Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro.

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Research Article

Human neutrophils from peripheral blood may physically interact with platelets in several settings including hemostasis, inflammation, and a variety of vascular disorders. A role for lipoxygenase (LO)-derived products has been implicated in each of these events; therefore, we investigated the formation of lipoxins during coincubation of human neutrophils and platelets. Simultaneous addition of FMLP and thrombin to coincubations of these cells led to formation of both lipoxin A4 and lipoxin B4, which were monitored by reversed-phase high pressure liquid chromatography. Neither stimulus nor cell type alone induced the formation of these products. When leukotriene A4 (LTA4), a candidate for the transmitting signal, was added to platelets, lipoxins were formed. In cell-free 100,000 g supernatants of platelet lysates, which displayed 12-LO activity, LTA4 was also transformed to lipoxins. Platelet formation of lipoxins was inhibited by the LO inhibitor esculetin and partially sensitive to chelation of Ca2+, while neither acetylsalicylic acid nor indomethacin significantly inhibited their generation. In contrast, neutrophils did not transform LTA4 to lipoxins. Cell-free 100,000 g supernatants of neutrophil lysates converted LTA4 to LTB4. These results indicate that neutrophil-platelet interactions can lead to the formation of lipoxins from endogenous sources and provide a role for platelet 12-LO in the formation of lipoxins from LTA4.

Lipoxin Formation during Human Neutrophil-Platelet Interactions

Evidence for the Transformation of Leukotriene A_4 by Platelet 12-Lipoxygenase In Vitro

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Abstract

Human neutrophils from peripheral blood may physically interact with platelets in several settings including hemostasis, inflammation, and a variety of vascular disorders. A role for lipoxygenase (LO)-derived products has been implicated in each of these events; therefore, we investigated the formation of lipoxins during coincubation of human neutrophils and platelets. Simultaneous addition of FMLP and thrombin to coincubations of these cells led to formation of both lipoxin A_4 and lipoxin B_4, which were monitored by reversed-phase high pressure liquid chromatography. Neither stimulus nor cell type alone induced the formation of these products. When leukotriene A_4 (LTA_4), a candidate for the transmitting signal, was added to platelets, lipoxins were formed. In cell-free 100,000 *g* supernatants of platelet lysates, which displayed 12-LO activity, LTA_4 was also transformed to lipoxins. Platelet formation of lipoxins was inhibited by the LO inhibitor esculetin and partially sensitive to chelation of Ca^{2+}, while neither acetylsalicylic acid nor indomethacin significantly inhibited their generation. In contrast, neutrophils did not transform LTA_4 to lipoxins. Cell-free 100,000 *g* supernatants of neutrophil lysates converted LTA_4 to LTB_4. These results indicate that neutrophil-platelet interactions can lead to the formation of lipoxins from endogenous sources and provide a role for platelet 12-LO in the formation of lipoxins from LTA_4. (*J. Clin. Invest.* 1990. 85:772–780.) eicosanoids • lipoxygenase products • transcellular-metabolism

Introduction

Lipoxygenase (LO), which are widely distributed in human tissues, transform nonesterified arachidonic acid to biologically active products that may play roles in both normal and pathophysiologic processes (1–3). The three major LOs (5-, 15-, and 12-LO) are compartmentalized within different cell types of peripheral blood. For example, upon stimulation human platelets activate an arachidonate 12-LO (4), while human neutrophils (PMN) possess both 5- and 15-LOs (5). Initial oxygenation of arachidonic acid (AA) via 5-LO leads to leukotrienes, which are held to play important roles in both inflammation and allergic reactions (1–3).

Interactions between the 5- and 15-LOs can lead to the formation of lipoxins (6). The lipoxins are a series of acyclic eicosanoids that contain a conjugated tetraene structure and three alcohol groups as characteristic features. The two main compounds of this series are positional isomers: one designated lipoxin A_4 (5S,6R,15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) and the other lipoxin B_4 (5S,14R,15S-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid) (7, 8). LXA_4 and LXB_4 each display profiles of activity that are distinct from those of other eicosanoids (reviewed in reference 1). Multiple pathways have been demonstrated for the biosynthesis of lipoxins (6–11). One route involves initial oxygenation by the 15-LO and formation of a 5(6)-epoxytetraene intermediate by human leukocytes to give LXA_4 and LXB_4 (8).

There is increasing appreciation that platelets play significant roles in modulating immunologic responses (12, 13). Interactions between platelets and PMNs can lead to the transcellular metabolism of eicosanoids (14) and the formation of products not generated by either cell type alone (15). During the course of the present investigation, Edenius et al. (16) reported that mixed platelet-granulocyte suspensions stimulated with the ionophore A23187 generate LXA_4 and four other lipoxins from endogenous sources of arachidonic acid. They also showed that LTA_4 is converted to LXA_4 by platelets and suggested that the 15-LO activity of platelets may be involved in this enzymatic conversion (16). Here, we report that simultaneous activation of PMNs and platelets by receptor-mediated agonists leads to the formation of both LXA_4 and LXB_4 and that LTA_4 is transformed by platelets to LXA_4, LXB_4, and the recently identified 7-*cis*-11-*trans*-LXA_4 (17). In addition, evidence is presented for the role of the platelet 12-LO and its ω-6-oxygenase activity in converting LTA_4 to lipoxins.

Glossary

ASA acetylsalicylic acid
Esculetin 6,7-dihydroxycoumarin
GC gas liquid chromatography
12-HETE 12S-hydroxy-5,8,14-cis-10-*trans*-eicosatetraenoic acid
15-HETE 15S-hydroxy-5,8,11-cis-13-*trans*-eicosatetraenoic acid
HMDS hexamethyldisilazane
HPETE hydroperoxyeicosatetraenoic acid
LO lipoxygenase
Lipoxin A_4, (LXA_4) 5S,6R,15S-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid
Lipoxin B_4, (LXB_4) 5S,14R,15S-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid
7-*cis*-11-*trans*-LXA_4 5S,6R,15S-trihydroxy-9,11,13-*trans*-7-*cis*-eicosatetraenoic acid
LTB_4, leukotriene B_4 5S,12R-dihydroxy-6,14-*cis*-8,11-*trans*-dihydroxyeicosatetraenoic acid
LTA_4, leukotriene A_4 5S-trans-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
Me_3Si trimethylsilyl
MS mass spectrometry
PGB_2 prostaglandin B_2
RP-HPLC reverse phase HPLC
TMCS trimethylchlorosilane

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Methods

FMLP, dextran, acetylsalicylic acid (ASA) and methyl formate were purchased from Sigma Chemical Co., St. Louis, MO. HPLC grade solvents were from American Scientific Products (McGaw Park, IL). Sep-Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA). Pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were from Pierce Chemical Co. (Rockford, IL), and diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitroguanidine purchased from Aldrich Chemical Co. (Milwaukee, WI). Synthetic 7-*cis*-11-*trans*-LXA_4 was from Dr. K. C. Nicolaou, University of Pennsylvania (Philadelphia, PA). Synthetic LTA_4-methyl ester, LXA_4, LXB_4, and esculetin (6,7-dihydroxycoumarin) were from Biomol Research Laboratories, Inc. (Philadelphia, PA). Other eicosanoids used as reference materials were prepared as described (7); these included 11-*trans*-LXA_4, 6S-11-*trans*-LXA_4, 8-*trans*-LXB_4 and 14S-8-*trans*-LXB_4. Saponification of LTA_4 methyl ester was performed in tetrahydrofurane under argon with the addition of LiOH at 4°C for 24 h. Before each experiment, the ultraviolet spectrum of LTA_4 was examined in MeOH, as well as its spectral shift (from 280 nm to 270 nm) upon addition of dilute HCl. The products formed after addition of HCl to LTA_4 in MeOH were analyzed by RP-HPLC (18).

Preparation of human PMN and platelet suspensions. For each experiment, venous blood (~ 100–120 ml) was drawn from healthy volunteers who had not taken aspirin or other medications for at least 7 d. PMN and platelet suspensions were prepared (14, 19, 20) from the same heparinized blood directly after venipuncture. Platelet-rich plasma was removed after centrifugation at 160 *g* for 15 min at room temperature. Washed platelet suspensions were prepared (14, 20), enumerated using a Coulter counter (model ZF; Coulter Electronics, Inc., Hialeah, FL) and suspended in PBS containing 0.1% albumin. In parallel, PMNs were isolated by dextran sedimentation of the remaining blood followed by Ficoll-Hypaque gradient centrifugation (19). The red cells were removed by hypotonic lysis followed by centrifugation, and the isolated PMNs were suspended in Dulbecco's phosphate-buffered saline, pH 7.4. These suspensions contained 98±1% PMNs as determined by light microscopy before coincubation with isolated platelets. The integrity of cells in suspension was monitored both before and during incubation conditions by determining their ability to exclude trypan blue. Under all experimental conditions, < 3% of the PMNs in suspension were permeable to trypan blue.

Cell-free 100,000 *g* supernatants of platelet lysates and PMN lysates were prepared (20). Briefly, freshly isolated cell suspensions were rapidly frozen in a dry ice/acetone bath and thawed to room temperature. This sequence was repeated three times followed by centrifugation at 100,000 *g* for 60 min at 10°C. All incubations with 100,000 *g* supernatants were performed directly after centrifugation.

Incubation conditions. PMNs (3.5×10^7 cells) were coincubated with autologous platelets at a ratio of 1:10 (PMN/platelet) in PBS (1 ml). After 5 min at 37°C, stimuli were added, which included either thrombin (0.1 U/ml) and FMLP (10^{-7} M), each added separately to individual cell populations, or cells incubated alone in the absence of stimuli. In coincubation experiments, platelets were treated with indomethacin (100 μM) for 20 min at 37°C before coincubations. In experiments with LTA_4 and intact platelets, cells (1.5×10^9 platelets/ml) were incubated 5 min at 37°C in PBS (1 ml) containing 0.1% albumin before addition of either LTA_4 (30 μM) alone or LTA_4 (30 μM) and stimuli (either ionophore A23187; 0.1 μM or thrombin; 1 U/ml). In these experiments platelets were treated with either ASA (100 μM), esculetin (100 μM), or indomethacin (100 μM) for 20 min at 37°C before incubation with LTA_4 and ionophore (0.1 μM).

Analysis of eicosanoids. Incubations were terminated by addition of cold ethanol (2 vol) containing PGB_2 as internal standard. The suspensions were placed at 4°C for a minimum of 30 min followed by centrifugation. Supernatants were removed and the remaining pellets were resuspended in MeOH (2 vol). This was repeated twice, and the resulting EtOH- and MeOH-containing supernatants were pooled and taken to dryness by rotoevaporation (17, 21). Materials were resuspended in MeOH/H_2O (1:45 vol/vol) by vortexing in a round-bottom flask (≈ 1–2 min) and transferred into a syringe followed by acidification to pH 3.5. Samples from each incubation were rapidly loaded into cartridges (C_{18} Sep-Paks), washed with H_2O and eluted with hexane and methyl formate. Materials eluted in the methyl formate fractions were concentrated under argon and examined for ultraviolet-absorbing materials before injection into a RP-HPLC system, which consisted of dual-pump gradient HPLC (LKB Instruments, Bromma, Sweden) equipped with an Ultrasphere-ODS (4.6 mm × 25 cm) column (Beckman Instruments, Inc., Fullerton, CA), injector, and solvent controller (LKB Instruments). The column was eluted with a gradient consisting of MeOH/H_2O/acetic acid (65:35:0.01) as phase one (t_0—20 min), a linear gradient with MeOH/acetic acid (99.99:0.01) as phase two (20–50 min) and a flow rate of 1.0 ml/min. This HPLC system was equipped with a photodiode array rapid spectral detector linked to an AT&T PC6300, and post-HPLC run analyses were performed utilizing a 2146-002 Wavescan program (LKB Instruments) and a chromatography data system (3000 series; Nelson Analytical, Paramus, NJ). LO products were quantitated from stored ultraviolet spectral data after RP-HPLC where baselines were achieved by computer-aided manipulations (21). The post-HPLC recovery of PGB_2 was 69±6% (n = 7 with 25 determinations; mean±SE). Ultraviolet spectra not recorded on-line during HPLC were recorded with a spectrophotometer (model 8452; Hewlett-Packard Co., Palo Alto, CA). Levels of significance, P, of the differences between platelets treated with inhibitors and transformation in those incubated in their absence were calculated using Student's t test.

After HPLC, collected material was treated with diazomethane and converted to trimethylsilyl derivative by exposure to hexamethyldisilazane and trimethylchlorosilane in pyridine (7). GC-MS was performed with a Hewlett-Packard 5988A MS equipped with an HP 59970A workstation, software and 5890 GC. A fused silica capillary SE-30 (Supelco, Inc., Bellefonte, PA) column 2-4004, 30 m, 0.25 mm i.d., 0.25 μM df was employed with a temperature program. The splitless on time was 0.90; initial temperature was 150°C (1 min), followed by 230°C (4 min), 240°C (8 min) and 245°C (12.0 min) with a 12.0-min solvent delay time.

Results

FMLP is a potent PMN-directed stimulus, whereas thrombin exerts its primary actions on platelets (22, 23). To further probe interactions between human PMNs and platelets, thrombin (0.1 U/ml) and FMLP (10^{-7} M) were added simultaneously to coincubations of platelets-PMNs (10:1) and the products generated were characterized. Analysis of methyl formate fractions by RP-HPLC revealed the presence of strongly absorbing materials when stored spectral data was recalled at 300 nm (Fig. 1). The presence of these chromophores suggested that conjugated tetraene-containing products were generated (6–8). Materials beneath the four peaks in Fig. 1 gave retention times identical to those of synthetic LXA_4, LXB_4, and their respective all-*trans*-isomers. In this RP-HPLC system 8-*trans*-LXB_4 and 14S-8-*trans*-LXB_4 coelute, as do 11-*trans*-LXA_4 and 6S-11-*trans*-LXA_4 (21). These products were not further resolved in the present studies and are denoted as trans-A and trans-B in the HPLC profiles.

Coincubations of platelets and PMNs without exposure to the combination of FMLP and thrombin did not generate these products. In addition, neither platelets incubated with thrombin alone nor PMNs incubated with either FMLP or thrombin alone generated detectable levels of these products (Table I). To determine if the platelet cyclooxygenase was involved in their formation, isolated platelets were treated with indomethacin (100 μM) and washed before their coincubation

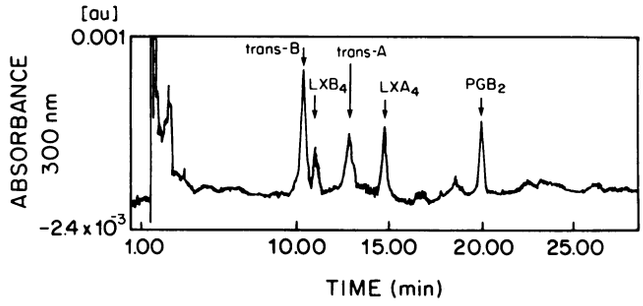

Figure 1. RP-HPLC profile of products obtained from coincubation of human PMNs and platelets stimulated with FMLP (10^{-7} M) and thrombin (0.1 U/ml). The ratio of PMNs to autologous platelets was 1:10 with 35×10^6 PMN/ml. This post-HPLC analysis of products is representative of profiles obtained from three different donors. Spectral analyses were performed with a Wavescan EG program 2146-*002* and a Nelson Analytical 3000 series chromatography data system. This chromatogram at 300 nm was recalled from spectral data collected by scanning between 230–330 nm. Arrows indicate the retention times of synthetic eicosanoids. Other products are not shown, i.e., TXB_2 eluted at 6.0 min, HHT at 33.1 min, and 12-HETE at 37.8 min in this system. *trans*-A denotes retention times of 11-*trans*-LXA_4 plus 6S-11-*trans*-LXA_4 and *trans*-B denotes 8-*trans*-LXB_4 plus 14S-8-*trans*-LXB_4.

with PMNs. Treatment with indomethacin did not result in a statistically significant reduction in the formation of the lipoxins (Table I).

Human PMN-derived eicosanoids can be transformed by platelets during cell-cell interactions (14, 24). In particular, LTA_4 has been shown to undergo transcellular metabolism in that it can be released from activated cells and enzymatically transformed to leukotrienes by a second cell type (25, 26). To determine if this PMN-derived product was involved in the formation of lipoxins during PMN-platelet interactions, isolated platelets were incubated with LTA_4 and the products formed were analyzed. After RP-HPLC, a similar profile of products was obtained as in the case of coincubations (Figs. 1 and 2). When the stored spectral data were recalled at 300 nm, several prominent peaks displaying strong absorbance at 300 nm were revealed (Fig. 2, *lower panel*). An isogram plot of the spectral data (240–330 nm; *upper panel,* Fig. 2) showed that each of the products separated by RP-HPLC carried a conjugated tetraene chromophore. Although the RP-HPLC profiles obtained from these incubations (cf. Figs. 1 and 2) were similar, they were not identical. Here, small amounts of material in Fig. 2 coeluted with synthetic LXB_4. In addition, a tetraene-containing product that coeluted with 7-*cis*-11-*trans*-LXA_4 (17) was formed (Fig. 2). Materials beneath the other three peaks coeluted with *trans*-B, *trans*-A, and LXA_4.

To further substantiate the identity of the product that was generated from LTA_4 by human platelets, the material that gave the retention time of LXA_4 was collected after HPLC and pooled from several incubations. The pooled samples were treated with diazomethane and converted to the trimethylsilyl derivative for analysis by GC/MS. This material eluted as a broad peak on GC with an equivalent chain length corresponding to 24.0–24.1. Its mass spectrum (Fig. 3) gave prominent ions at m/e 203 (base peak), 171, and 173 with weaker ions observed at m/e 289, 379, 492, and 582 (M). These ions and C-value are identical to those reported for both the leukocyte-derived and synthetic LXA_4 (6, 7).

The conversion of LTA_4 to lipoxins was examined next with platelets incubated in the presence or absence of stimuli and with cells treated with inhibitors (Table II). Exposure of platelets to either thrombin or ionophore enhanced the conversion of LTA_4 to lipoxins. In the case of ionophore, ~ 3.5% of the added LTA_4 was recovered as lipoxins. Esculetin, which inhibits the conversion of AA by the platelet 12-LO (27), completely inhibited the conversion of LTA_4 to lipoxins. In contrast, prior treatment of the cells with either ASA or indomethacin did not significantly block the formation of lipoxins.

Since human PMNs possess a 15-LO, it is possible the LTA_4 could be converted by this enzyme to a 15-hydroperoxy-derivative. Formation of this intermediate from LTA_4 could account for the formation of lipoxins (6–8). To test this hypothesis, PMNs (30×10^6 cells/ml) were incubated with either LTA_4 (30 μM) or LTA_4 with A23187 (2.5 μM). In neither case did PMNs generate lipoxins from exogenous LTA_4 (n = 3 different donors). Dose response studies with LTA_4 (0.1, 1, 10, 50, and 100 μM LTA_4) incubated with PMNs (20 min, 37°C) were also negative for lipoxin production from LTA_4 (n = 2). Together these findings suggest that LTA_4 is not converted to the 5(6)-epoxytetraene upon exposure to human PMNs.

In addition to 12-LO, human platelets also exhibit 15-LO activity (28), which may be involved in the conversion of LTA_4 to lipoxins. To examine further the conversion of LTA_4

Table I. Formation of Lipoxins by Human Platelets and Neutrophils

Incubation	LXA_4	*trans*-LXA_4	LXB_4	*trans*-LXB_4
Platelets + neutrophils (thrombin + *f*-Met-Leu-Phe)*	4.6±1.3	3.3±1.5	2.8±1.0	11.3±3.5
Platelets (indo) + neutrophils (thrombin + *f*-Met-Leu-Phe)‡	3.5±0.5§	9.7±2.4‖	1.8±0.3§	8.5±2.6§
Platelets + neutrophils (without stimuli)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Neutrophils + *f*-Met-Leu-Phe	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Platelets + thrombin	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Neutrophils + thrombin	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

* Neutrophils (3.5×10^7 cells) were coincubated with autologous platelets at a ratio of 1:10 (neutrophil/platelet) in 1 ml PBS (5 min, 37°C) followed by addition of thrombin (0.1 U) and FMLP (10^{-7} M). ‡ Platelets were treated with indomethacin (100 μM) for 20 min, 37°C before coincubation with neutrophils. After 5 min, thrombin (0.1 U) and FMLP (10^{-7} M) were added, and incubations were terminated at 20 min. Products were extracted and quantitated as described in Methods. Data represent the mean±SE of three separate experiments and are expressed in nanograms of product/1 ml of incubation. § Indicates $P < 0.5$ and ‖ $P < 0.1$ when compared to formation in the absence of indomethacin. The P values indicate NS.

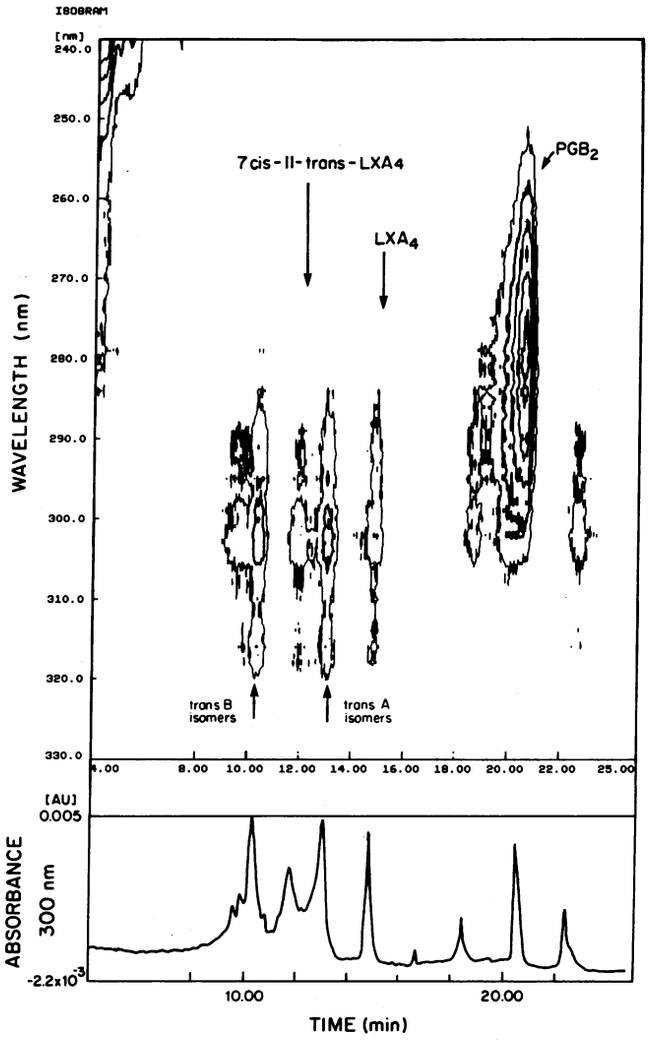

Figure 2. RP-HPLC isogram and chromatogram of products obtained from human platelets incubated with LTA_4. Platelets (1.5×10^9 cells) suspended in PBS (1 ml) containing albumin (0.1%) were incubated for 5 min at 37°C before addition of LTA_4 (50 μM) plus ionophore A23187 (0.1 μM) for 20 min at 37°C. The upper plot shows an isogram display of the stored spectral data (240–330 nm). Arrows indicate the retention time of synthetic standards. The lower plot shows the same spectral data recalled at 300 nm.

to lipoxins, 100,000 *g* supernatants were prepared from isolated cell lysates obtained by freeze-thawing. This technique provides supernatants rich in 12-LO (20). Incubation of LTA_4 with the 100,000 *g* supernatant of platelets led to the formation of lipoxins (Fig. 4 *A*). The profile of products was similar to that observed with LTA_4 and platelets (Fig. 2). The isolated platelet-derived 100,000 *g* supernatants were devoid of 15-LO, as evidenced by the finding that AA was converted solely to 12-HETE and 15-HETE was not detected (Fig. 4 *B*). Similar experiments were performed with 100,000 *g* supernatants prepared from isolated PMNs lysed by freeze thawing. In these experiments, LTA_4 was converted to LTB_4 (Fig. 4 *C*), and neither the ω-oxidation products of LTB_4 nor lipoxins were detected.

Activation of human platelets with thrombin results in a rise in intracellular free Ca^{2+}, which may play a role in 12-LO

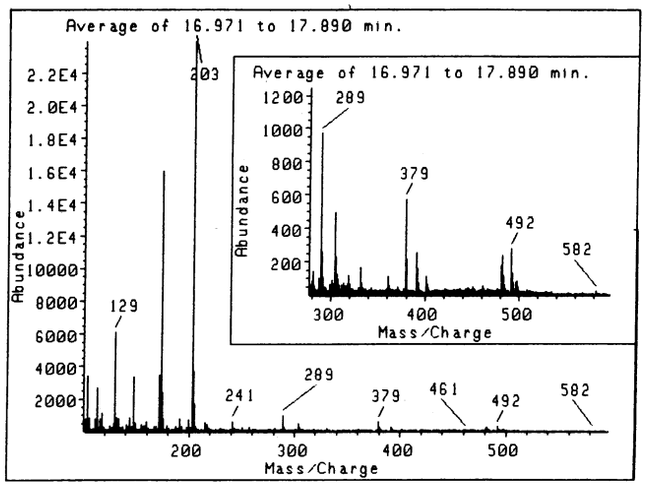

Figure 3. Mass spectrum of Me_3Si derivative of the platelet-derived product.

activity in these cells (23, 29). Therefore, the role of Ca^{2+} was assessed in the transformation of LTA_4 to lipoxins. Addition of EGTA (5 mM) significantly inhibited the transformation of LTA_4 to LXA_4. In contrast, inhibition of 7-*cis*-11-*trans*-LXA_4 was not statistically significant (Table III). Formation of 12-HETE from AA in these experiments was inhibited only 26% in the presence of EGTA (5 mM). When these supernatants were treated with esculetin, formation of lipoxins from LTA_4 was completely inhibited (Table III).

Discussion

In this paper we report the formation of lipoxins during coincubation of human PMNs and platelets. Their formation was initiated by exposure to both the chemotactic peptide FMLP and thrombin (Fig. 1). FMLP stimulates the release and oxygenation of AA in PMNs by receptor-mediated mechanisms (22, 30), while thrombin stimulates the release and oxygenation of AA in platelets (23). Neither stimulus alone provoked the formation of lipoxins (Table I, Fig. 1), indicating that activation of both cell types was required for the formation of lipoxins. Previous results have suggested that lipoxins can be generated by cell-cell interaction (6, 7). The present findings document for the first time that lipoxins can be generated by receptor-mediated activation of two cell types without addition of exogenous substrates. In addition, they both confirm and extend the findings of Edenius et al. with ionophore stimulated mixed platelet/granulocyte suspensions (16).

Granulocytes isolated from eosinophilic donors generate LXA_4 but not LXB_4 when challenged with the ionophore A23187 (31). Human PMNs generate small amounts of both LXA_4 and LXB_4 from endogenous sources in response to A23187, which are enhanced upon addition of 15-HETE (21). Lipoxins are also generated from AA by porcine leukocytes (32), mastocytoma cells (33), and bovine leukocytes (9). Although the results of these studies document the production of lipoxins from AA, the biosynthetic route(s) involved in their formation from endogenous sources remain to be fully elucidated.

In the case of human PMNs, cells incubated with FMLP alone did not generate detectable levels of lipoxins (Table I).

*Table II. Conversion of LTA_4 to Lipoxins by Platelets: Effects of Agonists and Inhibitors**

Incubation	LXA_4	7-*cis*-11-*trans*-LXA_4	*trans*-LXA_4	*trans*-LXB_4
LTA_4	21.7±7.8	6.7±3.6	52.5±25.0	35.7±18.0
LTA_4 + A23187 (0.1 μM)	64.0±23.2	19.4±10.9	157.1±74.8	106.9±44.1
LTA_4 + thrombin (1 U)	48.0±16.4	2.6±1.4	32.0±15.2	136.5±68.0
ASA-treated cells plus LTA_4 + A23187	44.8 ± 16.5^{NS}	13.1 ± 6.3^{NS}	71.4 ± 34.1^{NS}	36.8 ± 15.2^{NS}
Esculetin-treated cells plus LTA_4 + A23187	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

* Platelets (1.5×10^9 cells) in 1 ml buffer were incubated 5 min at 37°C before addition of either LTA_4 (30 μM) or LTA_4 plus stimuli. All incubations were performed with cells in PBS with 0.1% albumin at 37°C for 20 min. Cells were treated with either esculetin (100 μM), indomethacin (100 μM), or ASA for 20–30 min at 37°C before incubation with LTA_4 (30 μM) and A23187 (0.1 μM). The *P* values show NS difference in the conversion of LTA_4 in the presence of ASA. Results are presented in nanograms of product/1 ml; mean±SE with between three and eight separate experiments.

When exposed, however, to FMLP and 15-HETE, they generate both LXA_4 and LXB_4 (7). Along these lines, 15-HETE, a major product of AA metabolism in a variety of cell types (reviewed in reference 2), is rapidly transformed to lipoxins by activated human PMNs. During this event an inverse relationship is observed between leukotriene and lipoxin formation (21). In this biosynthetic route, 15-HETE is converted to a 5(6)-epoxytetraene, which can be transformed to either LXA_4 or LXB_4 (7, 8). Since neither FMLP nor thrombin alone stimulated detectable levels of lipoxins without addition of exogenous substrates (Table I), evidence was sought for the involvement of other biosynthetic routes that could account for their production during costimulation.

PMN-derived eicosanoids can be transformed by platelets

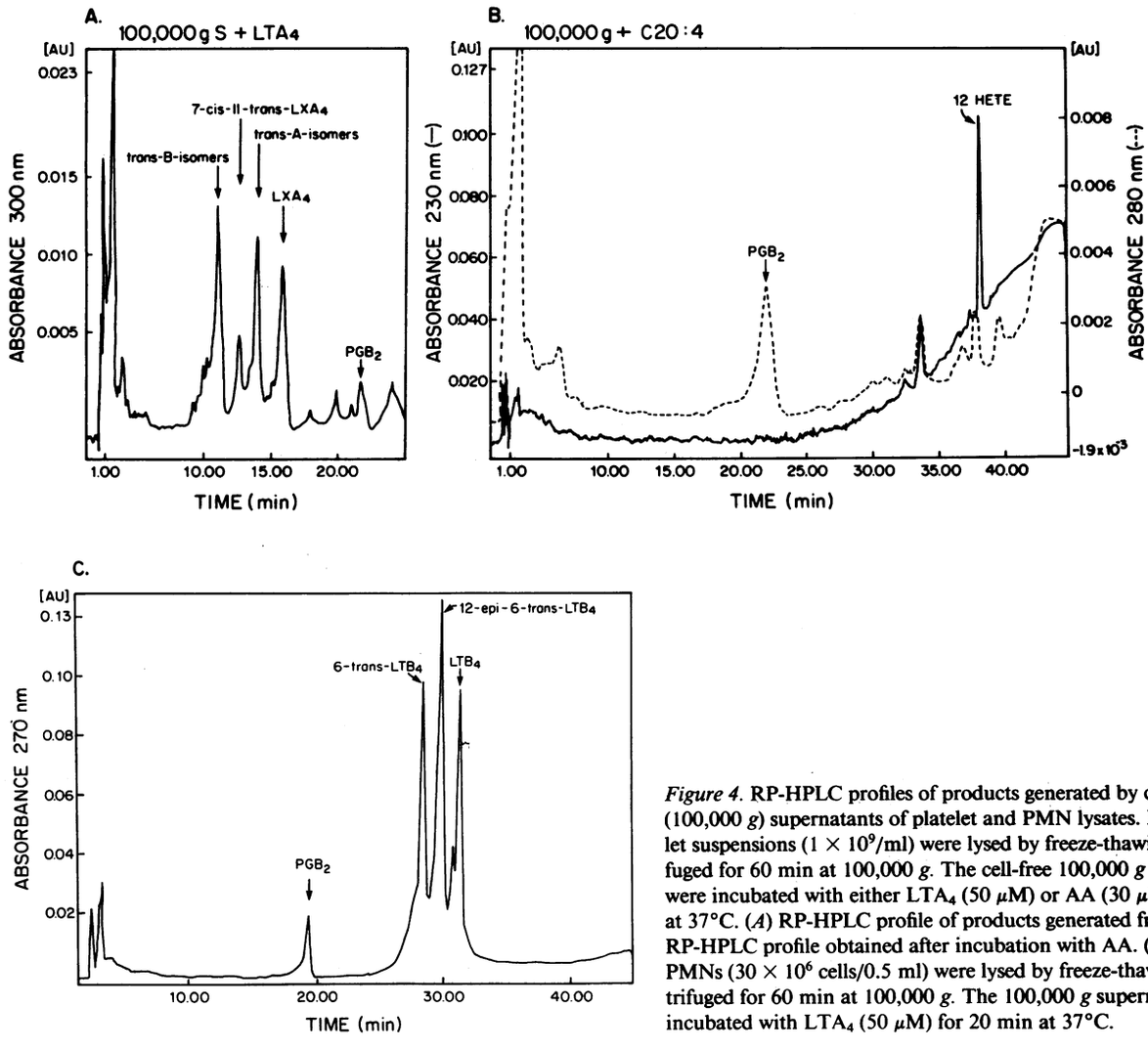

Figure 4. RP-HPLC profiles of products generated by cell-free (100,000 *g*) supernatants of platelet and PMN lysates. Isolated platelet suspensions (1×10^9/ml) were lysed by freeze-thawing and centrifuged for 60 min at 100,000 *g*. The cell-free 100,000 *g* supernatants were incubated with either LTA_4 (50 μM) or AA (30 μM) for 20 min at 37°C. (*A*) RP-HPLC profile of products generated from LTA_4. (*B*) RP-HPLC profile obtained after incubation with AA. (*C*) Isolated PMNs (30×10^6 cells/0.5 ml) were lysed by freeze-thawing and centrifuged for 60 min at 100,000 *g*. The 100,000 *g* supernatants were incubated with LTA_4 (50 μM) for 20 min at 37°C.

*Table III. Conversion of LTA_4 to Lipoxins by Cell-free Supernatants of Platelets**

Incubation	*n*	LXA_4	7-*cis*-11-*trans*-LXA_4	*trans*-LXA_4	*trans*-LXB_4
Supernatant plus LTA_4	5	28.8±5.6	10.8±2.0	32.5±5.9	34.1±8.2
EGTA treated	3	6.3±4.7‡	6.8±4.9^{NS}	9.1±8.3‡	5.8±4.4‡
Esculetin treated	3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

* Platelets (1.2×10^9 cells/ml) were lysed by freeze-thawing and 100,000 *g* supernatants were prepared as in Fig. 4, *A* and *B*. Supernatants were incubated in PBS (0.5 ml) containing 0.1% albumin and exposed to either EGTA (5 mM) or esculetin (100 μM) for 20 min at 37°C followed by addition of LTA_4 (50 μM) for 20 min at 37°C. Results are expressed in ng/1 ml; mean±SE. ‡ $P < 0.02$.

(14, 15, 24). In particular, LTA_4, an intermediate in the formation of leukotrienes within its cell of origin, can also be released and transformed by a second cell type (24–26). When LTA_4 was added to platelets, several products that displayed conjugated tetraene chromophores were resolved (Fig. 2), which coeluted with LXA_4, the *trans*-A isomers of LXA_4, 7-*cis*-11-*trans*-LXA_4 and the *trans*-B-isomers of LXB_4. In these incubations LXB_4 was formed in small amounts and 7-*cis*-11-*trans*-LXA_4, which was recently isolated (17), was also formed. Further analysis of the platelet-derived product showed that it displayed the identical properties on GC-MS as those of both synthetic and leukocyte-derived LXA_4 (cf. Fig. 3; reference 7). On the basis of these findings, we conclude that human platelets can transform LTA_4 to LXA_4. These observations extend those recently reported by Edenius et al. (16) and suggest that during receptor-mediated activation of PMNs and platelets incubated together, LTA_4 generated by PMNs can be transformed to lipoxins by platelets.

Transformation of LTA_4 to lipoxins by platelets was further characterized employing inhibitors with both intact cells and cell-free (100,000 *g*) supernatants. Treatment of platelets with either cyclooxygenase or LO inhibitors showed a differential effect on their formation (Tables I and II). Treatment of platelets with either ASA or indomethacin did not result in statistically significant inhibition of lipoxin formation. In contrast, esculetin completely blocked the transformation of LTA_4 to lipoxins (Table II), suggesting that LTA_4 is a substrate for lipoxygenation.

Although human platelets display 12-LO as their major LO pathway, an arachidonate 15-LO activity is observed (28). Since LTA_4 contains an available 1,4-*cis*-pentadiene, it was of interest to examine which platelet LO was responsible for the conversion of LTA_4 to lipoxins. To this end, cell-free 100,000 *g* supernatants were prepared from both isolated platelets and PMNs. Supernatants prepared from platelets lysed by the freeze-thawing technique convert AA to 12-HETE without detectable levels of 15-HETE (20). The present results confirm this observation, since addition of AA or ^{14}C-labeled AA to platelet 100,000 *g* supernatant led to formation of only 12-HETE and 15-HETE was not observed (Fig. 4 *B*). Aliquots of these same supernatants transformed LTA_4 to lipoxins (Fig. 4 *A*), which was inhibited by esculetin (Table III). Taken together these findings suggest a role for the platelet 12-LO or its ω-6 oxygenation activity in the transformation of LTA_4 to lipoxins.

In contrast, supernatants (100,000 *g*) obtained from PMN lysates transformed LTA_4 to LTB_4 (Fig. 4) and lipoxins were not detected. These results are consistent with the concept that the major route of LTA_4 metabolism within intact PMNs is its enzymatic conversion to LTB_4 by LTA_4 hydrolase (34). They do not, however, rule out the existence of other routes of metabolism for LTA_4.

During the interaction of PMNs and platelets, several routes of lipoxin formation may be operative. For example, with intact platelets LTA_4 may be a substrate for the 15-LO activity observed in these cells (28), which can generate a 5(6)-epoxytetraene to give lipoxins (16). Along these lines, we have found that LTA_4 is indeed a substrate for the soybean 15-LO and is quantitatively transformed to its 15-hydroperoxy-derivative (a 5(6)-epoxytetraene, unpublished observation). Although such a route of lipoxin biosynthesis is possible, several lines of evidence suggest a direct action of the platelet 12-LO on LTA_4: (*a*) the finding that intact platelets as well as their supernatants (100,000 *g*) that display 12-LO activity transform LTA_4 to lipoxins (Figs. 2–4); (*b*) the pattern of lipoxins formed in both cases was similar and 6S-LXA_4 was not observed in these HPLC profiles (Figs. 2 and 3). If the 5(6)-epoxytetraene was an intermediate in the biosynthesis of lipoxins in platelets, some nonenzymatic hydrolysis of the 5(6)-epoxytetraene would be expected to give rise to small amounts of 6S-LXA_4, which are found in incubations with activated human leukocytes and 15-HETE (7); and (*c*) previous results indicate that the human platelet 12-LO can act with high efficiency on substrates other than arachidonate, including both 15-HPETE and 5-HETE (14, 35, 36).

Although transcellular metabolism of LTA_4 and its transformation by 12-LO may account for the formation of LXA_4, only small amounts of LXB_4 were noted in incubations with platelets and LTA_4, while LXB_4 was found in a ratio of ~ 2:1 (LXA_4/LXB_4) in profiles obtained from coincubations (Table I). In addition, platelets incubated with LTA_4 also generated 7-*cis*-11-*trans*-LXA_4 (Figs. 2 and 4). These observations suggest that the formation of LXB_4 during PMN-platelet interactions may involve additional biosynthetic routes or that regulatory interactions between 12-LO and LTA_4 required for LXB_4 formation are disrupted in the cell-free system. Several routes of LXB_4 formation have been documented (8, 10, 11). Whether they play a role during PMN-platelet interactions or whether platelets simply serve as an additional source of AA for utilization by PMNs (14) remains to be determined.

Ca^{2+} plays an important role in the activation of the 5-LO in leukocytes (37). In contrast, Ca^{2+} chelation with EGTA does not completely inhibit the conversion of AA by 12-LO prepared from subcellular fractions of human platelets (29). In the present study, addition of EGTA (5 mM) to supernatants (100,000 *g*) only partially reduced the formation of 12-HETE (reduced by ~ 26%). The formation of LXA_4 as well as the all-*trans*-LX isomers was inhibited 70–80% in the presence of

EGTA (Table III), while its effect on 7-*cis*-11-*trans*-LXA_4 formation was not statistically significant. Since the orientation of substrates within the active site of LO plays a critical role in the chirality and the ratio of the products generated (38), these observations with EGTA treatment suggest a role for divalent cations in both the orientation and transformation of LTA_4 to lipoxins. In addition, both thrombin and ionophore enhanced the transformation of LTA_4 by platelets (Table II).

The human platelet LO stereoselectively abstracts hydrogen from carbon-10 of AA, which initiates an insertion of molecular oxygen at carbon-12 to form 12-HPETE (4). Since an alcohol group at carbon-15 is present in LXA_4 (6, 7), it is possible that the platelet 12-LO initiates lipoxin formation from LTA_4 by abstraction of hydrogen at carbon-13 of LTA_4 and thus displays its ω-6 oxygenase activity with LTA_4 as substrate. Indeed, results with purified 12-LO from porcine leukocytes indicate that this enzyme can catalyze oxygenations at either ω-6 or ω-9 positions depending upon the substrate (39). It is also likely that the opening of the 5(6)-epoxide intermediate to give a 5S,6R configuration as in LXA_4 is directed by the enzyme, since the 6S-isomer of LXA_4 (6S-LXA_4) was not formed in these incubations. The all-*trans* isomers of LXA_4 and LXB_4 can be formed upon isolation of lipoxins and by non-enzymatic hydrolysis of the 5(6)-epoxytetraene intermediate (7, 8). Their appearance here can, in part, reflect the formation of a delocalized cation intermediate after oxygenation of LTA_4 by the 12-LO. Formation of a similar structure (i.e., an enzyme-bound delocalized cation generated by proton transfer) has been proposed in the biosynthesis of LXA_4 and LXB_4 from a common intermediate (40). However, the detailed mechanism as well as the role of cofactors involved in the transformation of LTA_4 by platelets awaits further studies with purified human 12-LO.

The finding that platelets can generate biologically relevant levels of these products during coincubation with PMNs is of interest in view of their bioactions. For example, at submicromolar concentrations, lipoxins stimulate endothelial-dependent relaxation of aortic strips (41). LXA_4 also provokes vasodilation (in vivo) (42–44) and stimulates the formation of prostacyclin by human endothelial cells, albeit at higher concentrations (45). In addition to extracellular roles, LXA_4 may also serve an intracellular function since it activates isolated protein kinase C (46) and selectively stimulates its γ-subspecies (47).

Counterregulatory actions have been observed with lipoxins. In the nanomolar range, LXA_4 can antagonize both the cellular and in vivo actions of leukotriene D_4 in renal microcirculation (48). Prior exposure of glomerular mesangial cells to LXA_4 blocks both the binding of leukotriene D_4 to its receptor and the generation of inositol-triphosphate in mesangial cells which can produce LXA_4 (48, 55). Agonist-antagonist responses are also observed with human leukocytes (49, 50). LXA_4 promotes chemotaxis of granulocytes (10^{-9} M), while recent results show that prior exposure of PMNs to LXA_4 (10^{-10} M) inhibits the chemotactic response to either LTB_4 or FMLP. In these studies, LXA_4 also blocked both PI hydrolysis and calcium mobilization in human PMNs (50).

Although platelets can generate lipoxins (Figs. 1–4), these products do not stimulate platelet aggregation (41). Their role(s), if any, in platelet function remains to be determined. In summary, the present results demonstrate that incubation of PMNs and platelets with FMLP and thrombin leads to the formation of lipoxins by receptor-mediated mechanisms. Although FMLP acts primarily on PMNs, the actions of thrombin are not exclusively directed toward platelets. Thrombin is also reported to induce chemotaxis, aggregation (51) and degranulation (52) with human PMNs. In addition, thrombin-stimulated platelets adhere to PMNs (53) and activated platelets can enhance the functional responses of PMNs (54). Thus, the present results add to the complex repertoire of events that occurs during PMN-platelet interactions and they also provide evidence for a novel biosynthetic route for the formation of lipoxins, namely the conversion of LTA_4 by the platelet 12-LO. Moreover, they suggest that lipoxins can also be formed after initial oxygenation by the 5-LO and transcellular metabolism. Since lipoxins have been found to possess vasoactive functions (41–45), their formation during the interaction of PMNs with platelets may have implications in the regulation of hemostasis, inflammation, and the interactions of these cells with the vessel wall.

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