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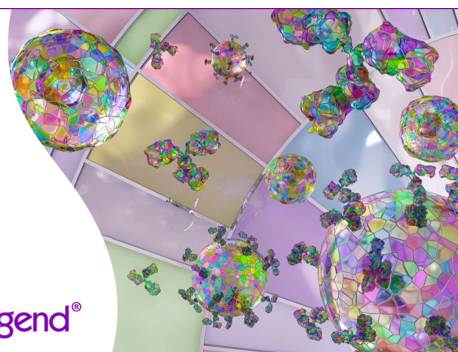

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Lipoxin A_4 and Aspirin-Triggered 15-epi-Lipoxin A_4 Inhibit Human Neutrophil Migration: Comparisons Between Synthetic 15 Epimers in Chemotaxis and Transmigration with Microvessel Endothelial Cells and Epithelial Cells[1]

Iolanda M. Fierro,[2]* Sean P. Colgan,* Giovanni Bernasconi,† Nicos A. Petasis,† Clary B. Clish,* Makoto Arita,* and Charles N. Serhan[3]*

Lipoxins (LX) are bioactive eicosanoids that can be formed during cell to cell interactions in human tissues to self limit key responses in host defense and promote resolution. Aspirin treatment initiates biosynthesis of carbon 15 epimeric LXs, and both series of epimers (LX and aspirin-triggered 15-epi-LX) display counter-regulatory actions with neutrophils. In this study, we report that synthetic lipoxin A_4 (LXA_4) and 15-epi-LXA_4 (i.e., 15(*R*)-LXA_4 or aspirin-triggered LXA_4) are essentially equipotent in inhibiting human polymorphonuclear leukocytes (PMN) in vitro chemotaxis in response to leukotriene B_4, with the maximum inhibition (~50% reduction) obtained at 1 nM LXA_4. At higher concentrations, 15-epi-LXA_4 proved more potent than LXA_4 as its corresponding carboxyl methyl ester. Also, exposure of PMN to LXA_4 and 15-epi-LXA_4 markedly decreased PMN transmigration across both human microvessel endothelial and epithelial cells, where 15-epi-LXA_4 was more active than LXA_4 at "stopping" migration across epithelial cells. Differences in potency existed between LXA_4 and 15-epi-LXA_4 as their carboxyl methyl esters appear to arise from cell type-specific conversion of their respective carboxyl methyl esters to their corresponding carboxylates as monitored by liquid chromatography tandem mass spectrometry. Both synthetic LXA_4 and 15-epi-LXA_4 as free acids activate recombinant human LXA_4 receptor (ALXR) to regulate gene expression, whereas the corresponding methyl ester of LXA_4 proved to be a partial ALXR antagonist and did not effectively regulate gene expression. These results demonstrate the potent stereospecific actions shared by LXA_4 and 15-epi-LXA_4 for activating human ALXR-regulated gene expression and their ability to inhibit human PMN migration during PMN vascular as well as mucosal cell to cell interactions. ***The Journal of Immunology,* 2003, 170: 2688–2694.**

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Lipoxins (LX)[4] are lipid-derived mediators generated at sites of vascular and mucosal inflammation where they down-regulate the further recruitment and functions of polymorphonuclear leukocytes (PMNs) (1). In humans, three main biosynthetic pathways are described for LX formation; each involves transcellular biosynthetic use of intermediates between distinct cell types that are in close proximity during inflammatory responses (1).

The more recently discovered third major pathway for generating LX was uncovered in studies of the impact of aspirin (ASA) and the actions in both the well-appreciated cyclooxygenase (COX)-2 and 5-lipoxygenase pathways during acute inflammation (2). In addition to inhibiting prostanoid biosynthesis, aspirin acetylates COX-2, switching its catalytic activity and enabling the conversion of endogenous arachidonic acid to 15*R*-hydroxyeicosatetraenoic acid (HETE) in lieu of prostanoid intermediates (3). The acetylated form of COX-2 remains active in vascular endothelial cells and mucosal epithelial cells to produce 15*R*-HETE. This novel mechanism of action for the ability of aspirin to regulate leukocyte function may explain in part the beneficial anti-inflammatory impact of aspirin (2), particularly in reducing the early PMN-mediated consequences of inflammation in vivo, as aspirin itself is not very effective at directly regulating isolated PMN responses in vitro (4). The ASA-COX-2-triggered 15*R*-HETE generated by endothelial or epithelial cells is transformed by leukocyte 5-lipoxygenase and subsequent reactions to a series of 15-epimer LXs (also termed the aspirin-triggered lipoxins (ATL)) to recognize their unique biosynthetic origins in vivo. ATL are the endogenous 15*R* enantiomers of lipoxin A_4 (LXA_4) and LXB_4, respectively, and to date they appear to share many of the bioactivities evoked by LX, including dampening PMN-dependent acute inflammation and reperfusion-initiated second organ injury (5, 6). The carbon 15 epimer denoted ATL is ~50% less effectively enzymatically converted to the biologically inactive group

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[4] Abbreviations used in this paper: LX, lipoxin; ASA, aspirin; ATL, aspirin-triggered 15-epi-lipoxins; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; LC/MS/MS, liquid chromatography tandem mass spectrometry; LXA_4, lipoxin A_4; 15-epi-LXA_4, 15 epimeric LXA_4; PMN, polymorphonuclear leukocytes; LTB_4, leukotriene B_4; ALXR, LXA_4 receptor; HEK293, human embryonic kidney 293.

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of 15-oxo-metabolites than native LXA_4 (7). This finding indicates that the dehydrogenation step at the carbon 15 position is highly stereospecific and suggested that when ATL are generated in vivo their biologic half life can be increased by about 2-fold greater than that of native LXA_4, which carries its alcohol at the carbon 15 position in the *S* configuration (6, 7).

The predominant hydroperoxide fatty acid products of most major human lipoxygenases carry oxygen in the *S* configuration as a result of the ability of the enzyme to stereoselectively abstract hydrogen in an antarafacial mechanism (reviewed in Refs. 6 and 8). The importance of stereochemistry in these and related lipid-derived mediators (i.e., small molecules) is most clearly illustrated by the results obtained with the different biologically generated isomers of leukotriene B_4 (LTB_4) (9). The stereoisomers of LTB_4 are 100-1000 times less potent than the natural LTB_4 in three different biological assays (10). Also, the platelet lipoxygenase product 12*S*-HETE and its *R* enantiomer that can be generated by either a 12*R*-lipoxygenase or a P450 mechanism (11) serve as another interesting case study for the importance of chirality in evoking the actions of lipid mediators. The 12*R*-HETE enantiomer proved to be a more potent neutrophil agonist than 12*S*-HETE in stimulating calcium mobilization and polymerization of actin (12). Using human PMN, Bittleman and Casale (13) drew attention earlier to the importance of the 5*R* and 5*S* enantiomers of 5-HETE and demonstrated that the *R* form is significantly more potent than 5*S*-HETE in stimulating neutrophil transmigration through endothelial and epithelial barriers (13). Hence, chirality of alcohol groups in eicosanoids contributes to the potency of these molecules.

In this work we report that exposure of PMN to nanomolar concentrations of either LXA_4 or its natural 15 epimer (15-epi-LXA_4) generated with aspirin treatment leads to inhibition of isolated PMN chemotaxis as well as transendothelial and transepithelial PMN migration. Both synthetic LXA_4 and 15-epi-LXA_4 proved to be essentially equipotent inhibitors at 1 nM, although each gave a different pattern of action particularly as their corresponding carboxy methyl esters, which were sharply concentration-dependent.

Materials and Methods

Human PMN chemotaxis

PMN from healthy volunteers were obtained as originally described (14), with modifications (7), and suspended in RPMI 1640 (BioWhittaker, Walkersville, MD) at 1×10^6 cells/ml. LTB_4 (Cayman Chemicals, Ann Arbor, MI) at 10 nM or vehicle was added to the lower wells of a 48-well chemotaxis chamber (NeuroProbe, Cabin John, MD). The wells were overlaid with a 5-μm pore size polycarbonate filter. PMN (50 μl) were placed in the upper wells, and the chamber was incubated (37°C, 5% CO_2) for 1 h. Following incubation, filters were removed, scraped of cells from the upper surface, fixed, and stained with Diff-Quik (Dade Behring, Newark, DE). For each incubation, performed in triplicate, cells that migrated across the filter toward the lower surface were enumerated by light microscopy. Four distinct fields were counted at high magnification. To assess inhibition, PMN were suspended in RPMI medium with vehicle or increasing concentrations of the eicosanoid (native LXA_4, LXA_4 methyl ester, 15-epi-LXA_4, or 15-epi-LXA_4 methyl ester) and incubated for different time intervals at 37°C before placement in the chamber.

Transmigration

PMN transepithelial and transendothelial migration was performed as described in Refs. 7 and 15. Briefly, human PMN were isolated from healthy volunteers and suspended in modified HBSS (without Ca^{2+} and Mg^{2+}, with 10 mM HEPES, pH 7.4; Sigma-Aldrich, St. Louis, MO) at 5×10^7 cells/ml. Before addition of PMN, either the T_{84} intestinal epithelial monolayers or human microvascular endothelial cell monolayers were extensively rinsed in HBSS to remove residual serum components. PMN were pre-exposed to synthetic LX (native LXA_4, LXA_4 methyl ester) or 15-epi-LX (15-epi-LXA_4 or 15-epi-LXA_4 methyl ester) for 15 min at 25°C. Transmigration was initiated by the addition of PMN (40 μl) to HBSS (containing Ca^{2+} and Mg^{2+}, 160 μl) in the upper chambers and chemoattractant (100 nM or 1 μM LTB_4 in HBSS for endothelia or epithelia, respectively) was added to the opposing (lower) chambers. PMN were not washed free of LXA_4, 15-epi-LXA_4, or their carboxyl methyl ester analogs before addition to monolayers, and thus 5-fold dilutions of synthetic LX were present during the transmigration incubations. PMN (1×10^6) were added at time 0, and transmigration was allowed to proceed for 60 or 120 min for endothelia and epithelia, respectively. All experiments were performed in a 37°C incubator room to ensure that endothelial/epithelial monolayers, solutions, plasticware, etc. were maintained at uniform temperature.

The number of PMN transmigrated was quantitated by assessing the PMN azurophilic granule marker myeloperoxidase as in Ref. 7. Following each transmigration assay, nonadherent PMN were extensively washed from the surface of the monolayer, and PMN cell equivalents estimated from a calibration curve were assessed as the number of PMNs that had completely traversed the monolayer (i.e., across the monolayer into the reservoir bath).

LXA_4 and 15-epi-LXA_4 methyl ester conversion

All incubations were stopped (60 min, 37°C) with the addition of 2 vol of cold methanol containing deuterium-labeled LTB_4 as the internal standard for extraction recovery. Samples were extracted with solid-phase C18 cartridges, and resulting methyl formate eluants were taken to dryness with a stream of N_2 and resuspended in methanol (100 μl) (16) before liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis as in Ref. 17. LXA_4, 15-epi-LXA_4, and their corresponding methyl esters were prepared by total organic synthesis, qualified as reported in Ref. 7, and then used throughout. Formation of the free acids was monitored by LC/MS/MS because the methyl esters did not give a molecular ion minus hydrogen fragmentation, and both methyl esters and free acids gave characteristic UV absorbance for the conjugated tetraene present and were used by quantitation.

Luciferase reporter assay

Human embryonic kidney (HEK)293 cells were seeded into 24-well plates at the density of 1×10^5 cells per well and cultured overnight in DMEM-10% FCS before transfection. A total of 0.05 μg of NF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA) was cotransfected with 0.4 μg of expression plasmid pcDNA3 (Invitrogen, San Diego, CA) encoding human LXA_4 receptor (ALXR) or mock using Superfect transfection reagent (Qiagen, Valencia, CA). At 24 h after transfection, the cells were incubated with LXA_4 or LXA_4 methyl ester for 30 min and then exposed to TNF-α (1.0 ng/ml) for 5 h. Luciferase activity was measured by the Dual-Luciferase reporter assay system (Promega, Madison, WI).

Statistical analysis

Values are presented as mean ± SEM. Comparisons among groups were performed by ANOVA. For all analyses, findings were considered statistically significant and reported when $p < 0.05$.

Results

*Contribution of 15*R *vs 15*S*-hydroxyl chirality for LX and ATL in inhibition of human PMN chemotaxis*

LXA_4 and 15-epi-LXA_4 each inhibit both PMN adhesion and transmigration (2, 7), indicating that a systematic side-by-side analysis of their actions was deemed necessary to determine the relative contribution of the alcohol chirality at carbon 15. To this end, the activities of LXA_4 and 15-epi-LXA_4 were directly compared with each other as their native carboxylic acids or their corresponding carboxy methyl ester forms with compounds prepared by total organic synthesis. Incubation of PMN with increasing concentrations (0.01–100 nM) of LX (30 min, 37°C) resulted in a concentration-dependent inhibition of cell migration to the chemoattractant LTB_4 (Fig. 1). The inhibition curves for both sets of compounds were essentially identical within the 0.01–1.0 nM concentration range, with a maximum inhibition (~50%) achieved at 1 nM. At concentrations of LXA_4 >1 nM, there was a statistically significant reduction in the inhibitory activity of the native LXA_4 (a bell-shaped dose-response curve was obtained). In contrast, 15-epi-LXA_4 showed a different pattern of action with isolated human PMN that displayed a maximal inhibition at 1 nM (Fig. 1*A*). When added as its corresponding methyl ester, 15-epi-LXA_4 methyl ester

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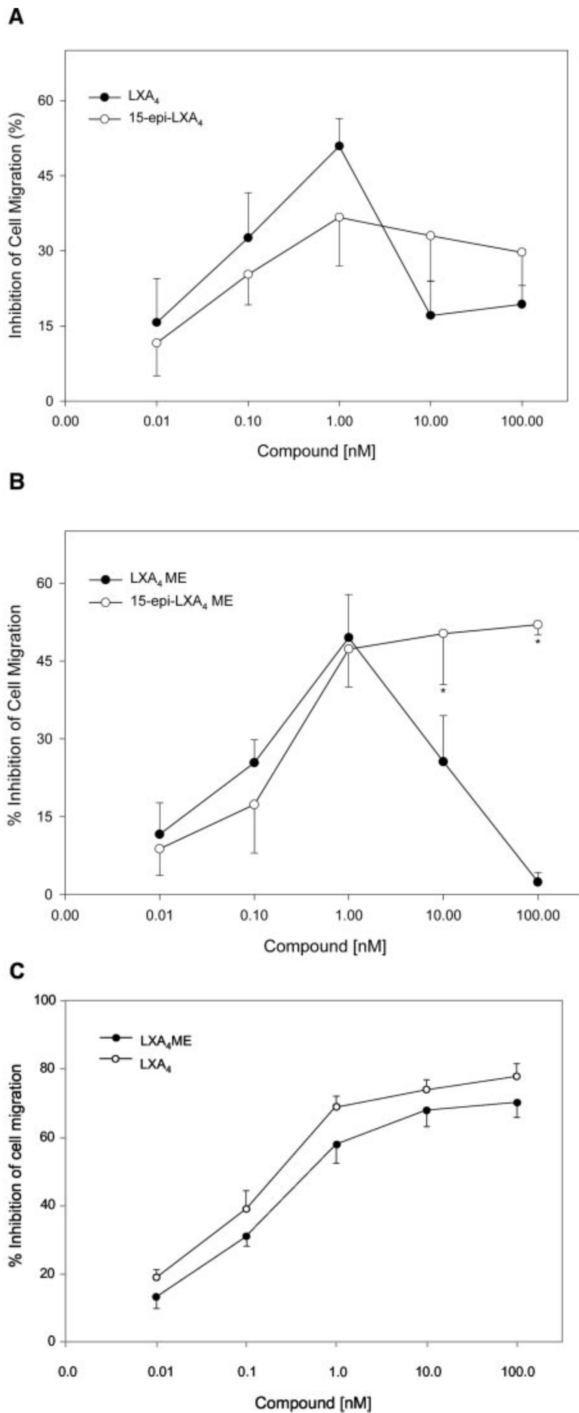

FIGURE 1. Direct comparison of the inhibitory actions of synthetic LXA$_4$ and 15-epi-LXA$_4$ on PMN chemotaxis. Human PMN were incubated with indicated concentrations of synthetic LXA$_4$ or 15-epi-LXA$_4$ for 30 min at 37°C before placement in the chemotaxis chamber. *A*, Percent inhibition for LTB$_4$- (10 nM) induced PMN chemotaxis by synthetic compounds as their carboxylic acids. *B*, Percent inhibition for LTB$_4$- (10 nM) induced PMN chemotaxis by their respective carbon position 1 carboxyl methyl esters. *C*, Percent inhibition for fMLP- (10 nM) induced PMN chemotaxis by LXA$_4$-free acid and methyl ester. Results are the mean ± SEM of three separate experiments performed in triplicate. The p values were <0.05 when compared with PMN exposed to chemoattractant with vehicle.

showed inhibition activity similar to its free acid. In contrast, LXA$_4$ methyl ester was less potent than its free acid at higher concentration (100 nM) (Fig. 1*B*). Essentially similar results were obtained using *N*-formylated peptide fMLP as a chemotactic agent, in that LXA$_4$-free acid showed a greater trend for increased potency than LXA$_4$ methyl ester (Fig. 1*C*).

Next, a time course for PMN exposure to the four synthetic compounds was performed (Fig. 2). The inhibition of PMN migration by 1 nM of LXA$_4$ and 15-epi-LXA$_4$ was time dependent with a maximum observed within 15 min (Fig. 2*A*). Each of their respective corresponding methyl esters gave a similar degree of inhibition (Fig. 2*B*).

Direct comparison of synthetic LX and ATL inhibition of PMN transendothelial and transepithelial migration

PMN exposed to LXA$_4$ and 15-epi-LXA$_4$ (0.01–100 nM) markedly decreased PMN transmigration induced by LTB$_4$ (Fig. 3). At concentrations of 1 nM or greater, 15-epi-LXA$_4$ showed a greater trend for increased potency than LXA$_4$ in inhibiting PMN transepithelial migration. Hence, with the two biologically relevant cell surfaces, namely epithelial or endothelial cells, both LXA$_4$ and 15-epi-LXA$_4$ inhibit PMN transmigration with an apparent potency difference observed between 15-epi-LXA$_4$ vs LXA$_4$ with epithelial cells.

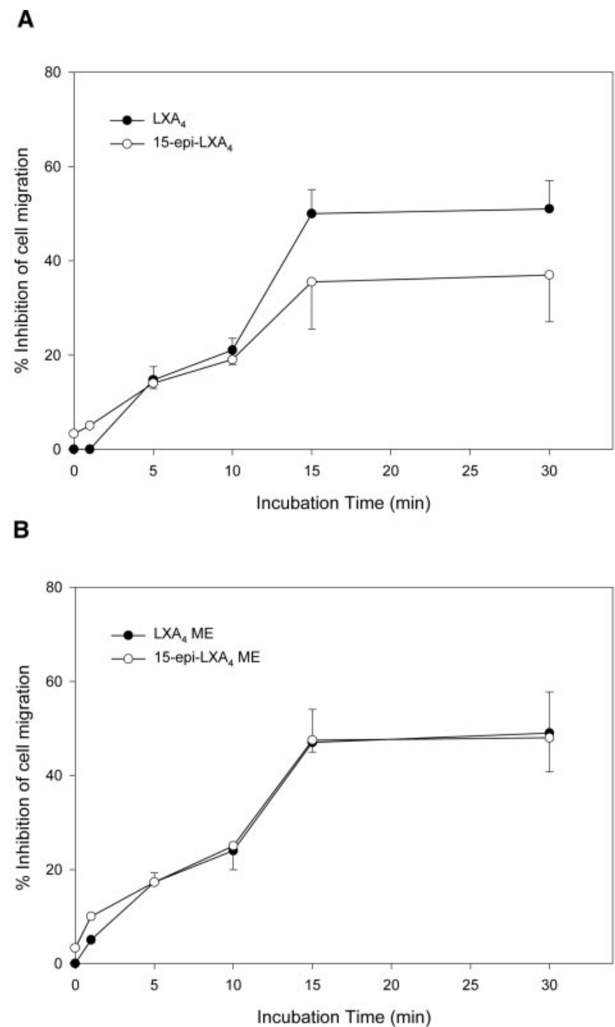

FIGURE 2. Time-dependent inhibition: LXA$_4$ vs 15-epi-LXA$_4$ inhibition of LTB$_4$-stimulated PMN chemotaxis. Human PMN were incubated with either LXA$_4$ (1 nM) or 15-epi-LXA$_4$ (1 nM) for different time intervals at 37°C before placement in the chemotaxis chamber. The synthetic C15 epimers were tested as their respective carboxylic acids (*A*) vs their position 1 carboxyl methyl esters (*B*). Results are the mean ± SEM of three separate experiments performed in triplicate.

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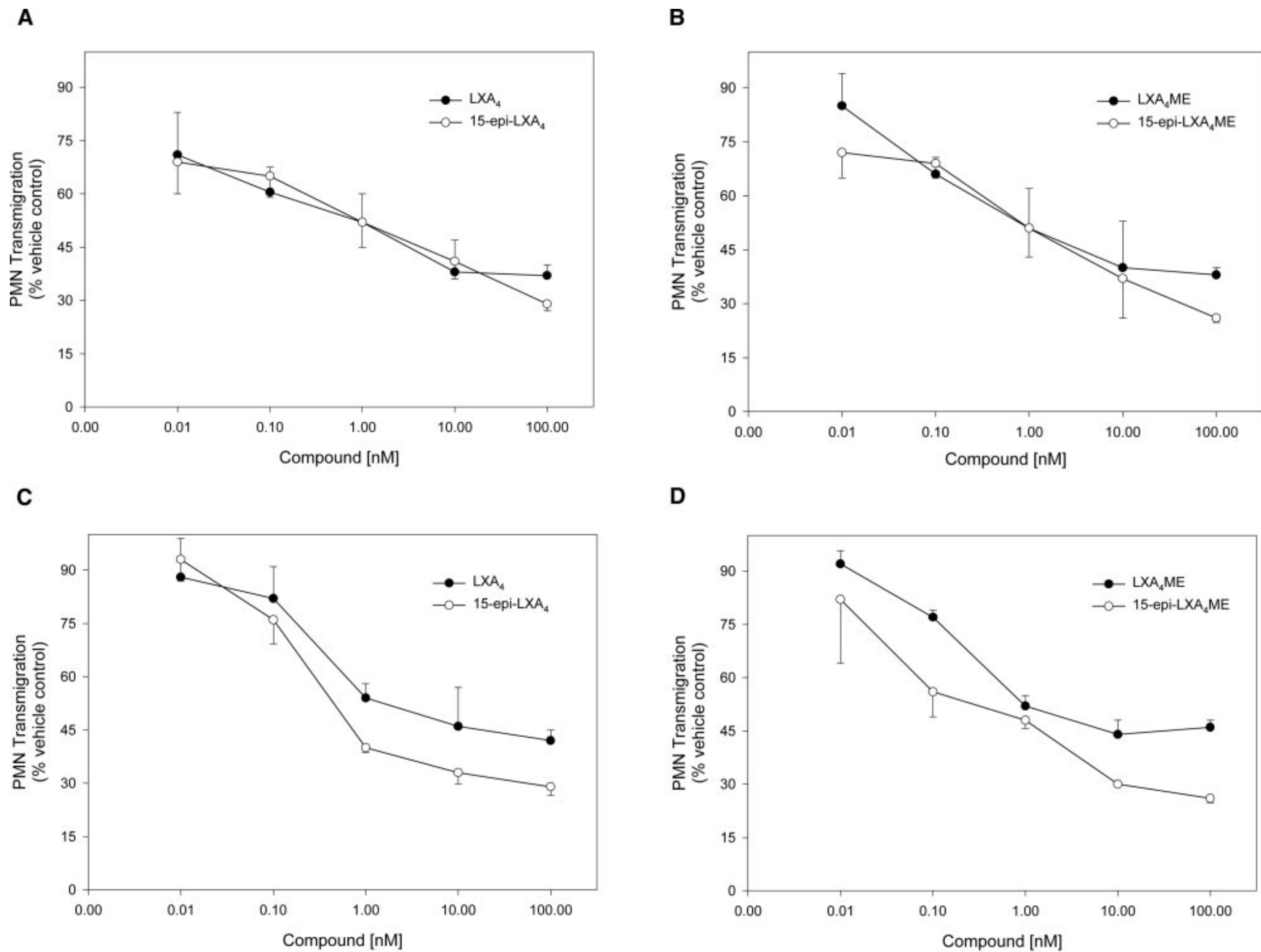

FIGURE 3. LXA_4 and 15-epi-LXA_4 inhibit PMN transmigration. LXA_4 and 15-epi-LXA_4 each gave concentration-dependent inhibition of LTB_4-stimulated PMN transmigration across microvessel endothelial (*A* and *B*) and epithelial (*C* and *D*) monolayers. Both series of compounds were directly compared as their carboxylic acids with their respective position 1 carboxyl methyl ester denoted LXA_4-Me and 15-epi-LXA_4-Me. PMN were exposed to LX for 15 min at 37°C. Transmigration was assessed by quantitation of PMN myeloperoxidase (see *Materials and Methods*). Results are the mean ± SEM of transmigration for three separate experiments for each panel.

Conversion of LXA_4 and 15-epi-LXA_4 methyl esters to their corresponding carboxylic acid: cell type-dependent conversion

PMN or T_{84} epithelial monolayers were each incubated with LXA_4 methyl ester or 15-epi-LXA_4 methyl ester (100 nM), because both are potent inhibitors of PMN recruitment in vivo (18, 19), to determine whether these cells can differentially convert the methyl esters to their corresponding free acids. Table I reports the percent conversion of the methyl ester for each cell type to its corresponding carboxyl-free acids as monitored by LC/MS/MS (see Fig. 4). When compared directly, the carboxyl methyl ester of 15-epi-LXA_4 gave approximately three times less than the conversion of LXA_4 methyl ester. These results indicate that the carboxyl methyl ester of 15(*R*) epimeric LXA_4 was not as readily converted as was LXA_4 methyl ester to its corresponding acids and illustrates the stereospecific conversion of this pair of epimers as their corresponding carboxyl methyl esters. Of interest, the methyl ester of ATL analogs proved a potent inhibitor of PMN recruitment in vivo in human and murine whole blood (cf Refs. 16 and 18) and is rapidly hydrolyzed to carbon 1 position free carboxylic acids in vivo (19).

Table I. *Differential conversion of carboxyl methyl esters of the C15 epimer*[a]

Cell Types	LXA_4-Me	15-epi-LXA_4-Me
PMN	70.7	24.2
T_{84} epithelia cells	11.5	23.2
PMN plus T_{84} epithelia cells	97.5	30.4

[a] Cells were incubated with either synthetic LXA_4 methyl ester or 15-epi-LXA_4 (100 nM; 60 min; 37°C) and percent loss was determined following extractions and LC/MS/MS analysis (see *Materials and Methods* and Fig. 4) to quantitate the respective free acids at m/z = 351. Results are representative of direct comparisons with duplicate incubations for each cell type and coincubation.

LXA_4 activates recombinant ALXR-regulated gene expression: dramatic reduction in potency with LXA_4 methyl ester

LXA_4 interacts specifically with its cognate G protein-coupled receptor, denoted ALXR (Ref. 20; for review see Ref. 21). LXA_4, its stable analogs, and their corresponding methyl esters have been shown to be potent inhibitors of leukocyte recruitment in vivo, and the carboxyl methyl ester of LXA_4 as noted (vide supra) is rapidly converted when administered i.v. systemically to its corresponding free acid (19). LXA_4 mediates its inhibitory actions by acting as an agonist at its receptor to regulate leukocyte responses. To determine whether there was a difference between LXA_4 receptor interactions when presented to cells in vitro as its free acid vs its corresponding methyl ester, we set up a reporter gene assay using HEK293 cells transiently transfected with human ALXR. Similar

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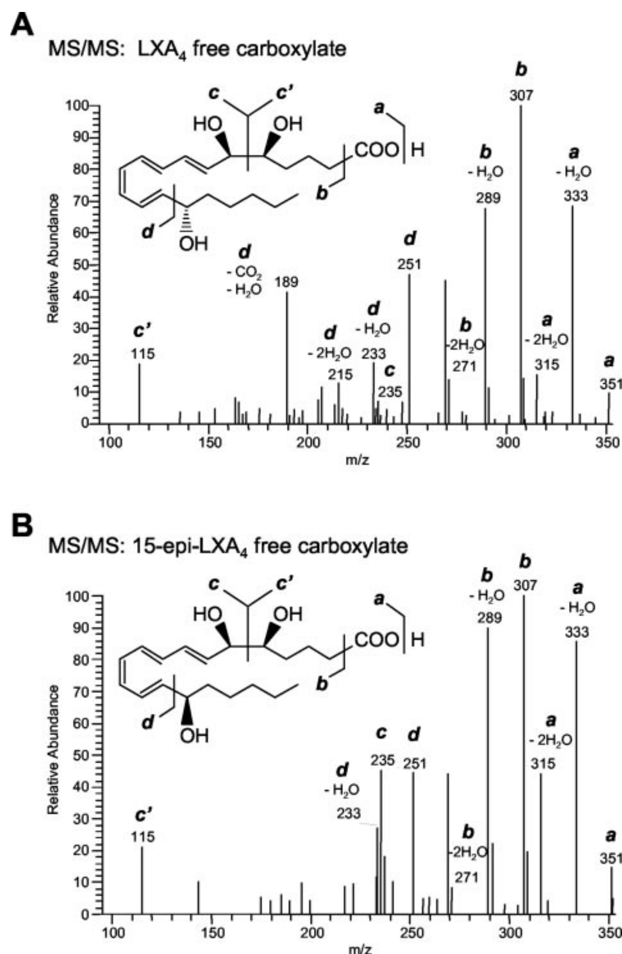

FIGURE 4. LC/MS/MS identification of LXA$_4$ and 15-epi-LXA$_4$. Following the incubation of LXA$_4$-Me or 15-epi-LXA$_4$-Me to cells using the conditions in Table I, products of interest were extracted and analyzed by LC/MS/MS using deuterium-labeled LTB$_4$ as the internal standard to calculate recovery and for normalization as in Ref. 17. *a-c* and *c′* denote prominent diagnostic ions used for identification (see *inset*). *a*, (molecular ion minus hydrogen) = *m/z* 351; *b*, -H-CO_2; *c′*, *m/z* = 115 (from carbon 1 through C5 fragmentation). *A*, MS/MS spectrum of LXA$_4$. *B*, MS/MS spectrum of 15-epi-LXA$_4$. This C15 epimer gave similar fragmentation with different relative intensities and retention time compared with LXA$_4$. The presence of m/z 351 in both spectra indicates the conversion of both LXA$_4$ and 15-epi-LXA$_4$ methyl esters to their respective free acids.

to the results obtained recently with HeLa cells expressing ALXR (22), this system permitted the direct assessment of TNF-α-stimulated NF-κB activation and its potential regulation by recombinant ALXR interactions with its lipid ligand LXA$_4$ in its native vs methyl ester form. Results in Fig. 5*A* demonstrate a sharp difference between the ability of LXA$_4$ methyl ester to activate, in a receptor-dependent fashion, the inhibition of NF-κB signal transduction vs the native form of LXA$_4$, namely as the corresponding carboxylic acid. Moreover, increasing amounts of LXA$_4$ methyl ester competed with the actions of LXA$_4$-free acid on NF-κB regulation (Fig. 5*B*). It was previously reported that radiolabeled LXA$_4$ methyl ester competes as effectively for LXA$_4$ recognition and specific binding to the recombinant ALXR and with human PMN as the carboxylic acid at 4°C (23, 24). These results demonstrate that the carboxylate LXA$_4$ is a preferred ligand to activate its receptor, and LXA$_4$ methyl ester acts as a partial antagonist by competing for receptor binding with LXA$_4$-free acid with apparently equal affinity. The carboxy methyl ester of LXA$_4$ transmits only a partial signal to inhibit NF-κB activation at 37°C. The carboxyl-free acid of 15-epi-LXA$_4$ gave results similar to those of LXA$_4$-free acid (results not shown). This is of particular interest because the methyl esters of LX and ATL are bioactive in vivo (18, 19) and increase the chemical stability and bioavailability of these compounds (18). Together, these findings indicate that LXA$_4$ and 15-epi-LXA$_4$ as their native carboxyl-free acids are the preferred ligands for activation of the ALXR and suggest that pharmacologic addition of LXA$_4$ methyl ester and/or its analogs as carboxyl methyl esters in vitro and in vivo models (19) requires conversion to free acid to evoke receptor-mediated gene regulation and biologic actions.

Discussion

At sites of acute inflammation, PMN function may be regulated by a diverse range of endogenous inflammatory signals, including both protein- and lipid-derived signals. It is well appreciated that LX and ATL display selective actions on leukocytes that include inhibition of neutrophil chemotaxis (25) and transmigration between and across both endothelial and epithelial cell monolayers (7). In this report, we evaluated the impact and contribution of synthetic LXA$_4$ and 15-epi-LXA$_4$ stereochemistry in PMN migration and activation of recombinant receptor ALXR in isolated cell systems in vitro. Both C15 epimers regulated PMN migration and were essentially identical in potency when directly compared within a physiologically relevant concentration range (i.e., <1 nM). At concentrations >1 nM, 15-epi-LXA$_4$ (i.e., 15(*R*)-epimer) in its aspirin-triggered form found in vivo (17) proved more potent than LXA$_4$ at inhibiting human PMN chemotaxis and PMN transepithelial migration. Differences between eicosanoid enantiomers with human leukocytes were also described for the carbon 5 position alcohol in 5-HETE, with the *R* isomer being significantly more potent as a stimulus of chemotaxis in vitro (13). Earlier studies addressed the contributions of tetraene geometry to the actions of LXs and found that they are highly stereospecific because 7-*cis*, 11-*trans*-LXA$_4$, which differs only in the geometry of two double bonds from native LXA$_4$, is more active than LXA$_4$ in stimulating the formation and release of PGI$_2$ by endothelial cells (26). Hence, the stereochemical requirements for LXs, as in other eicosanoids, are highly specific.

Along these lines, results using recombinant enzyme (7) indicated that the chirality of the alcohol at C-15 was important for optimal conversion, because 15-epi-LXA$_4$ gave only approximately one-half the rate of conversion of LXA$_4$ to its biologically inactive 15-oxo-LXA$_4$ metabolite. This finding implies that 15-epi-LXA$_4$, once generated in vivo when aspirin is used, might not be enzymatically inactivated as rapidly as LXA$_4$ and thus can exert a longer biological half life. Indeed, the aspirin-triggered epimer 15-epi-LXA$_4$ is more potent in blocking the PMN adhesion step with isolated cells in vitro than native LXA$_4$ (2, 7).

The presence of a methyl ester at the carbon 1 position instead of the natural form carboxylic acid group of LXA$_4$ (i.e., LXA$_4$-Me) enhanced its rate of conversion by isolated recombinant dehydrogenase (7). In the present experiments, we found that both PMN and T$_{84}$ epithelial cells efficiently convert both LXA$_4$ methyl ester and 15-epi-LXA$_4$ methyl ester to their corresponding carboxylic acids (Table I) identified by LC/MS/MS (Fig. 4). This conversion likely involves esterases that apparently are highly active and hydrolyze the methyl esters to carboxylic acids when both cell types are in contact (Table I). Also of interest, PMN were the most effective cell type, and 15-epi-LXA$_4$ methyl ester was converted to a lesser extent than native carbon 15 *S*-containing LXA$_4$ methyl ester, which may contribute to the long-lasting and more potent

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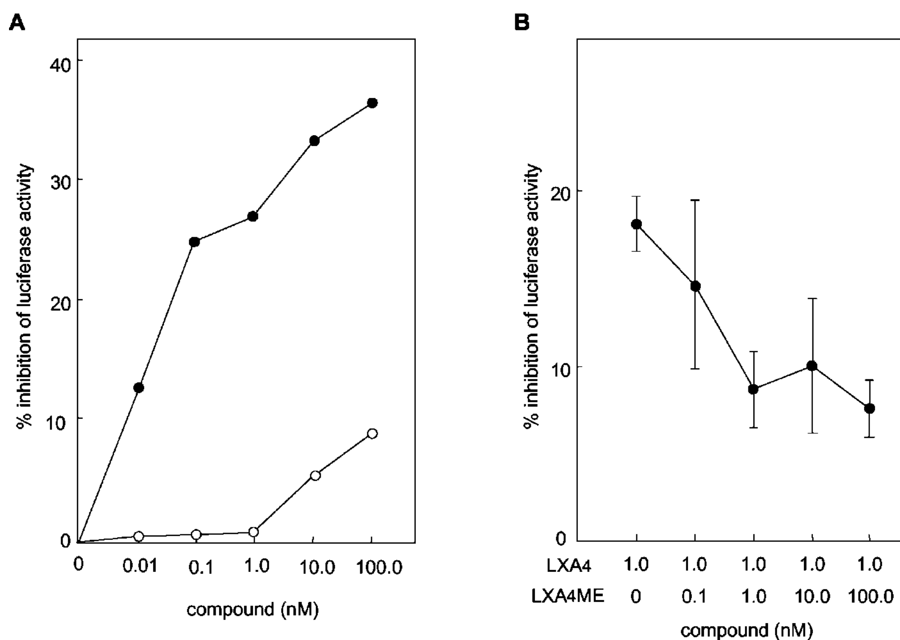

FIGURE 5. Comparison of the inhibitory actions of LXA_4 vs LXA_4 methyl ester in TNF-α-mediated NF-κB-luciferase gene expression. *A*, HEK293 cells were transiently transfected with human ALXR and κB-luciferase reporter plasmid. Cells were treated with indicated concentrations of LXA_4 (●) or LXA_4 methyl ester (○) for 30 min and then stimulated with TNF-α (1.0 ng/ml) for 5 h, and luciferase activity was measured. Results are representative of three separate experiments performed in duplicate. *B*, HEK293 cells transfected with human ALXR and κB-luciferase reporter plasmid were incubated for 30 min with LXA_4-free acid (1.0 nM) in the presence of indicated concentrations of LXA_4 methyl ester and were then stimulated with TNF-α (1.0 ng/ml) for 5 h, and luciferase activity was measured. Results are the mean ± SEM of four experiments.

actions of this epimer and related stable analog structures observed in murine studies in vivo (see Ref. 18; reviewed in Ref. 6).

The bioactions of LXA_4 and 15-epi-LXA_4 are specifically transduced in leukocytes by interacting with a high-affinity-specific LXA_4 receptor that recognizes both epimers (18). In the present experiments, incubation of PMN with either ATL or LXA_4 (from 0.1 to 100 nM at 37°C) did not affect subsequent specific binding of labeled LTB_4 to its high affinity receptor (not shown, similar for two separate PMN donors in duplicate), indicating that prior exposure of human PMN to LXA_4 or ATL did not merely downregulate the B_{max} for leukotriene B_4 receptors on these cells. Hence, it is of particular interest that recombinant human ALXR, when activated in a ligand-dependent fashion, regulated NF-κB signaling with TNF-α as an agonist. Both synthetic LXA_4 and 15-epi-LXA_4 were effective ligands in this system with recombinant human ALXR to regulate gene expression. However, the LXA_4 methyl ester proved to be far less effective if not devoid of activity in vitro for activating LXA_4 receptor-mediated gene expression. Moreover, increased concentration of LXA_4 methyl ester competed with the effect of active agonist LXA_4-free acid by 50% at maximum, suggesting that the methyl ester form of LXA_4 acts as a partial antagonist for ALXR. These results suggest that receptor activation in vivo with stable analog mimetics of LX and ATL requires conversion of carbon 1 position carboxyl methyl esters to corresponding free acids. In view of earlier findings, radiolabeled LXA_4 methyl ester binds effectively at 4°C to the ALXR and competes with carboxyl-free acid as effectively as the methyl ester (22). These results, taken together with the present results, indicate that, although recognized by the ALXR, the methyl ester ligand of LXA_4 as a ligand receptor pair is not as productive to regulate gene expression as the carboxyl-free acid or the native form of LXs and ATL. These results are in line with earlier findings with PG receptors (27) and the LTB_4 receptor (28, 29), which require carboxyl-free acids for individual eicosanoids to interact specifically with their corresponding G protein-coupled receptors. In vivo, topical application of methyl esters of LXs and their stable analogs as well as the ATLs proved to be very effective for delivery. These methyl esters are converted to the corresponding free acids that are very likely in view of the present results to be responsible for the in vivo gene expression regulation and leukocyte trafficking impact (17–19).

In summary, LXA_4 and 15-epi-LXA_4 each stop PMN chemotaxis in vitro as well as transmigration in vitro in a stereospecific fashion. These results are consistent with and extend those reported earlier (Refs. 2, 7, and 18; for review see Refs. 1 and 6) and indicate that human PMN responses to carboxyl methyl esters of LX and ATL are stereospecific at pharmacologic levels, i.e., >1 nM in vitro. This inhibitory activity appeared to be dependent in part on the degree of conversion of these respective carboxyl methyl esters. These findings provide a basis for assessing the relative contribution of the two separate biosynthetic pathways, namely for LX and the aspirin-triggered COX-2 pathway, in regulating PMN responses of interest in host defense, inflammation resolution, and tissue injury. It is noteworthy that the free acid form of LXA_4 and ATL stimulates human recombinant ALXR to block NF-κB-mediated gene expression. The LXA_4 methyl ester was not able to fully activate this receptor but does specifically compete at the ligand binding sites (22) and proved to serve as a partial receptor antagonist. Together, these findings indicate the contribution of the position 1 carboxyl methyl ester to the potency of LX and ATL with ALXR activation in vivo, which can now be subject to design modifications and therapeutic potential of these compounds as well as their systemic delivery to specific tissue sites in vivo. Moreover, they provide the first evidence for specific ALX receptor antagonists.

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