Lipoxin A_4 Receptor Activation Is Distinct from That of the Formyl Peptide Receptor in Myeloid Cells: Inhibition of CD11/18 Expression by Lipoxin A_4−Lipoxin A_4 Receptor Interaction†

Stefano Fiore and Charles N. Serhan*

Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Lipoxin A_4 (LXA_4) interacts with high-affinity receptors in human neutrophils and differentiated HL-60 cells. Recently, we characterized a myeloid-derived cDNA that encodes a LXA_4 high-affinity receptor (LXA_4R) [Fiore, S., Maddox, J. F., Perez, H. D., and Serhan, C. N. (1994) *J. Exp. Med. 180,* 253−260] denoted earlier as a related *N*-formyl peptide receptor (RFP). To examine the selectivity of this receptor we tested its preference for specific binding of ^{3}H-LXA_4 versus ^{3}H-*N*-formylmethionyl-leucyl-phenylalanine (^{3}H-FMLP). When receptor-transfected Chinese hamster ovary cells were exposed to either ^{3}H-LXA_4 or ^{3}H-FMLP, the receptor affinity for LXA_4 exceeded by 1000-fold that of FMLP (6.1 nM vs 5 μM). Upon differentiation, HL-60 cells acquire high-affinity binding sites and respond to both LXA_4 and FMLP. Northern blot analysis of differentiated HL-60 cells using an RFP probe showed a characteristic band at 2.1 kb. Differentiated HL-60 cells exposed to an RFP antisense oligonucleotide selectively lost ^{3}H-LXA_4 binding as well as LXA_4-stimulated lipid remodeling that paralleled the loss of mRNA for LXA_4R. In contrast, the specific mRNA for the FMLP receptor, ^{3}H-FMLP specific binding, and FMLP-induced phospholipase D activity were still observed. Treatment of human neutrophils with antisera raised against a peptide in the LXA_4R third extracellular domain also resulted in selective abrogation of ^{3}H-LXA_4 specific binding with polymorphonuclear leukocytes (PMN) without blocking ^{3}H-FMLP binding. FMLP-stimulated CD11b upregulation as well as homotypic aggregation of PMN was inhibited by LXA_4 (which at 10^{-9} M gave ~1 log unit shift to the right in the FMLP dose-response curve). The addition of LXA_4R antisera did not alter FMLP-induced responses in PMN but completely blocked LXA_4 actions. These results indicate that altering the expression of the LXA_4R protein by blockage of transcriptional mechanisms or hindrance of the LXA_4R extracellular domains leads to loss of LXA_4 specific binding and blockage of LXA_4 signaling. Moreover, they indicate that in myeloid cells LXA_4−LXA_4R interactions are dissociable from those of FMLP and that LXA_4 regulates CD11/18 on the PMN surface.

Lipoxins are trihydroxytetraene-containing eicosanoids that modulate leukocyte function [as reviewed in Samuelsson et al., (1987) and Serhan (1994)], inhibit chemotaxis of human PMN (Lee et al., 1989), modify smooth muscle tone (Dahlén et al., 1991), and may play relevant roles in the pathophysiology of asthma (Christie et al., 1992) and inflammation and wound healing (Serhan, 1994). Lipoxin A_4 displays a selective profile of bioactions with both PMN and differentiated HL-60 cells that includes stimulation of phospholipase A_2 and phospholipase D. These activities are linked to specific high-affinity receptors and a G-protein, pertussis toxin-sensitive mechanism of signal transduction (Fiore et al., 1992, 1993).

Interaction of LXA_4 with its cognate receptor in PMN does not trigger homotypic aggregation or degranulation (Nigam et al., 1990), which sharply contrasts with PMN responses to agonists such as LTB_4 and the synthetic chemotactic peptide FMLP. LTB_4 and FMLP elicit chemotaxis, degranulation, and O_2^- generation and mobilize intracellular Ca^{2+}, which are responses linked to host defense and the pathophysiology of inflammation (Samuelsson et al., 1987; Weissmann et al., 1980). Lipoxin A_4 inhibits PMN responses to LTB_4- and FMLP-stimulated chemotaxis and transmigration (Colgan et al., 1993; Lee et al., 1989). It also inhibits LTC_4- and LTD_4-mediated bioactions in both *in vivo* and *in vitro* models (Badr et al., 1989; Christie et al., 1992). The inhibition of LTD_4-induced vasoconstriction by LXA_4 has been linked to direct competition at the LTD_4 receptor level in mesangial cells (Badr et al., 1989) and in human vascular

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* To whom correspondence should be addressed at Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia, 75 Francis St., Boston, MA 02115.

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[1] Abbreviations: αMEM, minimum essential medium; antiLXA_4-R1 and 2, rabbit sera 8180 and 8185 raised against LXA_4R peptide; CHO, Chinese hamster ovary cells; DPBS, Dulbecco's phosphate-buffered saline; $DPBS^{2-}$, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+}; FACS, fluorescence-activated cell sorting; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; FPR, formyl peptide receptor; leukotriene B_4 (LTB_4), 5*S*,12*R*-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; leukotriene D_4 (LTD_4), 5*S*-hydroxy-6*R*-(*S*-cysteinylglycyl)-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; lipoxin A_4 (LXA_4), 5*S*,6*R*,15*S*-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXA_4R, lipoxin A_4 receptor; $pLXA_4R^+$, plasmid construct (pINF114) containing related formyl peptide receptor cDNA; $pLXA_4R^-$, plasmid construct (pINF) without related formyl peptide receptor cDNA; PLD, phospholipase D; PMN, polymorphonuclear leukocyte(s); RA, *all-trans*-retinoic acid; RFP, related formyl peptide receptor; RIPA, radioimmunoprecipitation assay.

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endothelial cells (Fiore et al., 1993), a mechanism that has been excluded for its ability to inhibit LTB_4 action with human PMN (Grandordy et al., 1990; Nigam et al., 1990). LXA_4 does not compete for ^{3}H-LTB_4 specific binding sites in PMN (Nigam et al., 1990; Fiore et al., 1994). Mechanisms underlying LXA_4 inhibitory actions on PMN remain of interest because regulation of this cell is pivotal in the outcome of an inflammatory response.

Recently we identified a myeloid-derived cDNA, previously termed RFP (related formyl peptide receptor), that encodes a LXA_4 high-affinity receptor (LXA_4R) (Fiore et al., 1994). RFP was originally reported as an orphan cDNA obtained by screening myeloid cell-derived libraries with FMLP receptor probes (Boulay et al., 1990; Murphy et al., 1992; Perez et al., 1992a). A 70% homology is observed between RFP and the FMLP receptor sequences; however, the ligand for RFP was not known. Expression of RFP cDNA in CHO cells gave the appearance of both high-affinity binding (K_d = 5 nM) and functional responses to LXA_4, which included the stimulation of signal-transduction events (i.e., increased GTPase activity and activation of phospholipases) (Fiore et al., 1994). When this receptor is expressed in stably transfected fibroblasts it is also reported to act as a "low-affinity" receptor for FMLP, giving intracellular Ca^{2+} mobilization that requires concentrations of 10−100 μM of the putative ligand FMLP (Ye et al., 1992). Lipoxin A_4 and FMLP receptor-mediated events have been demonstrated in both human PMN and retinoic acid-differentiated HL-60 cells (Fiore et al., 1993; Imaizumi & Breitman, 1986). Since these cells express both receptors, namely, LXA_4R and FPR, which are highly homologous proteins with 67% identity and 80% similarity, we addressed the selectivity of their interactions with the eicosanoid LXA_4 and bacterial peptide surrogate FMLP. Results from the present experiments indicate that LXA_4 actions in both HL-60 cells and PMN require expression of the seven-transmembrane-domain receptor LXA_4R, previously termed RFP, and that it is the preferred ligand.

EXPERIMENTAL PROCEDURES

Materials. Tritiated LXA_4 ([11,12-^{3}H]LXA_4, 40.5 Ci/mmol) was obtained as a custom tritiation of the 11,12-acetylenic LXA_4 methyl ester carried out at Du Pont−New England Nuclear tritiation laboratory (Boston, MA) and purified by HPLC as described in Fiore et al. (1992). ^{3}H-FMLP (53.6 Ci/mmol), ^{3}H-palmitate (30.0 mCi/mmol), ^{35}S-labeled cysteine−methionine mix (EXPRE^{35}S^{35}S, ~1000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Du Pont−NEN. Synthetic LXA_4 and LXB_4 (stocks 280 μM in EtOH) were obtained from Cascade Biochem Ltd. (Reading, Berkshire, U.K.). DPBS and cell culture reagents were from Whittaker M. A. Bioproducts (Walkersville, MD), and cell culture plasticware was from Marsh Biomedical Products (Rochester, NY). FMLP (10 mM stock in EtOH), salmon sperm DNA, and oligo(dT)−cellulose were from Sigma Chemical Co. (St. Louis, MO), and silicon oil was from Huls America (Bristol, PA). cDNA for the FPR and the plasmid construct pINF were generous gifts from Dr. H. D. Perez of Berlex Biosciences (Richmond, CA). Mouse monoclonal antibodies anti-human CD11b and CD11c, and anti-mouse IgG_1 and IgG_2 were purchased from Accurate Chemicals & Scientific Corp. (Westbury, NY) [fluorescein isothiocyanate- (FITC-) conjugated MOAb), and from Becton Dickinson (San Jose, CA) (phycoerythrin- (PE-) conjugated MOAb).

PMN Isolation. Human PMN were obtained by the modified Böyum method (Böyum, 1968) from fresh heparinized blood after venipuncture of healthy normal volunteers. When intended for subsequent FACS analysis experiments (as in Figures 6 and 7), the entire cell isolation was carried out at 4 °C. Suspensions in DPBS were monitored for cell number and viability by their ability to exclude trypan blue (n = 15; 96.4 ± 2.5% of the PMN were viable).

HL-60 Cell Culture and Differentiation. HL-60 cells were seeded in RPMI medium supplemented with 100 units/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. Individual flasks containing ~50 × 10^6 cells/mL were cultured in the presence of retinoic acid (1 μM for 120 h). Nitro blue tetrazolium reduction was performed to monitor induction of the polymorphonuclear phenotype as in Imaizumi and Breitman (1986). Before binding assays were performed, cells were washed twice in phosphate-buffered saline ($DPBS^{2-}$). After their viability was determined (~75−85%), cells were suspended at 20 × 10^6 cells/mL in $DPBS^{2+}$ (pH 7.4).

Chinese Hamster Ovary Cell Culture. Cells were cultured in petri dishes (100 mm) incubated in a 5% CO_2 atmosphere at 37 °C. CHO cells were grown in αMEM supplemented with adenosine, deoxyadenosine, and thymidine (0.2 mg/mL each), serum (10% FBS), and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). By the DEAE-dextran procedure (Lopata et al., 1984) for transient expression, cells were transfected with plasmids (pINF) containing either the RFP insert ($pLXA_4R^+$) or without ($pLXA_4R^-$) (Fiore et al., 1994). At 48 h after transfection (10 μg of DNA/dish), cells were detached by using $DPBS^{2-}$ containing 5 mM EDTA (3 min, 20 °C) and centrifuged (200g, 10 min) after addition of complete αMEM (2:1 v/v). Ligand binding assays were performed with intact cells suspended in $DPBS^{2+}$.

Ligand Binding Assays. ^{3}H-LXA_4 binding was carried out as in Fiore et al. (1992). Briefly, cell suspensions (5 × 10^6 cells/0.5-mL aliquot) placed onto a silicon oil cushion were incubated (4 °C) with the indicated labeled putative ligand concentrations in the presence or absence of 1−3 log units excess unlabeled ligands to determine total and specific binding. After reaching binding equilibrium, samples were centrifuged at high speed (4 °C, 30 s, 12000g). Pellets were next resuspended in scintillation fluid and radioactivity was determined using a Wallac 1409 β Counter (LKB-Pharmacia). Results obtained from binding experiments were analyzed with the Ligand program (Biosoft Elsevier).

Phospholipase D Activity. HL-60 cells were incubated with ^{3}H-palmitic acid (30 × 10^6/mL, 90 min at 37 °C). Cell uptake and distribution of the ^{3}H-palmitic acid label were similar to those previously reported (Fiore et al., 1992). Incubations (2 × 10^6 cells/mL) were performed at 37 °C with agonist additions in PBS or with PBS plus EtOH (EtOH final concentration = 0.5% v/v) to monitor phosphatidylethanol (PEt) formation (Fiore et al., 1993). Incubations were stopped after 30 s by adding 3.5 ice-cold $CHCl_3$/MeOH (2/5 v/v). Samples were extracted by the Bligh and Dyer method and concentrated organic phases were developed on thin-

layer chromatography as previously described (Fiore et al., 1992). All values for PEt formation were calculated by subtracting the disintegrations per minute obtained in the presence of agonist(s) alone from those measured in the presence of agonist(s) plus 0.5% EtOH.

Oligonucleotide and AntiLXA$_4$R Sera Design. Oligonucleotides were selected from a region of the RFP cDNA sequence which gave low homology with the formyl peptide receptor (vide infra). The following sense and antisense oligos to the 1−15 bp of the open reading frame were selected: 5′ ATGGAAACCAACTTC (sOl_{ATG}) and 5′ GAAGTTGGTTTCCAT ($asOl_{ATG}$). Phosphorothioate oligos were synthesized by and purchased from the Molecular Biology Core Facility of Dana Farber Cancer Institute (Boston, MA). From the molecular analysis of LXA$_4$R deduced amino acid residues, a region with a high antigenic index was selected [according to the Jameson−Wolf method (Wolf et al., 1988)] in the third extracellular domain, ASWGGTPEERLK (with only a ~33% homology with FPR). Custom polyclonal antibodies against the LXA$_4$R peptide were obtained by subcutaneous inoculation of emulsified (1:1 with Freund's adjuvant) multiple antigen peptide (MAP)-linked synthetic peptide in New Zealand rabbits (Research Genetics, Huntsville, LA). Sera obtained after two antigen boost injections gave titers of 6334 (rabbit 8180, serum antiLXA$_4$R1) and 15770 (rabbit 8185, serum antiLXA$_4$R2), respectively, when assayed by enzyme-linked immunosorbent assay (ELISA) against the specific MAP-linked peptide (values provided by supplier). Computer-assisted analysis (BLASTN and BLASTX program algorithms (Altschul et al., 1990)) indicated that both oligonucleotide and peptide sequences matched only against their cognate entries in genetic databases.

Northern Blot Analysis. Poly(A)$^+$ RNA was obtained from total RNA by the guanidine isothiocyanate method, electrophoresed in denaturing agarose gels (5−10 μg/lane), transferred onto nylon filters, and UV cross-linked, as in Perez et al. (1992b). Next, the following probes were labeled with [α-^{32}P]dCTP by random oligo priming: a LXA$_4$R *Eco*RI 1.7-kb fragment, a FPR *Eco*RI−*Xba*I 1.35-kb fragment, and an α-actin open reading frame (ORF) 700-bp fragment. Multiple nylon filter hybridizations with each probe were performed under high stringency conditions [50% (v/v) formamide, 42 °C, overnight]. No apparent cross hybridization was observed with the LXA$_4$R and FPR-derived probes. Multiple bands were observed with both probes as previously reported (Fiore et al., 1994) with major bands at ~2.1 kb for LXA$_4$R and 1.4 kb for FPR specific signals. An *Eco*57I−*Bsr*I 117-bp LXA$_4$R ORF-derived fragment (bp 475−592 ORF) gave identical results to the 1.7-kb *Eco*RI probe.

LXA$_4$R Radioimmunoprecipitation. Thirty-six hours after transfection of CHO cells with either pLXA$_4$R$^+$ or pLXA$_4$R$^-$, αDMEM was removed and replaced with methionine/cysteine-free αDMEM containing ^{35}S-methionine/cysteine (100 μCi/mL, 5 mL/plate). After 12−16 h, medium was removed and plates were rinsed with 10 mL of DPBS. Similarly, undifferentiated or RA- (1 μM, 120 h) differentiated HL-60 cells (both resuspended at 3.5 × 10^6 cells/mL) were labeled for 12−16 h by suspending them in methionine/cysteine-free αDMEM (3.5 × 10^6 cells/mL) containing ^{35}S-methionine/cysteine (100 μCi/mL, 15 mL/flask). Next, with CHO cells 500-μL aliquots of lysis buffer [500 mM NaCl, 10 mM Tris (pH 7.5), and 0.5% NP40] were added and plates were kept at 4 °C for 3−5 min before scraped material was transferred into microcentrifuge tubes. For HL-60 cells a similar procedure was applied. After washing cells (1100 rpm, 10 min), cell pellets were resuspended in 1.5 mL of lysis buffer and 500-μL aliquots were processed as follows. Samples were placed on ice for 30 min and then centrifuged (12000*g* for 30 min at 4 °C). Both supernatants and pellets (resuspended with a lysis buffer, 2% NP40) were used. In parallel, unlabeled CHO cells that were not transfected were processed to obtain cold lysates. Protein A-Sepharose (in DPBS, 10% w/v) was equilibrated with cold lysate (4 °C, 4−6 h, 2/5 v/v) while labeled lysate samples were incubated (2−4 h at 4 °C) with serum (20 μL) and cold lysate [100 μL, or an equivalent volume of 2% bovine serum albumin (BSA)]. Next, aliquots from the protein A mix (80 μL) were added to labeled lysate samples (200 μL), and the tubes were placed on a rocking platform and incubated at 4 °C overnight. Samples were centrifuged (12 000*g* at 4 °C for 10 s), washed five times [four times with ice-cold lysis buffer and the last rinse with ice-cold NaCl (150 mM) and Tris (pH 7.2, 50 mM)], and pellets were resuspended in 60 μL of 2× Laemmli SDS sample buffer. After ^{35}S content was determined, equal amounts of labeled material (60−100 × 10^3 dpm) were loaded onto a gradient SDS−polyacrylamide gel (10−15%). Autoradiography of the fixed gel (treated with autoradiography enhancer, Du Pont−NEN) was obtained with X-OMAT/AR films developed after 12−48 h with a M35A X-OMAT processor (Kodak, Rochester, NY).

FACS Analysis. PMN suspensions obtained after isolation at 4 °C were adjusted to ~22 × 10^6 cells/mL in DPBS^{2+} (pH 7.4) and maintained at 4 °C. At 3 min before stimuli or vehicle additions, the cells (in 450-μL aliquots) were placed at 37 °C. Next, samples were exposed for 3−5 min to either LXA$_4$ at the indicated concentrations (25 μL/sample) or equivalent volumes of buffer containing vehicle (EtOH, concentration did not exceed 0.1%) before addition of FMLP (25 μL). In selected experiments, in parallel to the above treatments, cells were incubated for 5 min at 37 °C with antiLXA$_4$R or control rabbit sera (20 μL/mL of cell suspension) before exposure of samples to LXA$_4$ (vide infra). At the indicated time points, 50-μL aliquots were transferred from samples into microcentrifuge tubes containing 2.0−2.5 μg (in 50 μL of ice-cold DPBS) of either anti-mouse IgG_1 and IgG_2 mouse MOAb (staining negative controls), anti-human CD11b mouse MOAb or anti-human CD11c mouse MOAb. Samples were gently mixed and placed at 4 °C for 30 min. Antibody incubations were stopped by addition of ice-cold DPBS (1 mL) and centrifugation at 500g (10 min at 4 °C). After removal of supernatants, cell pellets were resuspended and centrifuged twice before final resuspension in 500 μL of DPBS containing 2% paraformaldehyde (Lanier et al., 1985). Samples were stored at 4 °C for 24−48 h before FACS analysis was performed with a Becton-Dickinson FACScan.

PMN Aggregation. Homotypic aggregation of PMN was monitored by changes in light transmittance (4-channel aggregation profiler, Model PAP-4, Biodata Co., Hatbow, PA). After isolation, cells were suspended in DPBS (5 × 10^6/mL) and kept at 4 °C. Cell aliquots (1 mL) were placed into siliconized cuvettes, warmed at 37 °C for 3 min, and exposed for 5 min to either buffer alone, control serum, or antiLXA$_4$R2. Next, either vehicle alone or LXA$_4$ (at

indicated concentrations) was added for 3 min at 37 °C. Cuvettes with continuous stirring (800 rpm) were placed in the aggregometer wells and, after addition of FMLP (10^{-9}-10^{-6} M), PMN aggregation was monitored by continuous tracings until a plateau was reached.

RESULTS

LXA_4R Ligand Binding: Comparison between FMLP and LXA_4. CHO cells transfected with $pLXA_4R^+$ display a high-affinity binding for LXA_4, among other eicosanoids tested (Fiore et al., 1994). To evaluate and compare the affinity of transfected LXA_4R for LXA_4 and the peptide FMLP, the specific binding of ^{3}H-LXA_4 and ^{3}H-FMLP was measured with each label in competition assays using either its unlabeled homo- or heteroligand (Figure 1). Incubations in the presence or absence of 1−3 log units excess unlabeled LXA_4 or FMLP were conducted for 5 min (^{3}H-LXA_4, Figure 1, panel A) or 30 min (^{3}H-FMLP, Figure 1, panel B), until binding equilibrium for each ligand was attained. Tritiated LXA_4 (40 nM) binding to $pLXA_4R^+$-transfected CHO cells showed that LXA_4 (30−3000 nM) displacement was ~1000-fold greater than that obtained with FMLP (500−50 000 nM) (Figure 1, panel A). When ^{3}H-FMLP (40 nM) binding was measured after competition with either unlabeled LXA_4 or FMLP, the IC_{50} of LXA_4 also proved to be ~1000-fold greater than that of FMLP (Figure 1, panel B). In agreement with these displacement results, the binding affinities calculated for each of the two labels indicate that LXA_4 is the preferred ligand with a K_d value (6.1 nM) that is ~3 log units better than with FMLP (K_d = 5 μM). These data indicate a highly selective interaction for LXA_4 with this seven-transmembrane-domain receptor. FMLP "cross-interactions" with this receptor are demonstrable but are less effective than native LXA_4.

Time Course of LXA_4R mRNA Expression in HL-60 Cells. Lipoxin A_4 and FMLP bioactivity profiles and ligand binding are characterized for HL-60 cells (Fiore et al., 1993; Imaizumi & Breitman, 1986), which offered a system to examine their interactions with their respective receptors. Poly(A)$^+$ RNA isolated from HL-60 cells at indicated times (0−120 h) during retinoic acid- (RA-) induced differentiation was used in Northern blot analysis. The appearance of distinct mRNAs, observed for both LXA_4R (2.1 kb) and FPR (1.45 kb) (Figure 2A), was detectable as early as ~72 h for LXA_4R and 96 h for FPR (Figure 2B). These induction profiles provided the opportunity to modify LXA_4R expression by use of antisense oligonucleotides. At 48 h after addition of RA, a selective LXA_4R antisense oligonucleotide ($asOl_{ATG}$) directed to the first 15 bp of the open reading frame resulted in inhibition of LXA_4R transcription with loss of the LXA_4R mRNA at 2.1 kb (Figure 3). After LXA_4R antisense oligo treatment, expression of the 1.45-kb mRNA band specific for FPR was still observed at substantial levels (Figure 3). When the complementary sense oligonucleotide (sOl_{ATG}) was added, no apparent effect was obtained on either LXA_4R or FPR messenger RNA (Figure 3). These findings indicated that the LXA_4R antisense oligo selectively blocked LXA_4R transcription, sparing that of FPR.

Impact of LXA_4R Antisense Oligo Treatment on LXA_4 and FMLP Actions. Since $asOl_{ATG}$ treatment of HL-60 cells specifically blocked LXA_4R transcription, RA-treated HL-60 cells exposed to either sense or antisense oligonucleotides

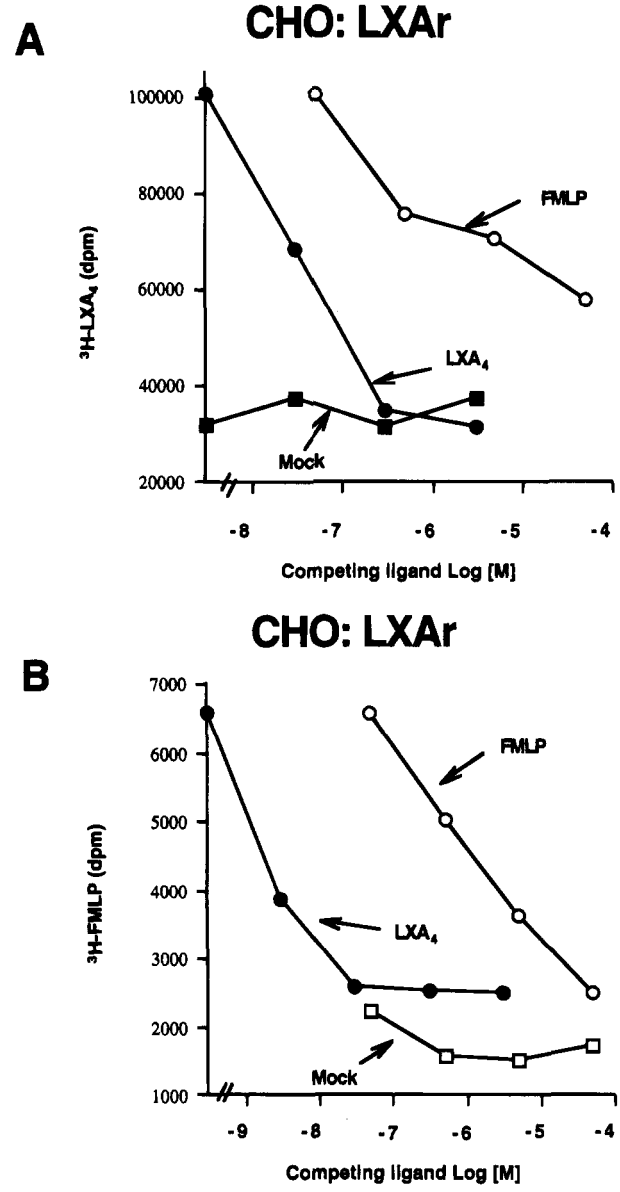

FIGURE 1: LXA_4R affinity for ^{3}H-LXA_4 and ^{3}H-FMLP: specific binding in CHO transfected cells. After transfection (48 h), with either $pLXA_4R^+$ (circles) or $pLXA_4R^-$ (squares), CHO cells were detached with $DPBS^{2-}$ (plus 5 mM EDTA), washed twice, resuspended in DPBS at 50 × 10^6 cells/mL, and placed at 4 °C. Next, aliquots (100 μL) were added to 400 μL containing either ^{3}H-LXA_4 (panel A) or ^{3}H-FMLP (panel B) (final concentrations of 40 nM for both), and specific binding was assessed at 4 °C in the presence or absence of 3−3000 nM unlabeled LXA_4 (filled symbols) or 50−50 000 nM unlabeled FMLP (open symbols). After 5 min (panel A) or 30 min (panel B), cells were pelleted through a silicon oil cushion and cell-associated radioactivity was measured by β scintillation. Results are the average of duplicate determinations from a representative experiment of n = 5 separate transfections.

were used to determine specific binding with ^{3}H-LXA_4 or ^{3}H-FMLP and the ability to stimulate phospholipase D activity (Table 1). Both LXA_4 and FMLP rapidly stimulate PLD activity in differentiated HL-60 cells and PMN (Fiore et al., 1993). Results indicate that $asOl_{ATG}$ but not sOl_{ATG} caused a loss of ^{3}H-LXA_4 binding and LXA_4-stimulated PLD activation, while both ^{3}H-FMLP binding and FMLP-stimulated PLD activation were preserved. Thus in RA-differentiated HL-60 cells the selective loss of LXA_4R blocked the appearance of LXA_4-mediated signal transduction events without affecting the FMLP-stimulated responses

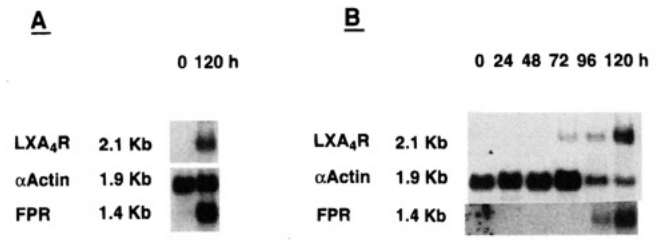

FIGURE 2: Time course of LXA_4R and FPR mRNA appearance in HL-60 cells. HL-60 cells were cultured in RPMI (10% FBS) with or without RA (1 μM). At indicated time intervals (0−120 h), total RNA was obtained from 2×10^8 cells. Poly(A)$^+$ RNA (5 μg/lane) was electrophoresed in denaturing agarose gel, blotted onto nylon filters, and UV cross-linked. Next, membranes were hybridized with either a ^{32}P-labeled LXA_4R probe, an FPR probe, or an α-actin probe. Results are representative of $n = 3$.

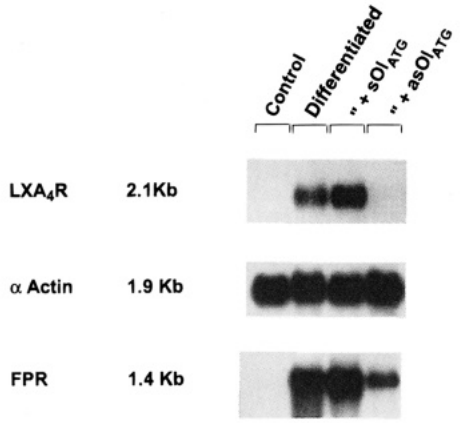

FIGURE 3: Expression of LXA_4R and FPR mRNAs: Impact of antisense ($asOl_{ATG}$) oligo directed against LXA_4R. HL-60 cells were cultured in RPMI (10% FBS) with or without retinoic acid (1 μM) for 48 h. Next, $asOl_{ATG}$ or sense oligonucleotide sOl_{ATG} was added to selected incubations. Total RNA was obtained after 120 h from both undifferentiated HL-60 and antisense-treated differentiated cells. Poly(A)$^+$ RNA from 2×10^8 cells (5 μg/lane) was electrophoresed in denaturing agarose gel and blotted onto nylon filters. Next, membranes were hybridized with a ^{32}P-labeled LXA_4R probe, an FPR probe, or an α-actin probe (ORF fragment). Results are representative of two separate experiments.

(Table 1). These results indicate a selective dependence of LXA_4 actions in these cells on the expression of LXA_4R.

Presence of LXA_4R and Its Relationship to LXA_4- and FMLP-Stimulated Responses in PMN. To evaluate the consequences of LXA_4R occupancy and associated responses in PMN, specific antisera (antiLXA_4R1 and antiLXA_4R2) were raised against a peptide in the third extracellular domain of LXA_4R, a region where homology with the formyl peptide receptor was ~33%, which represents one of the areas with lowest homology between these two receptors. Radioimmunoprecipitation assays with $pLXA_4R^+$-transfected CHO cells using these antisera showed specific precipitation of a protein of apparent molecular mass ~70 kDa (Figure 4, top). When material obtained from HL-60 cells was processed through the same radioimmunoprecipitation, the two antisera also identified two specific protein bands at ~66 and 41 kDa, respectively (Figure 4, bottom). These results are in close agreement with the molecular sizes found for the glycosylated forms of the FMLP receptor in HL-60 cells (Tardif et al., 1993). Addition of antiLXA_4R1 and 2 in competition binding assays with ^{3}H-LXA_4 and ^{3}H-FMLP binding with human PMN (where high-affinity receptors for both LXA_4 and FMLP are present) resulted in a selective loss of the

Table 1: Antisense Oligonucleotide against LXA_4R Blocks LXA_4, but Not FMLP, Specific Binding and PLD Activation with HL-60 Cells[a]

	specific binding (dpm)		PLD activity (PEt, dpm)	
	^{3}H-LXA_4	^{3}H-FMLP	LXA_4 (10^{-9} M)	FMLP (10^{-6} M)
HL-60 (undiff)	0	0	0	0
diff HL-60	1772	8854	7530	4059
diff HL-60+ $asOl_{ATG}$	0	7380	0	5403
diff HL-60+ sOl_{ATG}	1531	5515	4477	4805

[a] HL-60 cells were cultured in RPMI (10% FBS) in the absence or presence of retinoic acid (1 μM). After 48 h, either buffer or antisense or sense oligos (2 μg/mL phosphorothiolate oligos, bps 1−15 of the LXA_4R ORF) were added to cells exposed to RA. After 120 h, the cells were washed twice, suspended in DPBS (pH 7.4), and used either for binding assays with ^{3}H-FMLP and ^{3}H-LXA_4 or labeled with ^{3}H-palmitate [5 μCi/(30×10^6 cells), 90 min at 37 °C] (see Experimental Procedures). Next, ^{3}H-palmitate-labeled HL-60 cells were resuspended (2×10^6 cells/mL), and aliquots (1 mL) were taken for monitoring the formation of ^{3}H-PEt. Levels of ^{3}H-PEt formation were determined before and after exposure (30 s at 37 °C) to indicated concentrations of FMLP and LXA_4 in the presence or absence of 0.5% EtOH (see Experimental Procedures). Tritium content was measured by β counting. Specific binding values (dpm/10^7 cells, left two columns) represent those obtained after subtraction of the nonspecific binding. PLD activity data (dpm/10^7 cells) are corrected by subtracting ^{3}H-PEt values for vehicle ± EtOH alone (right two columns). Data are the average of two separate antisense experiments with duplicate determinations in each.

characteristic LXA_4 competition curve identifying ^{3}H-LXA_4 high-affinity binding sites (Figure 5A). Parallel treatments of PMN did not affect FMLP displacement with its homoligand (Figure 5B). Control rabbit preimmune serum was without effect on the receptor binding of either ligand (Figure 5). Thus the results indicated that both LXA_4R antisera blocked ^{3}H-LXA_4 binding without affecting that of ^{3}H-FMLP.

Since LXA_4 receptor occupancy leads to activation of specific cellular events including lipid remodeling via stimulation of phospholipases A_2 and D (Fiore et al., 1993; Nigam et al., 1990), we examined whether the loss of LXA_4−receptor interactions had an impact on LXA_4 bioactivities. Upregulation of PMN surface expression of CD11/CD18 molecules was measured by FACS analysis after addition of FMLP (Figure 6, panel A), a known potent agonist for CD11/18 upregulation (Molad et al., 1994). The observed 2−3-fold increase of CD11b expression reached plateau at ~5 min and proved concentration-dependent with an EC_{50} for FMLP of ~ 10^{-8} M (Figure 6, panel B). The magnitude of the values for FMLP regulation of CD11b is in close agreement with those reported (Molad et al., 1994). In parallel experiments, PMN exposure to LXA_4 (10^{-9} M, 3 min at 37 °C) resulted in 30−50% inhibition of the FMLP-stimulated CD11b upregulation (Figure 6). This inhibitory LXA_4 activity was concentration-dependent (Figure 6, panel C) with maximal activities between 10^{-9} and 10^{-7} M. LXA_4 added alone to PMN did not modify baseline surface expression of CD11b (Figure 6, panels A and C). Results confirming this LXA_4 activity were obtained in parallel experiments using monoclonal antibodies against CD11c to monitor FMLP-stimulated upregulation of this molecule on the PMN surface. LXA_4 inhibition of FMLP-stimulated CD11c upregulation (data not shown) had characteristics and

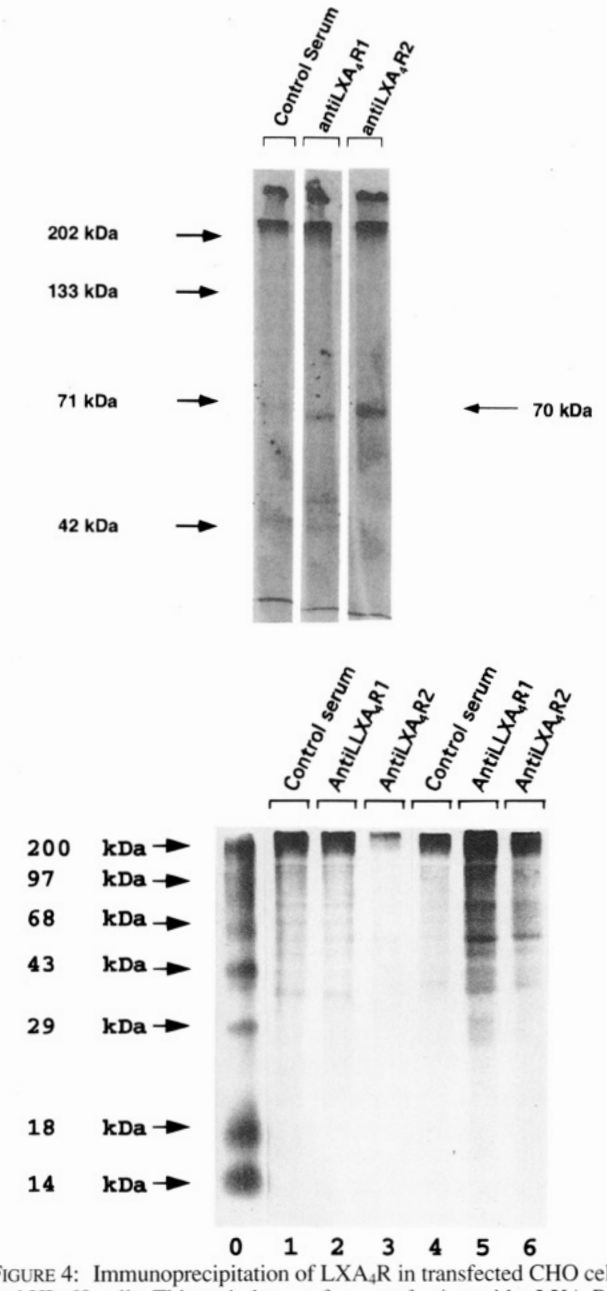

FIGURE 4: Immunoprecipitation of LXA_4R in transfected CHO cells and HL-60 cells. Thirty-six hours after transfection with $pLXA_4R^+$, CHO cells were cultured in the presence of ^{35}S-methionine and ^{35}S-cysteine. A 12−16-h labeling period was also used with undifferentiated or RA differentiated HL-60 cells. Antisera designated antiLXA4R1 and antiLXA4R2 directed against the LXA_4R peptide ASWGGTPEERLK were used to immunoprecipitate cell lysates of either $pLXA_4R^+$-transfected CHO cells (top panel), undifferentiated HL-60 cells (bottom panel, lanes 1−3), or RA-differentiated HL-60 cells (bottom panel, lanes 4−6) (see Experimental Procedures for details). The radioimmunoprecipitated material (~60 000 dpm/lane) was electrophoresed onto a 10−15% gradient SDS−polyacrylamide gel, and protein bands were visualized by autoradiography. Molecular sizes were obtained by comparison with molecular weight standard mixtures applied to the same electrophoretic runs (top panel, rainbow protein markers, high molecular weight; bottom panel, ^{14}C-labeled high molecular weight protein markers, lane 0). Results are representative of $n = 3$.

magnitude similar to those observed with CD11b (Figure 6).

AntiLXA4R2 blocked the LXA_4 inhibitory activity without altering the response to FMLP (Figure 7A). Similar results were obtained using antiLXA4R1 (data not shown). Treatment with either LXA4R2 antisera or control serum did not alter the FMLP-stimulated response, while antiLXA4R2 specifically reversed the inhibitory actions of LXA_4 on FMLP-stimulated CD11b upregulation (Figure 7). Together these results indicate that antiLXA4R2 blockage of ^3H-LXA_4 binding selectively abrogates LXA_4 actions on PMN surface expression of CD11b.

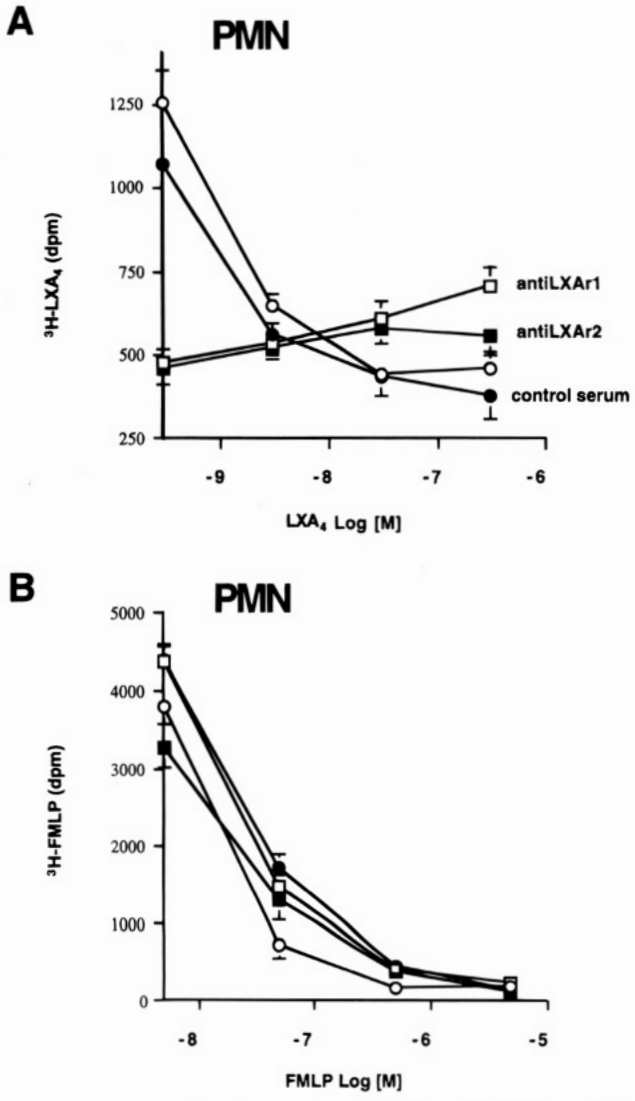

FIGURE 5: Impact of antisera against LXA_4R on ^3H-LXA_4 and ^3H-FMLP binding with PMN. PMN suspended in DPBS (5×10^7-cells/mL) were incubated at 37 °C for 5 min in the presence of buffer alone or rabbit sera (50 μL/mL of cell suspension). Next, cells were placed at 4 °C for 5 min before aliquots (100 μL) were taken to measure either ^3H-LXA_4 (0.3 nM) (panel A) or ^3H-FMLP (5 nM) (panel B) specific binding with PMN. Competition curves with each PMN incubation: buffer alone (open circles), control serum (filled circles), antiLXA4R1 (open squares), or antiLXA4R2 serum (filled squares) were measured in the presence of 1−3 log order excess unlabeled ligands. Results are expressed as the mean ± SEM of $n = 3$ separate experiments with duplicate determinations.

Since $\beta 2$ integrins play a fundamental role in leukocyte homotypic aggregation, we also determined the impact of LXA_4, FMLP, and antiLXA4R2 on PMN aggregation. Results in Figure 8 indicated that LXA_4 (10^{-9} M) inhibited FMLP-stimulated aggregation. Again, addition of antiLXA4R2 to PMN before LXA_4 led to the loss of LXA_4 inhibitory actions on FMLP-stimulated aggregation (Figure 8B). Together, results with LXA_4R antisera and PMN indicate that

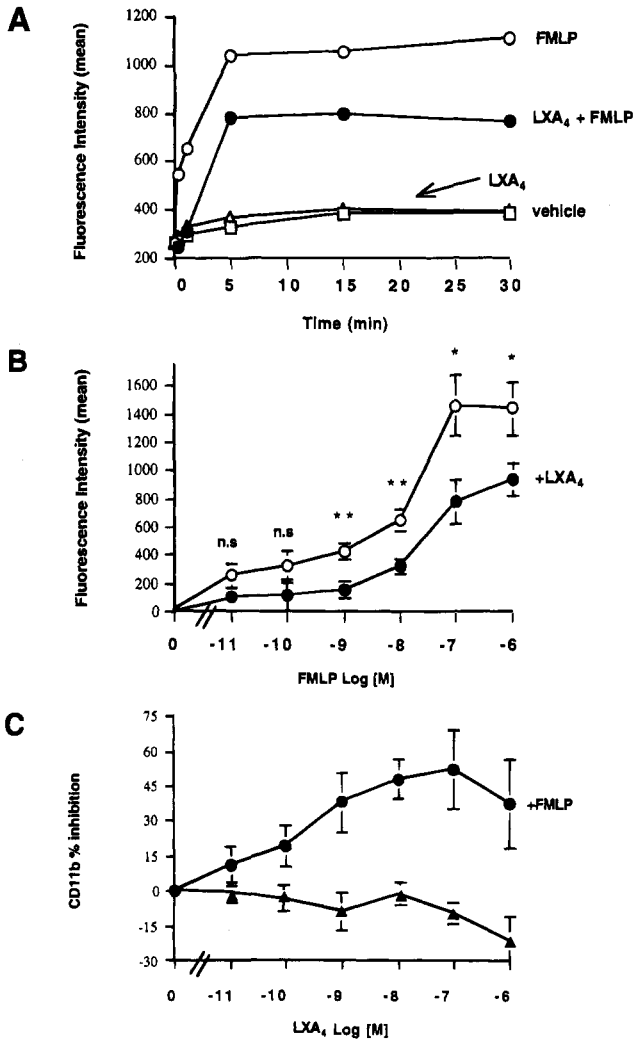

FIGURE 6: Lipoxin A_4 inhibits FMLP-induced CD11b upregulation in PMN. After isolation PMN were suspended in DPBS (2.2×10^7 cells/mL) and kept at 4 °C. Cell aliquots (450 μL) were then placed at 37 °C for 3 min and samples were exposed to either buffer containing vehicle (EtOH < 0.01% final volume) or LXA_4 (10^{-9} M) (25-μL additions) for an additional 3 min before FMLP was added (25-μL additions). Results are expressed as the mean fluorescence intensity for CD11b expression for, (panel A) time course and (panel B), dose–response, using sample exposed to either vehicle alone (open squares), FMLP (open circles), or LXA_4 (10^{-9} M) and FMLP (filled circles). Panel C: Percent inhibition of increasing concentrations of LXA_4 (10^{-11}-10^{-6} M) on the FMLP-(10^{-8} M) induced CD11b upregulation. Paired *t*-test analysis for FMLP and LXA_4 plus FMLP curves is reported for *P* values of <0.05 (*) and <0.01 (**); ns denotes no significant difference between paired values. Results (CD11b values above baseline obtained with vehicle alone) are (panel A) the average of duplicate determinations from a representative of $n = 3$ or (panels B and C) expressed as the mean ± SD of $n = 3$.

selective blockage of LXA_4 specific binding results in modification of LXA_4 bioactivities without affecting FMLP binding or its subsequent responses in these cells.

DISCUSSION

In the present study we have addressed the cellular events following exposure of myeloid cells, in particular human PMN, to LXA_4 and FMLP and we have evaluated their potential interactions and biological consequences initiated by these ligands. Since PMN and differentiated HL-60 cells both express these receptors (Fiore et al., 1993; Imaizumi & Breitman, 1986), CHO cells specifically transfected with $pLXA_4R$ were used to further characterize interactions of LXA_4 and its relation to FMLP ligand binding. Results in Figure 1 clearly indicated that the preferred ligand in CHO cells expressing LXA_4R is LXA_4, whose affinity (K_d = 6.1 nM) exceeds by ~1000-fold that observed for FMLP (K_d = 5 μM). Consistent with this receptor's low-affinity binding of ^{3}H-FMLP, we found that when $pLXA_4R$-transfected CHO cells were tested for ligand-mediated mobilization of arachidonate, FMLP stimulation was observed at micromolar concentrations compared to the subnanomolar concentrations of LXA_4 that elicited a response of the same magnitude (data not shown). Our results with FMLP are consistent with those reported with this receptor in stably transfected fibroblasts that respond only with micromolar concentrations of FMLP (Ye et al., 1992). Thus, on the basis of the present results, namely, (1) the preferred specific binding with ^{3}H-LXA_4 (Figure 1), (2) blocking antisense oligo and LXA_4R antisera (Table 1; Figures 6 and 7), and (3) the impact of these agents on LXA_4 bioactivity (Figure 8), we conclude that LXA_4R,

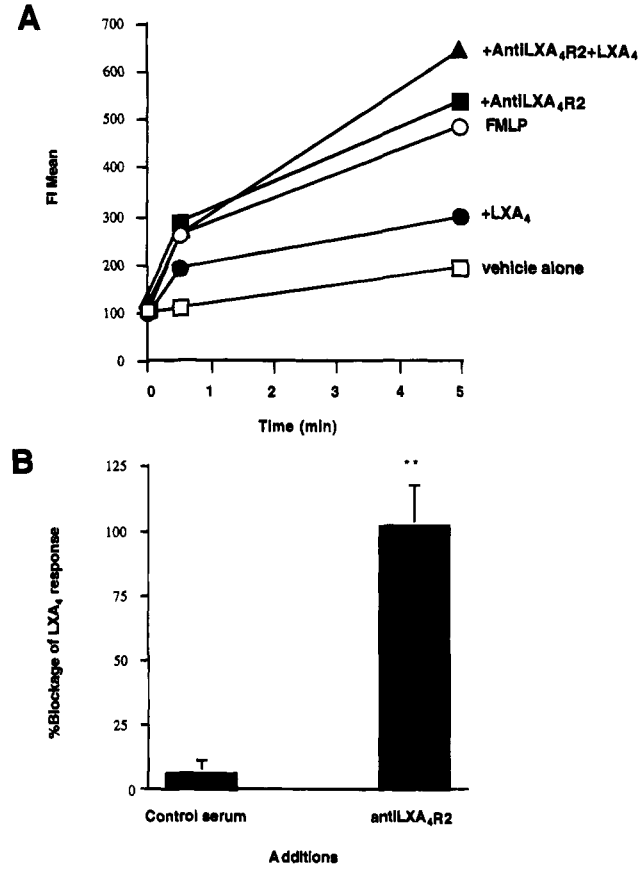

FIGURE 7: AntiLXA$_4$R2 antiserum blocks LXA_4 regulation of FMLP-stimulated CD11b expression. PMN (2.2×10^7cells/mL, in DPBS) were kept at 4 °C. Next, cells were warmed at 37 °C for 3 min before either vehicle or antiLXA$_4$R2 serum was added. For panel A, cell aliquots (450 μL) were added with either vehicle (open squares) or LXA_4 (10^{-9} M) and incubated for an additional 3 min before FMLP was added (10^{-7}M). AntiLXA$_4$R2 serum with cells was exposed to LXA_4 (3–5 min, 37 °C) and FMLP (filled triangles) or without LXA_4 (filled squares). PMN were exposed to FMLP alone (open circles) and after LXA_4 (3–5 min, 37 °C) (filled circles). Panel B shows the percent blockage of LXA_4 response by sera. Paired *t*-test analysis of samples with and without added antiLXA$_4$R2 (panel B) gave a *P* value of < 0.01 (**). Results are expressed as the mean fluorescence intensity of duplicate determinations of a representative experiment of $n = 3$ (panel A) or the mean ± SD of $n = 3$ (panel B).

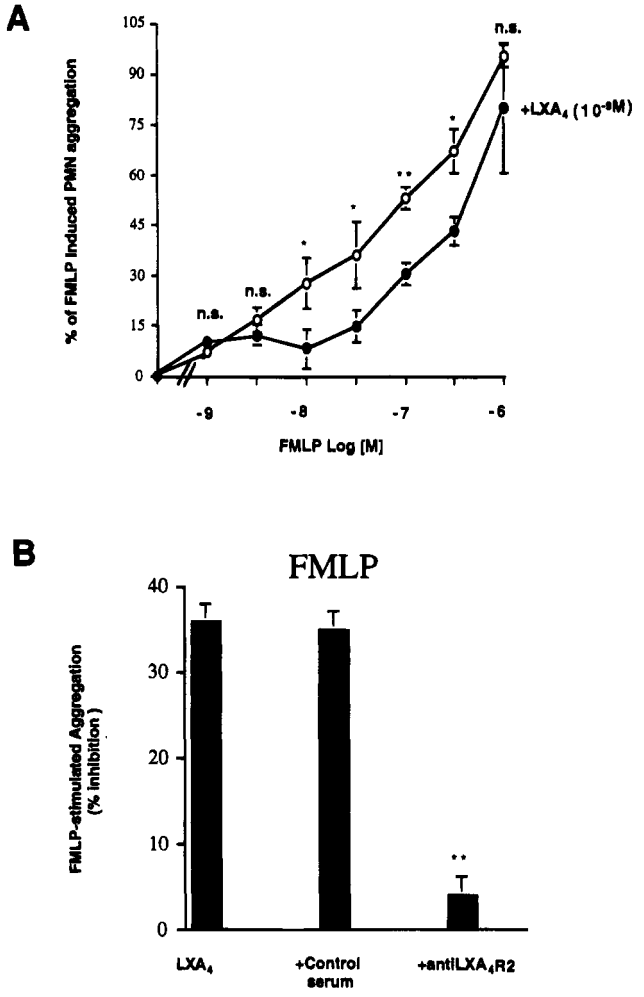

FIGURE 8: LXA_4 inhibits FMLP-stimulated PMN aggregation: Reversal by antiLXA_4R2. Cells were suspended in DPBS (5×10^6 cells/mL) and kept at 4 °C; aliquots (1 mL) were placed into siliconized cuvettes and placed at 37° for 3 min before being exposed to either vehicle (panel A, open circles) or LXA_4 (10^{-9} M) (panel A, filled circles). After an additional 3 min, cuvettes were placed in the aggregometer wells (stirred at 800 rpm), and PMN aggregation, triggered by adding the indicated concentrations of FMLP, was monitored with continuous tracings until a plateau was reached. In parallel determinations cells were exposed to either buffer alone, control serum, or antiLXA_4R2 followed by addition of LXA_4 (10^{-9} M). LXA_4 inhibition of FMLP-stimulated (10^{-7} M) PMN aggregation is determined as the percent of the FMLP response (panel B, black bars). Paired *t*-test analysis for the FMLP and LXA_4 plus FMLP curves (panel A) and for samples with or without added antiLXA_4R2 (panel B) are reported for *P* values of <0.05 (*) and <0.01 (**); ns denotes not significant differences between paired values. Results are the mean ± SD of three separate experiments.

formerly termed RFP, FPR2, or FPRL-1 (Murphy et al., 1992; Perez et al., 1992a; Ye et al., 1992), is required for LXA_4 receptor-mediated actions in myeloid cells and acts as its cognate receptor in these cells.

The bioactivity profile of several agonists that require binding to G-protein-coupled serpentine receptors is linked to the availability of specific transduction pathways (Uhing et al., 1992). Mammalian expression systems, used here for ligand binding experiments, may lack several of the necessary components needed to reconstitute native signal transduction pathways. Thus, these systems might afford valid examination of receptor occupancy (i.e., binding) but still fail to assess a putative ligand's ability to "activate" the receptor and transduce signal. In light of these considerations, further characterization of the interactions occurring between LXA_4, FMLP, and their receptors (LXA_4R and FPR) was carried out in differentiated HL-60 cells and PMN, cells where both agonists have specific receptor-mediated interactions (Fiore et al., 1993; Imaizumi & Breitman, 1986). Northern hybridizations revealed, as expected, the specific mRNAs present in RA-differentiated HL-60 cells for both LXA_4R and FPR (Figure 2A). The time course results indicate that the LXA_4R mRNA-specific signal at 2.1 kb appears as early as 48−72 h (Figure 2B). A specific FPR mRNA signal was detected ~96 h after exposure to retinoic acid (Figure 2B). Selective modification of this pattern of receptor expression during differentiation was achieved by exposing HL-60 cells to LXA_4R specific antisense oligos (Figure 3). This approach has been shown to be highly effective in modifying gene expression by altering transcriptional as well as subsequent events, even for constitutively expressed genes (Manfredini et al., 1993; Skorski et al., 1994). Treatment with $asOl_{ATG}$, but not the complementary sense oligo, led to loss of LXA_4R mRNA while expression of the FPR mRNA signal was preserved. The selectivity of $asOl_{ATG}$, revealed at the nucleic acid level in these experiments, was further analyzed in parallel to evaluate whether the impaired ability of ^3H-LXA_4 to bind with differentiated HL-60 cells correlated with the loss of LXA_4 receptor-stimulated events. PLD activation, one of the lipid remodeling pathways stimulated by LXA_4 interactions with its receptor (Fiore et al., 1993), was found to be completely abrogated with $asOl_{ATG}$ but not with sOl_{ATG} treatment (Table 1). Conversely, the preserved expression of FPR mRNA combined with unaltered ^3H-FMLP binding and FMLP-stimulated PLD activation underscores the high degree of selectivity of treatment with $asOl_{ATG}$ (Table 1). Thus, the presence of LXA_4R in HL-60 cells is selectively associated with LXA_4-linked signaling events, while it is not required for those events initiated by FMLP.

Study of gene expression using techniques that are applicable to cell lines such as HL-60 cells is hindered in PMN by the reduced transcriptional activity of these cells. The development of specific antisera has been reported to be a highly selective tool in the characterization of G-protein-coupled serpentine receptors such as those for PAF and IL-8 (Chuntharapai et al., 1994; Thivierge et al., 1993). Both blocking antibodies and stimulating antibodies that specifically interact with the target receptor proteins have been developed (Chuntharapai et al., 1994; Thivierge et al., 1993). Along these lines, we obtained rabbit antisera toward a specific peptide of the LXA_4R mapping to the third extracellular domain in the deduced amino acid sequence. After the selectivity of the antisera was characterized by radioimmunoprecipitation assays with either $pLXA_4R$-transfected CHO cell lysates (Figure 4, top) or HL-60 cells (Figure 4, bottom), specific binding for both ^3H-LXA_4 and ^3H-FMLP with PMN was monitored. The obtained sera gave inhibitory actions on the high-affinity binding of LXA_4 but not with FMLP high-affinity binding with PMN (Figure 5).

The blocking action of the antiLXA_4R sera was also monitored with LXA_4- and FMLP-elicited PMN responses. Evaluation of LXA_4 bioactions in both *in vivo* and *in vitro* models such as PMN extravasation and transmigration through epithelial or endothelial monolayer (Colgan et al., 1993; Hedqvist et al., 1989; Papayianni et al., 1994) indicates

that LXA_4 blocks PMN adhesiveness. Since in many of these processes a major role is played by the surface adhesion molecules CD11/18 β2 integrin complex in PMN, we next determined whether LXA_4 modified FMLP-stimulated upregulation of CD11b, which is considered a well-recognized marker of PMN activation (Molad et al., 1994). FACS analysis of PMN stained with fluorescein isothiocyanate- (FITC-) or phycoerythrin- (PE-) conjugated monoclonal antibodies against CD11b is a relatively rapid and accurate means of monitoring changes in surface levels of this β2 integrin complex (Molad et al., 1994). Lipoxin A_4 inhibited FMLP-stimulated CD11b upregulation in a concentration-dependent fashion (Figures 6 and 7). AntiLXA_4R sera specifically reversed LXA_4 activity without altering the PMN response to FMLP (Figure 7). In addition to heterotypic cell−cell interactions, the CD11/18 complex is also involved in sustaining PMN homotypic aggregation. After exposure to LXA_4 (10^{-9} M), ~35% inhibition of homotypic aggregation was observed (Figure 8), which was reversed with antiLXA_4R antisera (Figure 8B). These new findings with CD11b suggest that the ability of LXA_4 to inhibit CD11 expression on the PMN surface is a potential mechanism by which LXA_4 exerts its reported inhibitory actions in blocking PMN adhesion to endothelial cells (Papayianni et al., 1994) and migration on epithelial cells (Colgan et al., 1993) as well as *in vivo* inhibition of transmigration (Hedqvist et al., 1989).

Together, results of the present report indicate that LXA_4 responses evoked at nanomolar levels require specific receptor activation in myeloid cells. In these cells LXA_4R is linked to biological activities that can counteract leukocyte activation as monitored by LXA_4 regulation of CD11 expression, a response stimulated by the FMLP receptor. In addition, the specificity observed for the onset of biological responses after LXA_4 and FMLP occupancy of their respective cognate receptors suggests that, at physiologically relevant concentrations (i.e., subnanomolar), cross-interactions observed in mammalian expression systems might not be related to physiologic or pathophysiologic events. This is of particular relevance when it is taken into account that LXA_4 is an endogenous lipid-derived mediator whereas FMLP is an exogenous stimulus of PMN potentially derived from microbial proteins. It is likely that ligand cross-interactions observed *in vitro* at FMLP concentrations >1−10 μM are pharmacologic (Figure 1) in that, although LXA_4R registers a 67% identity and 80% similarity at the protein level to FPR, this receptor prefers a lipid-derived ligand, namely, LXA_4. Our findings do not preclude other peptides as potential ligands for other orphan members of this cluster or group of formyl peptide-related sequences (Boulay et al., 1990; Gao & Murphy, 1993). The present results also indicate that LXA_4 occupancy of LXA_4R can be an important means to control or regulate PMN cellular events that may be relevant during the onset of inflammation and tissue injury, namely, the regulation of adhesion complexes.

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