

Lipoxin A augments release of thromboxane from human polymorphonuclear leukocyte suspensions

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Lipoxin A (LXA) is a novel eicosanoid, generated by the interactions of lipoxygenases, which has a variety of biological actions. When added to human polymorphonuclear leukocytes, LXA stimulated thromboxane formation which was monitored as TxB₂ by radioimmunoassay. The compound augmented the formation of TxA₂ stimulated by the ionophore of divalent cations (A23187). Formation of thromboxane was inhibited by two non-steroidal anti-inflammatory drugs (i.e. indomethacin and proglumetacin). Results of the present study indicate that LXA can provoke the release and transformation of endogenous arachidonic acid to thromboxane. Moreover, they suggest a relationship between lipoxin A and the formation of cyclooxygenase pathway products.

Lipoxin; Thromboxane; Indomethacin; Polymorphonuclear leukocyte

1. INTRODUCTION

PMN leukocytes play a central role in the immune system and in inflammatory diseases. PMN leukocytes can release lysosomal enzymes, PGs, Txs and lipoxygenase products [1–4] when exposed to the calcium ionophore A23187. TxA₂ is a biologically active labile molecule which can be formed by PMN leukocytes as well as macrophages and platelets [5–7]. TxA₂ is enzymatically derived from arachidonic acid and in aqueous media a molecule of water is rapidly added to TxA₂ and the stable endoprocess TxB₂ is formed.

TxA₂ is believed to be the product of the

cyclooxygenase pathway in PMN leukocytes, to induce platelet aggregation, and provoke contraction in several tissues [8–10]. The conversion of arachidonic acid into various biologically active products is accomplished by cyclooxygenase and lipoxygenase pathways.

Recent studies have indicated that polyunsaturated fatty acids and their metabolites are involved in the functions of leukocytes [11–13]. The trihydroxytetraenes are a novel series of oxygenated derivatives formed from arachidonic acid by human leukocytes. The structure of the major compound was established and proved to be 5S,6R,15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid [11–17]. When added to human PMN leukocytes LXA stimulates chemotaxis without provoking aggregation [18]. Human natural killer cells exposed to LXA are unable to provoke target cell lysis [19].

Moreover, LXA also provokes contraction of parenchymal strips and stimulates microvascular changes [20]. In the present study we have determined the influence of LXA on TxA₂ (detected as

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Abbreviations: LXA, lipoxin A; Tx, thromboxane; PMN, polymorphonuclear; A23187, ionophore A23187; PG, prostaglandin; I, indomethacin; P, proglumetacin

release of TxB₂) by human peripheral blood derived polymorphonuclear cells in vitro.

2. MATERIALS AND METHODS

2.1. Collection of blood and isolation of PMN leukocytes

Blood was obtained from healthy donors and collected in polypropylene tubes containing sufficient heparin to obtain a final concentration of 20 U/ml. Tests were performed immediately after collection. PMN leukocytes were isolated as in [21]. Briefly, whole blood was centrifuged over Ficoll-Hypaque in 2% dextran for 30 min at room temperature [21]. After washing the cells twice with buffered saline, the red cells were lysed by distilled water, the remaining cells were washed and resuspended in Gey's buffer and viability examined by trypan blue-exclusion method. The cells were incubated at 10^6 per ml at 37°C in triplicate. The cell suspensions consisted of more than 94% PMN leukocytes. Prior to culture the cells were counted in a Bürker counting chamber. The cell preparation had a PMN to platelet ratio of 10:1 or less. Samples were treated with LXA at different concentrations (10^{-4} – 10^{-7} M), the controls received methanol at the same concentration of LXA (10^{-4}) and TxB₂ was determined utilizing the conditions described by Jakschik et al. [22]. The incubations were performed in triplicate and TxB₂ was quantitated by radioimmunoassay of the resulting supernatants [23].

2.2. Tested drugs

Indomethacin is a potent inhibitor of cyclooxygenase [24,25] and was employed as such in the present study to assess the cyclooxygenase dependence of LXA-induced TxA₂ formation. Here, incubations were exposed to indomethacin (Merck, Sharp & Dohme) at the indicated final concentrations (10^{-7} – 10^{-9} M), followed by determination of TxB₂ levels. Proglumetacin is a derivative in which the indolacetic group is linked by piperazinic bond to proglumide. This compound has been shown to be an effective anti-inflammatory agent, a cyclooxygenase inhibitor in vitro, and displays a gastroprotective activity [26]. All incubations were exposed to proglumetacin (Merck, Sharp & Dohme) at a final concentration of 3×10^{-7} M; followed by addition of either LXA

or A23187 and determination of TxB₂ level. Its complete mode of action as that of other non-steroidal anti-inflammatory drugs, remains to be fully elucidated.

2.3. Calcium ionophore A23187

Ionophore (Sigma) was dissolved in dimethylsulfoxide (DMSO) at 50 mg/ml. Dilutions of the ionophore were made directly in medium with various final concentrations. In separate tubes, in each experiment, cells were exposed to the vehicle alone (i.e. DMSO or ethanol) at identical concentrations, to determine non-specific formation or inhibition of TxA₂ generation by the vehicle.

The generation of TxA₂ was assessed by the appearance of its more stable product TxB₂ by radioimmunoassay as described [19]. Results are expressed as pg/ 10^6 cells. Each test and all controls were done in triplicate with each donor.

2.4. Preparation of lipoxin

LXA was a gift of Dr Charles N. Serhan, Hematology Division, Harvard Medical School, Boston, MA, USA. Briefly, LXA was isolated from suspensions of mixed human leukocytes (i.e., neutrophils, eosinophils, basophils, etc.) as described [14–17]. Human leukocytes prepared from healthy donors were warmed to 37°C (200 ml of 100×10^6 cells/ml). Next, 15-HETE (80–100 μ M) and A23187 (2.5 μ M) were added simultaneously in ethanol (1% final, v/v), and incubations were continued for 20 min. Incubations were stopped by addition of 2 vols methanol, extracted with ether, and subjected to silicic acid chromatography. Materials present in the ethyl acetate fraction were combined with 11,12,15-trihydroxy-5,8,13-[1-¹⁴C]eicosatrienoate and 11,14,15-trihydroxy-5,8,12-eicosatrienoate, treated with diazomethane, and then subjected to thin-layer chromatography. Thin-layer chromatography was carried out with plates coated with silica gel G and ethyl acetate-2,2,4-trimethylpentane (5:1, v/v) as solvent. The regions containing methyl esters exhibiting tetraene UV spectra (see [14]) but no radiolabel were collected, and the material was eluted from the gel with methanol. Samples were pooled and injected onto a reverse-phase HPLC column Altex ultrasphere-ODS 10 nm $\times$ 25 cm eluted with 65:35 (v/v) at 30 ml/min, with the UV detector set at 301 nm. After re-

chromatography in the same HPLC system, an aliquot of material was subjected to straight-phase HPLC employing a Bakerbond chiral column (J.T. Baker) with hexane/isopropanol as solvent (90:10, v/v). Free acids were prepared by LiOH saponification and ether extraction [24] followed by reverse-phase HPLC performed by using MeOH/H₂O/acetic acid (70:30:0.01, v/v).

The final purity of LXA was assessed by reverse-phase HPLC and proved to be 95% LXA with 5% of its all *trans*-isomer (11-*trans*-LXA). UV spectra of separated components were recorded in methanol with the use of a Hewlett-Packard 8450A spectrophotometer and an absorption coefficient of 50000 to determine concentrations. The compound was subject to analysis by gas chromatography/mass spectrophotometry.

3. RESULTS

Human PMN cells were assayed for TxA₂ formation in the presence or absence of LXA and calcium ionophore. Fig.1 shows the amounts of TxB₂ (pg/10⁶ cells) released by human PMN leukocytes after addition of calcium ionophore A23187 at varying concentrations (0.2, 2, 50, and 100 μ M). The amounts of TxB₂ released by the cell after 1 h incubation was maximum at both 2 and 50 μ M (269.81 $\pm$ 25.6 and 254.72 $\pm$ 40.2), and lower at 0.2 μ M (122.64 $\pm$ 32.4). At the highest concentration tested, A23187 (100 μ M) proved to be toxic for the cells, as determined by trypan blue exclusion and lower levels of TxB₂ were found (105.66 $\pm$ 30.1). At lower concentrations the ionophore was non-toxic and proved to stimulate TxA₂ formation in a dose- and time-dependent fashion. These values were significant when compared to those obtained from controls (35.85 $\pm$ 11.2).

Results in fig.2 show the amounts of TxB₂ detected in human PMN leukocyte suspension following addition of increasing concentrations of LXA. Here, TxB₂, detected after 1 h incubation, was enhanced by increments in the concentration of LXA added to the cell suspensions. Again, the formation of TxB₂ was dose-dependent. The effect of LXA was significant at the 10⁻⁴ or 10⁻⁵ M dose (266.35 $\pm$ 50.9 and 77.10 $\pm$ 20.2), while at 10⁻⁶ or 10⁻⁷ M it was not significant (51.31 $\pm$ 11.4 and 35.04 $\pm$ 13.0; the control was 35.05 $\pm$ 12.8).

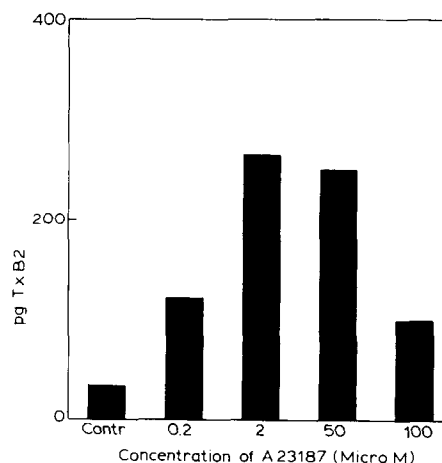


Fig.1. TxB₂ detected from human PMN leukocyte suspensions (10⁶ cell/ml) after 1 h incubation with calcium ionophore A23187 at four different concentrations (0.2, 2, 50 and 100 μ M). The experiments reported here were done in triplicate.

Fig.3 gives the amounts of TxB₂ detected after treatment with A23187 (2 μ M), LXA (10⁻⁴ M), A23187 (2 μ M) + LXA (10⁻⁴ M) and A23187 (2 μ M) + indomethacin (10⁻⁹ M) at different periods of incubation (15 and 60 min). The results indicate that after 15 min of incubation a significant stimulation of TxA₂ (monitored as TxB₂) is observed with A23187 (372.17 $\pm$ 41.9) compared with the control (40.09 $\pm$ 10.9).

Here, the very low dose of indomethacin (10⁻⁹ M) used, partially reduced the effect of A23187 (188.68 $\pm$ 19.9). At 60 min of incubation, the effect of A23187, A23187 + LXA and indomethacin were essentially the same as those at 15 min (313.68 $\pm$ 72.4, 358.49 $\pm$ 87.0 and 186.32 $\pm$ 50.9). The cells were also significantly affected by LXA (129.72 $\pm$ 24.5). The control value was 58.96 $\pm$ 20.9.

Fig.4 gives the percent of TxB₂ released following different treatments. Bars B and C show the release of TxB₂ after treatment with A23187 (2 μ M and 5 μ M, 223.47 $\pm$ 72.4 and 254.65 $\pm$ 51.2), respectively. A non-statistical significant difference was found between them. Bars D and E show the release of TxB₂ after treatment with LXA (10⁻⁴ and 10⁻⁵ M) (224.98 $\pm$ 57.0 and 188.54 $\pm$ 12.1), respectively. We found stimulation at 10⁻⁵ M only in this experiment. In F, G and H

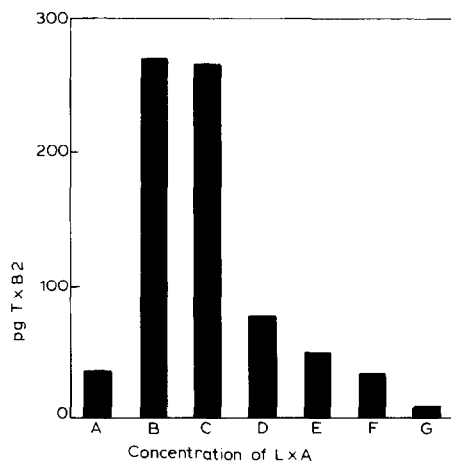


Fig.2. A, control; B, A23187 ($2 \mu\text{M}$); C, LXA (10^{-4} M); D, LXA (10^{-5} M); E, LXA (10^{-6} M); F, LXA (10^{-7} M); G, LXA (10^{-4} M) + I (10^{-7} M). TxB2 detected from human PMN leukocyte suspensions (10^6 cell/ml) after 1 h incubation with A23187 and LXA at four different concentrations (10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} M). Indomethacin (I) (10^{-7} M) strongly inhibits the TxA2 after stimulation with LXA (10^{-4} M) (G column). The experiments reported here were done in triplicate.

(105.54 ± 30.1 , 116.43 ± 29.9 and 108.54 ± 15.8), differences were not found compared to the control (99.53 ± 11.2). In I, 10^{-7} M indomethacin

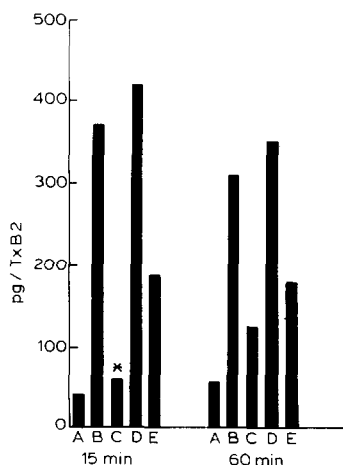


Fig.3. TxB2 detected at various times following incubation of human PMN suspensions (10^6 cell/ml) and different treatment: A, control; B, A23187 ($2 \mu\text{M}$); C, LXA (10^{-4} M); D, A23187 ($2 \mu\text{M}$) + LXA (10^{-4} M); E, A23187 ($2 \mu\text{M}$) + indomethacin (10^{-9} M). Triplicate cultures.

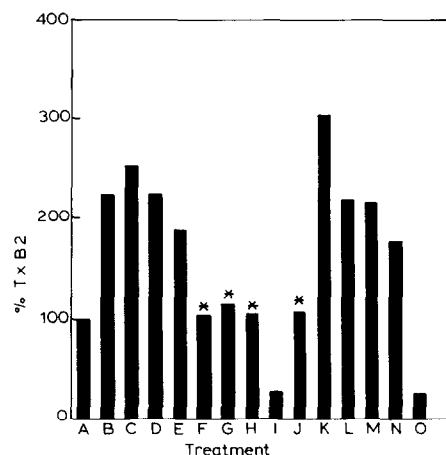


Fig.4. A, control; B, A23187 ($2 \mu\text{M}$); C, A23187 ($5 \mu\text{M}$); D, LXA (10^{-4} M); E, LXA (10^{-5} M); F, LXA (10^{-6} M); G, LXA (10^{-7} M); H, LXA (10^{-8} M); I, A23187 ($2 \mu\text{M}$) + I (10^{-7} M); J, A23187 ($2 \mu\text{M}$) + P ($3 \times 10^{-7} \text{ M}$); K, A23187 ($2 \mu\text{M}$) + LXA (10^{-4} M); L, A23187 ($2 \mu\text{M}$) + LXA (10^{-5} M); M, A23187 ($2 \mu\text{M}$) + LXA (10^{-6} M); N, A23187 ($2 \mu\text{M}$) + LXA (10^{-7} M); O, A23187 ($2 \mu\text{M}$) + LXA (10^{-4} M) + I (10^{-7} M). Percentage of TxB2 detected human PMNs after 1 h incubation with different treatments. Triplicate cultures. I, indomethacin; P, proglumetacin.

completely inhibited the release of TxB2 induced by either A23187 or LXA and reduced the levels observed in the absence of stimuli (31.92 ± 10.1). Here, proglumetacin ($3 \times 10^{-7} \text{ M}$) also inhibited TxA2 but to a lesser extent than that found with indomethacin (110.80 ± 20.7). Results in K, L, M and N show the enhancing action of LXA with A23187 which was observed with higher concentrations of LXA (307.60 ± 80.1 , 223.10 ± 12.1 , 221.60 ± 31.0 and 184.04 ± 11.1). These interactions were inhibited by treatment of the cells with indomethacin at 10^{-7} M (31.55 ± 15.0) before addition of either LXA or A23187.

4. DISCUSSION

Inflammation is, in part, mediated by neutrophils which when activated generate arachidonate-derived oxygenation products, oxygen radicals and release lysosomal enzymes [27–29]. Results of several investigations suggest that products of arachidonic acid metabolism have modulatory effects on the development of cellular

immunity [30–32]. Lipoxin A (5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid), a newly isolated compound derived from the oxygenation of arachidonic acid in human leukocytes, exerts little or no response in provoking neutrophil aggregation, but stereospecifically stimulates chemotaxis [15,18]. This compound also inhibits the cytotoxic activity of human natural killer cells [19]. In this respect it is well known that prostaglandins (i.e. PGE₂) can inhibit many immunological functions in vitro and in vivo [33–35].

In the present study, we have utilized human PMN leukocytes as a model system to monitor stimulus-induced cyclooxygenase-product formation and demonstrate here that human PMN leukocytes, exposed to LXA, release relatively high amounts of TxA₂. Addition of indomethacin to the cell suspensions blocked TxA₂ formation. Based on this observation it is likely that LXA can provoke the formation of cyclooxygenase-derived products in other cell types. Lipoxin-induced prostaglandin formation may be of importance in regulating other immunological responses. In addition, these results suggest that PMN leukocytes may be an additional source of cyclooxygenase products in immunopathology.

Since the stimulation of TxA₂ by LXA demonstrates that there is a relationship between the cyclooxygenase and lipoxygenase pathways we hypothesize that the LXA-induced block of natural killer cytotoxicity found by Ramsted et al. [19] can also be achieved, in part, by prostaglandins which may be released by these or other mononuclear cells after formation of LXA. Although the stimuli which provoke lipoxin formation in vivo remain to be determined, the finding that LXA can provoke the release of cyclooxygenase products may be of more general importance.

Further experiments are currently underway to clarify the mechanisms of action of lipoxin A.

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