Design of Lipoxin A_4 Stable Analogs That Block Transmigration and Adhesion of Human Neutrophils†

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ABSTRACT: Lipoxins (LX) are bioactive eicosanoids that carry a tetraene structure and serve as regulators of inflammation, in part by inhibiting neutrophil migration and adhesion. Lipoxin A_4 is rapidly regulated by conversion to inactive LX metabolites via local metabolism that involves dehydrogenation as the predominant route. Here, several LXA_4 analogs were designed that resisted rapid conversion by both differentiated HL-60 cells and recombinant 15-hydroxyprostaglandin dehydrogenase, systems where native LXA_4 is degraded within minutes. The rank order of conversion by recombinant dehydrogenase was LXA_4 methyl ester > PGE_2 ≈ PGE_2 methyl ester > LXA_4 >>> the novel LXA_4 analogs. In addition, 15(*R/S*)-methyl-LXA_4, 15-cyclohexyl-LXA_4, and 16-phenoxy-LXA_4 proved to retain LXA_4 bioactivity and inhibited neutrophil transmigration across polarized epithelial cell monolayers as well as adhesion to vascular endothelial cells. These results indicate that LXA_4 analogs can be designed using these criteria to resist rapid transformation and to retain biological actions of native LXA_4. Moreover, the results suggest that LXA_4 stable analogs can be useful tools both *in vitro* and *in vivo* to evaluate LXA_4 actions and therapeutic potential.

Eicosanoids play important roles in regulating essential multicellular processes including inflammation, thrombosis, and tissue repair. Lipoxins (LX) are a specific series of eicosanoids whose members carry a conjugated tetraene structure (Samuelsson et al., 1987). They are generated by unique pathway assemblies that can involve cell−cell interactions and/or transcellular biosynthetic routes, and compounds of this series have recently been shown to display intriguing new biological actions. In mammals, lipoxins are produced in hematologic, vascular, pulmonary, and renal tissues at sites where they appear to exert potent actions [for recent review, see Serhan (1994)]. Their disease association in humans led to the proposal that the LX are potential biomarkers of asthma (Chavis et al., 1995) and rheumatoid arthritis (Thomas et al., 1995).

One member of the lipoxins, namely LXA_4,[1] has been studied in both *in vitro* and *in vivo* models, where it displays actions consistent with a vasodilatory and counterregulatory role. This is in sharp contrast to the actions of most other lipid-derived mediators that are considered primarily "proinflammatory" mediators (Serhan, 1994). LXA_4, for example, stimulates cytokine-mediated myeloid colony formation (Stenke et al., 1991) and inhibits both neutrophil and eosinophil chemotaxis *in vitro* in the nanomolar concentration range (Lee et al., 1989, 1991; Soyombo et al., 1994). In coincubations, LXA_4 inhibits PMN transmigration across epithelial cells (Colgan et al., 1993) and endothelial cell monolayers (Papayianni et al., 1994). Such actions are also demonstrable *in vivo*, where LXA_4 is vasodilatory (Badr et al., 1989; Dahlén, 1989; Hedqvist et al., 1989), blocks both PMN diapedesis from postcapillary venules (Raud et al., 1991), and inhibits PMN entry in inflamed renal tissues during glomerulonephritis in animal models (Papayianni et al., 1995; Mayadas-Norton et al., 1995). A potential important role for LXA_4 in humans is emphasized by the finding that LXA_4, administered via inhalation, blocks airway

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[1] Abbreviations: DPBS, Dulbecco's phosphate-buffered saline; $DPBS^{2-}$, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}; 15-OH-PGDH, 15-hydroxyprostaglandin dehydrogenase; FMLP, *N*-formylmethionylleucylphenylalanine; HL-60 cells, human acute promyelocytic leukemic cell line; leukotriene B_4 (LTB_4), 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; lipoxin A_4 (LXA_4), 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 15-epi-LXA_4, 5(*S*),6(*R*),15(*R*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; Me, methyl ester; PGE_2, prostaglandin E_2; PMN, polymorphonuclear leukocyte(s); T_{84} cells, intestinal epithelial cells. LXA_4 analogs are abbreviated in accordance with the nomenclature of LXA_4, namely: 15(*R/S*)-methyl-LXA_4, 5(*S*),6(*R*),15(*R/S*)-trihydroxy-15-methyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 15-cyclohexyl-LXA_4, 15-cyclohexyl-16,17,18,19,20-pentanor-LXA_4; 16-phenoxy-LXA_4, 16-phenoxy-17,18,19,20-tetranor-LXA_4.

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constriction in asthmatic subjects (Christie et al., 1992). These results, in addition to an inverse relationship noted earlier in the biosynthesis of leukotrienes and lipoxins, raised the possibility that LX serve as endogenous chalones [reviewed in Serhan (1994)] and/or may act as novel "anti-inflammatory molecules" (Badr & Lakkis, 1994; Lee, 1995). Not only do LX serve counterregulatory roles in the normal events of inflammation, but modified LX may also be actual effectors of well-established anti-inflammatory therapies. We recently found that aspirin triggers the transcellular biosynthesis of a novel series of bioactive 15-epimeric LX, which appears to mediate some of aspirin's beneficial effects (Clària & Serhan, 1995). The 15-epi-LX are generated by novel route(s) that can involve cyclooxygenase II in endothelial cells and activation of the 5-lipoxygenase in human leukocytes.

An essential component in evaluating the role of LX as putative local mediators *in vivo* centers on understanding their time course of action. In general, eicosanoids are rapidly generated, exert their actions and are inactivated in their local microenvironment, and thus act as autacoids. The bioactivity of most linear eicosanoids is regulated via further rapid metabolism that can result in either increasing potency, as is the case for 5(*S*)-hydroxyeicosatetraenoic acid (5-HETE) conversion to 5-oxo-ETE (Powell et al., 1993), or decreasing their bioactivities in certain target tissues, as with ω oxidation of LTB_4 by PMN [reviewed in Diczfalusy (1994)]. LX are subject to both ω oxidation in isolated organelle preparations (Boucher et al., 1991; Sumimoto et al., 1993; Mizukami et al., 1994) and selective dehydrogenation/reduction in macrophage/monocytes to products that resemble the prostaglandin dehydrogenase-derived metabolites with respect to their side chain alterations, for example, 15-oxo-LXA_4, 13,-14-dihydro-15-oxo-LXA_4, and 13,14-dihydro-LXA_4 [see Serhan et al. (1993)]. Recently, we reported that dehydrogenation of LX is the major route of further metabolism in human leukocytes that results in their inactivation (Maddox & Serhan, 1995). In view of several low-molecular weight prostaglandin stable analogs that have therapeutic value (Collins & Djuric, 1993), LX analogs that would resist rapid inactivation and thus might retain potential beneficial actions of LX were envisioned. Here, we report the design criteria for LXA_4 analogs that enable them to resist rapid conversion and block PMN migration in two different coincubation systems.

EXPERIMENTAL PROCEDURES

HL-60 cells were from American Type Culture Collection (Rockville, MD), and cell culture reagents were purchased from BioWhittaker (Walkersville, MD). Synthetic LTB_4 and LXA_4 were from Cascade Biochem Ltd. (Reading, U.K.), and each was analyzed by RP-HPLC and UV. Their concentrations, as well as those of LXA_4 analogs, were determined using appropriate molar extinction coefficients (i.e., ~50 000 cm^{-1} M^{-1} for tetraene chromophores) prior to bioassays. Each eicosanoid was stored in methanol, and working stock solutions were prepared on the day of bioassay in ethanol, followed by dilutions in buffer solutions, to achieve final concentrations of <0.05% ethanol (v/v) when added in cell incubations. These daily working stocks were routinely discarded after use.

HL-60 Cell Culture and Differentiation. HL-60 cells were seeded in RPMI medium supplemented with 100 u/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and incubated at 37 °C with 5% CO_2 atmosphere in 250 mL flasks. The cells in individual flasks containing ~50 × 10^6 cells/flask or ~10^6 cells/mL were cultured in the presence of phorbol 12-myristate 13-acetate (PMA) as in Serhan et al. (1993). Nitro blue tetrazolium reduction was performed to monitor induction of phenotype (Imaizumi & Breitman, 1986). Before performing binding assays, cells were washed twice in phosphate-buffered saline ($DPBS^{2-}$). After their viability (>95%) was determined, differentiated cells were suspended at 10^6 cells/mL in $DPBS^{2+}$ (pH 7.4).

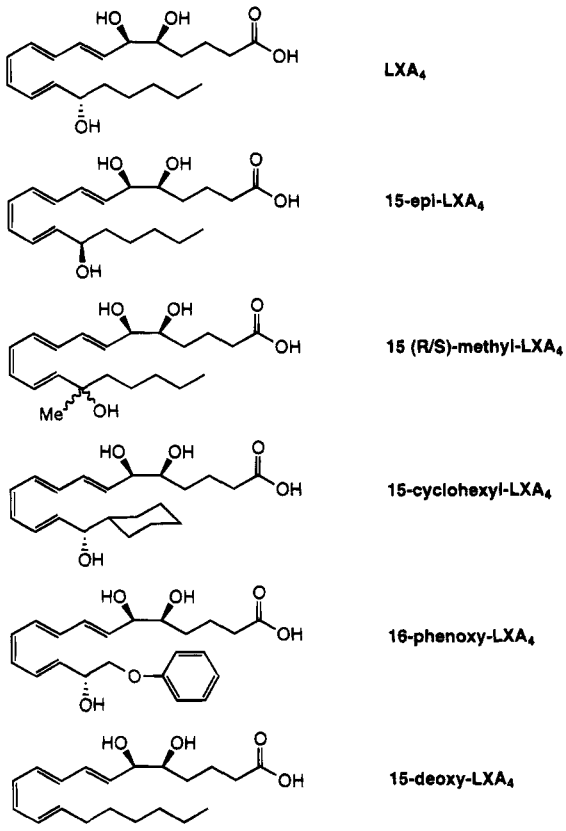
Culture of Human Umbilical Vein Endothelial Cells (HUVEC). Human umbilical vein endothelial cells were used at passages 1 and 2 and were isolated by collagenase digestion (0.1% collagenase, CLS3; Worthington Biochem. Corp., Freehold, NJ) and propagated on gelatin-coated (1%) tissue culture plates (Costar Corp., Cambridge, MA) in RPMI 1640 cell culture medium (BioWhittaker Inc., Walkersville, MD) supplemented with 15% bovine calf serum (BCS) (Hyclone Laboratories, Logan, UT), 15% NU-serum (Collaborative Research Inc., Lexington, MA), 50 μg/mL endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA), 8 u/mL heparin, 50 u/mL penicillin, and 50 μg/mL streptomycin.

Epithelial Cell Culture. T_{84} cells were grown in a 1:1 mixture of Dulbecco's modified Eagle medium and Hams F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 14 mM $NaHCO_3$, 40 μg/mL penicillin, 8 μg/mL ampicillin, 90 μg/mL streptomycin, and 5% newborn calf serum (Dharmsathaphorn et al., 1990). For apical-to-basolateral transmigration experiments, T_{84} monolayers were grown on collagen-coated, polycarbonate permeable supports (inserts) with a surface area of 0.33 cm^2 (Costar Inc., Cambridge, MA) as previously described. For physiologically directed, basolateral-to-apical neutrophil transmigration experiments, T_{84} cells were plated on the underside of 0.33 cm^2 polycarbonate filters that had been lightly coated with rat-tail collagen as previously described (Parkos et al., 1991). This permitted growth of inverted monolayers, which thus allowed neutrophils to settle by gravity into the immediate subepithelial compartment.

LXA_4 and Analog Analysis. LXA_4 and synthetic analogs were isolated and analyzed in two separate RP-HPLC systems and GC−MS as in Serhan et al. (1993). Briefly, the RP-HPLC system consisted of an LKB gradient dual pump equipped with an Altex Ultrasphere-ODS column (4.6 mm × 25 cm) with a flow rate of 1 mL/min and a linear gradient program. GC−MS was performed with a Hewlett-Packard 5971A mass detector quadrupole equipped with a HPG1030A workstation and GC5890. Methyl esters of the synthetic analogs of LXA_4 were converted to trimethylsilyl derivatives with bis(trimethylsilyl)trifluoroacetamide from Pierce Chemical Co. (Rockford, IL). Each gave permanent ions in its mass spectrum consistent with the parent structures shown in Scheme 1 and the results of NMR analysis. Each LX analog was synthesized by modifications of procedures reported by Webber et al. (1988). Their complete total synthesis will be reported in detail elsewhere.

The physical values for these compounds and their precursors were as follows. *15−Cyclohexyl-11,12-dehydro-16,17,18,19,20-pentanor-LXA_4 Methyl Ester (15-Cyclohexyl-11,12-dehydro-LXA_4-Me).* ^1H NMR (500 MHz, C_6D_6): δ

Scheme 1: LXA_4 Analogs Designed and Used in These Experiments[a]



[a] Nomenclature for the analogs uses LXA_4 basic structure for the template; i.e., carbon 1 (C-1) is the carboxylic acid, and C20 is the ω end of native LXA_4. 11,12-Acetylenic and corresponding methyl esters were prepared for each.

6.71 (dd, J = 15.4 and 10.9 Hz, 1H), 6.21 (m, 2H), 5.92 (d, J = 15.9 Hz, 1H), 5.82 (d, J = 15.6 Hz, 1H), 5.68 (dd, J = 15.3 and 6.5 Hz, 1H), 3.92 (br m, 1H), 3.63 (br m, 1H), 3.47 (m, 1H), 3.33 (s, 3H), 2.11 (t, J = 7.3 Hz, 2H), 1.82–1.52 (br m, 5H), 1.39–1.20 (br m, 4H), 1.12–1.10 (br m, 4H), 0.95–0.85 (br m, 2H). ^{13}C NMR (500 MHz, C_6D_6): 173.86, 145.40, 141.16, 134.73, 131.67, 112.31, 110.71, 91.67, 89.95, 76.53, 75.43, 74.11, 51.10, 43.95, 33.74, 31.48, 29.02, 28.40, 26.74, 26.43, 26.39, 31.53. UV: λ_{max}^{EtOH} = 295.5 and 312.5 nm. HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 7.9 min. Selective Lindlar catalytic hydrogenation of this acetylenic precursor gave the *15*-cyclohexyl-LXA_4 methyl ester, which gave the tetraene chromophore. UV: λ_{max}^{EtOH} = 288.1, 301.0, and 315.4 nm. HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 12.3 min.

15(R/S)-Methyl-11,12-dehydro-LXA_4 Methyl Ester. ^1H NMR (500 MHz, C_6D_6): δ 6.69 (dd, J = 15.4 and 10.9 Hz, 1H), 6.21 (m, 2H), 6.05 (dd, J = 15.9 and 2.1 Hz, 1H), 5.83 (dd, J = 15.6 and 1.8 Hz, 1H), 5.67 (dd, J = 15.3 and 6.5 Hz, 1H), 3.95 (br m, 1H), 3.51–3.56 (br m, 1H), 3.33 (s, 3H), 2.10 (t, J = 7.2 Hz, 2H), 1.41–1.12 (br m, 12H), 0.88 (t, 7.2 Hz, 3H). ^{13}C NMR (500 MHz, C_6D_6): 173.84, 150.45, 140.96, 131.67, 112.41, 108.03, 91.84, 89.88, 75.43, 74.11, 72.93, 51.08, 42.72, 33.75, 32.54, 31.46, 27.99, 22.92, 21.54, 19.82, 14.25. UV: λ_{max}^{EtOH} = 294.7 and 312.1 nm. HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 9.6 min. Hydrogenation with Lindlar catalyst gave *15(R/S)*-methyl-LXA_4 methyl ester. UV: λ_{max}^{EtOH} = 288.4, 301.0, and 315.4 nm. RP-HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 16.2 min.

16-Phenoxy-11,12-dehydro-17,18,19,20-tetranor-LXA Methyl Ester (16-Phenoxy-11,12-dehydro-LXA_4-Me). ^1H NMR (500 MHz, C_6D_6): δ 7.20–7.12 (m, 3H), 6.83–6.77 (m, 2H), 6.48 (dd, J = 15.4 and 10.9 Hz, 1H), 6.24 (dd, J = 15.4 and 10.9 Hz, 1H), 6.09 (dd, J = 15.9 and 5.4 Hz, 1H), 5.95 (d, J = 16.9 Hz, 1H), 5.73 (dd, J = 15.3 and 6.8 Hz, 1H), 5.64 (dd, J = 15.5 and 1.9 Hz, 1H), 4.51 (br s, 1H), 4.07 (br m, 1H), 3.91 (dd, J = 9.4 and 3.5 Hz, 1H), 3.78 (dd, J = 9.4 and 7.5 Hz, 1H), 3.61–3.58 (m, 1H), 3.55 (s, 3H), 1.78–1.55 (br m, 2H), 1.39–1.28 (br m, 2H), 0.87 (t, J = 7.4 Hz, 2H). ^{13}C NMR (500 MHz, C_6D_6): 174.15, 158.24, 140.73, 140.09, 133.38, 132.10, 129.57, 121.41, 114.61, 112.20, 112.09, 90.35, 90.09, 75.18, 73.74, 71.21, 70.48, 52.60, 33.65, 31.32, 21.00. UV: λ_{max} = 295.1 and 312.5 nm. HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 5.1 min. Lindlar hydrogenation gave 16-phenoxy-LXA_4 methyl ester with characteristic UV: λ_{max}^{EtOH} = 288.1, 301.0, and 315.4 nm. RP-HPLC: 25% water/methanol, 4 mL/min, Dynamax C18 80-299-C5, 6.3 min.

Preparation of Recombinant 15-OH-PGDH in Escherichia coli. The plasmid pGBT-PGDH, containing the complete coding region of 15-OH-PGDH, a generous gift from Dr. C. M. Ensor and Dr. H. H. Tai (University of Kentucky, Lexington, KY), was transformed into the *E. coli* strain TOP10F′ (Invitrogen, San Diego, CA). Transformants carrying the pGBT-PGDH plasmid were selected and cultured in broth containing ampicillin (50 μg/mL) and tetracycline (15 μg/mL) to A_{600} = 0.5, induced with isopropyl 1-thio-β-D-galactopyranoside (1 mM), and grown overnight in a rotary shaker at 37 °C (Ensor & Tai, 1991). 15-OH-PGDH was partially purified using initial procedures as in Krook et al. (1990). Overnight cultures (3 L) were pelleted (4000*g* for 20 min at 4 °C) and resuspended in 35 mL of potassium phosphate buffer (40 mM) (pH 7.0) containing EDTA (1 mM), dithiothreitol (0.1 mM), and 20% glycerol. The cells were sonicated and centrifuged for 20 min at 10 000*g* at 4 °C. The supernatant was loaded on a DEAE-Sephacel column (2.5 × 7.5 cm) (Sigma, St. Louis, MO) and eluted with a 400 mL gradient of 40 to 250 mM potassium phosphate buffer. Fractions were tested for 15-OH-PGDH activity via NAD^+ reduction, using PGE_2 as substrate (vide infra); those fractions containing the highest activity were pooled, and protein concentration was measured using the method of Bradford (1976).

Assay of 15-OH-PGDH Activity. Individual and pooled fractions from the DEAE-Sephacel column were assayed for activity by measuring the formation of NADH from NAD^+ spectrophotometrically at 340 nm as in Änggård and Samuelsson (1966). Briefly, buffer containing Tris-HCl (0.1 M) (pH 9.0) and NAD^+ (0.5 mM) in a total volume of 0.7 mL was warmed to 37 °C. Potential substrates (2.5–28 μM) and enzyme (20.9 μg in 50 μL) were added sequentially, and increments in absorption at 340 nm were recorded every 5 min for a total of 20 min at 37 °C. Reaction rates were calculated via first order regression curves computer-fitted to the change in absorption as a function of time.

Isolation of Polymorphonuclear Leukocytes (PMN). Human PMN were isolated from heparinized venous blood drawn from healthy volunteers by standard procedures (Böyum, 1968) of ficoll-hypaque density gradient centrifuga

tion and dextran sedimentation, as used in Takata et al. (1994). Contaminating red blood cells were removed by hypotonic lysis. The final pellet was suspended in Dulbecco's phosphate buffered saline (DPBS, pH 7.4) and contained 96 ± 3% PMN, as determined by light microscopy. Suspensions in which PMN showed signs of cellular activation (>10% of PMN in clumps of two or more cells) or loss of membrane integrity (>3% of PMN accumulating trypan blue) were routinely discarded. For studies of adhesion, PMN were labeled with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein pentaacetoxymethyl ester (BCECF-AM, Calbiochem, San Diego, CA) (4 μM, 40 min, 37 °C), washed three times in DPBS, and suspended in DPBS containing 1% bovine calf serum (BCS).

Transmigration Assay. The PMN transepithelial migration assay used in the present experiments has been previously detailed (Colgan et al., 1993; Parkos et al., 1991). Briefly, human PMN were suspended in modified Hanks' balanced salt solution (HBSS) (without Ca^{2+} and Mg^{2+}, with 10 mM Hepes, pH 7.4, Sigma) at a concentration of 5×10^7 cells/mL. Prior to addition of PMN, T_{84} (intestinal epithelial cell) monolayers were extensively rinsed in HBSS to remove residual serum components. Transmigration assays were performed by addition of PMN (40 μL) to HBSS (containing Ca^{2+} and Mg^{2+}, 160 μl) in the upper chambers after chemoattractant [FMLP (1 μM) in HBSS] was added to the opposing (lower) chambers. PMN exposed to either LXA_4 or LXA_4 analogs were not washed before addition to monolayers, and therefore, a 5-fold dilution of lipoxin was present during the transmigration assay. PMN (1×10^6) were added at time 0. Transmigration was allowed to proceed for 120 min. All experiments were performed in a 37 °C room to ensure that epithelial monolayers, solutions, plasticware, etc. were maintained at a uniform 37 °C temperature.

Transmigration was quantitated by assaying for the PMN azurophilic granule marker enzyme myeloperoxidase (MPO) as in Parkos et al. (1991). Following each transmigration assay, nonadherent PMN were extensively washed from the surface of the monolayer and PMN cell equivalents (PMN CE), estimated from a standard curve, were assessed as the number of PMN which had completely traversed the monolayer (i.e., across the monolayer into the reservoir bath).

PMN–Endothelial Cell Adhesion. In experiments on the influence of LXA_4 and its analogs on $\beta 2$ integrin-mediated PMN adhesion, BCECF-labeled PMN were coincubated with confluent endothelial monolayers (passages 1 and 2) grown on 96-well plates (4×10^5 PMN in 0.2 mL DPBS supplemented with 1% BCS) in the presence of agonist (10^{-7} M, 30 min). Following coincubation, the medium was aspirated and monolayers were washed gently with 1 mL of DPBS to remove nonadherent cells. The contents of each well were solubilized with 0.1% sodium dodecyl sulfate (SDS)/0.025 M NaOH; fluorescence was assessed in a Cytofluor 2300 fluorescence plate reader, and the number of adherent PMN was calculated from the fluorescence of the original PMN suspension.

Statistical Analyses. Results were analyzed using paired or unpaired Student's t-tests and assigning significance at $p < 0.05$.

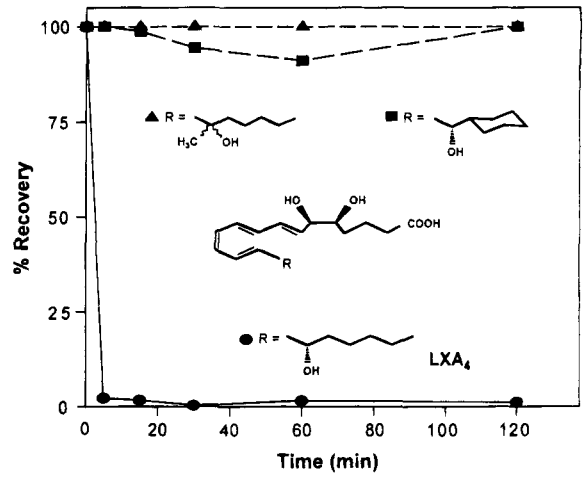


FIGURE 1: Time course of recovery of LXA_4 and LXA_4 analogs from HL-60 cells. HL-60 cells were treated with PMA (15 nM for 72 h) to induce the monocyte/macrophage-like phenotype. Differentiated HL-60 cells were harvested, washed in DPBS, and incubated (10^6 cells/mL) with either native LXA_4 (●) or analogs (1 μg/mL), 15(*R/S*)-methyl-LXA_4 (▲), or 15-cyclohexyl-LXA_4 (■). At the indicated intervals, aliquots were removed, placed in cold MeOH (2 volumes), and quantified. Results are representative of $n = 3$ separate experiments.

RESULTS

LXA_4 is rapidly converted by initial dehydrogenation at carbon 15 (C-15) to 15-oxo-LXA_4 (Serhan et al., 1993), which is biologically inactive (Maddox & Serhan, 1995). Therefore, a series of analogs was designed with bulky substitutions placed on the lower side chain at carbon 15 as well as at the carbon 20 (or ω end) of the native LXA_4 structure (Scheme 1). Also, 15-deoxy-LXA_4, a compound that did not carry an alcohol at carbon 15, was constructed and included in this series to evaluate the contribution of substituents at the C-15 position. To test the resistance of these compounds to conversion, each was added to PMA-differentiated HL-60 cells, a system that rapidly metabolizes LX. As expected, addition of LXA_4 to monocyte/macrophage-like HL-60 cells resulted in the rapid loss of LXA_4 within minutes of its exposure [cf. Serhan et al. (1993)]. Less than 10% of the added LXA_4 was recovered within the first 15 min of the incubation periods. In contrast, >95% of each of the LXA_4 analogs was recovered from PMA-induced HL-60 cells at time intervals up to 2 h (Figure 1). Thus, placement of a racemic methyl group at C-15 as in 15(*R/S*)-methyl-LXA_4 or either a cyclohexyl at carbon 15 (Scheme 1) or a phenoxy group at carbon 16 in place of the remaining carbons at the ω end (C-20) of LXA_4 (Figure 1 and data not shown) resulted in almost quantitative recovery of these compounds from incubations with differentiated HL-60 cells. Also, 15-deoxy-LXA_4 was not metabolized or significantly converted by these cells during the time course evaluated ($n = 3$, $T_0 - 2$ h). These findings indicated that the analogs were stable to myeloid cell-mediated conversion.

Further metabolism of LXA_4 by both differentiated HL-60 cells and human monocytes involves dehydrogenation by an enzyme that is similar to 15-hydroxyprostaglandin dehydrogenase in both reaction mechanism (Serhan et al., 1993) and physical properties (Maddox & Serhan, 1995). The human placental form of this enzyme has been cloned (Ensor & Tai, 1991). Therefore, we overexpressed the recombinant enzyme to both determine and compare the rate of conversion

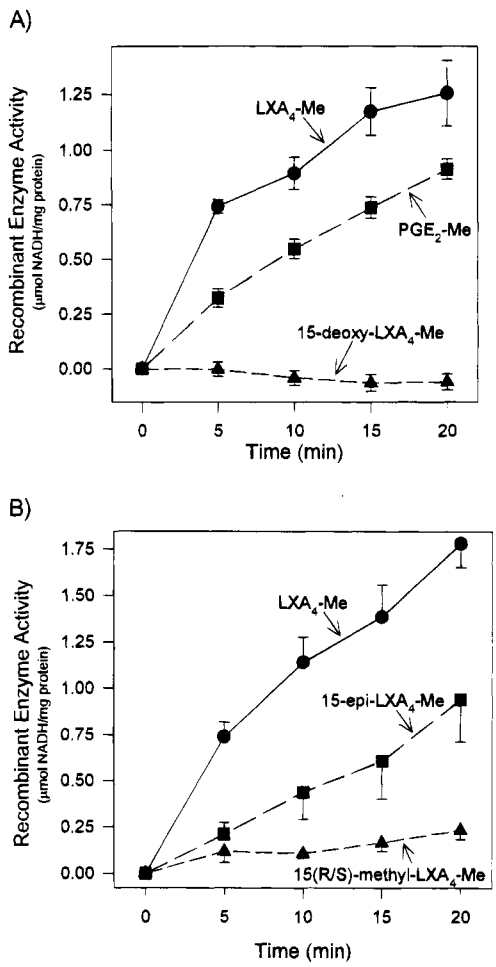


FIGURE 2: Conversion of LXA_4 and synthetic analogs by recombinant enzyme. Recombinant 15-OH-PGDH activity with (A) LXA_4, PGE_2, and 15-deoxy-LXA_4 and (B) LXA_4, 15-epi-LXA_4, and 15(*R/S*)-methyl-LXA_4. Time course of NADH formation was monitored at A_{340} using 28 μM (A) or 2.5 μM (B) of substrate or analogs (each compound was assayed as its corresponding methyl ester) as described in Experimental Procedures. Values are expressed as the mean ± standard error of $n = 3$ separate experiments.

Table 1: Conversion of Synthetic LXA_4 Analogs by Recombinant 15-OH-PGDH[a]

substrate	relative reaction rate
LXA_4-Me	100
PGE_2	66.53 ± 7.15
PGE_2-Me	60.14 ± 4.26
LXA_4	44.55 ± 3.01
15-cyclohexyl-LXA_4-Me	18.72 ± 7.82
16-phenoxy-LXA_4-Me	17.33 ± 0.79
15(*R/S*)-methyl-LXA_4-Me	16.36 ± 5.72
15-deoxy-LXA_4-Me	0.18 ± 0.25

[a] Absorption at 340 nm was measured every 5 min for a total of 20 min at 37 °C in a system containing 20.9 μg of partially purified recombinant enzyme, Tris (100 mM) (pH 9.0), NAD^+ (0.5 mM), and 28 μM of the substrate as in Experimental Procedures. Values represent means ± standard errors of $n = 3$ separate experiments and are expressed as percents of the reaction rates obtained with LXA_4-Me.

of LXA_4 and the designed synthetic analogs by the recombinant 15-OH-PGDH. The carboxymethyl ester of LXA_4 (LXA_4-Me) proved to be the best substrate for this enzyme even when directly compared to its well-established substrate PGE_2 (Figure 2A and Table 1). Conversion of PGE_2 was essentially equal to conversion of its corresponding methyl ester (PGE_2-Me). In contrast, the presence of a methyl ester

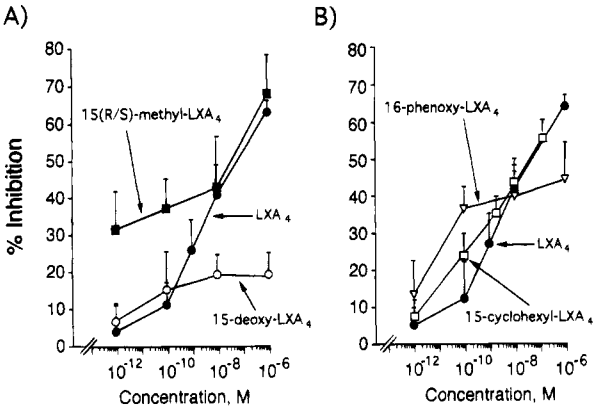


FIGURE 3: Impact of LXA_4 analogs on human PMN transmigration across polarized epithelial monolayers. PMN (1×10^6/monolayer) were exposed to vehicle-containing buffer (control), LXA_4 (●), 15-deoxy-LXA_4 (○), 15(*R/S*)-methyl-LXA_4 (■), 15-cyclohexyl-LXA_4 (B, □), or 16-phenoxy-LXA_4 (B, ▽) at indicated concentrations for 15 min at 37 °C. PMN were layered on the basolateral surface of T_{84} monolayers and driven to transmigrate in the physiological direction by a 10^{-6} M FMLP gradient for 2 h at 37 °C. Transmigration was assessed by quantitation of PMN MPO. Data were normalized relative to the vehicle controls and are expressed as percent inhibition ± mean standard error of transmigration ($n = 6-9$ monolayers per condition).

at the carbon 1 position instead of the carboxylic acid group of LXA_4 (Scheme 1) enhanced its rate of conversion by the recombinant enzyme. Elimination of the alcohol group at C-15, as in 15-deoxy-LXA_4-Me, resulted in little to no demonstrable activity, suggesting that this enzyme prefers alcohols at C-15 rather than those at either C-5 or C-6 positions in LXA_4 (Figure 2A and Table 1). The cyclohexyl, phenoxyl, and 15(*R/S*)-methyl analogs of LXA_4-Me were metabolized at rates of less than 20% of that of the parent compound, and native LXA_4 gave between 65 and 70% of the conversion rate of PGE_2 (Table 1). Similar results were obtained with each of the 11,12-acetylenic versions of these LXA_4 analogs ($n = 3$, data not shown). These values for LXA_4 and PGE_2 are consistent with those obtained from PMA-induced HL-60 cells [cf. Serhan et al. (1993)]. When directly compared to the conversion of LXA_4-Me, addition of a methyl group at the C-15 position resulted in a virtual loss of substrate activity for 15(*R/S*)-methyl-LXA_4-Me (Figure 2B). Of particular interest, the methyl ester of 15-epi-LXA_4 (carrying a C-15 alcohol in the *R* configuration), one of the new compounds triggered by aspirin treatment (Clària & Serhan, 1995), gave only approximately half the rate of conversion of LXA_4-Me (Figure 2B). This finding indicates that the 15(*R*) epimeric LXA_4 is not as rapidly inactivated as native LXA_4, which may lead to an increased tissue survival and activity of this natural aspirin-triggered LXA_4 analog. Together, results from both intact cells (i.e., HL-60 cell recovery) and isolated enzyme (i.e., conversion with recombinant enzyme) are consistent and indicate that the designed LXA_4 analogs were stable to metabolic conversion in conditions where the native LXA_4 or its methyl ester is rapidly transformed.

LXA_4 inhibits both transmigration of PMN across polarized monolayers of columnar epithelial cells (Colgan et al., 1993) and PMN adherence to vascular endothelial cells (Papayianni et al., 1994), which are sites of two important immune events in host defense and inflammation. To determine if the designed LXA_4 analogs retain these biologi-

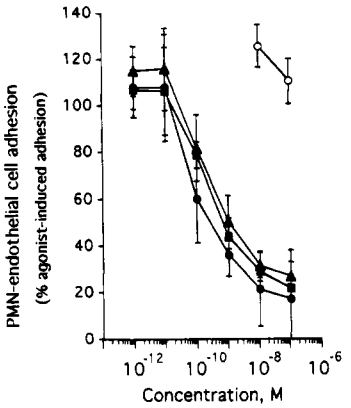


FIGURE 4: Inhibition of adhesion of human PMN and endothelial cells by LXA_4 analogs. BCECF-labeled PMN were exposed to LXA_4 or analogs for 15 min, washed twice with PBS, and coincubated with confluent endothelial monolayers grown on 96-well plates (see Experimental Procedures) in the presence of LTB_4 (10^{-7} M, 30 min). PMN integrity was not altered within this time course of exposure to LXA_4 (●) or LXA_4 analogs, as determined by exclusion of Trypan blue and ability to retain BCECF. 15(*R/S*)-Methyl-LXA_4 (■), 15-cyclohexyl-LXA_4 (▲), and 15-deoxy-LXA_4 (○). Data are expressed as the mean ± standard error of n = 3−6 separate experiments, each conducted in triplicate or quadruplicate.

cal activities of native LXA_4, we evaluated each analog compared to LXA_4 in both bioassay systems. Each of the LXA_4 analogs proved to be a potent inhibitor of PMN transmigration across epithelial monolayers (Figure 3). In this coincubation system, 15(*R/S*)-methyl-LXA_4 was the most potent inhibitor compared to LXA_4, and again, 15-deoxy-LXA_4 displayed the least activity (Figure 3A). The phenoxy and cyclohexyl analogs of LXA_4 were also potent inhibitors of PMN transepithelial migration (Figure 3B). A similar trend of activity was observed for PMN adhesion to vascular endothelial cells (Figure 4). In this assay, each of the LXA_4 analogs gave potencies in the range of that of native LXA_4, except for the 15-deoxy-LXA_4 that was essentially inactive. Thus, these results indicate that the designed LXA_4 analogs depicted in Scheme 1 retained the bioactivity of native LXA_4.

DISCUSSION

The present results indicate that LXA_4 analogs designed to carry substituents at the carbon 15 through ω 20 end of the native LXA_4 structure resist conversion by both intact myeloid cells and recombinant enzyme (Figures 1 and 2). Of particular importance, these newly designed analogs retain the bioactive properties of native LXA_4 in leukocyte migration assays and in this respect proved to be potent inhibitors of both PMN transmigration and adhesion (Figures 3 and 4). Together, these are the first criteria to allow for rational LX analog synthesis and evidence to establish that the native LX structures can be used as a backbone or template to develop LX structure-based analogs that possess potent biological activities.

Rapid inactivation of LX by myeloid cells (Maddox & Serhan, 1995) implies that LX, like other potent lipid and peptide mediators, are further metabolized in their local milieu. Each of the LXA_4 analogs resisted conversion by intact cells that rapidly transformed native LXA_4 (Figure 1). The resistance of these analogs was confirmed with recombinant enzyme (Figure 2), where addition of a bulky group at carbon 15 and/or the C-20 end of LXA_4 prevented rapid transformation yet retained the bioactivity of native LXA_4 observed in both transmigration and adhesion bioassays (Figures 3 and 4). Of note, the 15-deoxy-LXA_4 that did not contain either an alcohol at C-15 or bulky substituents at either the ω end or the C-15 side chain neither was converted nor carried bioactivity in the concentration range of the active LXA_4 analogs. These results indicate that a LXA_4 "trihydroxytetraene or an equivalent" conformation is a requisite for bioactivity of the LXA_4 analogs.

15(*R/S*)-Methyl-LXA_4, 15-cyclohexyl-LXA_4, and 16-phenoxy-LXA_4 proved to be poor substrates for recombinant 15-OH-PGDH when directly compared to either PGE_2 or native LXA_4 (Table 1). Whereas addition of a methyl group at position C-15 blocked conversion by the dehydrogenase, and placement of a methyl ester at carbon 1 (C-1) enhanced conversion of LXA_4 (i.e., LXA_4-Me), which now appears to be the best substrate presently known for this enzyme. The chirality of the alcohol at C-15 was also important for optimal conversion, since 15-epi-LXA_4 (i.e., 15(*R*)-LXA_4) was converted at less than half the rate of LXA_4. This finding implies that, when generated *in vivo*, 15-epi-LXA_4 will not be inactivated as rapidly as LXA_4 and thus may possess a longer *in vivo* biological half-life. Since 15-epi-LX are generated after aspirin treatment and 15-epi-LXA_4 proved to be a potent inhibitor of PMN adhesion (Clària & Serhan, 1995), the ability of 15-epi-LXA_4 to escape conversion, by approximately half the rate as compared to LXA_4, suggests that this natural analog that can be generated *de novo* (i.e., 15-epi-LXA_4) is also a potentially useful lead structure.

The sequence of events by which leukocytes infiltrate tissues encompasses rolling, arrest of cells, adherence and transmigration through intercellular junctions (diapedesis) of vascular endothelium, and often subsequent migration across columnar epithelia (Majno, 1982). This cascade is well-appreciated as including key elements in host defense, inflammation, and hypersensitive reactions. Small molecules (i.e., molecular weight < 600, preferably with few chiral centers) that can regulate these processes are of interest, particularly those of endogenous origin. Inflammation is normally a self-limited event in healthy individuals and is followed by wound healing. The finding that LXA_4, an endogenous mediator, can regulate these events both *in vitro* (Lee et al., 1989; Colgan et al., 1993; Papayianni et al., 1994) and *in vivo* in animal models (Raud et al., 1991; Papayianni et al., 1995) not only provides evidence toward understanding the endogenous role of these molecules but also gives model systems for evaluating their pharmacologic impact on PMN−epithelial cell transmigration and PMN−endothelial cell adhesion. Since native LXA_4 is a potent inhibitor of PMN transmigration and adhesion, we tested each of the stable analogs in these assays to determine whether the analogs retained function. It was possible that altering the structure of LXA_4, such as in either 6(*S*)-LXA_4 or 11-*trans*-LXA_4, would diminish or abolish bioactivity [reviewed in Serhan (1994)]. It is noteworthy that neither 6(*S*)-LXA_4 nor 11-*trans*-LXA_4 has been shown to carry biological activity in the systems evaluated to date. The 15(*R/S*)-methyl-LXA_4 proved to be the most potent inhibitor of transmigration with intestinal epithelial cells (Figure 3A). 16-Phenoxy-LXA_4 and 15-cyclohexyl-LXA_4 were well within the potency range of native LXA_4 (Figure 3B), indicating that these analogs are potent inhibitors of transmigration. They also inhibited

adhesion to vascular endothelial cells in a trend similar to native LXA_4. Thus, the criteria for LX analog development set forth, namely that the structures should delay or resist inactivation, retain bioactivity, and be of low molecular weight (i.e., <600 MW), which would allow for delivery and bioavailability, are fulfilled by these LX analogs (Scheme 1). In addition, these results confirm the activity of LXA_4 and several of its stable analogs disclosed in Scheme 1 in two separate coincubation settings that model two important biological processes involving PMN. Since transmigration on epithelium and adherence to endothelium are distinct sites of PMN function *in vivo*, these LXA_4 analogs may serve a "double hit" at blocking overt PMN recruitment to these sites.

Evidence from Belluzzi et al. (1994), who have studied acute pouchitis in humans, and Christie et al. (1992), who tested LXA_4 in the airways of asthmatics, suggests that native LXA_4 administration may have a beneficial outcome in humans. Some of these *in vivo* actions may be related, in part, to the vasodilatory properties of LXA_4 and its ability to block peptido-leukotrienes (LTC_4 and LTD_4) at the receptor level [reviewed in Badr and Lakkis (1994)], whereas its ability to block LTB_4-induced inflammation is not related to competition between LXA_4 and LTB_4 at the LTB_4 receptor (Serhan, 1994). The lung, kidney, and placenta are rich in 15-OH-PGDH activity [reviewed in Ensor and Tai (1991)] that could, along with monocytes (Maddox & Serhan, 1995), rapidly inactivate LXA_4. Thus, the design of LXA_4 analogs that utilize these criteria and either delay or resist further metabolism (Figures 1 and 2) as well as carry bioactivity (Figures 3 and 4) may provide useful leads not only in further evaluating the actions of LX in appropriate model systems but also in aiding in the development of agents that could have therapeutic impact. The design of specific LXA_4 analogs based on these molecules would be of interest, particularly if they possess the reported actions of native LX in hematopoietic, vascular, and pulmonary studies [reviewed in Serhan (1994)].

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