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# Leukotriene $B_5$ is formed in human neutrophils after dietary supplementation with icosapentaenoic acid

(leukocytes/$n-3$ 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

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**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 $B_5$ ($LTB_5$) from polymorphonuclear leukocytes (PMNL) stimulated with ionophore A23187. $LTB_5$ was identified on HPLC by UV absorption and by GC/MS and showed a behavior identical to that of *in vitro* synthesized $LTB_5$ 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. $LTB_5$ increased from undetectable values to 70.2 ± 18.7 pmol of $LTB_5$ per $10^7$ PMNL during the experimental period. Synthesis of $LTB_4$ did not change significantly (control, 218.8 ± 89.1; icosapentaenoic acid-enriched diet, 253.6 ± 18.7 pmol per $10^7$ PMNL). The ratio of $LTB_4/LTB_5$ corresponded to the ratio of arachidonic acid/icosapentaenoic acid in PMNL phospholipids. Our findings prove that $LTB_5$, which is 10 to 30 times less potent than $LTB_4$ 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 $B_4$ ($LTB_4$) via the 5-lipoxygenase pathway (1, 2). $LTB_4$ 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 $LTB_5$ is at least 1 order of magnitude less potent in stimulating PMNL chemotaxis and aggregation (12, 13). Our study provides the first evidence that $LTB_5$ 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.

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## 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 $B_2$ ($PGB_2$) were from Serva (Heidelberg, F.R.G.); [$^3$H]$LTB_4$ (specific activity, 59.0 Ci/mmol) was purchased from New England Nuclear. Synthetic $LTB_4$ was a gift from J. Pike (Upjohn, Kalamazoo, MI). Cod liver oil was from Møller (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 ($1 \times 10^7$) were preincubated at 37°C for 10 min in 200 μl of phosphate-buffered saline. Then, 10 μM ionophore A23187, dissolved in HBSS containing $Ca^{2+}$, was added to give a final volume of 0.5 ml and a $Ca^{2+}$ 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 $LTB_4$ and $LTB_5$.** The ethanolic solution was centrifuged, purified, and extracted using SEP-PAK $C_{18}$ cartridges as described (15). Reversed-phase (RP)-HPLC was carried out with a Nucleosil 5 $C_{18}$ column using MeOH/$H_2O$/acetic acid, 70:30:0.01 (vol/vol; pH adjusted to 5.7 with $NH_4OH$) as the mobile phase at 1 ml/min. For quantification of biosynthesized $LTB_4$ and $LTB_5$, $PGB_2$ (100 ng) or [$^3$H]$LTB_4$ ($2 \times 10^5$ 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.

$LTB_4$ and $LTB_5$ and the corresponding 6-*trans* isomers of

Abbreviations: PMNL, polymorphonuclear leukocytes; RP-HPLC, reversed-phase HPLC; $LTB_4$ and $LTB_5$, leukotrienes $B_4$ and $B_5$, respectively; HBSS, Hank's balanced salt solution; $PGB_2$, prostaglandin $B_2$.

*To whom reprint requests should be addressed.

each compound were separated, and the Me,$Me_3Si$-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 $LTB_4$ and $LTB_5$. Retention times were 13.1 min for $LTB_4$ and 14.5 min for $LTB_5$. 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 $LTB_4$ 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 $LTB_4$ 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 ($LTB_5$). $LTB_4$ 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 $LTB_5$.** For identification of the new compounds as 5,12-dihydroxy analogues, including $LTB_5$ 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 icosapentaenoic acid (50–100 μM), the formation of $LTB_4$ and its stereoisomers from cellular arachidonic acid was suppressed (Fig. 1 *Bottom*). In analogy to $LTB_4$ and its stereo-

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. *$P < 0.01$.

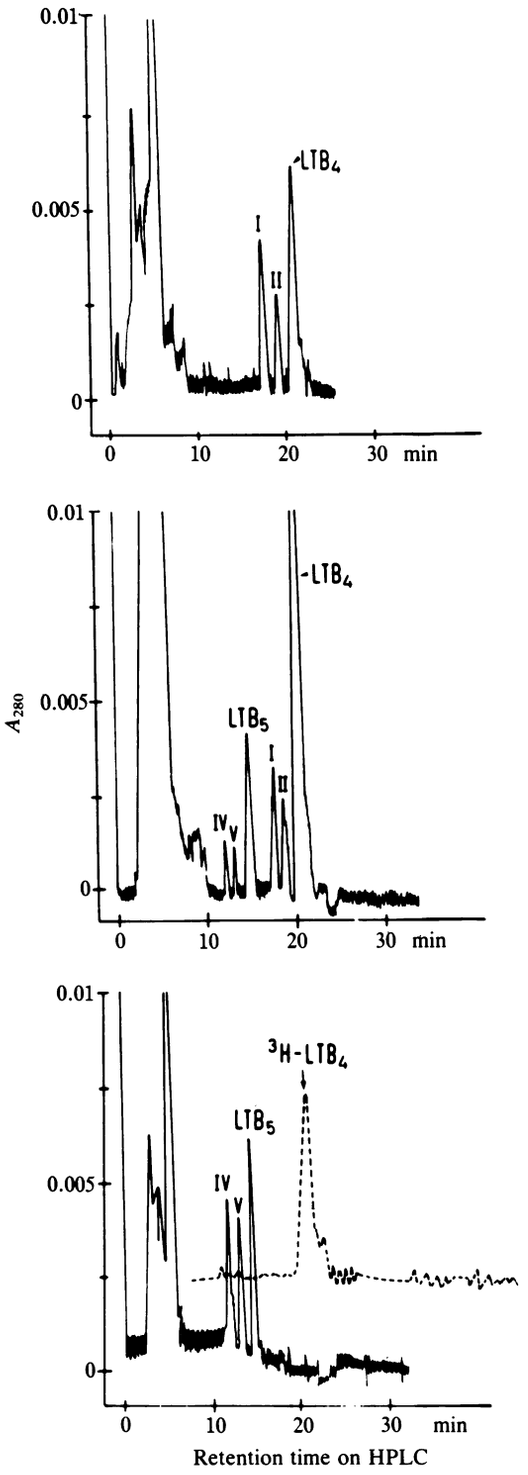


FIG. 1. Formation of $LTB_4$ and $LTB_5$ and their corresponding stereoisomers in human PMNL ($1 \times 10^7$ cells) from cellular precursor fatty acids arachidonic acid and icosapentaenoic acid before (*Top*) and after (*Middle*) a 4-wk period of cod liver oil (40 ml/day; ≈4 g of icosapentaenoic acid) added to an otherwise unchanged Western diet. Formation of $LTB_5$ and its stereoisomers after incubation of human control PMNL with exogenous 20:5, $n - 3$ fatty acid (*Bottom*); $4 \times 10^7$ PMNL were used for analysis of $LTB_5$ and $LTB_4$ after the 20:5, $n - 3$ fatty acid-enriched diet (*Middle*). The dotted line in the bottom panel indicates the elution profile of [$^3$H]$LTB_4$ added to this sample.

isomers, the compound appearing as the last peak of the new triplet (at 14.7 min) was designated $LTB_5$ and its structure was characterized by subsequent GC/MS. The Me,$Me_3Si$-derivative of $LTB_5$ revealed a longer retention time on capillary GC than did $LTB_4$ (14.5 versus 13.1 min) and a fragmentation pattern on MS characteristic for a 5,12-dihydroxy-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 $LTB_4$ ($M^+ = 494$). $LTB_5$ formed from endogenous cellular icosapentaenoic 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 $LTB_5$, and $m/z$ 383 and 293, both common to $LTB_5$ and $LTB_4$, suggesting an identical structure from $C_1$ to $C_{16}$ for both compounds. After catalytic hydrogenation, $LTB_5$ cochromatographed on capillary GC with authentic hydrogenated $LBT_4$, showing an identical fragmentation pattern; $m/z$ 487 ($M^+ - 15$); 389 ($M^+ - 113$); 299 [$M^+ - (113 + 90)$]; 215($Me_3Si{-}O^+{=}CH{-}(CH_2)_7{-}(CH_3)$). The two compounds eluted from the HPLC column prior to $LTB_5$ (peaks IV and V in Fig. 1 *Middle* and *Bottom*) had a different retention time on capillary GC as compared with $LTB_5$ but showed a fragmentation pattern comparable to that of $LTB_5$.

**Fatty Acids in PMNL Phospholipids and Formation of $LTB_4$ and $LTB_5$.** Comparison of the conversion rates of cellular arachidonic acid and icosapentaenoic acid in PMNL after the icosapentaenoic acid-enriched diet showed a ratio of $LTB_4$ to $LTB_5$ 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 $LTB_5$, the 5-lipoxygenase product of icosapentaenoic acid, is synthesized in human PMNL from endogenous icosapentaenoic acid incorporated into cellular phospholipids during dietary supplementation. The identity of $LTB_5$ was proven by RP-HPLC and GC/MS. It was shown that $LTB_5$ synthesized by human PMNL from endogenous icosapentaenoic acid has the same characteristics as

Table 2. Formation of $LTB_4$ and $LTB_5$ with corresponding stereoisomers in human PMNL under control conditions as compared to icosapentaenoic acid-enriched diet

| | 5-Lipoxygenase products, pmol per $10^7$ PMNL | |
|---|---|---|
| LTB in PMNL | Control diet | Icosapentaenoic acid-enriched diet |
| $LTB_4$ | 218.8 ± 89.1 | 253.6 ± 18.7 |
| Compound I | 76.3 ± 43 | 83.3 ± 39.6 |
| Compound II | 65.7 ± 51 | 79.4 ± 62 |
| $LTB_5$ | ND | 70.2 ± 18.7 |
| Compound IV | ND | 25.1 ± 13.6 |
| Compound V | ND | 18.7 ± 11.6 |

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

$LTB_5$ 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 $LTB_5$ 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 $LTB_5$ 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 $LTB_4$ 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

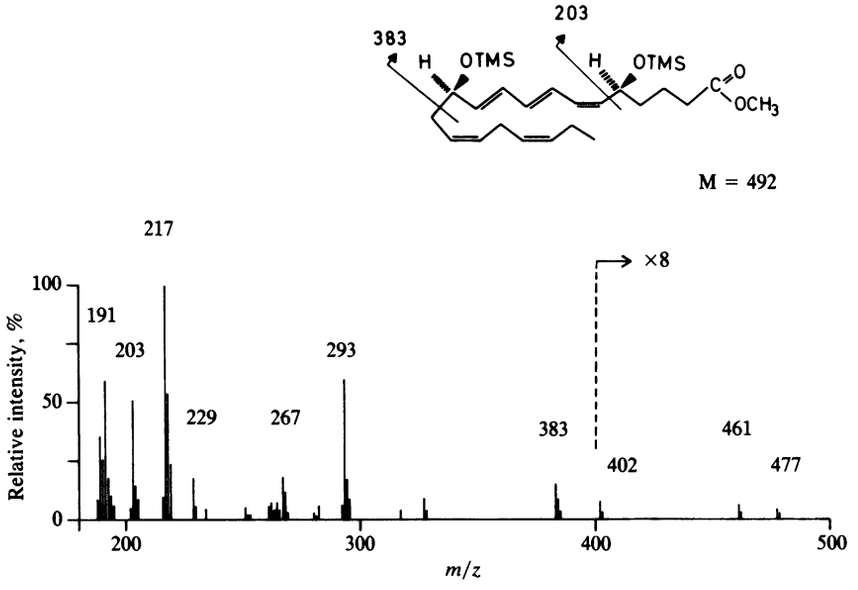


FIG. 2. Partial mass spectrum of the Me,$Me_3Si$-derivative of $LTB_5$, biosynthesized by human PMNL from exogenous 20:5, $n - 3$ fatty acid. PMNL ($1 \times 10^7$) were prepared as indicated and incubated with icosapentaenoic acid (100 μM) together with ionophore A23187 (10 μM) for 10 min at 37°C. The fragmentation pattern for $LTB_5$ was: $m/z$ 477 ($M^+ - 15$), 461 ($M^+ - 31$), 402 ($M^+ - 90$), 383, 293 (383 − 90), 267, 229, 217, 203, and 191.

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 $LTB_4$ and $LTB_5$ occurs at a comparable rate (Tables 1 and 2).

The biological effects of $LTB_4$, 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 $LTB_4$ in physiological and pathological responses of PMNL in processes like inflammation and immunological reactions. In two recent studies, $LTB_5$, 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 $LTB_4$ (12, 13). This demonstrates the functionally reduced activity of $LTB_5$ 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 the icosanoid 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 $LTB_4$ in washed PMNL after the icosapentaenoic acid-supplemented diet. Recent animal experiments suggest that a longer dietary supplementation of icosapentaenoic acid will decrease cellular arachidonic acid to a greater extent, with a concomitant decrease of $LTB_4$ 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 $LTB_5$ 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.

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