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THE EFFECTS OF LIPOXIN A AND LIPOXIN B ON FUNCTIONAL RESPONSES OF HUMAN GRANULOCYTES

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Lipoxin A and lipoxin B (LXA and LXB) are formed from arachidonic acid by leukocyte 5- and 15-lipoxygenases. We have assessed the effects of synthetic lipoxins on functional responses of human granulocytes. LXA stimulated migration at 1 nM. The effect was highly stereospecific, since e.g.6S-LXA and LXB were less active than LXA. Neither synthetic LXA nor several of its stereoisomers provoked degranulation or aggregation. LXB and its isomers did not induce any of these functional responses. These results indicate that migratory granulocyte responses to LXA are highly stereospecific.

Arachidonic acid is metabolized mainly by lipoxygenation in human granulocytes. Among a variety of compounds generated by

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Abbreviations:
LXA, (5S,6R,14S)-5,6,15-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; 6S-LXA, (5S,6S,15S)-5,6,15-trihydroxy-7,9,13--trans-11-cis-eicosatetraenoic acid; 11-trans-LXA, (5S,6R,15S)--5,6,15-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid; 6S-11-trans-LXA, (5S,6S,15S)-5,6-15-trihydroxy--7,9,11,13-trans-eicosatetraenoic acid; LXB, (5S,14R,15S)-5,14,15-trihydroxy-6,10,12-trans-8-cis--eicosatetraenoic acid; 8-trans-LXB, (5S,14R,15S)-5,14,15-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid; 14S-8-trans-LXB (5S,14S,15S)-5,14,15-trihydroxy-6,8,10,12-trans-icosatetraenoic acid; 15-HETE, 15-hydroxy-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-eicosatetraenoic acid; HBSS, Hanks' balanced salt solution; LTB_4, leukotriene B_4; HSA, human serum albumin, fMLP, formyl-methionyl-leucyl-phenylalanine; N-t-Boc-L-Ala-pNP, N-tert-butoxycarbonyl-L-alanine-p-nitrophenyl ester; TLC, thin layer chromatography

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the 5-lipoxygenase pathway leukotriene B_4 (LTB_4) plays an important role for granulocyte physiology (1-9).

Recently, a new series of lipoxygenation products from ionophore A23187 stimulated granulocytes were reported and termed lipoxins. They are the result of interaction between the 5- and 15- lipoxygenase pathways and contain a novel conjugated tetraene structure as the distinguishing feature (10). Both lipoxin A (LXA) and lipoxin B (LXB) appear to possess discrete biological activities. LXA, obtained from incubations of leukocytes, was observed to cause the generation of superoxide anion and the release of lysosomal elastase from human granulocytes, whereas it did not initiate aggregation (10). Moreover, LXA provoked contraction of lung strips, stimulated microvascular changes (11) and inhibited the cytotoxic activity of human NK cells (12). In each of these systems it appeared that the biological activities of LXA and LXB differed from those of either leukotrienes or prostaglandins.

Recently, the complete stereochemistry of LXA and LXB and several of their isomers have been determined (13,14). In addition to LXA, identified as 5S,6R,15S-trihydroxy- 7,9,13-trans--11-cis-eicosatetraenoic acid, both 11-trans-LXA and 6S-11-trans-LXA were present and identified in biologically derived materials. Moreover, improved isolation procedures permitted the identification of the 6S-isomer of LXA. When added to human NK cells (15), guinea pig lung strips or hamster cheek pouch (11,13), synthetic LXA (13) exhibits biological activities similar to those observed with the biologically derived material.

In this report we present data on some effects of synthetic lipoxins on several human granulocyte functional responses.

MATERIALS AND METHODS

Eicosanoids. Synthetic lipoxins were prepared as in (16,17). Lipoxins were used as free acids or methyl esters, as indicated. In some experiments LXA was isolated from suspensions of mixed human leukocytes (i.e. neutrophils, eosinophils, basophils etc.) as described (10,13,14). All lipoxins were kept at -70°C in methanol, and were diluted in HBSS just prior to use. Only freshly made solutions were used. - Synthetic LTB_4 was obtained from BioMol Research Lab. (Philadelphia, PA). LTB_4 samples were handled as described for lipoxins. In addition to synthetic LXA (and in some experiments biologically derived LXA) we used its 6S-LXA, 11-trans-LXA and 6S-11-trans-LXA isomers, Lipoxin B (5S,14R,15S) 5,14,15-trihydroxy-6,8,12-trans--trans-8-cis-eicosatetraenoic acid, LXB) and three isomers, 8-trans-, 14S-8-trans- and 14S-LXB) were also examined.

Chemicals. Percoll and dextran T500 were from Pharmacia (Upsala, Sweden), A23187 from Calbiochem (La Jolla, CA), fMLP from Peninsula Lab. (San Carlos, CA) and Hanks' balanced salt solution (HBSS) from Natl. Bacteriol. Lab. (Stockholm, Sweden). All other reagents were from Sigma Chem. Co. (St. Louis, MO).

Cell isolation. Granulocytes were isolated from human blood by a one-step Percoll technique (5). Platelets represented approximately one in ten granulocytes (5). In some experiments cells were treated with cytochalasin B (5µg/ml) for 3 min. at +37° C.

Functional assays. Granulocyte chemotaxis was measured with a miniaturized Boyden chamber technique, (Neuroprobe Inc., Bethesda, MD). Granulocytes, suspended in HBSS (supplemented with fatty acid free 0.2 % human serum albumin) at a concentration of 2.5 x 10^6 granulocytes/ml, were allowed to migrate into 3µm pore size cellulose nitrate filters for 45 min. at 37°. HBSS alone, fMLP and LTB_4 were used as standard chemotactic stimuli. Previous studies showed that maximum migration occurred with 10 nM fMLP and LTB_4. Migration was expressed as the mean distance migrated (in µm) by the leading 5 cells in 2 microscope fields in each of 4 replicate wells; migration was also expressed as the mean numbers of cells per field, counted at a depth of 50 µm into the filters, for 2 fields in each of 4 replicate wells. Results are expressed as net migration or cell numbers, i.e. stimulated migration minus spontaneous migration.

Granulocyte aggregation was measured in a platelet aggregometer (5). Stimuli were added to granulocyte suspensions containing 0.5 % HSA. The resulting peak change in light transmission was recorded as Δ T.

Elastase release was monitored as described (9). After the addition of cytochalasin B to granulocytes, N-t-Boc-L-Ala-pNP (10 µM) was added to both reference and sample cuvettes in ethanol (0.1%), and baselines were recorded at 360-390 nm for appr. 1 minute. All measurements were performed at 37°C with continous stirring. Stimuli were subsequently added and changes in absorbance were followed for 3 min.

Shape changes were followed by interference contrast microscopy (18).

Statistical methods: Students' t-test was used for analyses.

RESULTS

When purified granulocytes were exposed to synthetic lipoxins as chemoattractants, stimulated migration was observed for LXA free acid with a peak effect at 1 nM ($p < 0.001$ compared with migration against HBSS). Stimulation was evident both as leading front measurements and as number of cells at 50 nm into the filters (Fig. 1). Neither the methyl ester of LXA nor 6S-LXA, 6S--11-trans-LXA or 11-trans-LXA conferred significantly stimulated

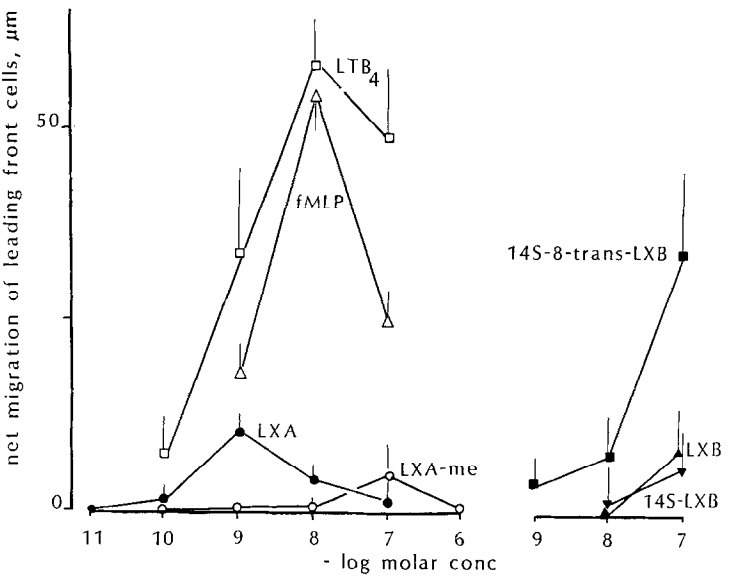

Figure 1. Migration of granulocytes in modified Boyden chambers in response to synthetic lipoxins, fMLP and LTB_4. The results are given as the distance to the leading front cells and expressed as net migration, i.e. stimulated minus spontaneous migration (to HBSS), the latter being 41+/-2 nm (n=20). Each mean value is based on 4-19 separate experiments; more specifically, for peak migration values in response to fMLP (△) and LTB_4 (□) n was 18, for LXA (●) n=11, for LXA methyl ester (LXA-me, 0) n=8, for LXB (▲) n=3, for 14S-8trans-LXB (■) n=4 and for 14S-LXB (▼) n=4. The number of granulocytes having migrated 50 µm into the cellulose nitrate filters was for HBSS 1.3+/- 0.7 per high power magnification field, for 10 nM of LTB_4 and fMLP the net number of cells (i.e. values for spontaneous migration have been subtracted) 49+/-10 and 69+/-13, respectively, for 1 nM of LXA 4.2+/-2.6, for 100 nM of LXA-me 2.7+/-2.5, for LXB and 14S-LXB (both at 100 nM) none, and, for 14S-8trans-LXB, also at 100 nM, 30+/-10 cells (means +/- SE values). Not shown in this figure are the values for 6S-LXA, which never exceeded spontaneous migration when assessed between 0.01 and 100 nM (n=3). Likewise, no response was noted for 6-trans isomers of LXA. ***=P<0.001, **=P<0.01 and *P<0.05.

migration (Fig. 1). A significant effect was seen with 14S-8--trans-LXB at 0.1 µM ($p<0.01$) but not with LXB or 14S-LXB. However, in all experiments LTB_4 and fMLP induced a considerably more pronounced migration, assessed both as leading front distances and accumulation of cells within the filters (Fig. 1). Biologically derived LXA methyl ester and LXB-free acid were also active, but only at 0.1 µM, where mean (+/- SE) net migration was 19 +/2 nm (n=4 $P<0.01$), and 15 +/- 6 nm (n=4;$p<0.05$), respectively.

Table 1. Granulocyte aggregation. Mean and SE values

	μM	mm	n
HBSS		1 +/- 1	6
LXA	10	9 +/- 9	7
-"-	1	2 +/- 2	2
6S-LXA	10	33 +/- 1	4
-"-	1	16 +/- 13	2
LTB_4	0.1	30 +/- 6	6

Granulocytes were treated with cytochalasin B, as described, and subsequently exposed to the stimulants. The ensuing maximal deflection from the baseline, measured in mm, was used for calculations of aggregation.

When granulocyte aggregation was assessed a small aggregating response could be observed to 10 μM of 6S-LXA, whereas LXA did not evoke such a response (Table 1). In addition, no obvious shape changes could be seen by light microscopy in response to 1 μM LXA. Elastase release was not evident from cytochalasin B treated granulocytes exposed to synthetic LXA free acid at 10 μM (n=4). In contrast, fMLP and LTB_4 conferred responses in both assays, the former causing a brisker response at equimolar concentrations.

DISCUSSION

Lipoxins have been shown to affect functional responses in several mammalian cell types (10-12, 15). In some systems stimulation is evident, e.g. guinea pig lung strip contraction (11), and protein kinase activation (19). In contrast, inhibitory effects have been observed for human NK-cell activity and microvascular tone (11). It has also been shown that effects of lipoxins appear to be induced by mechanisms that are distinct from those of either leukotrienes or prostaglandins (11). Whether this reflects stimulus specific sets of surface receptors remains to be demonstrated. LXA was also recently shown to cause protein kinase activation in isolated preparation of the enzyme (19).

There are a number of characteristic features for migratory response to LXA. Firstly, the peak migratory response occurred at a concentration one tenth of that observed for LTB_4 and fMLP. At that concentration, 1 nM, the distance migrated by leading front cells was considerably shorter and the number of migrating cells (at 50 μM into the filter) were fewer than observed for either LTB_4 or fMLP. This suggests that LXA may cause migration in a subset of granulocytes present in the cell preparations. Alternatively, LXA may be rapidly transformed by granulocytes so that a stable chemotactic gradient is not established. Since the metabolic fate(s) of LXA still is unknown, this question remains unanswered.

Secondly, a definite structure activity relationship for lipoxins was established. Both synthetic and biologically derived LXA were effective as chemoattractants. Here, neither 6S-LXA nor the two trans LXA isomers had any such effect; in addition, the methyl ester of LXA showed little to no effect. These findings indicate that both the 6R chirality and the free carboxylic acid are important determinates in provoking granulocyte activation with LXA. The nature of this clearly evident stereochemical specificity is intreaging, albeit the response is less than that observed with LTB_4.

Thirdly, the finding of a discrete LXA concentration at which migration was stimulated (whereas both higher and lower concentrations failed to do so) is in agreement with what is observed for both LTB_4 and fMLP. These two latter stimuli are believed to exert their chemotactic effect by means of high affinity surface receptors (20,21). In response to supraoptimal chemoattractant concentrations migration is inhibited, probably because of loss of or functional rearrangement of the high affi-

nity receptors. The finding of a similar change of responsiveness of granulocytes exposed to various LXA concentrations, as well as to LTB_4 or fMLP, suggests the presence and active expression of cellular receptors for the migratory response to LXA.

In original isolations of LXA from leukocytes the biologically derived material provoked granulocyte activation as evidenced by the generation of active oxygen species and the release of lysosomal elastase (10) but not aggregation suggesting that LXA is a selective secretagogue in granulocytes. Results of the present study with LXA prepared by total synthesis also indicate that LXA is a selective activator of granulocytes, for example, promoting chemotaxis. In contrast to the results of earlier studies with biologically derived LXA, which provoked some degranulation (10), synthetic LXA did not provoke the release of lysosomal elastase.

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