5(S),15(S)-Dihydroxyeicosatetraenoic Acid and Lipoxin Generation in Human Polymorphonuclear Cells: Dual Specificity of 5-Lipoxygenase towards Endogenous and Exogenous Precursors

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Summary

5-Lipoxygenase activation of human blood polymorphonuclear cells (PMN) from asthmatic patients (asthmatics) was studied to investigate whether differences may exist with healthy subjects (controls). The respective cell capacities to produce lipoxins (LXs), leukotrienes, and 5(S),15(S)-dihydroxyeicosatetraenoic acid [5(S),15(S)-diHETE] were compared under *in vitro* stimulation by ionophore A23187, with or without exogenous 15(S)-hydoxyeicosatetraenoic acid [15(S)-HETE]. Eicosanoids were analyzed by elution with an isocratic reverse-phase high performance liquid chromatography system, and their profiles, detected by simultaneous monitoring at 302, 280, and 246 nm, were evaluated on the basis of chromatographic behavior: UV spectral characteristics and coelution with synthetic standards. In the presence of exogenous 15(S)-HETE, human PMN were able to produce LXs and 5(S),15(S)-diHETE. Moreover, the total amount of LXs biosynthesized by PMN from asthmatics was twofold higher than that biosynthesized by PMN from controls. In the absence of exogenous 15(S)-HETE, PMN from asthmatics were able to produce 5(S),15(S)-diHETE, and LXs from endogenous sources, whereas in the same experimental conditions, no detectable amounts of these compounds were released by PMN from controls. The levels of 5(S),15(S)-diHETE, and LXs biosynthesized from endogenous arachidonic acid were highly correlated. Two different LX patterns were observed involving two possible metabolic pathways: (*a*) via the intermediate 5,6-epoxytetraene alone for LXs generation from exogenous 15(S)-HETE; and (*b*) via 5,6- and/or 14,15-epoxytetraenes leading to the formation of an enzyme-bound delocalized carbocation for LXs generation from endogenous arachidonate, respectively. The enhanced 5-lipoxygenase activation of blood PMN from asthmatics and the metabolism of exogenous 15(S)-HETE may reflect a priming induced by various mediators released from environmental cells, and could be considered as a model of transcellular signalization between PMN and endothelial cells.

The generation of arachidonic acid metabolites by lipoxygenase-catalyzed reactions is associated with activation of a wide range of human cell types that are involved in both physiologic and pathophysiologic events (1).

Human blood PMN, which are able to biosynthesize eicosanoids from the 5-lipoxygenase (5-LO)[1] pathway (2), are likely to be involved in inflammatory disorders such as asthma and rheumatoid arthritis (3–6). Moreover, previous

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[1] *Abbreviations used in this paper:* AM, alveolar macrophages; 5-HETE, 5(S)-hydroxyeicosatetraenoic acid; 12-HETE, 12(S)-hydroxyeicosatetraenoic acid; 15(S)-HETE, 15(S)-hydroxyeicosatetraenoic acid; 5(S),15(S)-diHETE, 5(S),15(S)-dihydroxyeicosatetraenoic acid; 15(S)-HPETE, 15(S)-hydroperoxyeicosatetraenoic acid; LT, leukotriene; LTA₄, 5(S)-5,6-oxido-11,14-cis-7,9-trans-eicosatetraenoic acid; LTB₄, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; LTC₄, 5(S),6(R)-5-hydroxy-6-(S-glutathionyl)-7,9-trans-11,14-cis-eicosatetraenoic acid; LX, lipoxines; LXA₄, (5S, 6R, 15S)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; 6S-LXA₄, (5S, 6S, 15S)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid; 11-trans-LXA₄, (5S, 6R, 15S)-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid; 6S-11-trans-LXA₄, (5S, 6S, 15S)-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid; 7-cis-11-trans-LXA₄, (5S, 6R, 15S)-trihydroxy-9,11,13-trans-7-cis-eicosatetraenoic acid; LXB₄, (5S, 14R, 15S)-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid; 8-trans-LXB₄, (5S, 14R, 15S)-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid; 14S-8-trans-LXB₄, (5S, 14S, 15S)-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid; RP-HPLC, reverse-phase high performance liquid chromatography.

studies demonstrated that 5-LO activity of PMN from patients with inflammatory diseases is enhanced as compared to 5-LO activity of cells from healthy subjects (controls) (7–10). This high level of 5-LO activity may occur as a result of specific cellular activation due to cooperation with surrounding blood cells. It is now well established that transcellular metabolism resulting from the cell–cell interactions lead to lipoxin (LXs) biosynthesis as a result of the interaction between several lipoxygenases (11, 12). Moreover, Serhan demonstrated the importance of LX involvement in respiratory disease, suggesting their chalone-like role in the inflammatory response and their impact in cell regulation (13).

Considering that human lung tissues displayed high levels of 15-LO activity (14) and that LXs were found in bronchoalveolar lavage from patients with severe pulmonary diseases (15), we designed, in a previous study, a model of cellular cooperation between alveolar macrophages (AM) and bronchial epithelial cells (16). We have demonstrated that AM from asthmatic patients (asthmatics), incubated in the presence of exogenous 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), produced in vitro twofold higher lipoxin amounts than AM from healthy subjects (controls). Simultaneously, Levy et al. demonstrated that in cytokine-primed human AM, 15-LO activity was induced so that the cells produced lipoxins from exogenously added arachidonic acid or LTA_4 (5(S)-5,6-oxido-11,14-cis-7,9-trans-eicosatetraenoic acid) (17). More recently, we demonstrated that LX generation by human PMN was correlated with the severity of inflammatory diseases (18, 19). Nanogram (picomole) levels of lipoxins are generated after (a) human whole-blood stimulation (20) without the addition of exogenous substrate; (b) receptor-mediated activation of both PMN and platelets during coincubation conditions (21); and (c) ionophore stimulation of PMN alone after exposure to exogenous 15(S)-HETE (22). According to this background, we decided to investigate whether the generation of LXs and 5,15-dihydroxyeicosatetraenoic acid [5(S),15(S)-diHETE] is related to the severity of asthma. Since PMN are able to respond to various stimuli through the synthesis of arachidonic acid–derived mediators involved in acute and chronic inflammatory processes, the aim of this study was to determine the mechanisms that induce 5(S),15(S)-diHETE and LXs generation, and to investigate whether these mediators may be considered as potential biomarkers of asthma more specific than leukotrienes (LTs).

PMN activation via the 5-LO pathway was estimated by analysis of the structure and level of the eicosanoids produced after cell stimulation by Ca^{2+} ionophore (A 23187) alone or together with 15(S)-HETE. The ionophore of divalent cation is not cell type specific, but it is a useful tool for the generation of individual eicosanoid in amounts that facilitate their isolation and structural determination by physical methods.

Materials and Methods

Reagents. Culture materials, nutritive medium, and FCS came from Flow Laboratories (Courbevoie, France). All solvents were of

HPLC grade and obtained from Farmitalia Carlo Erba (Milan, Italy). LTC_4 (5(S),6(R)-5-hydroxy-6-(S-glutathionyl)-7,9-trans-11,14-cis-eicosatetraenoic acid), LTB_4 (5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid), 20 OH-LTB_4, 5S-, 12S-, and 15(S)-HETE, 5(S),15(S)-diHETE, LXA_4 ((5S, 6R, 15S)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid), and LXB_4 ((5S, 14R, 15S)-trihydroxy-6,10,12-trans-8-cis-eicosatetraenoic acid) were from Cayman Chemical Company (Ann Arbor, MI). 6S-LXA_4 ((5S, 6S, 15S)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid) was from Cascade (Reading, UK). 8-trans-LXB_4 ((5S, 14R, 15S)-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid), 14S-8-trans LXB_4 ((5S, 14S, 15S)-trihydroxy-6,8,10,12-trans-eicosatetraenoic acid), 11-trans LXA_4 ((5S, 6R, 15S)-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid), 6S-11-trans-LXA_4 ((5S, 6S, 15S)-trihydroxy-7,9,11,13-trans-eicosatetraenoic acid), and MK 886 were generous gifts from Dr. C.B. Pickett and Dr. A.W. Ford-Hutchinson (Merck Frosst, Montreal, Canada).

Subjects. The study included 7 healthy subjects (controls) and 38 asthmatic patients (asthmatics). None of the subjects were smokers and they were free of medication that might interfere with the study design. Theophylline and β agonists were stopped 1 wk before the study; nonsteroidal antiinflammatory drugs and corticosteroids had been discontinued for at least 1 mo. The seven controls were without any history of allergy or respiratory diseases. The clinical characteristic of the 38 asthmatics are described in Table 1. Asthma was defined according to the American Thoracic Society as previously described in detail (23). All patients had a reversible airways obstruction characterized by an increase of at least 12% in the FEV_1 value and an absolute value of 200 ml after inhalation of 200 μg of salbutamol (24). The study was performed after informed consent, and it fulfilled the criteria of the Ethics Commitee of the University of Montpellier.

Preparation of PMN. The cells were isolated and purified from heparinized (20 U/ml) venous blood (50 ml) by centrifugation of samples at 400 g for 20 min at 20°C over discontinuous Percoll gradient (25). The PMN suspension in Percoll was added to an equal volume of saline and then centrifuged at 400 g for 10 min. The pellet was resuspended into 20 ml of a solution of NH_4Cl/Tris/K_2CO_3 (0.130/ 0.01/0.016 M) (pH 7.4) to lyse contaminating erythrocytes. After centrifugation, 6.10^7–8.10^7 PMN were recovered. Cells were then resuspended into PBS (pH 7.4) containing $CaCl_2$ and $MgCl_2$ (final concentration = 2 × 10^{-3} and 0.5 × 10^{-3} M, respectively). PMN purity was evaluated after cytocentrifugation and May Grünwald staining, and it was always >95%. There was no statistical differences in the number of eosinophils in controls and asthmatics, as reported in previous works (26). Viability determined by the trypan blue exclusion test was >90%. PMN were adjusted to 10^7 cells/ml, and the cell suspensions were prewarmed at 37°C for 5 min before ionophore stimulation.

Table 1. *Characteristics of Healthy Subjects and Asthmatic Patients*

	Controls	Asthmatics
Number	7	38
Age (yr)	35.4 ± 3.2	37.2 ± 6.3
Gender	4 male/3 female	24 male/14 female
FEV_1 (% predicted)	100 ± 1.6	83.8 ± 7.7

FEV_1 is forced expiratory volume in 1 s expressed as the percentage of the predicted value. Data are expressed as mean values ± SEM.

Preparation of Washed Platelets. Blood from controls (50 ml) was collected in polypropylene tubes containing acid–citrate–dextrose anticoagulant as previously described (27) and centrifuged at 200 g for 15 min. The platelet-rich plasma was further centrifuged at 650 g for an additional 20 min. Cell pellet was washed in glucose buffer (Tris-HCl 15 mM, NaCl 134 mM, EDTA 1 mM, glucose 5 mM, pH 7.4), recentrifuged, and then adjusted to 1.10^9 cells/ml into Hepes-tyrode buffer to prevent aggregation. Aliquots of 0.50, 0.25, 0.10, and 0.01 ml were adjusted to 1 ml adding PBS with Ca^{2+} and Mg^{2+}, and prewarmed at 37°C for 2 min before ionophore stimulation of platelets alone.

Platelet-PMN Coincubations. Platelet suspension aliquots of 0.50, 0.25, 0.10, and 0.01 ml were added to 10^7 PMN (platelets/PMN ratios 50, 25, 10, and 1, respectively). The mixtures were centrifuged and the pellets were resuspended into 1 ml of PBS containing Ca^{++} and Mg^{++}, and incubated at 37°C for 2 min before stimulation with ionophore alone.

Stimulation Procedures. A 23187 (final concentration = 5 × 10^{-6} M) was added to the cell suspension with or without 15(S)-HETE (final concentration = 3 × 10^{-6} M) in ethanol (minimal concentration 0.1%), and incubation was continued for 5 min. When 15(S)-HETE was added, it was preincubated for 2 min at room temperature. PMN from controls were incubated both with and without 15(S)-HETE. PMN from asthmatics were incubated without 15(S)-HETE (n = 25) or in the presence of 15(S)-HETE (n = 13). Several experiments were carried out on cells prewarmed at 37°C in the presence of MK 886 (2.5 × 10^{-7} M). Simultaneous experiments with 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HPETE) were carried out on cells from several study patients, in the same conditions. Incubations of the same amounts of 15(S)-HETE and 15(S)-HPETE were carried out in PBS alone to test for the possibility of autoxidation. At the end of stimulation, the supernatant was separated from the cells by centrifugation at 2,000 g, and were added to 1 ml of ice-cold methanol to stop the reaction. The samples were stored at −20°C for further analysis of extracellular metabolites. The cell pellet was resuspended in 1 ml PBS, to which 1 ml of ice-cold methanol was added. The samples were stored at −70°C for further analysis of intracellular metabolites. After one night at −70°C, the cell suspensions were centrifuged and the supernatant was stored for the study of cell-associated free HETE. For covalently linked HETE analysis, the pellet was treated with 0.1 ml NaOH 2 N and 0.4 ml MeOH at 37°C for 1 h. Then the solution was neutralized by addition of 0.1 ml of acetic acid 2 N, and the volume was adjusted to 1 ml with additional MeOH. After centrifugation, the samples were directly injected onto the analytical column.

Metabolite Identification and Quantification. The stored samples were directly investigated by reverse-phase HPLC (RP-HPLC) analysis, as previously described (16).

(a) Dihydroxy- and trihydroxyeicosatetraenoic acid analysis: RP-HPLC was carried out on a Lichrospher 100 RP-18 column (150 × 3.9 mm, 5-μm particles; Merck, Darmstadt, Germany). LXs, LTs, and 5(S),15(S)-diHETE were eluted with methanol/water/acetic acid (65:35:0.1, vol/vol/vol, pH 5.6) containing 0.5% EDTA as the mobile phase; they were detected by simultaneously monitoring the following wave lengths: 302, 270, and 246 nm, corresponding to λ_{max} of conjugated tetraenes, trienes, and dienes, respectively.

(b) Monohydroxy-eicosatetraenoic acid analysis: RP-HPLC was performed on a Nucleosil C18 column (250 × 3.9 mm, 5-μm particles) and eluted with methanol/water/acetic acid (78:32: 0.01, vol/vol/vol) with the UV detector set at 237 nm.

(c) *Detection and quantification.* Arachidonic acid metabolites

were identified using cochromatography with synthetic standards and UV spectroscopy in stop-flow mode. They were quantified by external standard method, on the basis of molecular extinction coefficients of 55,000, 35,000 and 28,000 for conjugated tetraenes, trienes, and dienes, respectively. The sensitivity threshold was 0.2 ng at 302 nm and 0.5–1 ng at the other wave lengths.

Statistical Analysis. The results presented as nanograms per 10^7 cells were expressed as mean ± SEM. Statistical differences were determined using the Mann-Whitney U test for unpaired samples (experiments on PMN from controls, n = 7; experiments on PMN from asthmatics incubated without 15(S)-HETE, n = 25; experiments on PMN from asthmatics incubated with 15(S)-HETE, n = 13). Significance level was set at P <0.05. Correlations were evaluated using the Spearman rank correlation.

Results

5-LO activity in human PMN was investigated via LXs and 5(S),15(S)-diHETE biosynthesis under conditions of stimulation by A 23187 alone or together with exogenous 15(S)-HETE. Experiments with 15(S)-HPETE were carried out simultaneously on PMN from several asthmatics (the results were identical to those obtained with 15(S)-HETE). Some experiments were carried out in the presence of A 23187 alone on platelet–PMN mixtures and on platelet suspensions to compare the results with those obtained with PMN alone.

Two series of experiments were carried out: (a) without addition of exogenous substrate for PMN from 25 asthmatics; and (b) with exogenous added 15(S)-HETE for the cells from 13 asthmatics. The same experiments were simultaneously performed on PMN from seven controls.

Eicosanoid Biosynthesis from Endogenous Sources (PMN from 7 Controls and 25 Asthmatics). Stimulation of human PMN by A23187 alone generated products containing conjugated diene detected at 237 nm, and conjugated triene detected at 280 nm. Fig. 1 *A* reports the analysis results at 280 nm for 20-OH LTB₄, Δ6-*trans* LTB₄ isomers generated from nonenzymatic LTA₄ opening and LTB₄, with retention times of 6.80, 20.50, 20.22, and 24.70 min, respectively. All the metabolites were released mainly into the culture medium, but 20% of them remained associated in the cell pellet. No detectable amounts of LTC₄ were observed in controls or asthmatics. Simultaneous detection at 246 nm usually showed the same eicosanoid pattern with characteristic equal peak areas for the two Δ6-*trans*-LTB₄ isomers since the molecular extinction coefficients were identical for all LTB₄ metabolites containing conjugated triene chromophore. The asthmatics cell analysis of the supernatant and the cell pellet detected a new peak with a retention time of 22.40 min (Fig. 1 *B*). This product coeluted with authentic 5(S),15(S)-diHETE and showed the same UV spectrum as the reference standard, with a maximum at 246 nm and a shoulder at 235 nm, which is characteristic of a double-conjugated diene (28).

Simultaneous detection at 302 nm, which did not usually reveal any metabolites in these analysis conditions, showed for the asthmatics' PMN several peaks with UV spectra
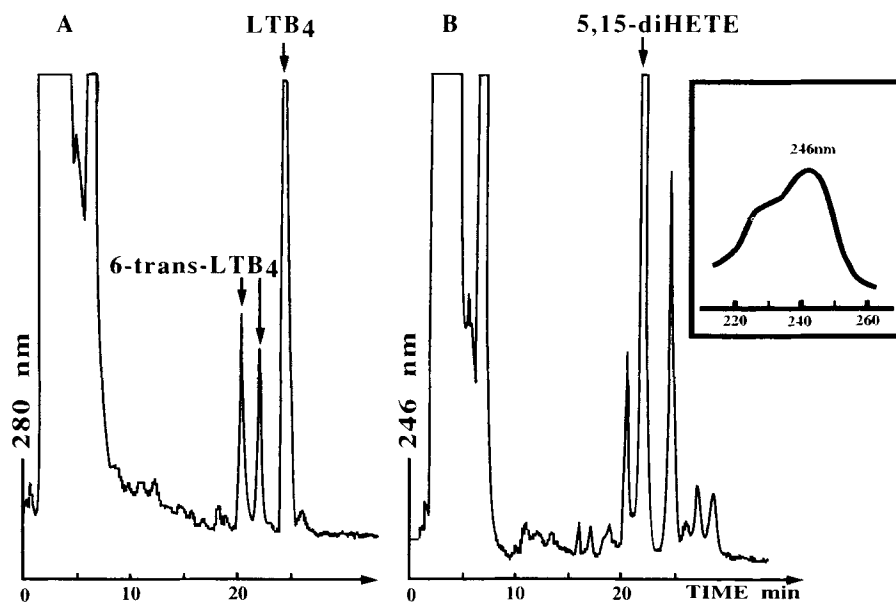
Figure 1. RP-HPLC profiles of dihydroxy arachidonic acid metabolites generated by human PMN. 10^7 cells/ml were incubated for 5 min at 37°C with A 23187 (5 μM) with and without 15(S)-HETE (3 μM) for controls cells, with 15(S)-HETE only for one group of asthmatics cells ($n = 13$), and without exogenous precursor for another group of asthmatics cells ($n = 25$). Sample aliquots were injected directly on RP-HPLC, and chromatography was carried out with eluting solvent methanol/water/acetic acid (65:35:0.1 vol/vol/vol) adjusted to pH 5.6, at a flow rate of 0.5 ml/min for 15 min and 1 ml/min from 15 to 65 min. Peaks were identified by comparison of their elution time with those of synthetic standards and by their UV spectra. The same metabolite pattern was obtained for the cells from asthmatics or controls stimulated in the presence of 15(S)-HETE and for the cells from asthmatics stimulated without 15(S)-HETE. (*A*) Recording at 280 nm corresponding to conjugated triene λ_{max}. (*B*) Recording at 246 nm corresponding to λ_{max} of double-conjugated diene 5(S)-15(S)-diHETE. *Inset*, stop-flow UV spectrum of 5(S),15(S)-diHETE (λ_{max} 246 nm with shoulder at 237 nm); *arrows*, the elution positions of authentic standards. Chromatograms *A* and *B* are derived from one representative experiment (cells from the same donor), and the experiment was repeated several times with similar results.

giving a triplet of absorption bands at 288, 302, and 316 nm, which is characteristic of the conjugated tetraene system of the LX structure (Fig. 2, *insert*). Fig. 2 displays expansion of the LX region monitored at 302 nm. The metabolite stereochemistries were assigned to the respective peaks by several successive chromatographic superimpositions with synthetic standards according to the method described in a previous work (16). The peak with a retention
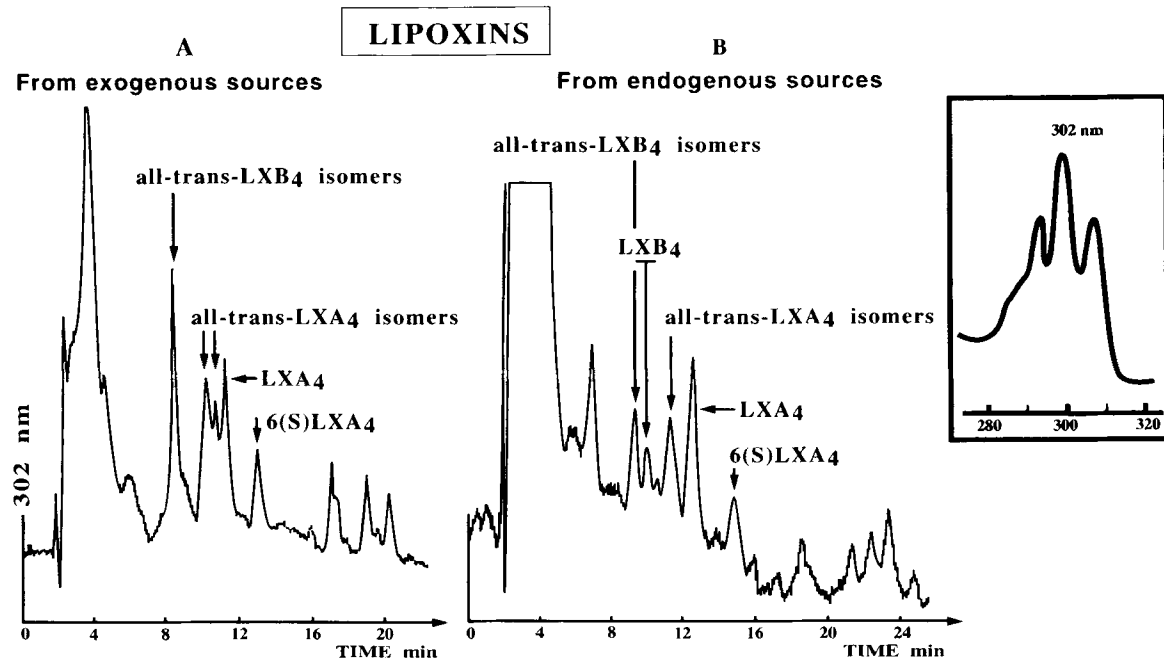

Figure 2. RP-HPLC profiles of trihydroxy arachidonic acid metabolites generated by human PMN. 10^7 cells/ml were incubated for 5 min at 37°C with A 23187 (5 μM) with and without 15(S)-HETE. Expansion of the RP-HPLC chromatograms were recorded at 302 nm corresponding to conjugated tetraene λ_{max}, under the conditions described in Fig. 1. (*A*) Focus on the LX region related to the PMN from asthmatics ($n = 13$) and from controls ($n = 7$) incubated with exogenous 15(S)-HETE. (*B*) Focus on the region related to the cells from asthmatics ($n = 25$) incubated without exogenous precursor. *Inset*, stop-flow characteristic UV spectrum of the conjugated tetraene system of the LX structure (λ_{max} 302 nm with shoulders at 288 and 316 nm). As reported for Fig. 1, chromatograms *A* and *B* are derived from one representative experiment, and the experiments were repeated several times with similar results.

time of 10.10 min coeluted with the natural LXB$_4$; the peak at 9.30 min coeluted with 14S-8-*trans*-LXB$_4$ isomers that were not resolved in this mobile phase. The peak at 12.60 min coeluted with natural LXA$_4$, the peak at 11.40 min coeluted with 11-*trans*-LXA$_4$ and 6S-11-*trans*-LXA$_4$ isomers, and the peak at 14.80 min with 6S-LXA$_4$.

Detection at 237 nm showed the presence of 5-HETE, as usually described for PMN, but failed to point out any detectable amounts of 15(S)-HETE, 12-HETE, or 12-hydroxyheptadecatrienoic acid, even after cell pellet hydrolysis.

Fig. 3 reports the amounts of all the metabolites generated in vitro by the two cell populations after A23187 stimulation. PMN from asthmatics released significantly into their culture medium higher levels of 20-OH-LTB$_4$, LTB$_4$, and LTB$_4$ isomers than PMN from controls: 203.5 ± 9.1 vs 139.4 ± 12.8 (P <0.0008), 173.1 ± 9.1 vs 153.7 ± 12.8 (P <0.0008), and 144.8 ± 6.3 vs 113.2 ± 6.8 ng/10^7 cells (P <0.0045), respectively. Besides the normally generated LTs, PMN from asthmatics were able to generate marked amounts of 5(S),15(S)-diHETE (135.4 ± 14.2 ng/10^7 cells), as well as LXs (6.1 ± 1.0 ng/10^7 cells), whereas no detectable amounts of these metabolites were found in

PMN from controls. Moreover, the metabolite levels were highly correlated, as shown in Fig. 4. Among the asthmatics cells this study shows evidence of two cell types: "high responders" (n = 13), which cells biosynthesized 5,15-di-HETE levels >150 ng and "mild responders" (n = 12), with correlation coefficients r = 0.803 (P <0.003) and 0.942 (P <0.0001) respectively.

PMN from asthmatics incubated in the presence of A23187 were able to biosynthesize from their endogenous arachidonic acid pool LTB$_4$, 20-OH-LTB$_4$, LTB$_4$ isomers, dihydroxy derivatives containing conjugated dienes identified as 5(S),15(S)-diHETE, and trihydroxy derivatives containing conjugated tetraene identified as LXB$_4$-*trans*-isomers, LXA$_4$-*trans*-isomers, and 6S-LXA$_4$ and the two natural LXA$_4$ and LXB$_4$, with relationships between dihydroxy acid levels and total LX amounts.

Eicosanoid Biosynthesis from Exogenous 15(S)-HETE (PMN from 7 Controls and 13 Asthmatics). Exposure of PMN to A 23187 in the presence of 15(S)-HETE resulted in the generation of products containing conjugated diene, triene,
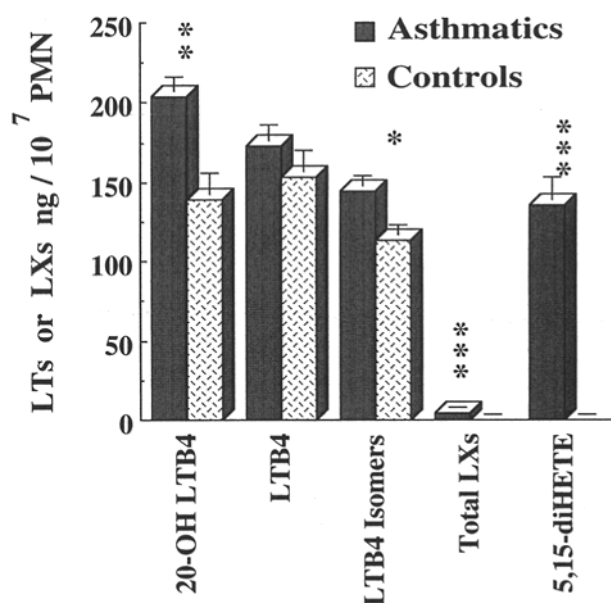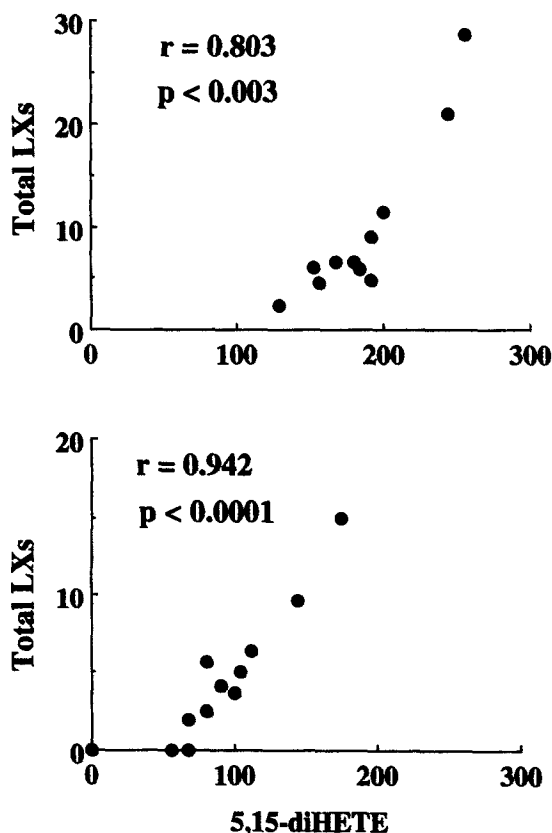

Figure 3. Di- and trihydroxy products generated from endogenous sources by human PMN. The cells from controls and asthmatics were stimulated by A 23187 (5 μM) at 37°C for 5 min. The total amounts of biosynthesized metabolites were identified by RP-HPLC, as outlined in Fig. 1. They were quantified by external standard method (comparison of the peak area to standard curves obtained in the same conditions of analysis on the basis of molecular extinction coefficients of 55,000, 35,000, and 28,000 for conjugated tetraenes, trienes, and dienes respectively). These results represent the total amounts of the metabolites released into the culture medium and of the metabolites associated to the cells. Results presented as nanograms per 10^7 cells were expressed as mean ± SEM from the number of subjects in each population. Filled bars, asthmatics (n = 25); hashed bars, controls (n = 7). Eicosanoid levels were higher in PMN from asthmatics with significant differences (except for LTB$_4$): *P <0.0043, **P <0.0008, ***P <0,0001 (Mann-Whitney U test). No detectable amount of LXs and 5(S),15(S)-diHETE were found in PMN from controls.

Figure 4. Correlations between LXs and 5(S),15(S)-diHETE levels generated by PMN from asthmatics (n = 25). The cells were stimulated by A 23187 (5 × 10^{-6} M for 5 min at 37°C) without exogenous 15(S)-HETE, in the conditions as described in Fig. 1 legend. Data are expressed as nanograms per 10^7 cells. The two cell populations were determined by the high 5(S),15(S)-diHETE levels (*upper panel*, 5(S),15(S)-diHETE levels >150 ng/10^7 cells, n = 13) and by the lower 5,5-diHETE levels (*lower panel*, n = 12). r = 0.803 with P <0.003 and r = 0.942 with P <0.0001, respectively (Spearman rank correlation).

and tetraene as previously reported (22, 29). They were detected simultaneously at 246, 280, and 302 nm on the same sample injection. The profiles obtained at 246 nm with exogenous added 15(S)-HETE did not differ from that obtained without 15(S)-HETE (same pattern as reported in Fig.1 B), but the profile recorded at 280 nm differed by the absence of Δ6-trans-LTB4 and by the twofold lower amounts of LTs. Fig. 2 A, which gives the profile of conjugated tetraene-containing products, also showed two marked differences with that obtained for biosynthesis from endogenous sources (Fig. 2 B): (a) the low amounts of natural LXB$_4$, undetectable in our experimental conditions of analysis and; (b) the generation of a product with a retention time at 11.40 min, which was identified as the 7-cis-11-trans-LXA$_4$, according to published data (30). When the cells were incubated in the presence of MK 886, none of the metabolites were detected.

PMN from asthmatics and controls, stimulated by A 23187 in the presence of 15(S)-HETE, were thus able to synthesize LTB$_4$ and its metabolites, and to transform exogenously added 15(S)-HETE into dihydroxy derivatives, 5(S),15(S)-diHETE, and into trihydroxy derivatives containing conjugated tetraene, all-trans-LXB$_4$ isomers, cis- and trans-LXA$_4$ isomers, LXA$_4$, and 6S-LXA$_4$.

In conclusion, we observed that the eicosanoid pattern obtained from experiments with exogenously added precursor differed from the profile obtained without exogenous 15(S)-HETE.

Biosynthesis of LXs and 5(S),15(S)-diHETE from Exogenously Added 15(S)-HETE in Asthma. Since 15(S)-HETE and related compounds have been described as human leukocyte 5-LO inhibitors (31), 15(S)-HETE was added at 3 μM (final concentration) to 10^7 PMN/1 ml PBS: this concentration may not be cytolytic and provided the best ratio of LXs vs LT biosynthesis. Dose–response experiments showed that exogenous 15(S)-HETE >6 μM failed to enhance LXs and 5(S),15(S)-diHETE levels in human PMN, while LTB$_4$ synthesis was strongly decreased; <3 μM, generated LX levels remained within the detection threshold range (data not shown).

Fig. 5 shows the results concerning all eicosanoids produced by 10^7 PMN in the presence of exogenous 15(S)-HETE. Comparing Figs. 3 and 5, partial inhibition of the LT biosynthesis occured for both the controls and asthmatics cells, with a highly significant twofold decrease (P <0.0001). 20-OH-LTB$_4$ and LTB$_4$ levels in PMN from asthmatics were higher (80.9 ± 10.2 and 52.2 ± 10.3 ng/ 10^7 cells) than those in PMN from controls (50.1 ± 26.5 and 30.1 ± 17.7), but the differences were not significant. It is noteworthy that Δ6-trans-LTB$_4$ formation was completely inhibited for both asthmatics and controls. Moreover, 15(S)-HETE transformation into 5(S),15(S)-diHETE by PMN from asthmatics was nearly twofold higher than by PMN from controls: 93.5 ± 3.1% vs 59.0 ± 7.6% formation from the total added 15(S)-HETE, with very significant differences (P <0.0005). Similarly, LXs, which were biosynthesized in the same level range as LTs (Fig. 6), were twofold more important in asthmatics than in controls:
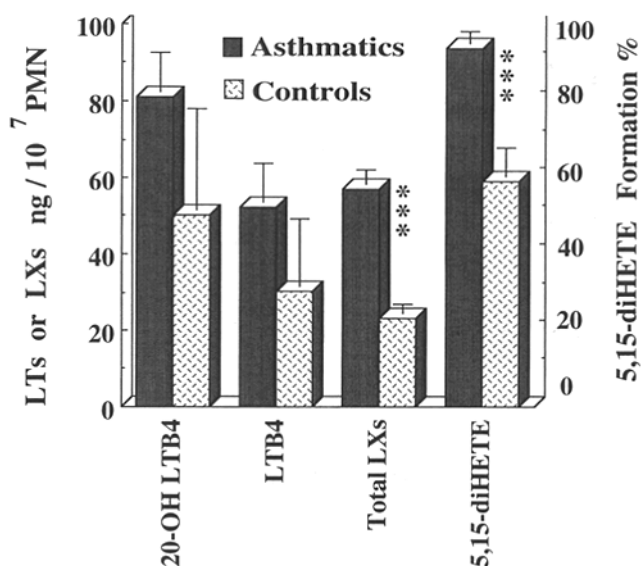

Figure 5. Di- and trihydroxy products generated by A 23187–stimulated PMN in the presence of 15(S)-HETE. PMN were stimulated A 23187 (5 μM) for 5 min at 37°C with 15(S)-HETE (3 μM) in the conditions described in Fig. 1 legend. The amounts of metabolites were determined by RP-HPLC analysis by external standard method, as outlined in Fig. 3. As reported in the Fig. 3 legend, they represented the whole-cell production. Results presented as nanograms per 10^7 cells for LTs and LXs are reported on the left vertical axis. Results presented as the percentages of 5(S),15(S)-diHETE formation from the initial added 15(S)-HETE amounts are reported on the right vertical axis. The results are expressed as mean ± SEM for the number of subjects in each population. Filled bars, asthmatics (n = 13); hashed bars, controls (n = 7). LT production was lower than in the cells incubated without 15(S)-HETE (see Fig. 3). Eicosanoid levels were higher in asthmatics' PMN than in controls' cells, with significant differences for the LX pathway only: ***P <0.0005 (Mann-Whitney U test).

56.8 ± 4.0 ng vs 23.0 ± 2.5 ng/10^7 cells, with highly significant differences (P <0.0001).

The amounts and distribution of trihydroxy compounds obtained for the cells from controls and asthmatics were detailed in Fig. 6. Total LXs levels were determined as the sum of the four groups of products resolved by HPLC elution: two LXB$_4$ isomers, two all-trans-LXA$_4$ isomers plus 7-cis-11-trans-LXA$_4$, LXA$_4$, and 6S-LXA$_4$ (Fig. 2 A), and were biosynthesized in the 3:3:3:1 ratio that is independent of the pathology. The level of every group of isomers was significantly higher in cells from asthmatics than in those from controls: LXB$_4$ isomers (16.4 ± 1.4 ng vs 8.1 ± 1.3 ng, P <0.0001), LXA$_4$ isomers (14.9 ± 1.9 ng vs 5.6 ± 0.6 ng, P <0.0005), LXA$_4$ (17.3 ± 0.8 ng vs 6.5 ± 0.8 ng, P <0.0001), and 6S-LXA$_4$ (6.1 ± 0.8 ng vs 2.4 ± 0.5, P <0.005). Thus, PMN from asthmatics were able to synthesize greater levels of total LXs than PMN from controls, but 16.3% of these metabolites remained inside the cells of asthmatics, whereas cell-associated LXs levels produced by cells from controls were significantly higher, 26.4% (P <0.045).

Stimulation of Mixed Platelet/PMN and Platelet Alone Suspensions by A 23187. These coincubations were performed to compare the results with those described previously. As
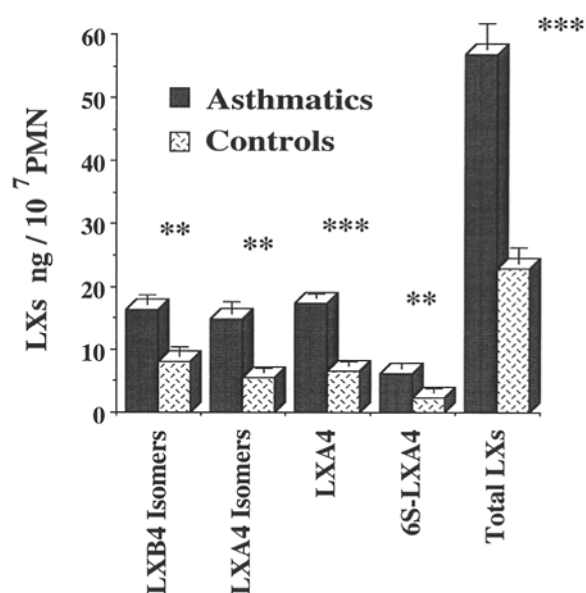
Figure 6. LXs biosynthesized from A 23187–stimulated PMN. PMN were stimulated by A 23187 (5 μM) for 5 min at 37°C with 15(S)-HETE (3 μM). The LX group corresponded to material resolved by HPLC analysis under the conditions described in Fig. 2 A, and quantified by external standard method under the conditions described in the Fig. 3 legend. Results presented as nanograms per 10^7 cells are expressed as mean ± SEM for the number of subjects in each population. Filled bars, asthmatics ($n = 13$); hashed bars, controls ($n = 7$). LX biosynthesis in PMN from asthmatics was twofold higher than in PMN from controls with highly significant differences. Statistical analysis (Mann-Whitney U test): LXB_4 isomers, $P < 0.0001$; LXA_4 isomers, $P < 0.0005$; LXA_4 $P < 0.0001$; 6S-LXA4, $P < 0.0005$; total LXs, $P < 0.0001$ (Mann-Whitney U test).

reported in the literature (32), a platelets/PMN ratio of 100 was required to produce 52 ng of LXs when 10^7 leukocytes were incubated with A 23187 (1 μM) at 37°C for 30 min. LX levels were produced linearly according to the platelet/PMN ratio, and 10^7 PMN required fivefold more platelets to produce 28.8 ng of total LXs with only 30 ng of 5(S),15(S)-diHETE (data not shown). In the same conditions, platelets alone (5 × 10^7) were able to produce 12-HETE (72 ng) and HHT (70 ng) without any detectable amounts of other metabolites.

Discussion

We investigated the generation of di- and trihydroxyeicosatetraenoic acids by A 23187–stimulated PMN incubated in the presence or absence of 15(S)-HETE. We demonstrated (a) that the total amounts of LXs biosynthesized by PMN from asthmatics were twofold higher than those biosynthesized by PMN from controls; and (b) in the absence of exogenous 15(S)-HETE, PMN from asthmatics were able to produce 5(S),15(S)-diHETE and LXs from endogenous sources, whereas in the same experimental conditions, no detectable amounts of these compounds appeared in the PMN from controls.

Human blood PMN were isolated and purified to avoid any contaminating cells such as erythrocytes and platelets, and thus eliminate any possible eicosanoid synthesis result-

ing from transcellular metabolism. RP-HPLC coupled with a multi–wave length spectrophotometer detector was useful in resolving and monitoring products containing conjugated triene and tetraene. Aliquots of samples were directly injected into an isocratic RP-HPLC system, and the analysis procedure did not involve solid-phase extraction, concentration, or derivatization. The samples only had to be deproteinized and centrifuged, thus minimizing double-bond isomerization, loss, and deterioration of eicosanoids. Moreover, for cis–double-bond isomerization, the samples had to be left overnight at room temperature, as previously reported (16). The presence and identity of UV chromophores were characterized by their retention times and spectra in the stop-flow mode.

The aim of our study was to demonstrate the PMN hyperactivation consecutive to asthma, measuring the cell capacity to generate, via the 5-LO pathway, LXs from endogenous arachidonic acid or from exogenously added 15(S)-HETE.

PMN from asthmatics exposed to A 23187 (5 μM) at 37°C for 5 min released higher levels of LTs than PMN from controls into the culture medium. In contrast to PMN from controls, they were able to synthesize high levels of 5(S),15(S)-diHETE and substantial amounts of products containing conjugated tetraene chromophore 14S-8-trans-LXB_4 and 8-trans-LXB_4, LXB_4, 6S-11-trans-LXA_4 and 6R-11-trans-LXA_4, LXA_4, and 6S-LXA_4. When exposed to 15(S)-HETE (3 μM), A 23187–stimulated PMN generated metabolites from endogenous and exogenous sources. PMN were able to synthesize 20-OH-LTB_4 and LTB_4 (without Δ6-trans-LTB4) and to transform 15(S)-HETE into conjugated diene derivative 5(S),15(S)-diHETE and into seven products containing conjugated tetraene chromophore: 14S-8-trans-LXB_4 and 8-trans-LXB_4, 6S-11-trans-LXA_4 and 6R-11-trans-LXA_4, 7-cis-11-trans-LXA_4, LXA_4, and 6S-LXA_4. 5(S),15(S)-diHETE was not the result of 15(S)-HETE autoxidation since they were not available in control incubations carried out in a cell-free system (data not shown). The presence of all trans-LXs isomers and the absence of LXB_4 may not have been the result of double-bond isomerization owing to the extraction procedure, since in the same analysis conditions, LXB_4 was clearly generated by asthmatics cells. The main important differences in the experiments carried out in this study are thus summarized by the following observations related to the LXs formation: (a) human PMN did not generate natural LXB_4 from exogenous sources in amounts that could be detected in our analysis conditions: and (b) only PMN from patients with pulmonary disease were able to biosynthesize LXs from endogenous sources with high LXB_4 levels, and to produce from exogenous sources twofold higher total LX levels than cells from controls.

The metabolic pathway involved to account for LXs generation by human PMN was reported in Scheme 1. In five experiments, 15-HPETE led to the same reaction product profile as obtained by Kim for rat AM (33, 34) and by Chavis et al. for human AM (16). Hence, the metabolic pathway previously suggested for human AM may explain LX generation by human PMN. As previously reported by

A) ENDOGENOUS SOURCES   **B) TRANSCELLULAR METABOLISM**
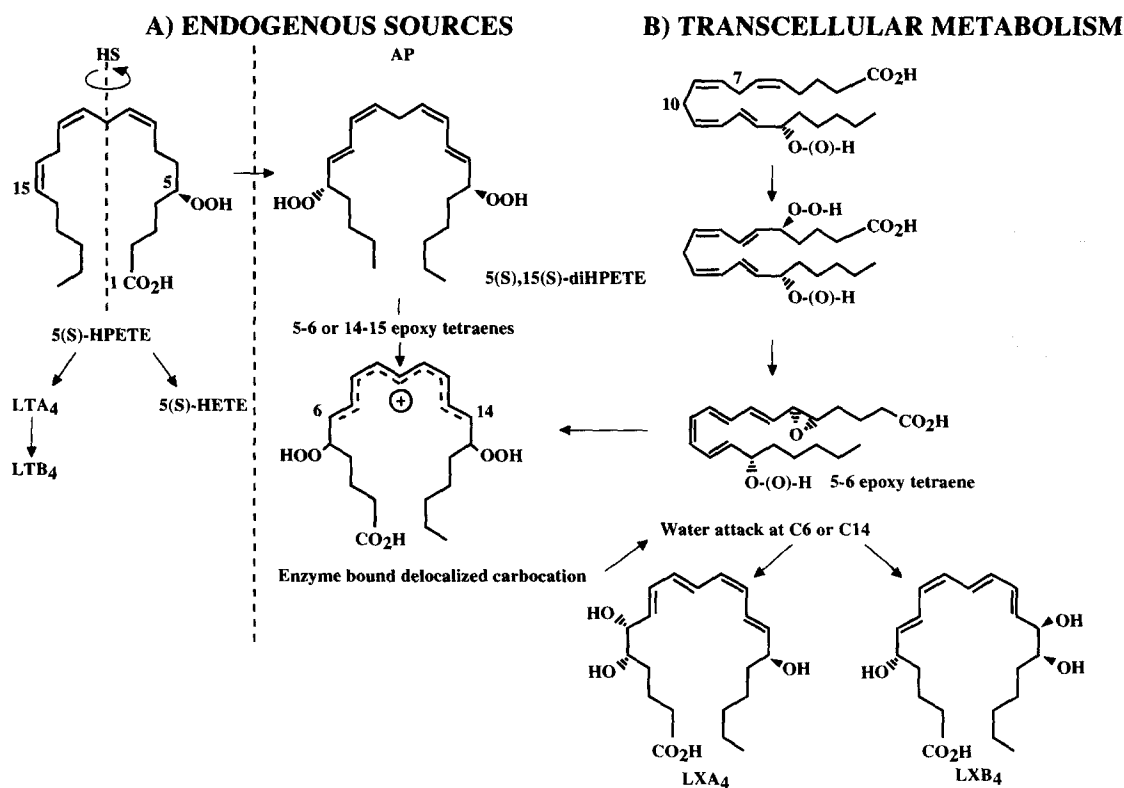
Scheme 1. Hypothetical pathways for LX generation by human neutrophils. (A) Generation of 5(S),15(S)-diHETE and LXs from endogenous arachidonic acid by the involvement of the delocalized carbocation intermedicate described by Corey et al. (39) and Serhan (13, 42) as a possible LX precursor in the metabolization of 15(S)-HETE via the formation of human leukocytes. (B) Metabolization of exogenous 15(S)-HETE via the formation of 5,6-epoxytetraene, according to Serhan et al. (12), Rokach et al. (38), and to previous results obtained with AM (16).

Serhan et al. (30, 35–37), LX biosynthesis occurred via formation of the epoxytetraene intermediate 15-OH-LTA$_4$ generated from 5-hydroperoxy,15-hydroxyeicosatetraenoic acid, whose enzymatic opening led to natural LXA$_4$ and nonenzymatic opening to *all-trans* LX isomers (38), as described in Scheme 1 B.

LX biosynthesis from endogenous sources was explained in Scheme 1 A. The 5-HPETE generated in a first step by the 5-LO activity led to 5-HETE and LTA$_4$. Even after cell pellet hydrolysis, no detectable amounts of 15(S)-HETE was recovered, showing that it was not reacylated into cellular lipid pools or membrane phospholipids. Although no evidence for 15(S)-HETE formation was shown, PMN from asthmatics should have displayed 15-LO activity: Nichols et al. (39) have indicated that the 15-LO in human PMN is a cryptic enzyme that needs to be stimulated to metabolize endogenous substrate. It is possible that 5-hydroxy-eicosanoids, biosynthezised by one pool of PMN, have acted as second messengers towards another pool, and that they may mimic an as yet unidentified physiological activator of the 15-LO. 15-LO would thus be able to generate 15-OH LTA$_4$ alone. This is similar, however, to the situation in the presence of exogenous substrate, where LXB$_4$ levels were too weak to be detected. 15-LO action on the small amounts of 5,6-diHETEs occurring from the nonen-

zymatic LTA$_4$ opening would be able to produce LXs in the same manner as platelets (40), but for the same reason, this mechanism would not be retained. Therefore, since 15(S)-HETE was not recovered, it was not biosynthesized or was rapidly catabolized by ω hydroxylation or β oxidation, being thus undetectable nor able to lead to LXs. A mechanism involving 5-LO action on the symmetric stable horseshoe conformation ascribed by Corey et al. to 5-HPETE (41) would thus explain the observed high amounts of LXB$_4$. After rotation of the molecule around its symmetry axis in the enzymatic site consecutive to "special" activation of the 5-LO, abstraction of the hydrogen atom at the C13 position, identical in this case to the C7 position specific to the 5-LO activity (42), led to 5,15-diHPETE. In a first step, gluthation peroxidase reduced 5,15-diHPETE to 5(S),15(S)-diHETE; in a second step, proton abstraction at the C10 position, specific of the 5-LO epoxysynthase activity (43), generated 5,6- and/or 14,15-epoxytetraenes, leading to delocalized enzyme-bound carbocation as described by Corey et al. (41) and in further works by Serhan (13, 44), explaining thus LXs formation by water attack from the top π face of the complex at the C6 or C14 positions. This mechanism pointed to the fact that the in situ endogenous 5(S)-HPETE generation was the key step for LXA$_4$ and LXB$_4$ formation from a common precursor in activated PMN:

this hypothesis was strengthed by the lack of LX generation from exogenously added 5-HETE, as previously reported by Serhan (22).

This new class of hydroxytetraenes was first isolated and characterized by Serhan et al. (11, 12), and then described using various incubation systems (1, 45). Substantial amounts of LXs were produced by A 23187–stimulated cells according to three different in vitro incubation systems, with or without exogenously added substrate. The synthesis of LXs generated by rat basophilic leukemia cells (46), human PMN (22, 30), or AM (16, 33, 34) incubated with 15(S)-HETE, by nasal polyps (47) and permeabilized human platelets (48) incubated with LTA_4, and by granulocytes from eosinophil donors incubated with arachidonic acid (49), all required the addition of exogenous substrate. In contrast, the synthesis of LXs generated by human cell–cell coincubations such as platelet granulocytes (32), GM-CSF–primed PMN and platelets (21), or cell–tissue coincubations such as granulocyte nasal polyps (47) did not require any exogenous addition since the substrate was provided by one cell type and transformed by the other by a transcellular metabolism mechanism. The synthesis of LXs generated by cells from hematopoietic organs, such as bone marrow cells (50) or, more recently, macrophage-like cells isolated from rainbow trout (51, 52), was proceeded by one cell type without the addition of any exogenous substrate and without the participation of surrounding cells.

This is the first time that the simultaneous generation of 5(S),15(S)-diHETE and LXs in vitro by human phagocytes has been reported without exogenous addition of any precursor and without in vitro cytokine priming. These results on LX generation from endogenous sources cannot be interpreted by a dual cell system involving platelet contamination: in fact, the platelet/PMN ratio required to generate the same metabolite level was from 50 to 100, which would be far beyond such a ratio because of the remaining adherent platelets after the purification process. Our analysis of experiments with asthmatics' PMN preparations did not show the presence of LTC_4 in contrast to the results reported by Maclouf et al. (53) for mixed platelet/PMN suspensions. Moreover, when 15(S)-HETE was added to the cells in the presence of MK 886, a specific inhibitor of PMN 5-LO translocation (54), metabolite biosynthesis was completely stopped, whereas if PMN suspensions had been contaminated, the action of the platelet 12-LO would have led to the formation of 8,15-diHETE after hydrogen abstraction at C10 on the 15(S)-HETE molecule. Finally, high levels of 5(S),15(S)-diHETE were not obtained when PMN were stimulated in the presence of various quantities of platelets in the absence of 15(S)-HETE. This suggested

that LX generation could arise neither from LX synthase activity of human platelet 12-LO (55) nor by stimulation of LX synthesis by endogenously formed 12-HETE in platelets (56). Therefore, 5(S),15(S)-diHETE and LXs are actually generated in vitro by the asthmatics blood PMN from their endogenous arachidonate pools. On the other hand, PMN from asthmatics metabolized twofold more 15(S)-HETE and produced twofold greater quantities of each LX species than PMN from controls. The higher levels of LXs in asthmatics' PMN and the metabolic steps involved in LXs formation indicated that in vitro 5(S),15(S)-diHETE and LXs generations reflected in vivo–priming of PMN: this priming may be induced in bronchial diseases by cytokines (21) or TNF-α released by other blood cells or by pulmonary cells involving interleukin or TNF-α/LX axis, similar to the TNF-α/LT axis described in lung endotoxin shock (57, 58). In conclusion, LXs and 5(S),15(S)-diHETE, already considered as biomarkers of inflammatory states (18, 19), may be more specific than LTB_4 in pulmonary diseases.

The data presented here demonstrate that the use of 15(S)-HETE as an exogenous substrate is a useful approach for assessing the PMN 5-LO activation status independently of endogenous arachidonic acid availability. Human PMN were thus shown to be 15(S)-HETE metabolizing cells, and the relationship between LX formation and 15(S)-HETE substrate availability suggested that in vivo there may be an interaction between PMN and surrounding inflammatory cells that could serve as 15(S)-HETE donors. Since transcellular metabolism is considered as a mechanism of eicosanoid biosynthesis (59), the production of LXA_4 identified in whole blood by Serhan et al. (20) may have been the result of a cell–cell cooperation between leukocytes and other cells. The primary sources of 15(S)-HETE, depending on the extent of inflammation, are likely eosinophils with active 15-LO activity (28). In bronchial diseases, however, vascular endothelial cells are now considered to be active inflammatory cells rather than a biological barrier to body fluids (60). The priming of human PMN, specifically asthmatics cells, might be explained by potential cellular interactions with vascular endothelial cells since several authors reported evidence of an LO-dependent mechanism in PMN adherence to endothelial cells (61–64). It will be of interest to proceed further with this work to elucidate whether cell-associated LX amounts have any significance, knowing the physiological properties of LXA_4 (13), and to investigate the mechanism of cell–cell interactions that could be involved in arachidonic acid metabolism by human cells from the hematovascular system, and to provide a link between cellular lipid mediators and cell-mediated immunity in the context of pulmonary diseases.

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