Brief Definitive Report

FUNCTIONAL CHARACTERIZATION OF SYNTHETIC LEUKOTRIENE B AND ITS STEREOCHEMICAL ISOMERS*

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Of the several possible double-bond stereoisomers of 5*S*,12*R*-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-di-HETE), based on physical measurements, one specific compound (Fig. 1) (1–3) has been preliminarily reported to correspond to the biologically generated neutrophil chemotactic factor, leukotriene B (LTB) (4, 5). Previous studies (4, 6) had suggested the presence of one *cis* and two *trans* ethylene bonds in its triene portion. We report the comparison of six synthetic stereoisomers of unambiguously defined geometry with natural LTB, using chemotactic assays in vitro and in vivo and spasmogenic activity.

Materials and Methods

Preparation of Natural LTB from Human Neutrophils. Neutrophils isolated from venous blood of normal subjects (5, 6) were incubated for 30 min at 37°C at a concentration of 5×10^7 cells/ml in Hanks' balanced salt solution containing 0.02 M Tris-HCl, pH 8, 10 μM indomethacin, 5 μM calcium ionophore A23187, and 0.5 mg/ml of arachidonic acid. LTB and the monohydroxyeicosatetraenoic acids (mono-HETE) were extracted and resolved from both the residual arachidonic acid and more polar products by silicic acid column chromatography (4, 5), and LTB was purified by reverse-phase high performance liquid chromatography on a C_{18} ODS column (Ultrasphere; Altex Scientific, Inc., subsidiary of Beckman Instruments, Inc., Berkeley, Calif.) that was developed isocratically at a flow rate of 1 ml/min with methanol:water:glacial acetic acid (78:22:0.01, vol/vol) (5). The purified LTB contained <1% of other mono- and di-HETE, as assessed by gas chromatography (6).

Synthesis of 5,12-di-HETE Stereoisomers, Leukotriene C (LTC), and Leukotriene D (LTD). 5*S*,12*R*-dihydroxy-6,14-*cis*,8,10-*trans*-eicosatetraenoic acid was synthesized, as previously described (1, 3), by two independent methods that produced the same substance. 5*S*,12*R*- and 5*S*,12*S*-6,8,10-*trans*,14-*cis* di-HETE and 5*S*,12*R*-6,8-*trans*-10,14-*cis* di-HETE (2) were also prepared as outlined previously. In the case of the C-12 diastereomers of the racemic 6,10-*trans*,8-*cis* di-HETE, the racemate having the longer elution time was shown by independent synthesis to be 5*S*,12*R* and also its enantiomer, using a reverse phase C_{18} column with 7:3 methanol:water for elution; the (±)-5*S*,12*S* diastereomer eluted earlier in this chromatographic system (2). LTC and LTD were prepared as previously described (7, 8).

In Vitro and In Vivo Assessment of Human Neutrophil Migration. Chemotaxis of human neutrophils of >96% purity was assayed by a modification of the Boyden technique in chambers with a 0.2-ml blind-end stimulus compartment (Neuroprobe, Inc., Bethesda, Md.) and filters with 3-μm

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pores (Sartorius; Beckman Instruments, Inc., Science Essentials Co., Mountainside, N. J.), with the period of migration at 37°C reduced from that of previous studies (5, 6) to 45 min. The chemotactic responses were assessed in duplicate and expressed as net neutrophils per high power field (hpf) after subtraction of the background level of migration in the absence of a stimulus.

In three separate experiments, 13–16 cutaneous sites on the anterior thorax of an anesthetized rhesus monkey were each injected i.d. with 0.1 ml of phosphate-buffered saline/2% ethanol, pH 7.4, alone, or containing LTC, LTD, natural LTB, or a synthetic 5,12-di-HETE isomer. 4 h later, the monkey was again anesthetized, and a 4-mm punch biopsy was obtained from each injection site. Each specimen was fixed in Karnovsky's mixture and the Epon-embedded tissue was cut to a thickness of 1 μm, as previously described (9). Tissue sections were stained with Giemsa's reagent and examined with a Leitz Dialux 20 microscope (Marcon Instruments, Norwood, Mass.) at magnifications up to × 1,000. Neutrophil infiltrates per total biopsy section were graded semiquantitatively from examination of ~40 hpf as 0 (<5 cells), 1+ (5–20 cells), 2+ (21–100 cells), and 3+ (>100 cells).

Measurement of Nonvascular Smooth Muscle Spasmogenic Activity. The 5,12-di-HETE stereoisomers and natural LTB were each assessed for isometric contractile activity on 6–12 guinea pig pulmonary parenchymal strips because of the exquisite sensitivity of this nonvascular smooth muscle to the leukotrienes of the slow-reacting substance of anaphylaxis (SRS-A) (8, 10) as well as for isometric and isotonic contractile activities, respectively, on guinea pig tracheal spirals and ileal smooth muscle (10, 11).

Results

In Vitro Neutrophil Migration. The maximum chemotactic response to natural LTB (Table I) was comparable to that evoked by a 1:100 dilution of a standard preparation of fragments of the fifth component of human complement (C5) (59.8 ± 2.9) (6). Of the four synthetic stereoisomers of 5,12-di-HETE having one *cis* ethylenic bond in the conjugated triene portion of the molecule, only the 6-*cis*-8,10-*trans* compound (Fig. 1)—designated here as c,t,t, to indicate configurations of the 6,8, and 10 triene linkages, respectively—elicited a response comparable to that elicited by natural LTB, i.e., detectable at 0.3 ng/ml (10^{-9} M) and maximum at 3–10 ng/ml ($1–3 \times 10^{-8}$ M). With this abbreviated (45 min) incubation period for the chemotactic assay, neither of the two racemic mixtures of the 6-*trans*,8-*cis*,10-*trans* (t,c,t) compounds, nor the 6,8-*trans*-10-*cis* (t,t,c) compound elicited a reproducible net neutrophil chemotactic response at 10^{-9} M, and, at concentrations up to 10^{-6} M, none evoked a maximum response similar in magnitude to that elicited by natural LTB or the c,t,t compound (synthetic LTB). Within the assessed range of concentrations, the maximum effects of 5*S*,12*S*-t,c,t and its enantiomer and the t,t,c compound were elicited at 300 ng/ml (10^{-6} M) and 100 ng/ml (3×10^{-7} M), respectively, and were comparable in magnitude to the responses evoked by both natural and synthetic (c,t,t) LTB at 1 ng/ml (3×10^{-9} M). The pure 5*S*-12*S*-dihydroxy-6,10-*trans*,8,14-*cis*-eicosatetraenoic acid synthesized by a different technique[1] was no more active than the racemic mixture. The two all-*trans* (t,t,t) C-12 diastereomers of 5*S*,12-di-HETE were each even less potent, with 5*S*,12*R* t,t,t being the least active (Table I). In two separate experiments (data not shown) the racemic 5*S*,12*R*-/5*R*,12*S*-t,c,t mixture was 3- to 10-fold less active over a 2.5 log dose range than was the 5*S*,12*S*-/5*R*,12*R*-racemate of the same olefinic bond stereoisomer. The unnatural stereoisomeric analogues were not

[1] Corey, E. J., A. Marfat, and B. C. Laguzza. Total synthesis of 5*S*,12*S*-dihydroxy-6,10-*E*,8,14-*Z*-eicosatetraenoic acid. Manuscript submitted for publication.

TABLE I

Chemotactic Activities In Vitro for Neutrophils by 5,12-di-HETE

Concentration	Net neutrophils/hpf (mean ± SD)*					
	Natural LTB	c,t,t (5*S*,12*R*)	t,c,t (5*S*,12*S* and 5*R*,12*R*)	t,t,c (5*S*,12*R*)	t,t,t (5*S*,12*R*)	t,t,t (5*S*,12*S*)
ng/ml						
0.3	9.0 ± 4.8	7.7 ± 2.6	3.2 ± 3.6	2.7 ± 5.1	0.8 ± 1.3	0.0
1.0	28.6 ± 5.4	32.8 ± 2.3	7.0 ± 6.0	12.2 ± 6.6	5.2 ± 1.9	3.1 ± 2.4
3.0	45.3 ± 9.1	55.6 ± 8.8	12.8 ± 5.6	13.9 ± 5.0	9.2 ± 3.3	5.7 ± 1.0
10	45.2 ± 12.7	56.0 ± 5.6	16.9 ± 7.8	15.2 ± 1.6	8.8 ± 1.1	6.8 ± 1.5
30	38.4 ± 11.1	49.3 ± 9.8	21.1 ± 8.6	19.3 ± 1.6	6.4 ± 0.9	12.1 ± 3.1
100	29.7 ± 14.3	44.5 ± 13.2	25.7 ± 10.4	27.5 ± 10.4	10.7 ± 2.0	20.3 ± 3.0
300	ND‡	ND	33.6 ± 4.9	ND	18.6 ± 3.0	30.4 ± 9.5

* Mean values are for 4 experiments, except for compound t,t,c (5*S*,12*R*), where n = 3, and for the highest dose tested for each of the other compounds, where n = 2.

‡ Not determined.

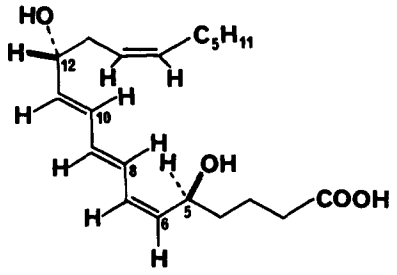

FIG. 1. Chemical structure of 5*S*,12*R*-dihydroxy-6,14-*cis*,8,10-*trans*-eicosatetraenoic acid, LTB.

only two orders of magnitude less potent than synthetic (c,t,t) LTB, but also exhibited a flatter dose-response curve that extended over two orders of magnitude and failed to achieve a maximum response equal to that observed with natural and synthetic (c,t,t) LTB.

In Vivo Cutaneous Response. In three experiments carried out to compare the histologic effects of five of the six synthetic 5,12-di-HETE, excluding 5*S*,12*R*-/5*R*,12*S*-t,c,t, at 100 ng/site, only c,t,t provoked an infiltrative response by neutrophils at 4 h (2-3+), as did natural LTB (3+). Both natural LTB and synthetic (c,t,t) LTB also provoked modest (1-2+) neutrophil infiltration at 10 ng intradermal doses, in contrast to the other assessed synthetic isomers of 5,12-di-HETE, at 10 and 100 ng/site, and LTC and LTD, at up to 500 ng/site, which were inactive.

Nonvascular Smooth Muscle Spasmogenic Effects. The guinea pig pulmonary parenchymal strips responded to natural or synthetic (c,t,t) LTB in a dose-response fashion beginning at 3×10^{-10} M and reaching 25% of that response elicited by 10^{-4} M histamine (EC_{25}) at 3×10^{-8} M; LTC and LTD achieved the same EC_{25} with 4×10^{-10} M and 2×10^{-11} M, respectively. None of the other synthetic 5,12-di-HETE analogues induced a contraction >5% of the 10^{-4} M histamine response at concentrations up to 3×10^{-8} M. Neither natural nor synthetic (c,t,t) LTB provoked a response on the guinea pig tracheal spirals at 2×10^{-7} M; relative to the contraction elicited

by 10^{-4} M histamine, the EC_{25} for LTD on this tissue was 3×10^{-8} M. The ileum, which responds to 2×10^{-10} M LTD with a contraction equal to that elicited by 4×10^{-8} M histamine, failed to respond to 2×10^{-6} M synthetic (c,t,t) LTB.

Discussion

LTB, the most potent known lipid chemotactic factor for the in vitro attraction of neutrophils (5), is structurally a 5*S*,12*R*-di-HETE, with one of its three conjugated ethylenic bonds in the *cis* arrangement and the other two in the *trans* (1–5). Direct comparison of natural LTB and synthetic stereoisomers meeting these structural requirements demonstrated that only the 5*S*,12*R*-dihydroxy-6,14-*cis*,8,10-*trans* eicosatetraenoic acid (Fig. 1), c,t,t, has neutrophil chemotactic activity comparable to that of the natural product. Specifically, for both natural LTB and synthetic (c,t,t) LTB, a threshhold effect was appreciable at 0.3 ng/ml, one-half maximum effect at 1 ng/ml, and maximum effect at 3–10 ng/ml (Table I). In contrast, the concentrations of the related synthetic t,c,t and t,t,c compounds eliciting a neutrophil response comparable to the one-half maximum LTB effect were each 100 ng/ml; thus, these isomers are two orders of magnitude less active than the natural product and approximately as equiactive as 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) (12). As previously recognized (6, 13), each of the t,t,t C-12 diastereomers was even less active.

Comparisons of natural LTB with five of the synthetic 5,12-di-HETE for local in vivo chemotactic effects in the skin of a rhesus monkey and for nonvascular smooth muscle spasmogenic effects in vitro on guinea pig pulmonary parenchymal strips were also discriminating. In each assay, natural LTB and synthetic (c,t,t) LTB produced an approximately equivalent dose-related response, whereas the other four analogues had little to no effect within the same concentration range. As natural LTB and synthetic 5*S*,12*R*-dihydroxy,6,14-*cis*,8,10-*trans*-eicosatetraenoic acid appear to be functionally identical in three assays, their identity is strongly implied. In both the rhesus monkey and the human (14), LTB is by itself capable of inducing a local cutaneous neutrophil infiltrate at 4 h and in the human appears to give an initial wheal and flare.

Natural and synthetic (c,t,t) LTB, like LTC and LTD, demonstrated a preferential action on guinea pig pulmonary parenchymal strips as opposed to tracheal spirals. However, the EC_{25} (relative to the effect of 10^{-4} M histamine) for LTB is 1.9 and 3.2 logs higher than for LTC and LTD, respectively, and this effect of LTB is apparently expressed indirectly through one or more cyclooxygenase products generated from the LTB-stimulated strip (15).

The structural basis for the 100-fold increased efficacy of LTB above that of 5-HETE in eliciting chemotaxis of human neutrophils must relate to the omega portion of the molecule, from C-6 to C-20, in which LTB incorporates a second hydroxyl group at C-12 and three, rather than two, ethylenic bonds in conjugation. The most stable conformation of LTB is expected to involve a coplanar arrangement of carbons 5–12 with s-*trans* stereochemistry about the 7,8- and 9,10- bonds, as shown in Fig. 1. This conformation is also indicated by an analysis of the 270 and 500 MHz proton magnetic resonance spectrum of the diacetate methylester of the c,t,t compound (P. B. Hopkins, unpublished data). It is likely that the remaining carbons prefer a maximally staggered, extended conformation. If it is assumed that this

preferred molecular geometry is involved in binding to the recognition site and that the geometrical preferences for other 5,12-di-HETE are similar (e.g., coplanar C-5 to C-12 s-*trans* triene system), it seems clear from tracing paper overlays of the molecular structures that the t,c,t structure is the closest match to c,t,t LTB and the t,t,t structure is the poorest one, with t,t,c being intermediate. This order is consistent with their relative chemotactic efficacies (Table I). We conclude that the structure of natural LTB is 5*S*,12*R*-dihydroxy-6,14-*cis*,8,10-*trans*-eicosatetraenoic acid (Fig. 1) and that its chemotactic potency is critically dependent upon the stereochemistry within the C-1 to C-12 domain.

Summary

Leukotriene B (LTB), a potent lipid chemotactic factor for neutrophils, is 5*S*,12*R*-dihydroxy-6,14-*cis*,8,10-*trans*-eicosatetraenoic acid (Fig. 1), based upon direct comparison of natural LTB with synthetic 5*S*,12*R*-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-di-HETE) stereoisomers in three biological assays. Of the six synthetic stereoisomers evaluated, only the 5*S*,12*R*,6,14-*cis*,8,10-*trans* compound had chemotactic potency for human neutrophils *in vitro* that was comparable to that of natural LTB, with a concentration of 3×10^{-9} M eliciting a one-half maximum response. In contrast, the racemic mixture of 5*R*,12*R*- and 5*S*,12*S*-6,10-*trans*,8,14-*cis*, the racemic mixture of 5*S*,12*R*- and 5*R*,12*S*-6,10-*trans*,8,14-*cis*, the 5*S*,12*R*-6,8-*trans*,10,14-*cis*, the 5*S*,12*R*-6,8,10-*trans*,14-*cis*, and the 5*S*,12*S*-6,8,10-*trans*,14-*cis* stereoisomers required concentrations of 3×10^{-7} to 1×10^{-6} M to elicit comparable responses. Only natural LTB and its synthetic counterpart elicited a local neutrophil infiltration when injected into the skin of the rhesus monkey at 10 ng and 100 ng per site. Natural and synthetic LTB at a concentration of 3×10^{-8} M each provoked an EC_{25} contractile response of guinea pig pulmonary parenchymal strips in vitro, whereas the other four tested stereoisomers of 5,12-di-HETE were inactive at this concentration. Structure-function analyses suggest that the neutrophil chemotactic activity depends critically upon the C-1 to C-12 domain, including the stereochemistry of the 6-, 8-, and 10-olefinic bonds and the presence of both hydroxyl groups.

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