upon catalytic hydrogenation, LTB3 cochromatographed on capillary GC with authentic hydrogenated LTB4, showing an identical fragmentation pattern; m/z 487 (M+ - 15); 389 (M+ - 113); 299 [M+ - (113 + 90)]; 215(Me3Si—O—CH(CH3)2—(CH3)]. The two compounds eluted from the HPLC column prior to LTB3 (peaks IV and V in Fig. 1 Middle and Bottom) had a different retention time on capillary GC as compared with LTB3 but showed a fragmentation pattern comparable to that of LTB3.

Fatty Aids in PMNL Phospholipids and Formation of LTB4 and LTB3. Comparison of the conversion rates of cellular arachidonic acid and icosapentaenoic acid in PMNL after the icosapentaenoic acid-enriched diet showed a ratio of LTB4 to LTB3 of 3.6:1, which corresponds quantitatively to the ratio of arachidonic acid to icosapentaenoic acid in PMNL phospholipids (see Tables 1 and 2).

DISCUSSION

Our study shows that LTB3, the 5-lipoxygenase product of icosapentaenoic acid, is synthetized in human PMNL from endogenous icosapentaenoic acid incorporated into cellular phospholipids during dietary supplementation. The identity of LTB3 was proven by RP-HPLC and GC/MS. It was shown that LTB4 synthetized by human PMNL from endogenous icosapentaenoic acid has the same characteristics as

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control diet</th>
<th>Icosapentaenoic acid-enriched diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTB4</td>
<td>218.8 ± 89.1</td>
<td>253.6 ± 18.7</td>
</tr>
<tr>
<td>Compound I</td>
<td>76.3 ± 43</td>
<td>83.3 ± 39.6</td>
</tr>
<tr>
<td>Compound II</td>
<td>65.7 ± 51</td>
<td>79.4 ± 62</td>
</tr>
<tr>
<td>LTB3</td>
<td>ND</td>
<td>70.2 ± 18.7</td>
</tr>
<tr>
<td>Compound IV</td>
<td>ND</td>
<td>25.1 ± 13.6</td>
</tr>
<tr>
<td>Compound V</td>
<td>ND</td>
<td>18.7 ± 11.6</td>
</tr>
</tbody>
</table>

Values are means ± SD from six volunteers. ND, not detectable.

LTB3 produced by PMNL in vitro from exogenous icosapentaenoic acid: identical retention times on RP-HPLC (Fig. 1 Middle and Bottom) and on capillary GC (14.5 min) and identical fragmentation patterns on MS (Fig. 2). A similar behavior on HPLC and GC/MS has been described for LTB3 synthesized from icosapentaenoic acid in a mouse mastocytoma cell line (17). The two metabolites with identical fragmentation patterns but different retention times on HPLC and GC/MS are most likely double-bond isomers of LTB3 with a 5,12-dihydroxy structure, as previously suggested (17). The fact that high concentrations of exogenously added icosapentaenoic acid completely suppressed the formation of LTB4 from cellular arachidonic acid may result from rapid binding of exogenous free icosapentaenoic acid to the 5-lipoxygenase, thereby preventing effective lipoxygenation of released cellular arachidonic acid.

After supplementation of the volunteers' Western diet with icosapentaenoic acid for 4 wk, the polyunsaturated fatty acids in PMNL phospholipids indicated a characteristic change. The significant increase of icosapentaenoic acid demonstrates the uptake and incorporation of this fatty acid into cellular phospholipids. Arachidonic acid and linoleic acid (18:2, n - 6) decreased slightly despite continued supply, suggesting a competition of these polyunsaturated fatty
acids with icosapentaenoic acid for common binding positions in PMNL phospholipids. Comparison of the conversion rates of cellular arachidonic acid and icosapentaenoic acid in PMNL after the icosapentaenoic acid-enriched diet demonstrates that transformation of cellular arachidonic acid and icosapentaenoic acid to LTB₄ and LTB₃ occurs at a comparable rate (Tables 1 and 2).

The biological effects of LTB₄, the 5-lipoxygenase product derived from arachidonic acid, towards human PMNL include chemokinesis and chemotaxis, aggregation, release of lysosomal enzymes, and stimulation of superoxide anion production (3, 18). They show the fundamental importance of LTB₄ in physiological and pathological responses of PMNL in processes like inflammation and immunological reactions. In two recent studies, LTB₃, synthesized from icosapentaenoic acid in vitro, was shown to possess only 1/10th to 1/30th of the chemotactic and aggregatory potency towards human PMNL as compared to LTB₄ (12, 13). This demonstrates the functionally reduced activity of LTB₃ in its agonist action on human PMNL. Epidemiological and experimental studies suggest that changes in the natural history of atherothrombotic and inflammatory disorders may be achieved by altering theicosanoid precursor availability (5–9). Formation of icosapentaenoic acid-derived icosanoids with a desirable spectrum of biological activities has been implicated in those beneficial effects (10, 11).

In our short term study, arachidonic acid in PMNL phospholipids was only slightly reduced. This could be one reason for the unreduced formation of LTB₄ in washed PMNL after the icosapentaenoic acid-supplemented diet. Recent animal experiments suggest that a longer dietary supplementation of icosapentaenoic acid will decrease cerebral arachidonic acid to a greater extent, with a concomitant decrease of LTB₄ formation (17, 19). In contrast to these animal experiments in which the normal n − 6 fatty acid diet was completely replaced by a n − 3 fatty acid diet, in our experiments an otherwise unchanged Western diet was supplemented with icosapentaenoic acid. The intake of n − 6 polyunsaturated fatty acids, including arachidonic acid, remained unchanged during the supplementation period. The present results, which prove the synthesis of LTB₃ in human PMNL from endogenous icosapentaenoic acid after dietary supply, in conjunction with previous animal experiments and in vitro studies, suggest that a long-term enrichment of our Western nutrition with higher doses of icosapentaenoic acid may modify the contribution of leukotrienes in reactions in which these products are of pathogenetic relevance.

We thank C. von Schacky for help with the volunteer study, I. Kurzmann and R. Böhlig for technical assistance, and W. Siess for criticism of the manuscript. This study was supported by Wilhelm-Sander-Stiftung (82.004.1).