Lipoxin Generation by Permeabilized Human Platelets†

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ABSTRACT: Human platelets convert leukocyte-derived leukotriene (LT) A_4 to lipoxins during transcellular lipoxin biosynthesis. Here, we examined lipoxin generation in intact human platelets and compared it with that elicited from permeabilized platelets. Conversion of LTA_4 to lipoxins by permeabilized cells exceeded (10–15 times) that to peptidoleukotrienes, while intact cells exposed to thrombin generated similar amounts of these two series (LT/LX). Permeabilized platelets also generated 3–5 times more lipoxins than intact cells. Lipoxin A_4 (LXA_4), lipoxin B_4 (LXB_4), and their respective all-trans isomers were identified by physical methods including HPLC and GC–MS. Chiral analysis of platelet-derived all-trans-containing LXs revealed that >69.5 ± 0.5% carried alcohol groups in the *R* configuration at carbons 6 and 14 (e.g., 11-*trans*-LXA_4 and 8-*trans*-LXB_4), respectively. More than 50% of these all-trans LX were formed by isomerization of native LXA_4 and LXB_4 during isolation. Lipoxin formation with permeabilized platelets gave an apparent K_m of 8.9 μM and V_{max} of 83.3 ng/(min·10^9 platelets) with maximal conversion in pH range 7–9. In addition, permeabilized platelets converted 14,15-LTA_4 and LTA_5, but not LTA_3, to lipoxins. Consecutive exposure to LTA_4 did not alter LXA_4 generation but inhibited LXB_4 by 40–50%, suggesting that LXB_4 formation can be regulated by suicide inactivation. Unlike platelets, human endothelial cells did not convert LTA_4 to lipoxins. These results indicate that lipoxin formation is a major route of LTA_4 metabolism in thrombin-activated platelets and those that have undergone a loss of membrane barriers. Moreover, they provide evidence for a novel pathway involving conversion of 14,15-LTA_4 and regulatory events in platelet lipoxin generation that may be relevant in vascular physiology.

Lipoxins are a distinct series of lipoxygenase (LO)[1] products within the eicosanoid family that contain a conjugated tetraene structure (Serhan et al., 1984). They possess potent bioactions and are generated via the interaction of individual human LOs [for reviews, see Samuelsson et al. (1987) and Serhan (1991)]. Lipoxin A_4 (Serhan et al., 1986b) and lipoxin B_4 (Serhan et al., 1986a) are the two main bioactive products of this series that have a stereoselective impact in several tissues and cell types (Serhan, 1991). Lipoxins can be generated by single cell types from endogenous sources of arachidonic acid (Pettitt et al., 1991) or by cell–cell interactions (Marcus, 1990) that initiate transcellular LX-biosynthetic pathways (Serhan, 1991). Multiple biosynthetic routes can lead to lipoxins in human cell types that involve initial lipoxygenation of arachidonic acid at either carbon 15 or carbon 5 with subsequent conversion to a 5(6)-epoxytetraene-containing intermediate or its equivalent. For example, one route involves conversion of 15-HETE by leukocytes (Serhan et al., 1986a,b), and another utilizes LTA_4, a leukocyte 5-LO product, for conversion to lipoxins by human platelet 12-LO (Serhan & Sheppard, 1990; Fiore & Serhan, 1990; Edenius et al., 1991) or human epithelial 15-LO (Edenius et al., 1990). Thus, in addition to the extracellular release of LTA_4 (Dahinden et al., 1985) for amplification of leukotriene biosynthesis by a second cell type [reviewed in Marcus (1990)], LTA_4 may also represent a pivotal intermediate for lipoxin production in human tissues.

Thrombosis, inflammation, and atherosclerosis are multicellular processes where platelets play an important role and appear to be an active milieu for transcellular biosynthesis of eicosanoids (Marcus, 1990; Hajjar et al., 1989). The platelet is not considered an inflammatory cell per se but plays a role in inflammation via its participation in transcellular metabolism (Marcus, 1990). Time lapse ultrastructural results with thrombosis in humans triggered by vascular injury show formation of platelet aggregates within seconds (Wester et al., 1979). By 10 min, these aggregates become larger and some platelets in the aggregates can express various levels of degranulation, swelling, and loss of plasma membrane integrity. Neutrophils appear and can adhere to these morphologically altered platelets (Wester et al., 1979). The viable

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[1] Abbreviations: ASA, acetylsalicylic acid; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; GC–MS, gas chromatography–mass spectrometry; 5-HETE, (5*S*)-hydroxy-8,11,14-*cis*-6-*trans*-eicosatetraenoic acid; 12-HETE, (12*S*)-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid; 15-HETE, (15*S*)-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid; LO, lipoxygenase; LT, leukotriene; LTA_3, leukotriene A_3, (5*S*)-*trans*-5,6-oxido-7,9-*trans*-11-*cis*-eicosatrienoic acid; LTA_4, leukotriene A_4, (5*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; 14,15-LTA_4, (14*S*)-*trans*-14,15-oxido-5,8-*trans*-10,12-*cis*-eicosatetraenoic acid; LTB_4, leukotriene B_4, (5*S*,12*R*)-dihydroxy-6,14-*cis*-8,11-*trans*-eicosatetraenoic acid; LTC_4, leukotriene C_4, (5*S*)-hydroxy-(6*R*)-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD_4, leukotriene D_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycine-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LXA_4, lipoxin A_4, (5*S*,6*R*,15*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 6*S*-11-*trans*-LXA_4, (5*S*,6*S*,15*S*)-trihydroxy-7,9,11,13-*trans*-eicosatetraenoic acid; 11-*trans*-LXA_4, (5*S*,6*R*,15*S*)-trihydroxy-7,9,11,13-*trans*-eicosatetraenoic acid; LXB_4, lipoxin B_4, (5*S*,14*R*,15*S*)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid; 8-*trans*-LXB_4, (5*S*,14*R*,15*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid; 14*S*-8-*trans*-LXB_4, (5*S*,14*S*,15*S*)-trihydroxy-6,8,10,12-*trans*-eicosatetraenoic acid; PBS, phosphate-buffered saline; PGB_2, prostaglandin B_2; RP-HPLC, reverse-phase HPLC; SP-HPLC, straight-phase HPLC.

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platelets then take part in clot retraction (Freiman, 1987; Zucker-Franklin, 1988). Although direct evidence is limited showing that platelets can lose membrane integrity during formation of a thrombus, Jørgensen et al. (1967) observed that, around the periphery of platelet aggregates in vivo, the platelets in some instances have gaps in their plasma membranes. Also, platelets in contact with collagen after injury to carotid arteries frequently show gaps in their membranes (Jørgensen et al., 1967). Along these lines, we have recently found that rupture of atheromatous plaques induced by percutaneous transluminal angioplasty in vivo triggers the intracoronary appearance of lipoxins and leukotrienes in humans (Brezinski et al., 1992). Since the peptidoleukotrienes, LTC_4 and LTD_4, are potent vasoconstrictors (Dahlén, 1989), their appearance may be linked to angioplasty-associated sequelae.

Although the role of lipoxins in the human vascular system is still being appreciated, it is known that LXA_4 induces arteriolar dilation in the hamster cheek pouch in vivo and that both LXA_4 and LXB_4 induce relaxation of guinea pig aorta and human pulmonary artery that were precontracted with either prostaglandin $F_{2\alpha}$ or endothelin (Dahlén, 1989). Lipoxins A_4 and B_4 dilate cerebral arterioles in pigs (Busija et al., 1989) and evoke an endothelium-dependent vasorelaxation in guinea pig, rat, and rabbit aortic smooth muscle (Lefer et al., 1988). LXA_4 also antagonizes the in vivo actions of LTD_4 in rats (Badr et al., 1989) and stimulates both human (Brezinski et al., 1989) and rat (Leszcynski & Ustinov, 1990) endothelial cells to generate prostacyclin. Chemotaxis of human neutrophils is inhibited by prior exposure to LXA_4 (Lee et al., 1991). Since these responses are elicited in the subnanomolar to micromolar range, it appears that lipoxin formation may be relevant at sites of vascular damage and in the further development of vascular lesions.

Lipoxin formation by human platelets is not inhibited by aspirin and involves conversion of leukotriene A_4 by 12-LO (Serhan & Sheppard, 1990). Recent results indicate that human megakaryocytes expressing 12-LO activity and COS cells transfected with human 12-LO can convert LTA_4 to lipoxins (Sheppard et al., 1992). Also, platelets from patients with myeloproliferative disorders lacking 12-LO activity are unable to produce lipoxins during blastic crisis (Stenke et al., 1991). Platelets involved in thrombotic, inflammatory, or atherosclerotic events can be activated; therefore, we examined lipoxin and leukotriene formation by permeabilized platelets. Here, we report that relieving the platelet membrane barrier results in a high level of LTA_4 conversion to lipoxins and characterized some regulatory events in their generation. In addition, evidence for a novel route of lipoxin biosynthesis is presented that involves enzymatic conversion of 14,15-LTA_4 to lipoxins by platelets.

MATERIALS AND METHODS

Acetylsalicylic acid (ASA), saponin, and LDH kit procedure No. 500 were purchased from Sigma (St. Louis, MO), and thrombin was from Enzyme Research Laboratories, Inc. (South Bend, IN). HPLC grade solvents were purchased from Doe and Ingalls (Medford, MA). Sep-Pak C_{18} cartridges were from Waters Associates (Milford, MA). Diazomethane was prepared from *N*-methyl-*N*-nitroguanidine purchased from Aldrich Chemical Co. (Milwaukee, WI). Arachidonic acid was obtained from NuCheck Prep (Elysian, MN). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce (Rockford, IL). Synthetic LTA_4 methyl ester, 14,15-LTA_4 methyl ester, LXA_4, 11-*trans*-LXA_4, LXB_4, 8-*trans*-LXB_4, and 12-*S*-HETE were purchased from Biomol Research Laboratories (Philadelphia, PA). LTA_3 methyl ester and LTA_5 methyl ester were from Cascade Biochem Limited (Reading, Berkshire, England). Saponification of LTA_4 methyl ester, LTA_3 methyl ester, and 14,15-LTA_4 methyl ester was performed in tetrahydrofuran under an atmosphere of argon with LiOH (0.1 mM) (4 °C for 24 h). To ensure that epoxides were intact, immediately before each experiment the ultraviolet spectra of LTA_3, LTA_4, and 14,15-LTA_4 were each examined in MeOH, and their spectral shift (i.e., 280–270 nm obtained) was determined upon addition of dilute HCl as in Rådmark et al. (1984). Iloprost was a gift from Dr. Schillinger, Schering (Berlin, Germany).

Preparation of Human Platelets. Venous blood was collected from healthy donors who denied taking medication for at least 10 days. Platelet-rich plasma was obtained by mixing blood with acid citrate dextrose (Romano & Hawiger, 1990) followed by centrifugation (250*g* for 15 min at room temperature). Platelets were isolated by centrifugation of the platelet-rich plasma (1100*g* for 15 min at room temperature) in the presence of EDTA (7 mM). Cells were washed twice with HEPES–Tyrode buffer (pH 7.4) in the presence of EDTA (7 mM) and suspended in this buffer containing 0.1% fatty acid free human albumin and $CaCl_2$ (1 mM), used throughout unless otherwise indicated. Platelets were enumerated using a Coulter counter (model ZF; Coulter Electronics, Inc., Hialeah, FL). The presence of contaminating cell types was assessed by light microscopy (leukocytes represented <1 in (2–4) × 10^4 platelets). Platelets were routinely permeabilized using rapid freezing in a dry ice–acetone bath, thawed to room temperature (full cycle ~30 min), and taken immediately for incubations and analysis by scanning electron microscopy (see Results). LDH release did not exceed 1.6 ± 0.3% (SEM) with intact cells incubated with thrombin (20 min, 37 °C) and was 21.6 ± 0.9% (SEM) in the freeze–thaw permeabilized cell incubations (20 min, 37 °C). These values are expressed as percent of total cellular LDH after treatment with Triton X-100 (final 2% v/v). Platelet homogenates in Table I were prepared as described by Marcus et al. (1966).

Incubation Conditions. Washed intact platelets [(0.1–10) × 10^9 cells/mL] in HEPES–Tyrode buffer (0.5 mL, with 0.1% albumin) were placed at 37 °C (5 min) and incubated (20 min at 37 °C) in the presence of thrombin (1 unit/mL) with either LTA_4 (0.3–50 μM), LTA_3 (20 μM), 14,15-LTA_4 (20 μM), arachidonic acid (0.7–20 μM), or vehicle. Permeabilized platelets from the same donors were incubated in parallel with each eicosanoid or vehicle alone. For suicide inactivation experiments, permeabilized platelets (10^8/0.5 mL buffer) were exposed successively to LTA_4 (50 μM) at 20 min intervals at 37 °C. Coincubations with endothelial cells were performed by adding platelets (5 × 10^8) in buffer (2 mL) to gelatin-coated 100-mm plastic Petri dishes (polystyrene; Nunclon) containing confluent human umbilical vein endothelial cells (Brezinski et al., 1989) which had been washed twice with PBS and treated with indomethacin (100 μM), to block prostacyclin formation, for 20 min at 37 °C in atmosphere supplemented with 5% CO_2. After 5 min, thrombin (1 unit/mL) was added to stimulate platelet aggregation for 3 min followed by addition of LTA_4 (13 μM) for 20 min in a 37 °C incubator with 5% CO_2.

Analysis of Eicosanoids. Incubations were terminated by addition of cold methanol (2 vol) containing either PGB_2 or 13-HOD (as an internal standard). Samples were extracted and eluted from Sep-Pak C_{18} cartridges [Waters Associates,

Milford, MA, as in Fiore and Serhan (1990)]. Methyl formate fractions were concentrated with a stream of N_2, scanned for ultraviolet-adsorbing materials (all spectra were recorded in MeOH) with a model 8452 spectrophotometer (Hewlett-Packard Co., Palo Alto, CA), and injected into RP-HPLC consisting of a model 484 UV detector (Waters) and a pump model 501 (Waters) with a Beckman Ultrasphere-ODS (4.5 cm × 25 cm) column. The column was eluted with MeOH/H_2O/acetic acid (65:35:0.01; v/v/v) at the flow rate of 1 mL/min (2×10^3 psi), and the UV detector was set at 300 nm to monitor conjugated tetraenes or 270 nm for trienes. Peptidoleukotrienes (LTC_4, LTD_4, LTE_4) eluted in the MeOH fractions (from Sep-Pak cartridges) were resolved by RP-HPLC. This system consisted of an LC-75 UV detector (Perkin-Elmer, Norwalk, CT) and a pump model 110A (Beckman Instruments, Berkeley, CA) equipped with an Altex Ultrasphere-ODS eluted with MeOH/H_2O/acetic acid (65:35:0.01; v/v/v), pH 5.7, at 1 mL/min (1×10^3 psi), and the UV monitor was set at 280 nm. For 12-HETE determinations, 13-HOD (80 ng) was prepared and used as internal standard. The monohydroxy products were resolved using the same equipment with an isocratic mobile phase MeOH/H_2O/acetic acid (75:25:0.01; v/v/v), and the UV detector was set at 235 nm. Chiral analyses were performed with a Bakerbond chiral column [see Kühn et al. (1987)] (4.6 mm × 25 cm, 5-μM particle size) eluted with *n*-hexane/*n*-propanol (91.5:8.5; v/v) at 1.2 ml/min.

GC–MS Analysis. Materials collected after HPLC were treated with diazomethane (20 min, RT) followed by exposure to BSTFA (~12 h, RT in the dark). Analyses were performed with a Hewlett-Packard 5890 gas chromatograph series II with a Hewlett-Packard 5971A mass-selective detector quadrupole equipped with an MS chemstation (Hewlett-Packard 1030A). The instrument was set to collect and store data at 1.6 scans/s. The column was a HP Ultra 2 (25 m × 0.2 mm × 0.33 μm), and all injections were made in the splitless mode with BSTFA (2 μL) as solvent. The GC temperature program was initiated at 150 °C and reached 250 °C at 10 min and 325 °C at 20 min.

After RP-HPLC and GC–MS, products were routinely identified and quantified by comparison of their retention times and peak areas with calibrations obtained using synthetic standards. In suicide inactivation experiments, the amounts of lipoxins formed during the second incubation were calculated by subtracting the quantities formed during the initial incubation with LTA_4 (50 μM; 20 min, 37 °C) from the total amount of lipoxins obtained following two consecutive incubations.

RESULTS

Conversion of LTA_4: Comparison between Permeabilized and Intact Platelets. To determine whether disruption of the plasma membrane barrier altered either lipoxin or peptidoleukotriene formation, platelets were permeabilized and exposed to LTA_4. Permeabilized platelets converted up to 58.3 ± 1.8% ($n = 3$) of LTA_4 to tetraene chromophores (Figure 1A). In parallel incubations, intact platelets exposed to thrombin and LTA_4 generated lower levels of tetraene chromophores corresponding to 20.4 ± 1.2% ($n = 3$) of the added LTA_4 (Figure 1B). It is important to point out that the fresh platelet suspensions used in these experiments were not preemptively activated during isolation procedures as monitored by the absence of platelet aggregates in the incubations. RP-HPLC analysis of materials generated with permeabilized and thrombin-activated platelets gave qualitatively

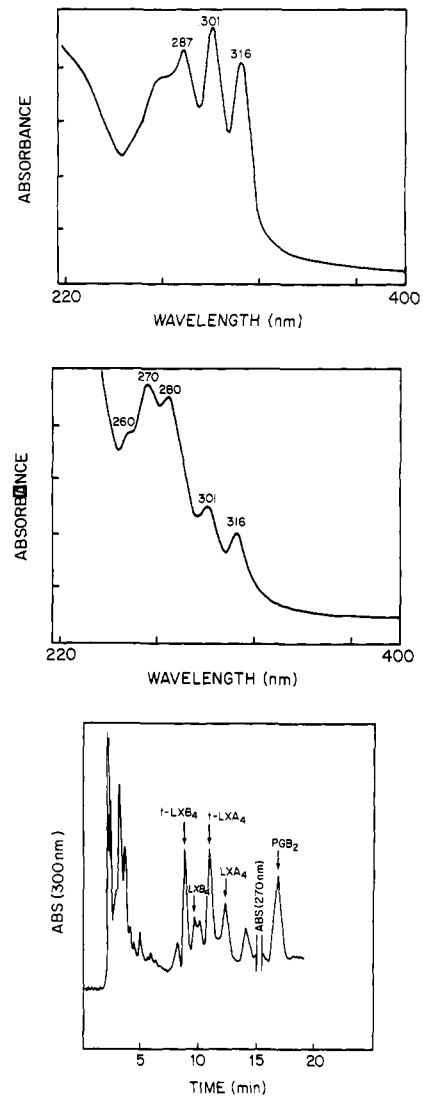

FIGURE 1: Products obtained from permeabilized (A, top) and intact (B, middle) platelets. Platelets (10^9 cells/0.5 mL of buffer) were permeabilized and incubated with LTA_4 (20 μM) or exposed simultaneously to thrombin (1 unit/mL) and LTA_4 (20 μM) for 20 min at 37 °C. After extraction (see Materials and Methods), UV spectra of products eluted in the methyl formate fractions were recorded in MeOH: (A, top) freeze–thaw permeabilized; (B, middle) intact. These materials were injected into RP-HPLC and eluted with MeOH/H_2O/acetic acid (65:35:0.01, v/v/v). (C, bottom) Representative chromatogram (arrows indicate retention times of authentic eicosanoids). Results are from $n = 11$ separate donors with 75 determinations.

similar profiles of products that eluted with the retention times of authentic LXA_4, LXB_4, and their all-trans isomers, respectively (Figure 1C). A comparison among platelet preparations for lipoxin generation is given in Table I. Platelets obtained from the same donors permeabilized by either saponin treatment or freeze–thaw generated similar amounts of lipoxins. Platelet suspensions disrupted by either sonication or homogenization without intact cells present also converted LTA_4 to lipoxins, albeit to slightly lower levels. The freeze–thawed platelets, unless otherwise indicated, were used throughout because it provided a rapid and simple method of introducing LTA_4 to permeabilized platelets.

In contrast to their enhanced ability to produce lipoxins, permeabilized platelets generated only low amounts (10–15 times less) of the peptidoleukotrienes (Figure 2A). Scanning electron microscopy with these preparations showed that permeabilized cells possessed ~0.03–0.18-μm gaps in their

Table I: Lipoxin Generation: Comparison among Platelet Preparations[a]

platelet incubation	% conversion to tetraenes	LXB_4 (ng)	LXA_4 (ng)
freeze–thaw	19.6	101.6	114.8
saponin	22.0	132.7	122.0
sonicate	18.3	93.4	100.8
homogenate	15.9	73.1	76.9

[a] Platelets (2.5×10^8) suspended in HEPES–Tyrode buffer containing human albumin (0.1%) were subjected to freeze–thaw (one cycle), treated with saponin (20 μg/mL, 3 min), sonicated (two cycles, 15 s), or homogenated as in Marcus et al. (1966). Each suspension was incubated with LTA_4 (20 μM) for 20 min at 37 °C. PGB_2 was used as internal standard, and products were extracted and quantitated following RP-HPLC. Values are expressed in ng/incubation and corrected for isomerization to *all-trans*-LX (see Materials and Methods). Results are representative of three separate experiments.

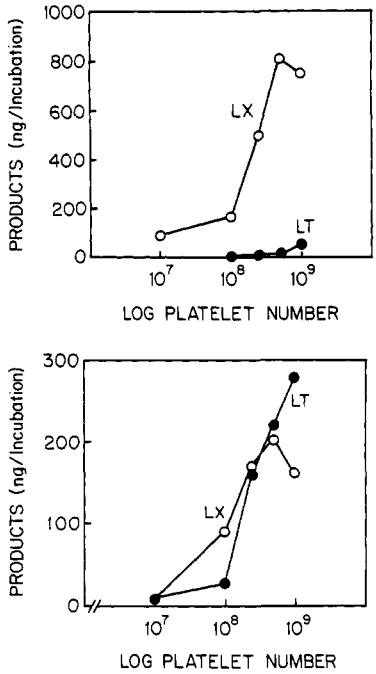

FIGURE 2: Relationship between lipoxins and peptidoleukotrienes. Comparison between permeabilized platelets (A, top) or intact cells exposed to thrombin (1 unit/mL) and (B, bottom) incubated (20 min, 37 °C) with LTA_4 (20 μM). Products were extracted and quantitated after RP-HPLC as described under Materials and Methods. LX (○) represents the sum of LXA_4, LXB_4, and their all-trans isomers, and LT (●) is the sum of LTC_4, LTD_4, and LTE_4. Results are representative from two separate donors.

membranes (not shown) and were not completely lysed. This was also evidenced by an LDH release value of 21.6 ± 0.9% taken following 20-min, 37 °C incubations, which is less than that of complete platelet lysates (100%). Intact platelets exposed to thrombin generated lipoxins and peptidoleukotrienes in comparable amounts (Figure 2B). In addition, washed platelet aggregates exposed to LTA_4 (e.g., 40, 60, or 80 min after the exposure to thrombin, 0.1 unit/mL) retained their ability to generate lipoxins, in particular, LXA_4 (results not shown). Together these findings indicate that perturbation of platelet membranes results in a substantial increase in the conversion of LTA_4 to lipoxins.

LXB_4 Generation by Platelets. Among the products formed by intact platelets incubated with LTA_4, LXA_4 has been identified by physical methods including GC–electron impact MS (Serhan & Sheppard, 1990), and both LXA_4 and LXB_4 have been identified by ED-UV coupled RP-HPLC and negative ion electron capture GC–MS (Fiore & Serhan, 1990). LXB_4 formation has not been observed in some platelet

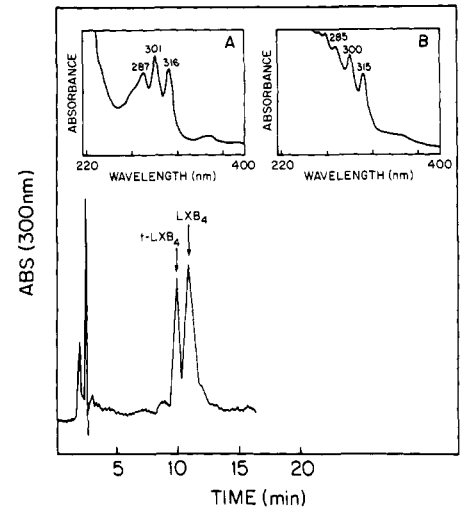

FIGURE 3: LXB_4 from permeabilized platelets. Materials from four separate incubations with permeabilized platelets (5×10^9) and LTA_4 (50 μM) were isolated and subject to RP-HPLC as in Figure 1C. Materials were collected and rechromatographed with MeOH/H_2O/acetic acid (65:35:0.01%, v/v/v). Arrows denote retention times of authentic 8-*trans*-LXB_4 (denoted as t-LXB_4) and LXB_4. Inserts show UV spectra of material beneath the peak with retention time of *all-trans*-LXB_4 (A) and LXB_4 (B) (recorded in MeOH).

preparations of others where LXA_4 is generated, and hence LXB_4 has been suggested to be a lipoxin solely derived from leukocytes (Edenius et al., 1991). Since LXB_4 carries biological actions in some cases different from those of LXA_4 (Dahlén, 1989; Lee et al., 1991), we took advantage of the considerable amounts of lipoxins generated by permeabilized platelets for further identification. To this end, materials formed during several incubations with permeabilized platelets and eluted beneath the peak with the retention times of synthetic lipoxins including LXB_4 were collected after RP-HPLC. These platelet suspensions were essentially devoid of leukocytes carrying 5-lipoxygenase as evidenced by the absence of 5-HETE generation upon incubation with arachidonic acid (20 μM, 20 min, 37 °C); 12-HETE was the major product in these incubations ($n = 2$). The collected materials from beneath the LXB_4 peak gave two peaks with the same retention times after second chromatography as LXB_4 and 8-*trans*-LXB_4, respectively (Figure 3). The UV spectrum of this material gave λ_{max}^{MeOH} at 300 nm (Figure 3, insert B), consistent with that of LXB_4, and the peak eluting with the retention time of *all-trans*-LXB_4 (as in Figure 1C) gave a λ_{max}^{MeOH} of 301 nm (Figure 3, insert A) (Serhan et al., 1986a). GC–MS analysis of the methyl ester, trimethylsilyl derivative showed a *C* value of 24 with prominent ions at m/z 173 (base peak) and 203 and weaker ions at m/z 582 (M), 492 (M-90), 482 (M-100), 275, and 171. The platelet-derived material coeluting with *all-trans*-LXB_4 gave two products in the GC profile, one with a *C* value of 28.3 with prominent ions at m/z 173 (base peak) and 379 and weaker ions at 492, 482, 409, 402, 329, 319, and 307 and the other at C28.0 with virtually identical prominent ions in its spectrum. These results are consistent with those documented (Serhan et al., 1986a) for LXB_4 (*C* value 24.0), 8-trans-LXB_4 (*C* value 28.3), and 14*S*-8-*trans*-LXB_4 (*C* value 28.0).

Chiral Analysis of All-trans-Containing Lipoxins. The relative amounts of individual *all-trans*-lipoxin isomers generated by platelets have not been defined. Previous studies have demonstrated that the *all-trans*-lipoxins isolated from leukocytes represent four major products, namely, 11-*trans*-LXA_4, 6*S*-11-*trans*-LXA_4, 8-*trans*-LXB_4, and 14*S*-8-*trans*-LXB_4 which elute beneath two peaks in most RP-HPLC

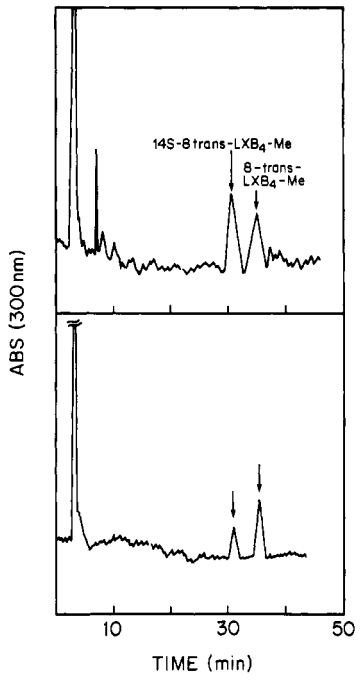

FIGURE 4: Separation of 8-*trans*-LXB_4 and 14*S*-8-*trans*-LXB_4. The region containing *all-trans*-LXB_4 was collected after RP-HPLC (as in Figure 1C) and treated with diazomethane. Products were suspended in hexane/isopropanol (50:50, v/v) and injected into a Bakerbond chiral column eluted with hexane/isopropanol (91.5:8.5, v/v), at a flow rate of 1.2 mL/min. (Upper panel) Authentic standards. (Lower panel) Platelet-derived products.

systems used to resolve lipoxins (Serhan et al., 1986a,b; Nicolaou et al., 1989; Serhan & Sheppard, 1990). To gain further insight into the amounts generated that may reflect biosynthetic routes, materials obtained following incubation of permeabilized platelets with LTA_4 were subjected to chiral analysis. Products from several incubations (as in Figure 1C) were pooled, treated with diazomethane, and chromatographed by RP-HPLC. All-trans-containing LXA_4 and LXB_4 isomers, chromatographed as their corresponding methyl esters, were collected following RP-HPLC (as in Figure 1C) and subjected to chiral column SP-HPLC. The *all-trans*-LXB_4 isomers that eluted beneath a single peak in RP-HPLC were resolved into two components eluting with the retention times of synthetic 8-*trans*-LXB_4-Me and 14*S*-8-*trans*-LXB_4-Me, respectively (Figure 4). 8-*trans*-LXB_4-Me represented 69.5 ± 0.5% of the *all-trans*-LXB_4-Me. The *all-trans*-LXA_4 isomers, 11-*trans*-LXA_4 and 6*S*-11-*trans*-LXA_4-Me, were also resolved in this system. 11-*trans*-LXA_4-Me constituted 75.5 ± 0.8% of the total *trans*-LXA_4-Me. These results indicate that the majority of lipoxins generated by platelets eluting beneath the all-trans fractions in RP-HPLC profiles are in the *R* configuration.

Platelet Isomerization of Lipoxins. Both LXA_4 and LXB_4 undergo isomerization to their all-trans counterparts during workup (Serhan et al., 1986b). To distinguish the percentage of the trans isomers originating from platelet biosynthesis and that formed from the native compounds during workup, LXA_4 and B_4 were added to platelet suspensions and the extent of isomerization was determined before and after incubation and extraction. Results in Table II indicate that >50% of LXB_4 and LXA_4 each isomerizes upon incubation and extraction. Since similar results were obtained when lipoxins were incubated with either buffer alone or heat-denatured (boiled, 100 °C, 60 min) platelets, it was concluded that isomerization of both LXA_4 and LXB_4 to their corresponding all-trans isomers was not likely to be an enzymatic event carried out by these cells. Thus, for quantitation of LXA_4 and LXB_4 generation in these experiments (*in vitro*), workup-induced isomerization should be taken into account. This finding was used throughout to assess the contribution of isomerization to the amounts of LXA_4 and B_4 formed by including control incubations to determine isomerization during extraction.

Kinetics of Lipoxin and 12-HETE Formation. We next evaluated the K_m and V_{max} for lipoxin production compared to 12-HETE generation using ASA-treated (0.5 mM, 20 min) permeabilized cells. A Lineweaver–Burk plot of lipoxin formation (after RP-HPLC) showed an apparent K_m of 8.9 μM and a V_{max} of 83.3 ng/(min·10^9 platelets). 12-HETE generation gave a K_m of 2.1 μM with a V_{max} of 376 ng/(min·10^9 platelets) (Figure 5), a K_m value in accordance with that previously reported (Lagarde et al., 1984). Stimulus-induced changes in intracellular pH play an important role in platelet activation (Siffert et al., 1987) and can alter 12-HETE generation (Lagarde et al., 1984). Therefore, we examined the pH dependence of lipoxin formation with LTA_4 and permeabilized platelets. Lipoxin formation was maximal in the pH range 7–9. In addition, lipoxin production by intact platelets was increased ~40% by raising the intracellular pH with the Na^+ ionophore monensin (10 μM, 1 min before exposure to LTA_4, 37 °C) (not shown).

Conversion of LTA Isomers to Lipoxins. To address the requirements in substrate specificity, leukotriene A_3 (LTA_3), 14,15-LTA_4, and LTA_4 were each incubated in parallel with permeabilized platelets (Table III). LTA_3, known to inhibit conversion of LTA_4 to leukotriene B_4 (LTB_4) (Evans et al., 1985), was neither converted (Table III) nor inhibited conversion of LTA_4 to lipoxins after prior exposure (5 min, 37 °C, $n = 2$, not shown). Of interest, conversion was observed with 14,15-LTA_4. RP-HPLC of the products revealed that, with 14,15-LTA_4, LXB_4 and its isomers were predominant although formation of LXA_4 and its isomers was observed in lesser amounts (Figure 6, Table III). Conversion of 14,15-LTA_4 to lipoxins was abolished by treatment of platelets with esculetin (a lipoxygenase inhibitor) (Sekiya et al., 1982). In contrast, treatment of platelets with ASA did not alter their capacity to transform 14,15-LTA_4 ($n = 2$). These results indicate that lipoxygenation at carbon 5 position of 14,15-LTA_4 (required to form lipoxins) is carried out by platelets and provide evidence that this is yet another biosynthetic route that can lead to lipoxin formation.

Suicide Inactivation. Suicide inactivation is a regulatory mechanism for several enzymes in the arachidonic acid cascade [reviewed by Walsh (1984)]. When permeabilized platelets were challenged with two subsequent additions of LTA_4 (50 μM), LXB_4 production but not LXA_4 was reduced 40–50% (Figure 7), indicating that LXA_4 formation is not subject to suicide inactivation and suggesting that the generation of LXB_4 by platelets can be regulated by this mechanism.

Endothelial Cell/Platelet Coincubations. To determine whether endothelial cells can alter lipoxin production, we examined their formation during coincubation of platelets with indomethacin-treated endothelial cells. Here, platelets maintained their ability to generate lipoxins. Also, endothelial cells did not generate lipoxins when exposed to LTA_4 (Table IV). Iloprost, a prostaglandin I_2 stable analog, treatment of platelets reduced lipoxin formation ~40–50% with intact cells exposed to thrombin and LTA_4.

DISCUSSION

Thrombosis and microemboli occurring as a consequence of vascular injury are characterized by initial formation of

Table II: Isolation-Induced Generation of 11-*trans*-LXA_4 and 8-*trans*-LXB_4[a]

conditions or incubations	LXB_4	8-*trans*-LXB_4	LXA_4	11-*trans*-LXA_4
LXA_4 + LXB_4 (direct injection)[b]	95.1 ± 1.9	4.9 ± 1.9	84.5 ± 1.6	15.1 ± 1.6
LXA_4 + LXB_4 + platelets	51.7 ± 0.1	48.6 ± 0.2	36.9 ± 1.8	62.1 ± 3.3
LXA_4 + LXB_4 + platelets (heat-denatured)[c]	49.7 ± 1.4	50.2 ± 1.4	35.8 ± 0.8	64.1 ± 0.8
LXA_4 + buffer			36.9 ± 3.6	63.0 ± 0.1
LXB_4 + buffer	48.5 ± 0.1	51.5 ± 0.1		

[a] LXA_4 (100 ng) and LXB_4 (100 ng) were added to either platelets (10^9), heat-denatured[c] platelets (10^9, 100 °C, 60 min), or buffer (0.5 mL) and incubated for 20 min at 37 °C. After extraction, materials were analyzed by RP-HPLC as in Figure 1C. Results are expressed as the percent of each individual compound present bef ore[b] (values obtained following direction injection) and after incubation and extraction. Data represent the mean ± SEM of three separate experiments.

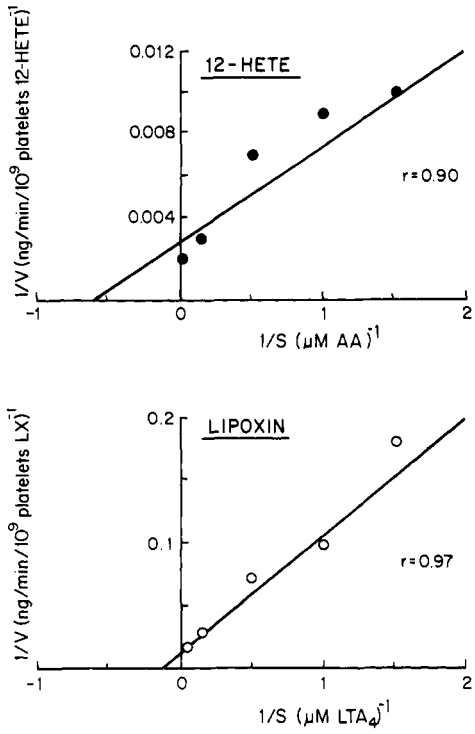

FIGURE 5: Lineweaver–Burk plot of lipoxin and 12-HETE formation. Permeabilized platelets (2.5×10^8) were incubated (20 min, 37 °C) with either arachidonic acid (upper) or LTA_4 (lower). Materials were extracted and quantitated following RP-HPLC.

Table III: Lipoxin Formation: Comparison with LTA_3, LTA_4, and 14,15-LTA_4[a]

incubation	% conversion to tetraene	LXB_4	LXA_4
platelets + LTA_3	ND	ND	ND
platelets + LTA_4	35.6 ± 2.1	75 ± 1.2	93.4 ± 9.3
platelets + 14,15-LTA_4	13.2 ± 1.2	24.9 ± 1	7.4 ± 1.4

[a] Permeabilized platelets (2.5×10^8) suspended in HEPES–Thyrode buffer (0.5 mL) containing human albumin (0.1%) were incubated with either LTA_3 (20 μM), LTA_4 (20 μM), or 14,15-LTA_4 (20 μM) for 20 min at 37 °C. Products were extracted, and materials were resolved by RP-HPLC. Values are expressed as ng/incubation and corrected for isomerization (values from Table I). Data (mean ± SEM) are from 3–4 separate experiments. [b] ND = not detected.

platelet aggregates followed by recruitment of leukocytes (Wester et al., 1979; Frink et al., 1988; Zucker-Franklin, 1988). Ultrastructural studies with thrombi show that, at sites of vascular damage, interactions between platelets and PMN are likely to occur when platelets have already aggregated, secreted the contents of their granules, and lost integrity of their plasma membrane (Wester er al., 1979; Jørgensen et al., 1967). Here, we report that permeabilized platelets generated high levels of lipoxins (Figure 1) while their ability to produce peptidoleukotrienes was considerably reduced compared to intact cells (Figure 2). Similarly, platelet aggregates retained

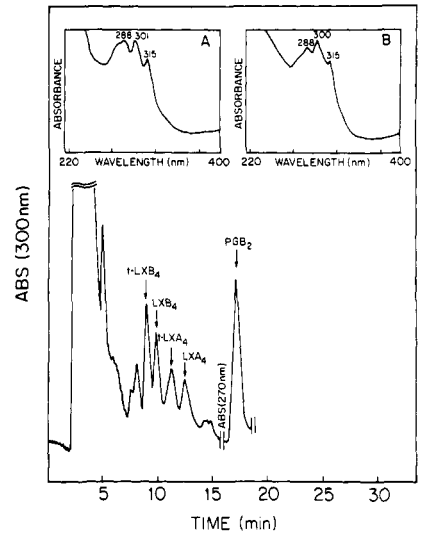

FIGURE 6: RP-HPLC profile of lipoxins obtained from 14,15-LTA_4. Materials extracted from incubation of permeabilized platelets (2.5×10^8) with 14,15-LTA_4 (20 μM) were chromatographed by RP-HPLC. The column was eluted with MeOH/H_2O/acetic acid (65:35:0.01, v/v/v) at 1 mL/min. Retention times of authentic eicosanoids are denoted by arrows. (Inserts) UV spectra of products collected beneath the peaks labeled *all-trans*-LXB_4 (A) and LXB_4 (B). Results are representative from four separate donors with duplicate determinations in each.

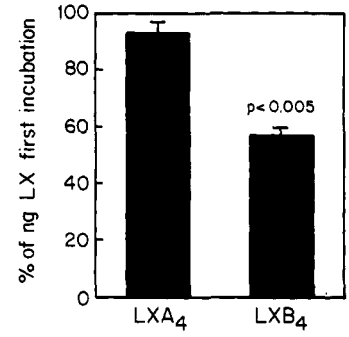

FIGURE 7: Suicide inactivation of LXB_4 formation. Aliquots of permeabilized platelets (10^8 cells/0.5 mL) were incubated at 37 °C with LTA_4 (50 μM). After 20 min, one incubation was stopped while another received a second addition of LTA_4 (50 μM) and continued for 20 min. Products were quantitated following RP-HPLC. Data are expressed in percent of product formed in nanograms. Results are the mean ± SEM from three separate donors.

their ability to convert LTA_4 to LXA_4 (see Results), suggesting that activated platelets can still utilize leukocyte-derived LTA_4.

Several points may contribute to the high capacity of permeabilized platelets to generate lipoxins: first, the platelet membrane barrier is relieved for LTA_4 uptake, allowing for substrate utilization (Figure 1); second, the human platelet 12-LO does not undergo rapid inactivation (Hamberg & Hamberg, 1980) and thus can convert newly available substrate (i.e., LTA_4 donated by leukocytes); and third, uncoupling of

Table IV: Platelet Lipoxin Formation during Coincubation with EC[a]

incubations (thrombin + LTA_4)	LXB_4 (ng/incubation)	LXA_4 (ng/incubation)
platelets[b]	37.4	41.7
platelets + EC[b]	30.1	37.7
EC	ND	ND
iloprost-treated platelets[c]	23.1	16.4

[a] Platelets (5×10^8) in buffer (2 mL) containing human albumin (0.1%) were added to either confluent endothelial cells (EC)[b] or gelatin-coated[b] Petri dishes. Thrombin (1 unit/mL) and LTA_4 ($\sim 13\ \mu M$) were added (20 min at 37 °C) and terminated with cold MeOH (3 vol). [c] Platelets were incubated with iloprost (10 μM) 5 min at 37 °C before adding LTA_4 and thrombin. Products were quantitated using RP-HPLC as described under Materials and Methods and corrected for isomerization with values obtained in Table II. Results are the mean of duplicate incubations and are representative of two separate experiments. ND = not detected.

LTC_4 biosynthesis may redirect conversion of LTA_4 toward lipoxins (Figure 2). Taken together, the present results suggest that, at sites of vascular injury where interactions between PMN and activated platelet aggregates occur, lipoxin generation may represent a main metabolic route for LTA_4 conversion by platelets. Indeed, substantial amounts of LXA_4 (i.e., pico- to nanogram levels) are generated in vivo in patients with coronary artery occlusion following plaque rupture (Brezinski et al., 1992). Lipoxins exert bioactions in the microvasculature (Dahlén, 1989; Badr et al., 1989) and have counterregulatory actions on PMN (Lee et al., 1991) that may be evoked following LX generation by platelets.

Conversion of LTA_4 to lipoxins by human platelets is triggered by the 12-LO (Serhan & Sheppard, 1990; Edenius et al. 1991). This has been confirmed recently with the finding that both 12-LO-expressing and transfected cells can generate lipoxins. Along these lines, we proposed that LTA_4 is converted by action of the 12-LO to a delocalized cation intermediate that is subsequently transformed to LXA_4 and LXB_4 (Sheppard et al., 1992), which is in accordance with a LX synthase model introduced by Corey and Mehrotra (1986). LXB_4 formation by isolated platelets has not been routinely observed by others (Edenius et al., 1991). The present results not only confirm that platelets generate both LXA_4 and LXB_4 (Figures 1C and 3 and Results) but also show that LXB_4 is subject to suicide inactivation (Figure 7) and that both lipoxins undergo isomerization (Table II). It is noteworthy that both LXA_4 and LXB_4 were formed by intact, permeabilized, and homogenized platelets (Table I). This is consistent with previous observations (Fiore & Serhan, 1990) and suggests that LXA_4 and LXB_4 originate predominantly by enzymatic transformation of a common intermediate. Further support for this scheme is provided by results from chiral analysis with the all-trans-containing lipoxins (Figure 4 and Results). The dominant appearance of isomers in the *R* configuration in $\sim$3:1 ratio with those in the *S* configuration is consistent with an enzymatic origin because nonenzymatic formation is expected to produce essentially equal amounts of the trans isomers in the *R* and *S* configuration. Also, $\sim$50% of the all-trans-containing lipoxins isolated from platelets are derived from isomerization of the native LXA_4 and LXB_4 during workup (Table II). Thus, the configuration of the all-trans-containing lipoxins reflects, at least in part, the biosynthetic pathway operative to form native lipoxins (i.e., LXA_4 and LXB_4).

Human platelet 12-LO possesses dual positional substrate specificity and can abstract hydrogen from either carbon 10 position of arachidonic acid to produce 12-HETE [see Hamberg and Hamberg (1980)] or carbon 13 position of LTA_4 to generate lipoxins (Serhan & Sheppard, 1990; Sheppard et al., 1992). The affinity of LTA_4 conversion proved to be comparable to that for arachidonic acid as shown by the values obtained for their apparent K_m (Figure 5). This finding suggests that arachidonic acid and LTA_4 are well positioned in the substrate-binding pocket of human 12-LO. Similarly, this type of switching in positional specificity has recently been observed following substitution of methionine for valine at position 418 in human 15-LO, rendering a mutant that carries out both 12- and 15-LO functions equally (Sloane et al., 1991). It is likely that the allylic methylene hydrogen at carbon 10 of arachidonic acid is more favorably positioned within the reaction center of the enzyme compared to the available hydrogen at carbon 13 in LTA_4. On the other hand a reduced availability of LTA_4, owing to its rapid rate of nonenzymatic hydrolysis (upon exposure to an aqueous environment), may contribute to the apparent lower rate of LTA_4 conversion. Conversion of LTA_4 to lipoxins also showed pH-dependence comparable to that reported for the generation of 12-HETE (Lagarde et al., 1984). In addition, monensin added to intact platelets led to a 40% increase in lipoxin production, suggesting that the rise in intracellular pH in platelets that is observed upon activation (Siffert et al., 1987) can contribute to optimal conditions for platelet LX generation.

Platelets converted 14,15-LTA_4 to lipoxins (Figure 7 and Table III). This represents a novel pathway of lipoxin generation by transcellular metabolism that implies cooperation between human 15- and 12-LO potentially without 5-LO involvement. 14,15-LTA_4 is formed by human 12-LO-catalyzed conversion of 15-HPETE (Maas & Brash, 1983). 15-LO mRNA has been localized in macrophage-rich areas of atherosclerotic lesions (Ylä-Herttuala et al., 1990), suggesting that 15-LO-derived products such as 15-HPETE may be generated by atheromatous lesions. Conversion of 14,15-LTA_4 to lipoxins was inhibited by esculetin, implicating involvement of 12-LO. Human platelets do not display 5-LO activity with exogenous arachidonic acid (Edenius et al., 1991; Hamberg & Hamberg, 1980) in that 5-HETE is not detected and in these cells 12-lipoxygenation of arachidonate is a major activity (Hamberg & Samuelsson, 1974). The present platelet suspensions were essentially devoid of contaminating cell types that can carry 5-LO (i.e., monocytes, lymphocytes, or neutrophils) as evidenced by the absence of 5-HETE generation in these suspensions (Results). To form lipoxins, the platelet enzyme must abstract hydrogen from carbon 7 position to insert oxygen at carbon 5 position. The 12-LO of platelets can abstract the methylene hydrogen at carbon 10 of arachidonic acid or at carbon 13 position of LTA_4. In 14,15-LTA_4, these are not present, and only the methylene group and hydrogen at carbon 7 position are available.

Since permeabilized platelets converted 14,15-LTA_4 to both LXA_4 and LXB_4 (Figure 5, Table III) that was not altered by aspirin but inhibited by esculetin, it appears that 12-LO was responsible for their formation. The bovine 12-LO has been shown to act on substrates in a cell-free system to give a product profile different from that in intact cells (Walstra et al., 1987a,b). To explain this, Walstra et al. have advanced the theory that the 12-LO in a cell-free setting can accept substrates in an inverted position within the catalytic site. In view of their work with the bovine 12-LO, it is possible that the human 12-LO abstracts hydrogen at carbon 7 in 14,15-LTA_4 to give 14(15)-epoxytetraene or its equivalent carbonium cation intermediate [e.g, a (5*S*)-hydroperoxy-15-hydroxynonatetraenyl cation] that can be converted to LXB_4

and LXA_4 [similar to that proposed in Sheppard et al., (1992)]. Further support for this biosynthetic route was obtained from GC–MS analyses of acid methanol trapping products isolated from incubations of permeabilized platelets with 14,15-LTA_4. A temporal inverse relationship was observed for the loss of 8-methoxy-15-hydroxyeicosatetraenoic acids (14,15-LTA_4-derived trapping products) and the appearance of 14-methoxy-5,15-dihydroxyeicosatetraenoic acids (14,15-epoxytetraene-derived trapping products), providing evidence that 14,15-LTA_4 is subject to 5-lipoxygenation (not shown). The present results do not exclude a possible role for platelet epoxide hydrolase(s) in the generation of both LXA_4 and LXB_4, and further studies are required to evaluate their involvement in platelet LX generation. LTA_5 was also converted by platelets to lipoxins of the 5 series [results not shown; see Wong et al. (1985)], while LTA_3 was not a substrate (Table III). Together these results indicate that conversion of LTA to lipoxins by permeabilized platelets requires only the presence of an available 1,4-*cis*-pentadiene unit in the epoxide precursors.

Several enzymes of the arachidonic acid cascade are subjected to suicide inactivation [reviewed in Walsh (1984)]. Inhibition of LXB_4 formation was observed in an incubation setting that supports this type of mechanism (Figure 7). Although LXA_4 and B_4 show distinct actions in some bioassay systems [reviewed in Serhan (1991)], the biological relevance of selective suicide inactivation for LXB_4 formation by platelets remains to be fully understood. It is noteworthy that, in platelets, formation of LXA_4 which induces vasodilation is not regulated by suicide inactivation, whereas that of thromboxane A_2, a potent vasoconstrictor, is controlled by a suicide mechanism (Jones & Fitzpatrick, 1991). These differences may serve to favor accumulation of vasodilatatory products such as LXA_4 during vascular events.

Endothelial cells regulate platelet functions in vivo by several mechanisms including the generation of prostacyclin (Marcus, 1990; Marcus et al., 1991). They also can convert exogenous LTA_4 to peptidoleukotrienes (Feinmark & Cannon, 1986) and are reported to possess 15-LO activity (Hopkins et al., 1984). It was of interest to determine lipoxin generation by endothelial cells and platelets during coincubations. Endothelial cells were treated with indomethacin to block PGI_2 generation because we found iloprost (a PGI_2 analog) to inhibit 40–50% of lipoxin formation by thrombin-stimulated platelets (Table IV). Evidence for lipoxin formation by cultured endothelial cells was not obtained (Table IV), indicating that they do not lipoxygenate LTA_4. In contrast, they did generate measurable amounts of peptidoleukotrienes (not shown), consistent with previous results (Feinmark & Cannon, 1986). These results indicate that human unbilical cord-derived endothelial cells lack the enzymatic machinery to generate lipoxins. Also, endothelial cells did not interfere with lipoxin formation by platelets during coincubations in that platelets were able to generate lipoxins regardless of the presence of indomethacin-treated endothelial cells (Table IV).

In summary, the present results indicate that disruption of the platelet membrane, an event that may occur in vivo (Wester et al., 1979; Frink et al., 1988; Jørgensen et al., 1967), substantially enhances lipoxin generation from LTA (Figure 1–5). They also suggest that activated platelets at sites of vascular injury are likely to contribute to lipoxin generation within vessels. Their role in vascular biology, however, remains to be fully appreciated.

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