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Endogenous Anti-inflammatory Mediators from Arachidonate in Human Neutrophils

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Eicosanoids have been historically involved in the pathogenesis of various inflammatory diseases. Lipoxins (LXs) and epi-LXs show physiological effects relevant to inflammation regulation. In this study, we focused on LX precursors based on the hypothesis that their entrance and metabolism into the cell may facilitate their targeting at the inflammation site. Because compound chirality is of considerable importance in the efficacy of therapeutic agents, our aim was to study the anti-inflammatory effects of various epimers of LXA_4 precursors compared to LXA_4. Blood polymorphonuclear cells (PMNs) were incubated with 15(S)- or 15(R)-hydroxyeicosatetraenoic acid (HETE), 14(R)-,15(S)-, or 14(S),15(S)-diHETE, and LXA_4 and then stimulated with the calcium ionophore A23187. We found that 15(R)-HETE rather than 15(S)-HETE was preferentially metabolized and that 15-epi-LXs were produced in larger amounts than LXs. In contrast, when PMNs were incubated with the diastereoisomers of 14,15(S)-diHETE, 14-epi-LXB_4 was produced in lower amounts than LXB_4. Enantiomers of 15-HETE and diastereoisomers of 14,15-diHETE and LXA_4 were able to significantly decrease LTB_4 release by PMNs. These results suggest a potential resolution of the inflammatory process through endogenous anti-inflammatory mediators released by the way of *trans*-cellular metabolism.

Key Words: **lipoxin; leukotriene B_4; hydroxyeicosatetraenoic acid; inflammation; neutrophils; anti-inflammatory mediators.**

Lipoxins (LXs) are products of the *trans*-cellular metabolism of arachidonate-derived mediators by lipoxygenases (LO) (1, 2). LXs are biologically active trihydroxytetraenes which play a role in numerous inflammatory diseases (3–6). Although LXA_4 and LXB_4 are involved in the stimulation of monocytes (7), they also act as endogenous anti-inflammatory mediators (8–13). Moreover they could participate to the anti-inflammatory action of the steroid-treatment in rheumatoid disease (14) since their levels increase during clinical recovery. LXs are the products of the precursor 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) which results from the usual pro-S hydrogen abstraction at C-13 position of arachidonic acid (AA) by 15-LO. Epi-LXs are derived from the precursor 15(R)-HETE, resulting from the pro-R hydrogen abstraction at C-13 position of AA by acetylated inducible cyclooxygenase 2 (COX-2) (15–17). These epi-LXs have been shown to present more potent anti-inflammatory properties than natural LXs (18–22).

5(S)-HETE, which is one of the natural precursor of LXs, was demonstrated to play a role in T-lymphocyte regulation (23), by inhibiting the pro-inflammatory 5-LO activity (24, 25), and showing some anti-inflammatory activities (26). Moreover, 15(S)-HETE is also involved in apoptotic processes (27). In contrast little is known about physiological 15(R)-HETE effects (28, 29) and nothing related to pro- or anti-inflammatory effect has been reported. 14(R),15(S)-diHETE is produced by the enzymatic hydrolysis of 15-OH LTA_4 (30) but also generated by human tracheal epithelial cells incubated with AA (31) or blood leukocytes incubated with 15(S)-HPETE (32). The physiological effects of 14(R),15(S)-diHETE involves inhibition of leukotriene B_4 (LTB_4)-induced superoxide anion generation (33) and human natural killer cell activity *in vitro* (34).

The present study will focus on the LX precursors, and we hypothesized that their entrance and their metabolization inside the cell may facilitate their targeting to the site of inflammation. Human polymorphonuclear cells (PMNs) are highly involved in inflammation (35–41). LTB_4 is a potent inflammatory mediator from the 5-LO pathway and human PMNs released high amounts of LTB_4. *In vitro* synthesis of LXs and epi-LXs was tested when PMNs were incubated with epimer precursors such as 15(S) or 15(R)-HETE, and

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diastereoisomer precursors such as 14(R),15(S) or 14(S),15(S)-di-HETE. Because compounds chirality is of considerable importance in the efficacy of therapeutic agents, we investigated potential anti-inflammatory properties of different epimers of LX precursors, and compared the modulation of *in vitro* LTB_4 release by A23187-stimulated PMN with the effects of LXA_4 itself.

METHODS

Reagents. Culture materials, nutritive medium and fetal calf serum (FCS) were obtained from Gibco. All solvents were of HPLC grade and obtained from Farmitalia Carlo Erba. Calcium ionophore A23187 was purchased from Sigma-Aldrich. LTB_4, 5(S),15(S)-diHETE, 14(R),15(S)-diHETE, 14(S),15(S)-diHETE, LXs, 15(S)-HETE, and 15(R)-HETE (stock solutions 100 μg/ml in absolute ethanol) were from Cayman Chemical Co.

Preparation of PMNs. Cells were isolated and purified from heparinized (20 U/ml) venous blood (50 ml) from volunteer subjects by centrifugation of samples at 400 g for 20 min at 20°C, over discontinuous Percoll gradient. PMNs 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 M/0.01 M/0.016 M) (pH 7.4) to lyse contaminating erythrocytes. After centrifugation, 6×10^7 to 8×10^7 PMNs were recovered. Cells were then resuspended into PBS (pH 7.4) containing $CaCl_2$ and $MgCl_2$ (final concentration 2×10^{-3} M and 0.5×10^{-3} M respectively). PMN purity was evaluated after cytocentrifugation and May–Grünwald staining and was always greater than 95%. Viability determined by the trypan blue exclusion test was greater than 90%. PMN were adjusted to 10^7 cells/ml and the cell suspensions were prewarmed at 37°C for 5 min before stimulation.

Stimulation procedures. PMNs were preincubated with vehicle (n = 33), 15(S)-HETE (n = 29), 15(R)-HETE (n = 16), 14(R),15(S)-diHETE (n = 19), 14(S),15(S)-diHETE (n = 12), or LXA_4 (n = 13) for 2 min (final concentration 3 μM) and then stimulated for 5 min at 37°C by A23187 (5 μM). The reaction was stopped by addition of ice cold methanol and the samples were stored at −70°C for further analysis. After centrifugation, aliquots of the samples were directly injected onto the analytical column.

Metabolite identification and quantification. Stored samples were directly investigated by reverse phase high performance liquid chromatography (RP-HPLC) analysis (42). RP-HPLC was carried out on a Lichrospher 100 RP-18 column (150 × 3.9 mm, 5-μm particles, Merck). LXs, LTs, and 14,15-diHETEs were eluted with methanol/water/acetic acid (65:35:0.1, v/v/v, pH 5.6) containing 0.5% EDTA as the mobile phase. They were detected by simultaneously monitoring the following wavelengths, 302 nm and 270 nm, corresponding to λ_{max} of conjugated tetraenes and trienes, respectively. Metabolites were quantified by external standard method, on the basis of molecular extinction coefficients of 55,000 and 35,000, respectively. The sensitivity threshold was 0.2 ng at 302 nm and 0.5 to 1 ng at the other wavelengths.

Statistical analysis. Results were presented as nanograms per 10^7 cells and expressed as means ± SEM. Statistical differences were determined using Wilcoxon test to compare paired samples. Significance level was set at $P < 0.05$.

Study design. Ability of human PBM to metabolize eicosanoids for the generation of LXs and epi-LXs was investigated using conditions of stimulation by A23187 with and without exogenous precursors. Simultaneously the effects of LXs and precursors on the release of LTB_4 and its metabolites were evaluated. The PMN incubations with inhibitors were proceeded using 3 μM as final concentration so that eicosanoid metabolism may be monitored using RP-HPLC UV monitoring above the threshold detection.

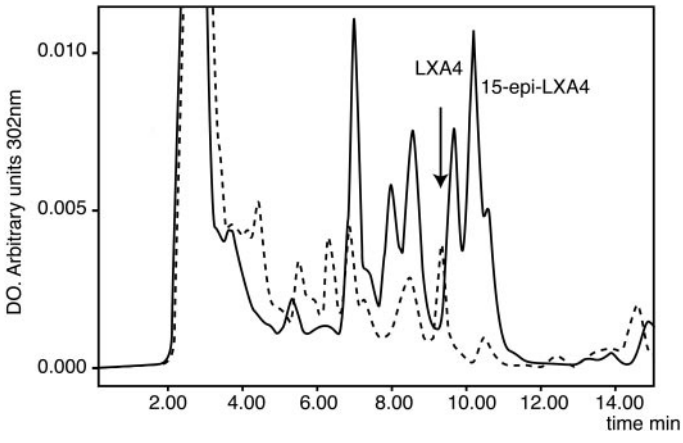

FIG. 1. RP-HPLC profiles of LXs and 15-epi-Lxs generated by human PMNs. 10^7 cells/ml were incubated for 5 min at 37°C with A23187 (5 μM) in the presence of 15(S)-HETE (dotted line) or 15(R)-HETE (full line). Aliquots of sample supernatant were directly injected on RP-HPLC column and chromatography was carried out with eluting solvent methanol/water/acetic acid (65/35/0.1 v/v/v) adjusted to pH 5.6, at a flow rate of 0.5 ml/min for 15 min and 1 ml/min from 15 to 30 min. Peaks were identified by comparison of their elution time with those of synthetic standards. The RP-HPLC chromatograms were recorded at 302 nm corresponding to conjugated tetraene λ_{max}. Chromatograms are derived from one representative experiment and the experiments were repeated several times with similar results.

RESULTS

Transformation of 15-HETEs and 14,15(S)-diHETEs

Figure 1 shows expanded scales of chromatograms recorded at 302 nm when PMNs were incubated with the epimers of 15-HETEs. LXA_4 and the all-*trans* isomers of LXA_4 and LXB_4, generated by 15(S)-HETE metabolization, were eluted between 6.5 and 10 min. 15-epi-LXA_4 and the all-*trans* isomers of 15-epi-LXA_4 and 15-epi-LXB_4, generated by 15(R)-HETE transformation, were eluted between 6.8 and 11 min. Figure 2 shows expanded scales of the chromatograms recorded at 302 nm when PMNs were incubated with the diastereoisomers of 14,15(S)-diHETEs. LXB_4, generated by 14(R),15(S)-diHETE metabolization, was eluted at 7.5 min. 14-epi-LXB_4, generated by 14(R),15(S)-diHETE transformation, was eluted at 11 min. Figure 3 reports the total amounts of LXs and epi-LXs generated by human PMNs incubated with the same final concentration of precursors. 15-epi-LXs were produced in higher amounts than natural LXs: 180 ± 19 compared to 33 ± 5 ng for 10^7 PMNs respectively ($P < 0.0001$). In contrast 14-epi-LXB_4 levels were significantly lower than LXB_4: 51 ± 13 compared to 69 ± 22 ng for 10^7 PMNs, respectively ($P < 0.001$).

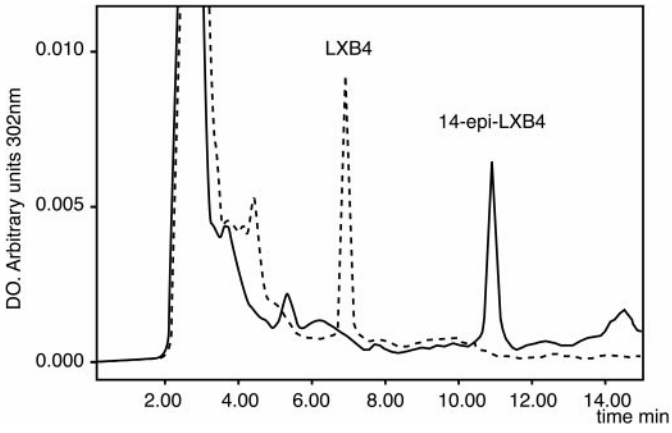

FIG. 2. RP-HPLC profiles of LXB_4 and 14-epi-LXB_4 generated by human PMNs. 10^7 cells/ml were incubated for 5 min at 37°C with A23187 (5 μM) in the presence of 14(R),15(S) or 14(S),15(S)-diHETE. Analysis conditions were the same as described for Fig. 1.

Effects of LX Precursors on LB_4 Generation

PMNs stimulated with A23187 in the absence of different LX precursor epimers biosynthesized LTB_4, 20-OH LTB_4 and LTB_4 isomers from their endogenous arachidonate pools. Figure 4 shows that the epimers of 15-HETE, diastereoisomers of 14,15(S)-diHETE and LXA_4 significantly decreased all the metabolites, except 20-OH LTB_4. Mean values of LTB_4 metabolite

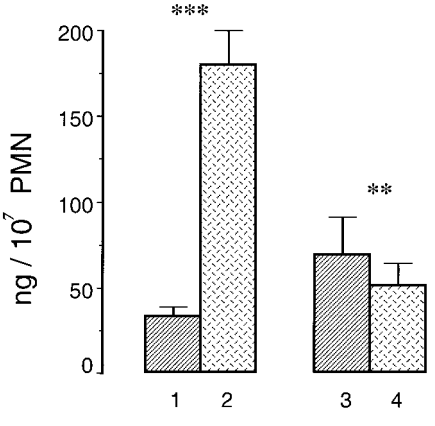

FIG. 3. LXs and epi-LXs generated by human PMNs. The cells were stimulated as described in the legend to Figs. 1 and 2. The total amounts of biosynthesized metabolites were identified by RP-HPLC as outlined in Figs. 1 and 2 and quantified by external standard method (comparison of the peak area to standard curves obtained in the same conditions of analysis on the basis of molecular extinction coefficients of 55,000 for conjugated tetraenes). Results presented as ng/10^7 cells were expressed as mean ± SEM. (**1**) LX amounts generated by incubation with 15(S)-HETE. (**2**) epi-LX amounts generated by incubation with 15(R)-HETE. (**3**) LXB_4 generated by incubation with 14(R),15(S)-diHETE. (**4**) 14-epi-LXB_4 generated by incubation with 14(S),15(S)-diHETE. Significant enhancement was observed for 15-epi-LX generation compared to that of LXs (***$P < 0.0001$). In contrast significant enhancement was observed for LXB_4 generation compared to that of 14-epi-LXB_4 (**$P < 0.001$).

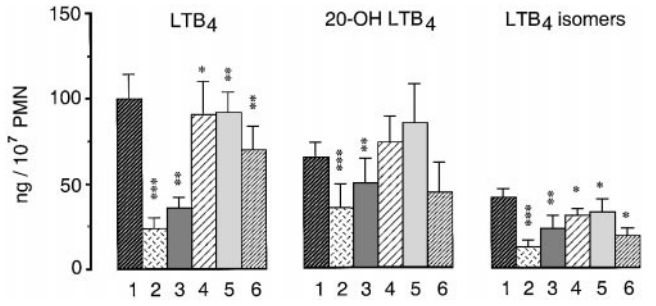

FIG. 4. Effects of HETEs and LXA_4 on LTB_4 metabolites released by human PMNs. The cells were stimulated as described above. The total amounts of biosynthesized metabolites were identified and quantified as outlined in Fig. 3. Results presented as ng/10^7 cells were expressed as mean ± SEM. (**1**) stimulation of PMNs with A23187 (5 μM) alone. (**2**) A23187-stimulation in the presence of 15(S)-HETE. (**3**) A23187-stimulation in the presence of 15(R)-HETE. (**4**) A23187-stimulation in the presence of 14(R),15(S)-diHETE. (**5**) A23187-stimulation in the presence of 14(S),15(S)-diHETE. (**6**) A23187-stimulation in the presence of LXA_4. Final concentration was 3 μM for all the inhibitors. Significant decreases of metabolite biosynthesis were observed except for the release of 20-OH LTB_4 when PMNs were incubated with 14,15(S)-diHETE and LXA_4 (***$P < 0.0001$, **$P < 0.005$, and *$P < 0.05$).

levels were expressed as ng/10^7 PMNs. Concerning LTB_4 isomers, mean values were decreased from 41 ± 5 to 12 ± 4 ($P < 0.0001$) for 15(S)-HETE, 23 ± 7 ($P < 0.005$) for 15(R)-HETE, 30 ± 4 (NS) for 14(R),15(S)-diHETE, 32 ± 8 (NS) for 14(S),15(S)-diHETE and 18 ± 5 ng ($P < 0.05$) for LXA_4. Mean values of 20-OH LTB_4 levels were significantly decreased from 65 ± 9 to 35 ± 14 ($P < 0.0001$) for 15(S)-HETE and 50 ± 14 ($P < 0.005$) for 15(R)-HETE but with the diastereoisomers 14(R),15(S) or 14(S),15(S)-diHETE, and LXA_4, mean values were no significantly different: 74 ± 15, 85 ± 23, and 44 ± 18 ng, respectively. The most dramatic decrease was observed for LTB_4 production. LTB_4 itself decreased from 100 ± 15 to 24 ± 6 for 15(S)-HETE, 36 ± 6 for 15(R)-HETE, 91 ± 20 for 14(R),15(S)-diHETE, 92 ± 26 for 14(S),15(S)-diHETE and 70 ± 14 for LXA_4. Paired experiments and the corresponding *P* values were reported in Fig. 5.

DISCUSSION

Transcellular metabolism of eicosanoid was studied incubating PMNs with mono and di-HETEs. Incubation with 15(S)-HETE led to LX pool which was constituted of natural LXA_4 and isomers of LXA_4 and LXB_4. Incubation with 15(R)-HETE led to 15-epi-LX pool including 15-epi-LXA_4 and isomers of epi-LXA_4 and epi-LXB_4. Incubation with 14(R),15(S) and 14(S),15(S)-diHETE led to LXB_4 and 14-epi-LXB_4, respectively. 15-epi-LXs were produced in higher amounts than natural LXs whereas 14-epi-LXB_4 was produced in lower amounts than LXB_4.

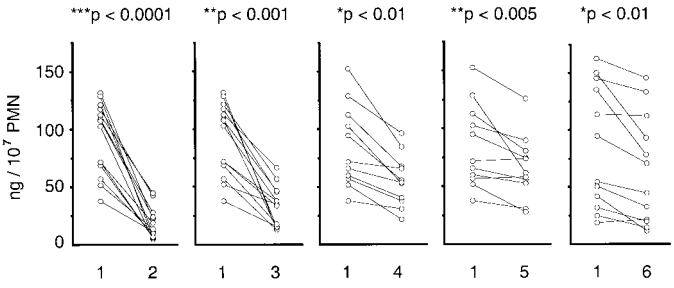

FIG. 5. Effects of 15-HETEs, 14,15-diHETEs and LXA_4 on LTB_4 released by human PMNs. Experimental conditions and graph design are the same as outlined in Fig. 4. The graph is representative of the effect of endogenous anti-inflammatory mediators on the biosynthesis of LTB_4 by human PMNs.

The present investigation describes for the first time that PMNs preferentially metabolized the non classical arachidonate-derived 15(R)-HETE rather than 15(S)-HETE and generated important amounts of epi-LXs. It is well known that 15(S)-HETE is rapidly reacylated in cellular phosphatidylinositol of human neutrophils (43) or pulmonary epithelial cells (44), and plays a role in cellular membrane remodeling (45). 15(S)-HETE is released by bronchial epithelial cells in asthma (46, 47). Therefore LXA_4 is generated by the way of *trans*-cellular metabolism (48) and released into the airways as endogenous anti-inflammatory mediator. Since higher levels of 15(R)-HETE were transformed by PMNs, 15(R)-HETE might be mainly reincorporated into phosphatidylcholine (PC) which is the preferential substrate for PLA_2. Considering that PC is the main constituent of pulmonary surfactant (49), a relation between its protecting role and the ability of PC to release 15(R)-HETE for *trans*-cellular metabolism into epi-LXs is suspected. 15(R)-HETE may be reincorporated into pulmonary surfactant to be vehicled to target cells and generate important amounts of epi-LXs. NSAID might be effective via this pathway. This model can explain why epi-LXs are more active than natural LXs to inhibit inflammatory mediators.

To estimate their anti-inflammatory properties, we assessed the action of both epimers of 15-HETEs, both diastereoisomers of 14,15(S)-diHETEs and LXA_4 on the *in vitro*-generation of LTB_4 and its metabolites released by human PMNs. A23187 stimulation alone led to LTB_4, 20-OH LTB_4 and LTB_4 isomers biosynthesis. 15(S)-HETE and 15(R)-HETE decreased the release of all LTB_4 metabolites. 14(R),15(S)-diHETE,14(S),15(S)-diHETE and LXA_4 significantly decreased LTB_4 and LTB4 isomer generation but not 20-OH LTB_4. The epimers of 15-HETE, diastereoisomers of 14,15(S)-diHETEs and LXA_4 were found to involve inhibitory effects with different potencies: 15(S)-HETE $>$ 15(R)-HETE $>$ 14,15(S)-diHETEs $=$ LXA_4. 15-HETEs were not only 5-LO substrates but also acted as 5-LO inhibitors, as reported by others for different cell types (24). 15-HETEs mainly acted by the switching-over of substrate utilization (25), whereas LXs possibly acted via a specific receptor (50–52). Nothing was known about 14,15(S)-diHETE properties, neither about its mechanism of action except that the 14(R) diastereoisomer inhibits NK cell function (34). Mono-HETEs may act as potent inhibitors, whereas di-HETEs and LXA_4 moderate inflammatory mediators, allowing the detoxification mechanism by the way of 20-OH LTB_4 release. It has been reported that the lack of LTB_4 production by PMNs, via PLA_2 inhibition, may result in the suppression of the infiltration of circulating cells into the site of inflammation, thus inducing a susceptibility to infection (53). The lack of LTC_4 synthase may be associated with neurometabolic disorders (54). Since the complete inhibition of inflammatory lipid mediators were reported to be deleterious, it is interesting that direct precursors such as di-HETEs, and LXs themselves, exert anti-inflammatory properties by modulating LTB_4 release rather than complete inhibition, so that biological effects resulting of the complete absence of LTB_4 may be avoided.

PMN, when triggered with appropriate stimulus, could not only generate endogenous anti-inflammatory mediators by the way of *trans*-cellular metabolism, but also may use their precursors for host defense via lipid mediator inhibition. The interest of this study was to demonstrate the importance of *trans*-cellular metabolism: 15-HETE metabolites are less potent inhibitors of inflammatory mediator biosynthesis but their mechanism of action may be beneficial by avoiding deleterious effects induced by complete inhibition of lipid mediators.

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