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Lipoxin A_4 Stable Analogs Are Potent Mimetics That Stimulate Human Monocytes and THP-1 Cells via a G-protein-linked Lipoxin A_4 Receptor*

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Lipoxins (LX) are bioactive eicosanoids that activate human monocytes and inhibit neutrophils. LXA_4 is rapidly converted by monocytes to inactive products, and to resist metabolism, synthetic analogs of LXA_4 were designed. Here, we examined the bioactivity of several LXA_4 analogs in monocytes and found, for chemotaxis, 15(*R/S*)-methyl-LXA_4 and 15-epi-LXA_4 were equal in activity, and 16-phenoxy-LXA_4 was more potent than native LXA_4. Both 15(*R/S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 were ~1 log molar more potent than LXA_4 in stimulating THP-1 cell adherence ($EC_{50} \approx 1 \times 10^{-10}$ M). Dimethylamide derivatives of the LXA_4 analogs also possessed agonist rather than antagonist properties for monocytes. Neither LXA_4 nor 16-phenoxy-LXA_4 affected monocyte-mediated cytotoxicity. We cloned an LXA_4 receptor from THP-1 cells identical to that found in PMN. Evidence of receptor-mediated function of LXA_4 and the stable analogs in monocytes included desensitization of intracellular calcium mobilization to a second challenge by equimolar concentrations of these analogs, but not to LTB_4. Increases in $[Ca^{2+}]_i$ by LXA_4 and the analogs were specifically inhibited by an antipeptide antibody to the LXA_4 receptor; and both LXA_4- and analog-induced adherence and increments in Ca^{2+} were sensitive to pertussis toxin. Together, these results indicate that the LXA_4 stable analogs are potent monocyte chemoattractants and are more potent than native LXA_4 in stimulating THP-1 cell adherence, at subnanomolar concentrations. Moreover, they provide additional evidence that the LXA_4 stable analogs retain selective bioactivity in monocytes and are valuable instruments for examining the functions and modes of action of LXA_4.

Lipoxins (LX)[1] are members of the eicosanoid family of bioactive lipid mediators with trihydroxytetraene structures (1). They are generated during cell-cell interactions and transcellular transfer of intermediates (2, 3) and lipoxin A_4 (LXA_4) was recently identified *in vivo* from human patients with rheumatoid arthritis (4) or asthma (5). The original pathways of LX formation identified were via lipoxygenase-lipoxygenase interactions and an additional route of novel LX formation was recently demonstrated (6). This pathway, via aspirin-triggered acetylation of cyclooxygenase-2 in human endothelial cell/neutrophil (PMN) coincubations, results in generation of 15*R*-epimers of LX (*e.g.* 15-epi-LXA_4). Thus, LX are the first products identified from both lipoxygenase-lipoxygenase and cyclooxygenase-lipoxygenase interactions.

Lipoxins display selective activities on human leukocytes that are either stimulatory or inhibitory, depending on the target cell type involved. In human PMN, LXA_4 induces chemokinesis but inhibits chemotaxis toward leukotriene B_4 (LTB_4) and *N*-formylmethionylleucylphenylalanine (FMLP) (7). LXA_4 also inhibits FMLP-induced PMN transmigration across intestinal epithelium (8). In addition, LTB_4- and LTC_4-induced PMN adherence to human umbilical vein endothelial cells (HUVEC) is reduced by ~70% by LXA_4 and LXB_4 (9) and aspirin-triggered 15-epi-LXA_4 also inhibits LTB_4-stimulated PMN adherence to HUVEC at nanomolar concentrations and is ~two times more potent than LXA_4 in this setting (6). LXA_4 and LXB_4 also down-regulate peptidoleukotriene-induced P-selectin expression on HUVEC (9). LXA_4 displays *in vivo* activity, with inhibition of PMN migration into the kidney in rat glomerulonephritis models (3) and inhibition of PMN diapedesis from postcapillary venules (10). In contrast to the down-regulation of PMN, LX exhibit selective stimulatory activities in the monocyte, as we recently described potent activation of human monocyte migration and adherence to laminin by both LXA_4 and LXB_4 (11).

Human monocytes were also found to rapidly convert more than 80% of added LXA_4 to novel metabolites via dehydrogenation and reduction of double bonds and the products were identified as 15-oxo-LXA_4; 13,14-dihydro-15-oxo-LXA_4 and 13,14-dihydro-LXA_4 (12). The oxo- and dihydro-LX produced by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U81501.

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[1] The abbreviations used are: LX, lipoxins; BCECF-AM, [2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester]; $DPBS^{2-}$, Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+}; $DPBS^{2+}$, Dulbecco's phosphate-buffered saline with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$; FMLP, *N*-formylmethionylleucylphenylalanine; HUVEC, human umbilical vein endothelial cells; LTB_4 (leukotriene B_4), (5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; LXA_4 (lipoxin A_4), (5*S*,6*R*,15*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 15-epi-LXA_4 (15-epi-lipoxin A_4), (5*S*,6*R*,15*R*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; LXB_4 (lipoxin B_4), (5*S*,14*R*,15*S*)-trihydroxy-6,8,12-*trans*-10-*cis*-eicosatetraenoic acid; Me, methyl ester; 15-(*R*/*S*)-methyl-LXA_4, (5*S*,6*R*,15*R*/*S*)-trihydroxy-15-methyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid; 16-phenoxy-LXA_4, 16-phenoxy-17,18,19,20-tetranor-LXA_4; PMN, polymorphonuclear leukocyte; PTX, pertussis toxin.

monocytes were essentially inactive in stimulating monocyte adherence, in contrast to the native compounds (11). The dehydrogenation of LX (*i.e.* production of oxo-LX) by human monocytes appears to be carried out by 15-hydroxyprostaglandin dehydrogenase, which is present in these cells as determined by reverse transcriptase-polymerase chain reaction and Western blotting (11) and was confirmed using recombinant enzyme (13).

We identified an orphan seven-transmembrane receptor cDNA as a high-affinity receptor for LXA_4 (14). LXA_4 binding to this receptor activates both phospholipase A_2 and phospholipase D (14, 15), responses that are inhibited by pretreatment of cells with pertussis toxin (PTX). The magnitude of Ca^{2+} mobilization via this receptor appears to be cell type-specific. For example, Ca^{2+} mobilization by LXA_4 in PMN is less than 10% of that induced by an equal concentration of FMLP (16), and in monocytic cells, LXA_4, but not LXB_4, induces increases in intracellular Ca^{2+} greater than 50% of that induced by equimolar FMLP (17). These findings imply different stimulation of second messengers with cell type specificity and are in concordance with the different responses of monocytes *versus* PMN to LX. They also suggest that LX could contribute to resolution of injury at sites of inflammation by suppressing PMN influx and stimulating a self-limiting (by monocyte metabolism) monocyte migration to promote healing.

In view of the rapid transformation and inactivation of the LX by monocytes and, potentially, other cells *in vivo*, it was highly desirable to design LX analogs that would resist this metabolism and maintain their structural integrity and potential beneficial biologic actions to use as tools in disease research. To this end, LXA_4 analogs have been synthesized, and they were evaluated for their structural stability in incubations with monocytic cells and with 15-hydroxyprostaglandin dehydrogenase and were resistant to conversion in both cell and enzyme incubations (13). Here, we examined the biologic activity of the synthetic LXA_4 analogs with monocytes and THP-1 cells and determined the relative potency of these compounds *versus* native LXA_4 in stimulating migration and adherence as well as Ca^{2+} mobilization in these cells. In addition, we provide the first evidence that the analogs act via the same G-protein-linked receptor as LXA_4.

EXPERIMENTAL PROCEDURES

LXA_4 and Analog Analysis—LXA_4 was purchased from Cascade Biochem Ltd. (Berkshire, United Kingdom) and synthetic analogs were prepared and characterized, including NMR spectroscopy, as in Ref. 13. The integrity and concentration of each synthetic LX analog was assessed before each series of experiments. Concentrations of analogs were determined using an extinction coefficient of 50,000.

Cell Culture and Isolation—The human acute monocytic leukemia cell line, THP-1 (ATCC, Rockville, MD), was maintained in RPMI (Biowhittaker, Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Biowhittaker) and antibiotics in a 37 °C incubator with 5% CO_2 atmosphere. Human monocytes were isolated using a modification of the method of Denholm and Wolber (18). Briefly, whole blood collected in acid citrate dextrose from healthy volunteers was centrifuged ($200 \times g$) at 25 °C for 15 min for removal of platelet-rich plasma. The plasma was aspirated and the cells were resuspended to the original blood volume with Dulbecco's phosphate-buffered saline ($DPBS^{2-}$, Biowhittaker), layered over Ficoll-Hypaque (Organon Teknika Corp., Durham, NC), and centrifuged ($500 \times g$) at 25 °C for 35 min. The mononuclear cell layer was collected, washed once, and resuspended in $DPBS^{2-}$ plus 0.1% bovine serum albumin. A Percoll:10 × Hanks' balanced salt solution (10:1.65) mixture was prepared and 8 ml mixed with 4 ml of mononuclear cells in a 10 × 1.5-cm round-bottom silanized polypropylene tube and centrifuged ($370 \times g$) at 25 °C for 30 min. Monocytes were collected from the upper 5 mm of the gradient and washed with 50 ml of PBS^{2-} before counting and viability assessment.

Chemotaxis—Chamber chemotaxis was evaluated using a microchamber technique according to the method of Falk *et al.* (19). Monocytes were isolated as above and suspended at 5×10^6/ml in $DPBS^{2+}$. Thirty μl of a chemoattractant solution or vehicle ($DPBS^{2+}$ plus 0.05% EtOH) were added to the lower wells of a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD). A polycarbonate membrane (5 μm pore size) was layered on top of the chemoattractant wells and 40 μl of monocytes added to the top wells and the chamber incubated at 37 °C for 90 min. After incubation, the membrane was removed, scraped of cells from the upper surface, and stained with modified Wright-Giemsa stain. Cells that migrated through the membrane in four high power fields were counted.

Laminin Adhesion—Monocytes were resuspended in $DPBS^{2-}$ (4×10^6/ml) and [2′,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein acetoxymethyl ester] (BCECF-AM, Calbiochem, La Jolla, CA) was added (1.0 μM) and incubated with cells for 20 min at 37 °C. Cells were washed once with $DPBS^{2-}$ and suspended (3.3×10^6/ml) in $DPBS^{2+}$ plus 0.1% bovine serum albumin. Aliquots (90 μl) of cells were added to each well of a 96-well flat-bottom tissue culture plate coated with laminin (Collaborative Biomedical Products, Bedford, MA) and allowed to settle for 10 min. Ten μl of agonist or vehicle were added to each well and plates incubated at 37 °C for 20 min. Following incubation, wells were aspirated and washed once with $DPBS^{2+}$ plus 0.1% bovine serum albumin. Adherent cells were solubilized with 100 μl of 0.025 M NaOH plus 0.1% sodium dodecyl sulfate and the plate stirred on a rotary shaker for 20 min, followed by fluorescence quantitation. Adherence of THP-1 cells with exposure to vehicle alone was 6.3–8.3% of total cells added.

Calcium Mobilization—Monocytes and/or THP-1 cells (2.5×10^6 cells/ml) were loaded with Indo-1 (2 μM) (Molecular Probes, Inc., Princeton, NJ) for 30 min, washed twice, and suspended in Hanks' balanced salt solution supplemented with Ca^{2+} (1 mM). Indo-1 excitation was at 358 nm, with detection of fluorescence at 405 nm (Photon Technology International Deltascan 4000, South Brunswick, NJ). The intracellular Ca^{2+} concentrations were calculated as described in Tsien *et al.* (20).

Cell-mediated Cytotoxicity—Monocytes (2.5×10^5 cells/50 μl) were added to 96-well tissue culture plates in RPMI plus 10% fetal bovine serum and incubated overnight at 37 °C with either vehicle ($DPBS^{2+}$ plus 0.1% EtOH), lipopolysaccharide (100 ng/ml) from *Escherichia coli* serotype O26:B6 (Sigma), LXA_4 (1 μM), 16-phenoxy-LXA_4-Me (1 μM), or a combination of lipopolysaccharide (100 ng/ml) and LX (1 μM). Following overnight incubation, target cells (THP-1 cells) were added (5.0×10^4 cells/50 μl) to achieve a ratio of 5 effector cells/1 target cell. Cells were incubated together overnight at 37 °C. Following the second overnight incubation, 50-μl aliquots of medium supernatant were removed for use in a lactate dehydrogenase based cytoxicity assay (Cyto Tox 96®, Promega Corp., Madison, WI).

Receptor Cloning—Total RNA was isolated from THP-1 cells using TRIzol™ reagent (Life Technologies, Inc.). One μg of total RNA was reverse-transcribed and used as the template for a PCR. PCR was performed using the sense primer 5′-CACCAGGTGCTGCTGGCAAG-3′ (corresponding to the immediate 5′ side of the starting codon ATG of the LXA_4 receptor cloned from HL-60 cells (21)) and antisense primer 5′-AATATCCCTGACCCCATCCTCA-3′ (corresponding to the immediate 3′ side of the stop codon TGA) for 30 cycles (94 °C for 30 s, 64 °C for 45 s, and 72 °C for 80 s) with TaqPlus™ DNA polymerase (Stratagene, La Jolla, CA). A single band of approximately 1.1 kilobase pairs was obtained and subcloned into the *Eco*RV site of pBluescript II KS(+) (Stratagene). Three independent clones were subjected to sequencing by an automated sequencer (ABI PRISM model 373A, version 1.20).

RESULTS

LXA_4 is rapidly transformed by monocytes by initial dehydrogenation at carbon 15 to 15-oxo-LXA_4, which is biologically inactive (11, 13). Therefore, a series of analogs was designed with bulky substitutions on carbon 15, or at the carbon 20 end of the molecule (Fig. 1). The aspirin-triggered compound, 15-epi-LXA_4, and free acid, methyl ester (Me), and dimethylamide forms of 15(R/S)-methyl-LXA_4 and 16-phenoxy-LXA_4 (Fig. 1) were synthesized and used in the present experiments.

Several of these LXA_4 analogs proved to be potent chemotaxins for monocytes with 15-epi-LXA_4, 15(R/S)-methyl-LXA_4, and 16-phenoxy-LXA_4 stimulating migration of monocytes at 100 nM concentrations (Fig. 2). 15-epi-LXA_4 and 15(R/S)-methyl-LXA_4 were similar in potency to LXA_4. 16-Phenoxy-LXA_4 stimulated greater numbers of monocytes to migrate than LXA_4 and was ~23% fewer than the number of cells migrating with FMLP at this concentration (Fig. 2).

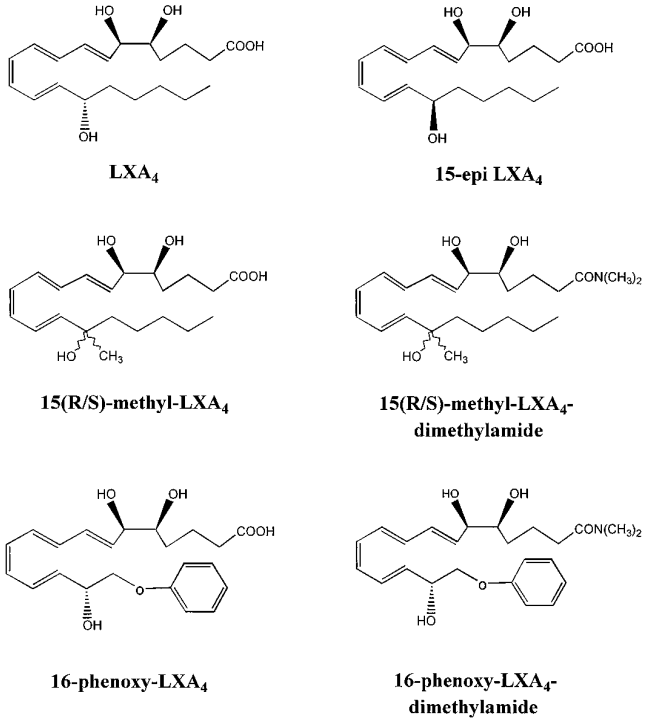

FIG. 1. **Structures of lipoxin A$_4$ and synthetic stable analogs used in these experiments.** Structures are depicted as free acids; the methyl esters of 15(*R*/*S*)-methyl-LXA$_4$ and 16-phenoxy-LXA$_4$ were also prepared and used in some experiments and are identified as such in figure legends.

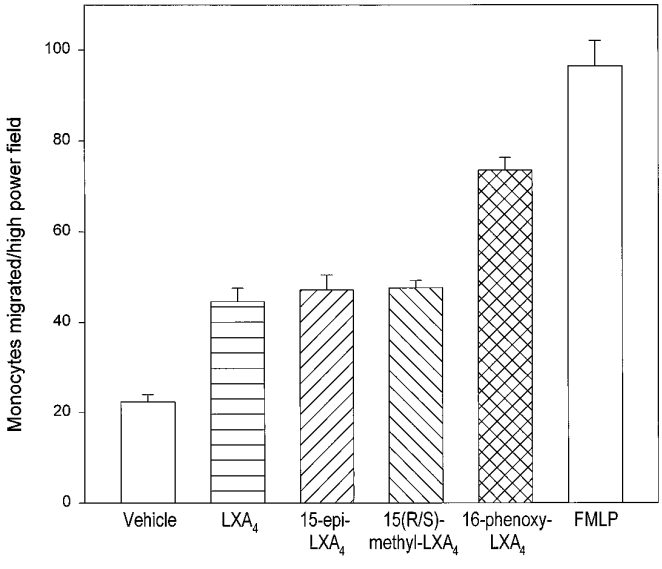

FIG. 2. **LXA$_4$ stable analogs stimulate human monocyte chemotaxis.** Chemotaxis was quantitated in chambers using agonists at equimolar concentrations (10^{-7} M). Values represent mean numbers (±S.E.) of monocytes that migrated through 5-μm polycarbonate filters and are expressed as cell counts per high power field for four separate experiments performed in triplicate. Values for all agonists are significantly higher than vehicle as analyzed using paired Student's *t* tests ($p < 0.001$).

LXA$_4$ analogs were also potent stimuli of THP-1 cell adherence to laminin (Fig. 3). 15(*R*/*S*)-methyl-LXA$_4$ and 16-phenoxy-LXA$_4$ were more effective than LXA$_4$ in stimulating THP-1 cell adherence, especially at concentrations less than 1 nM (EC_{50} analogs $\approx 8 \times 10^{-11}$ M, EC_{50} LXA$_4$ $= 8.3 \times 10^{-10}$ M).

A seven-transmembrane-spanning receptor cloned from neutrophilic HL-60 cells was recently characterized by our laboratory as a high-affinity LXA$_4$ receptor (14). LXA$_4$ activity with PMN appears to be mediated via binding to this receptor, confirmed by specifically inhibiting LXA$_4$ actions via treatment of cells with LXA$_4$-receptor antisense oligonucleotides or an antireceptor antibody (15). To determine if the same receptor is present in monocytic cells, we used primers from the PMN receptor sequence and reverse transcribed total RNA from THP-1 cells. One band of approximately 1.1 kilobase pairs in size was produced from this reverse transcriptase-polymerase chain reaction and, when sequenced (GenBank™ accession number U81501), proved to be identical to the LXA$_4$ receptor cloned from the differentiated (neutrophil-like) HL-60 cells. A band of identical size was also seen in reverse transcriptase-polymerase chain reaction using human peripheral blood monocyte RNA (data not shown). The deduced amino acid sequence in Fig. 4 shows the seven-transmembrane regions as well as the peptide sequence in the third extracellular domain to which specific antisera were raised (15). This domain was chosen for production of antiserum, because it is a region of high antigenicity and is an area predicted to interact with ligand.

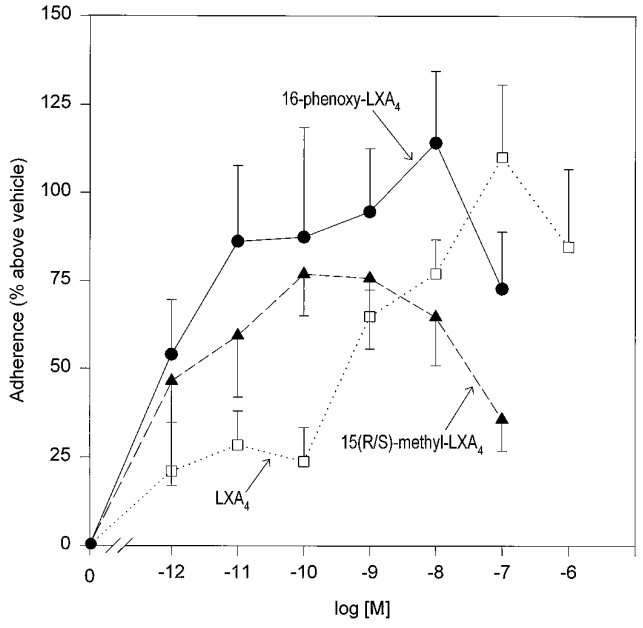

FIG. 3. **LXA$_4$ stable analogs increase THP-1 cell adherence to laminin.** THP-1 cells were labeled with BCECF-AM and incubated (3×10^5 cells in 100 μl) with each compound for 40 min at 37 °C. Values represent means ± S.E. for four separate experiments performed in quadruplicate and are expressed as the percent adherence above that with cells exposed to vehicle alone.

LXA$_4$ increases intracellular calcium in monocytes (17); therefore we examined the impact and potency of LXA$_4$ analogs in this system. Both 15(*R*/*S*)-methyl-LXA$_4$-Me and 16-phenoxy-LXA$_4$-Me stimulated concentration-dependent increases in intracellular Ca^{2+} in monocytes (Fig. 5). Similar results were obtained for both the methyl ester and free acid forms of LXA$_4$ and the analogs (data not shown). 16-Phenoxy-LXA$_4$-Me stimulated greater increments in Ca^{2+} than LXA$_4$, while 15(*R*/*S*)-methyl-LXA$_4$-Me mobilized less Ca^{2+} than LXA$_4$ within this concentration range. We recently determined that adherence of monocytes to laminin was not dependent on an increase in $[Ca^{2+}]_i$, because addition of an intracellular Ca^{2+} chelator to monocytes did not result in inhibition of LX-induced adherence (17). Changes in intracellular Ca^{2+} are, nevertheless, a reliable means to assess receptor-mediated signaling and may be connected with a specific Ca^{2+}-mediated function, which has not yet been identified; therefore, it was further employed to de-

FIG. 4. **Myeloid LXA$_4$ receptor.** Deduced amino acid sequence of the LXA$_4$ receptor. Transmembrane regions are indicated by *shaded boxes*, potential glycosylation sites are marked with an *asterisk*, and the third extracellular domain to which an antipeptide antiserum was raised is *overlined*. The cDNA sequence of the LXA$_4$ receptor was cloned from total RNA isolated from undifferentiated THP-1 cells, and 1 μg was reverse-transcribed and used as the template for PCR (see "Experimental Procedures").

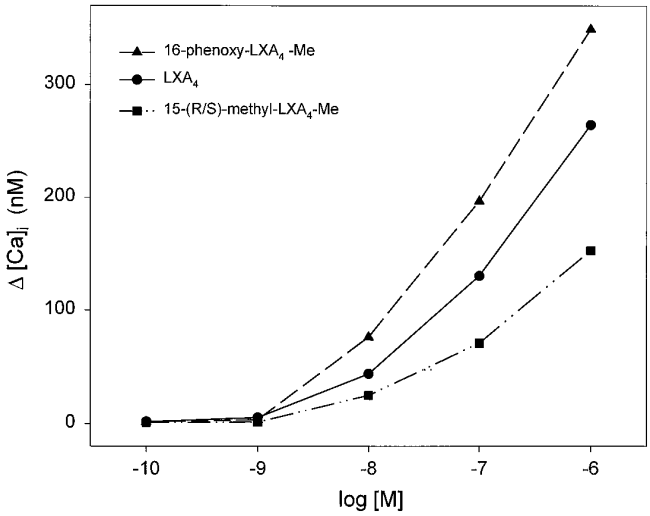

FIG. 5. **LXA$_4$ stable analogs mobilize intracellular Ca^{2+} in human monocytes.** Peripheral monocytes (2.5×10^6 cells/ml) loaded with Indo-1 (2 μM) were suspended in Hanks' balanced salt solution supplemented with 1 mM Ca^{2+} and exposed to LXA$_4$, 15(R/S)-methyl-LXA$_4$-Me, or 16-phenoxy-LXA$_4$-Me under constant stirring. Values are from a representative experiment performed in duplicate from $n = 3$ separate donors.

termine whether monocyte activation by LXA$_4$ and analogs is mediated by a common receptor site. We first examined homologous desensitization by LXA$_4$ and found that it completely down-regulates Ca^{2+} mobilization by a second equimolar addition of LXA$_4$ (Fig. 6*A*). The specificity of this response was determined by addition of LTB$_4$ to the cells approximately 60 s after LXA$_4$. The Ca^{2+} response to LTB$_4$ was unaffected by prior stimulation with equimolar LXA$_4$ (Fig. 6*A*). We next tested for cross-desensitization between LXA$_4$ and the individual analogs and found that addition of LXA$_4$, followed by either 15(R/S)-methyl-LXA$_4$-Me or 16-phenoxy-LXA$_4$-Me, or *vice versa*, completely desensitized the Ca^{2+} response to the second ligand (Fig. 6*B*).

To determine if the Ca^{2+} response to LXA$_4$, 15(R/S)-methyl-LXA$_4$-Me, and 16-phenoxy-LXA$_4$-Me was via the cloned LXA$_4$ receptor, we treated monocytes with the specific antiserum to the third extracellular domain of the receptor. Monocytes were exposed to anti-LXA$_4$ receptor antiserum (anti-LXA$_4$R) or preimmune rabbit serum (1:500) for 10–30 min prior to exposure to agonists. Pretreatment with the anti-LXA$_4$R specifically inhibited intracellular mobilization of Ca^{2+} by LXA$_4$ and both analogs, but did not affect the response to LTB$_4$ (Fig. 7).

PMN responses to LXA$_4$ are inhibitable by PTX, indicating linkage of the receptor to G proteins in this cell type (16, 22). To investigate this property of the receptor in monocytic cells, we treated both monocytes and THP-1 cells with PTX. Pretreatment of monocytes with PTX completely abrogated the Ca^{2+} response by LXA$_4$ and both stable analogs (Fig. 8*A*). Additionally, THP-1 cell exposure to PTX inhibited LXA$_4$- and analog-induced THP-1 cell adherence >90%, with no effect on response to phorbol 12-myristate 13-acetate, a stimulus which bypasses cell surface receptors (Fig. 8*B*). This result reinforces that both adherence and intracellular Ca^{2+} mobilization elicited by LXA$_4$ and the stable analogs are via binding to the G-protein-linked LXA$_4$ receptor.

Because monocytes are involved in chronic conditions such as atherosclerosis and rheumatoid arthritis, it was important to also examine potential antagonists of LX-induced monocyte stimulation. To this end, we prepared dimethylamide derivatives of 15(R/S)-methyl-LXA$_4$ and 16-phenoxy-LXA$_4$ that were modeled after the PGF$_{2\alpha}$ and LTB$_4$ dimethylamide analogs that are receptor-level antagonists of their respective native compounds (23, 24). We tested these compounds for potential agonist or antagonist activity in THP-1 cell adherence and found that both 15(R/S)-methyl-LXA$_4$-dimethylamide (Fig. 9) and 16-phenoxy-LXA$_4$ (data not shown) stimulated adherence of THP-1 cells with no statistically significant difference in potency from that of their corresponding free acids. Additionally, pretreatment or simultaneous addition of these dimethylamide analogs to THP-1 cells did not inhibit adherence stimulated by their respective free acids (data not shown). Mobilization of intracellular Ca^{2+} was also examined and 15(R/S)-methyl-LXA$_4$-dimethylamide increased $[Ca^{2+}]_i$ in monocytes in the same potency range as 15(R/S)-methyl-LXA$_4$-Me.

Monocytes are known to play important roles in cancer cell and immune surveillance. To further examine the range and specificity of monocyte activities that LXA$_4$ and stable analogs may induce, we examined their impact on cell-mediated cytotoxicity. LXA$_4$ and the analogs had no effect on monocyte cytotoxicity at concentrations of 1 μM and did not change cytotoxicity when added together with lipopolysaccharide (Table I).

DISCUSSION

LX have been shown, *in vitro*, to stimulate monocyte adherence and migration, while they inhibit the same responses in PMN (6, 8, 9, 11). Synthetic LX analogs that have longer half-lives *in vitro* and *in vivo* than native LX were designed to further evaluate the impact of LX on monocytes and aid in discerning signal transduction via the LX in monocytes. The LXA$_4$ stable analogs used in these determinations were 15(R/S)-methyl-LXA$_4$ and 16-phenoxy-LXA$_4$ (Fig. 1), which we previously showed had longer half-lives in incubations with monocytic cells and with recombinant 15-hydroxyprostaglandin dehydrogenase than native LXA$_4$. These LXA$_4$ stable analogs were shown to have similar bioactivity to native LXA$_4$ in inhibiting PMN adhesion and migration (13), and here is the first

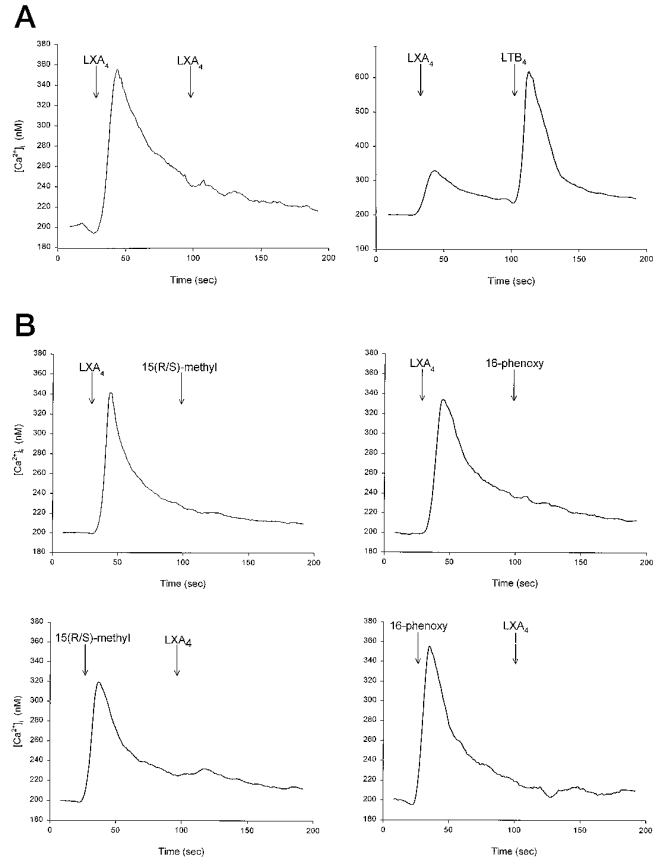

FIG. 6. **Monocyte desensitization.** *A*, LXA_4 intracellular Ca^{2+} mobilization is selectively desensitized. Peripheral monocytes were treated as in Fig. 5. LXA_4 (100 nM) or LTB_4 (100 nM) was added at time indicated by *arrows*. *B*, LXA_4 and analogs cross-desensitize for intracellular Ca^{2+} mobilization. LXA_4, 15(*R*/*S*)-methyl-LXA_4-Me, or 16-phenoxy-LXA_4-Me (100 nM) was added at time indicated by *arrows*. Values are from a representative experiment performed in duplicate from $n = 3$ separate donors.

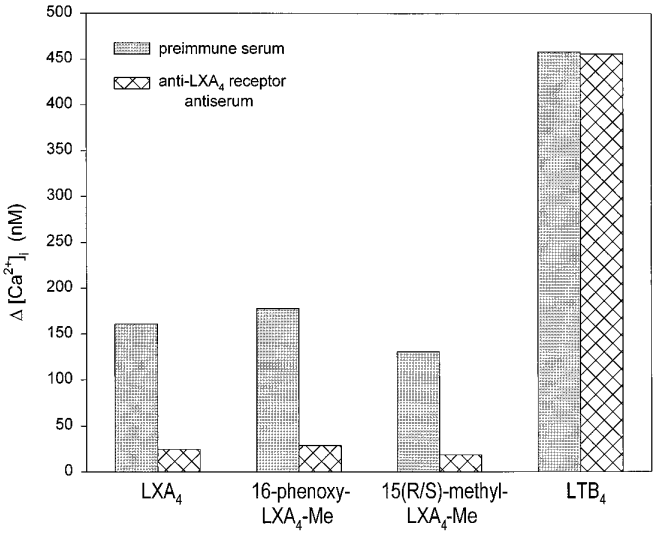

FIG. 7. **Antipeptide antibody to LXA_4 receptor down-regulates LXA_4- and analog-induced Ca^{2+} response.** Peripheral blood monocytes were treated as in Fig. 5 and exposed to either preimmune rabbit serum or anti-LXA_4 antiserum (1:500) for 10 min prior to agonist exposure (100 nM). Values are expressed as percent of the maximal (100%) Ca^{2+} mobilization and are from a representative experiment performed in duplicate from $n = 3$ separate donors.

report of monocyte stimulation by 15-epi-LXA_4 (aspirin-triggered LXA_4), 15(*R*/*S*)-methyl-LXA_4, and 16-phenoxy-LXA_4. These analogs were potent monocyte chemoattractants at 100 nM concentrations and 15(*R*/*S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 were more potent in stimulating THP-1 cell adherence to laminin than native LXA_4. These results suggest that structural changes made to the native molecule have decreased conversion to inactive products by monocytic cells because, as we recently reported, the monocyte products of LXA_4, namely, 15-oxo-LXA_4, 13,14-dihydro-15-oxo-LXA_4, and 13,14-dihydro-LXA_4, were virtually inactive in stimulating monocyte adherence (11).

We cloned an LXA_4 receptor from the monocytic cells which, when sequenced, proved to be identical to the receptor on neutrophilic cells. These results imply that the different LX responses seen in monocytes *versus* PMN are not due to actions via a different receptor. Other eicosanoids, for example prostaglandin E_2, have alternate receptor forms in different tissues (25) to explain the diversity of their bioactions. Alternate forms of the LXA_4 receptor have not yet been identified; however, this does not preclude their existence and is an area we are pursuing in other cell types.

These LX stable analogs also mobilized intracellular Ca^{2+} in monocytes in a dose-dependent manner, and the differences in the relative potency of the analogs (16-phenoxy-LXA_4 > LXA_4 > 15(*R*/*S*)-methyl-LXA_4) in this assay *versus* adherence (16-phenoxy-LXA_4 ≈ 15(*R*/*S*)-methyl-LXA_4 > LXA_4) reinforces our recent findings, in that addition of an intracellular Ca^{2+} chelator to THP-1 cells had no effect on LX-induced cell adherence, suggesting that Ca^{2+} mobilization and adherence are independent events in these cells (17). The increase in $[Ca^{2+}]_i$ stimulated by LXA_4 showed homologous desensitization and the analogs also proved to desensitize the cells to an LXA_4 response, but not an LTB_4 response. This cross-desensitization implicated a common receptor site on the monocyte for LXA_4, 16-phenoxy-LXA_4, and 15(*R*/*S*)-methyl-LXA_4.

Specific antibodies to G-protein-linked seven-transmembrane receptors have been produced before, such as a polyclonal antibody to the platelet-activating factor receptor (26) and a monoclonal antibody to the receptor for the anaphylatoxin C5a (27). The C5a receptor antibody was shown to specifically inhibit Ca^{2+} transients and functional responses of both neutrophils (27) and eosinophils (28). We showed previously that the anti-LXA_4R antiserum was selective, as characterized by immunoprecipitation. It specifically blocked LXA_4 binding with PMN and also inhibited LXA_4 activity in PMN (15). Here, the antipeptide antibody to the LXA_4 receptor specifically inhibited $[Ca^{2+}]_i$ increments by LXA_4 and the individual LX analogs in monocytes, which suggests that these compounds are acting specifically via the cloned monocyte LXA_4 receptor.

This receptor appears to be coupled to a PTX-sensitive G-protein in monocytes, because both adherence and Ca^{2+} mobilization were inhibited by PTX treatment. LXA_4 responses in PMN, including phospholipase A_2 activation and release of arachidonic acid (16) and activation of phospholipase D (22), are also PTX-sensitive. This indicates that the receptor coupling in monocytes and PMN is similar to this point, although there could be different PTX-sensitive G-protein subtypes that are coupled to the receptor and must diverge downstream in the signal transduction pathway to stimulate monocytes and inhibit PMN.

Leukocyte chemoattractants have been classified in two categories, the "classical" chemoattractants, including FMLP, LTB_4, and C5a, which activate phospholipases, induce Ca^{2+} increments, trigger generation of reactive oxygen products, and release of granule enzymes, and the "pure" chemoattractants, including substance P and transforming growth factor β1,

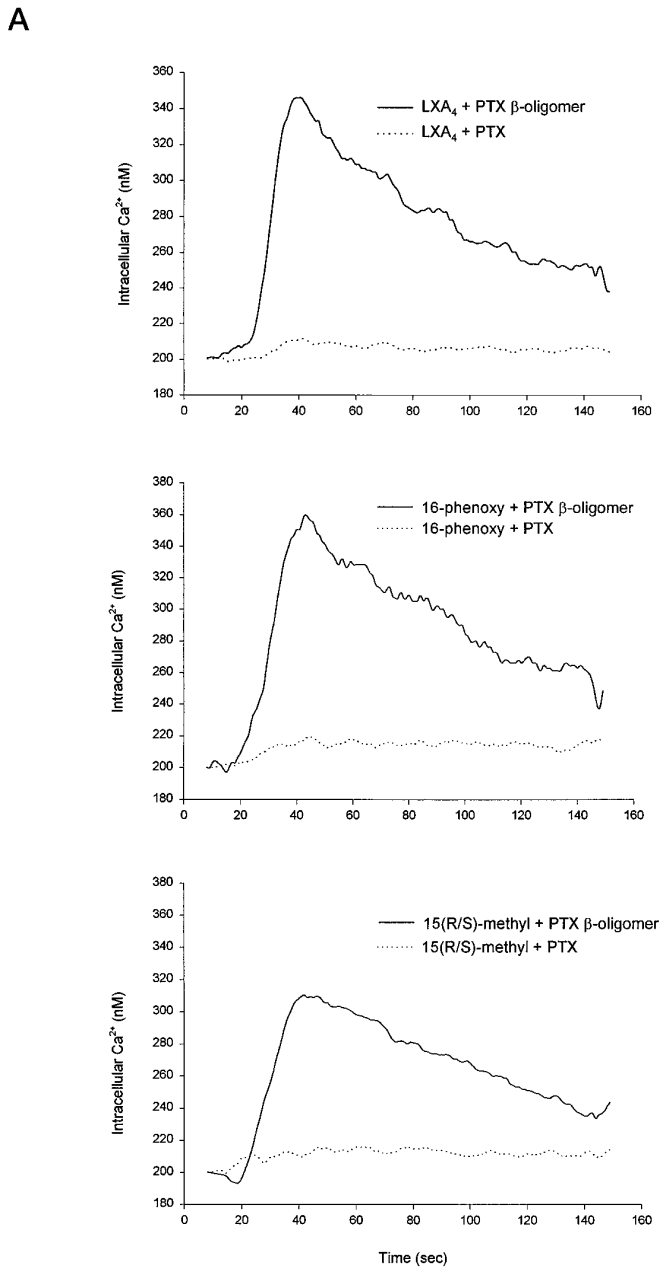

FIG. 8. **PTX inhibits LXA$_4$- and analog-induced responses.** *A*, intracellular Ca^{2+} mobilization: monocytes were loaded with Indo-1 as in Fig. 5 and incubated with PTX (2 μg/ml) or the inactive β-oligomer of PTX (2 μg/ml) for 1 h before agonist (100 nM) exposure. Values are from a representative experiment performed in duplicate from $n = 2$ separate donors. *B*, THP-1 cell adherence to laminin: cells were exposed to PTX (100 ng/ml) for 16 h and labeled with BCECF-AM prior to incubation (3×10^5 cells in 100 μl) with LXA$_4$ analogs (10 nM) or phorbol 12-myristate 13-acetate (150 nM) for 40 min at 37 °C in laminin-coated plates. Values are expressed as percent adherence above vehicle and are from a representative experiment performed in quadruplicate from $n = 3$ separate experiments.

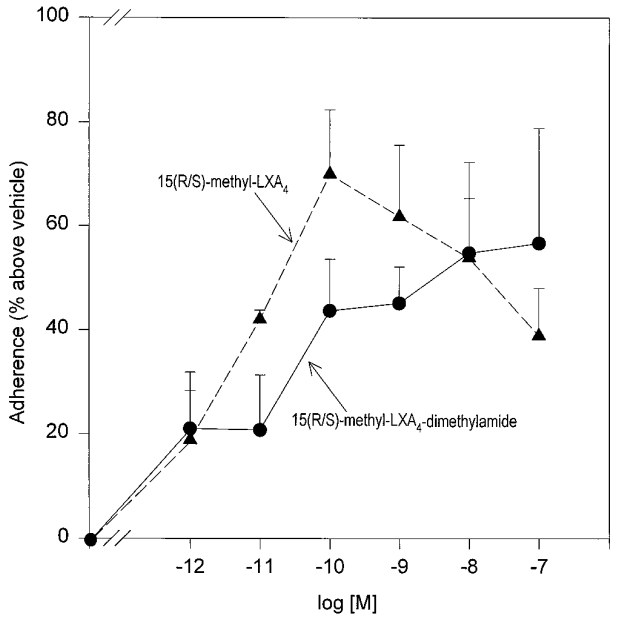

FIG. 9. **15(*R/S*)-methyl-LXA$_4$-dimethylamide stimulates THP-1 cell adherence.** THP-1 cells were labeled with BCECF-AM and incubated (3×10^5 cells in 100 μl) with each compound for 40 min at 37 °C. Values represent means ± S.E. for four separate experiments performed in quadruplicate and are expressed as the percent adherence above that with cells exposed to vehicle alone.

TABLE I
LXA$_4$ and 16-phenoxy-LXA$_4$-Me do not enhance cell-mediated cytoxicity

Cytotoxicity is reported as the percent of target cells killed by peripheral blood monocytes, quantitated by lactate dehydrogenase release. Monocytes were exposed overnight to LXA$_4$ (1 μM), 16-phenoxy-LXA$_4$-Me (1 μM), or vehicle (DPBS^{2+} plus 0.1% EtOH) with and without LPS (100 ng/ml) before addition of target cells (see "Experimental Procedures"). Values are from a representative experiment performed in quadruplicate from $n = 2$ separate donors.

	Vehicle	LXA$_4$	16-Phenoxy-LXA$_4$-Me
Control	8.4%	8.7%	7.9%
LPS	30.4%	30.1%	27.8%

which do not mobilize Ca^{2+}, activate phospholipases, stimulate superoxide, or enzyme release (29). In common, both of these classes of chemoattractants act via G-protein-coupled receptors that are sensitive to PTX. Results of the present and recent reports with human monocytes (11, 17) on the activity of LXA$_4$ and its stable analogs place these LX in a unique class of chemoattractants due to the following characteristics, namely: 1) stimulation of monocyte chemotaxis and adherence, 2) Ca^{2+} mobilization, 3) no impact on cell-mediated cytotoxicity, 4) no generation of superoxide anion, and 5) action via PTX-sensitive receptors. The LX lie between the classical and pure chemoattractants in their effects, by inducing Ca^{2+} increments and activating phospholipases without initiating production of reactive oxygen species. Interestingly, in PMN, LX also inhibit some of these same activities of the classical chemoattractants

FMLP and LTB_4, including inhibition of chemotaxis and adherence (reviewed in Ref. 1). Therefore, LXA_4 has distinctive actions compared with known molecules that interact with leukocytes.

Dimethylamide versions of two of the more potent LXA_4 analogs were prepared as potential antagonists, based on the models of LTB_4- and $PGF_{2\alpha}$-dimethylamides that can act as specific antagonists to their parent compounds (23, 24), but these dimethylamide-LX analogs proved to have agonist properties as potent as their corresponding free acids and methyl ester analogs. It is, therefore, apparent that there is little effect of modifying the LXA_4 molecule at the carbon 1 position carboxylic acid and implies that the ligand interaction of LXA_4 with its receptor differs from that of LTB_4 and $PGF_{2\alpha}$. Nevertheless, these data provide us with a basis of evidence to develop other analogs that could prove to be either potent agonists or antagonists to the actions of LXA_4 on monocytes. Developing antagonists to LX actions with monocytes is of interest, because it is possible that, in certain chronic inflammatory conditions or viral infections, when monocytes instead of PMN are the prominent leukocytes involved, inhibition of overt LX chemotactic properties may be desirable to the outcome of disease.

These data provide new evidence on the activity of novel LXA_4 analogs in human monocytes and indicate that they act via the cloned G-protein linked LXA_4 receptor. The increased potency (~1 log molar) of these LX analogs indicates that structural modifications made to the carbon 20 end of LXA_4 both inhibited dehydrogenation and oxidation of the molecule, and thus blocked inactivation, and did not hinder receptor-ligand interactions. Together, the results of the present report indicate that the LXA_4 analogs are potent activators of monocytes that mimic the activity of native LXA_4, but are active at even lower concentrations than the parent compound. This increased potency and decreased transformation of the analogs *in vitro* provides better tools for dissecting the differences in signal transduction between monocytes and PMN that lead to the selective effects of LX in these two cell types and, additionally, has important implications as far as longer half-lives and increased potency *in vivo*. Moreover, our results suggest that LX analogs will be vital tools in determining the impact of these and related compounds in selective monocyte and PMN trafficking in inflammatory diseases.

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Lipoxin A_4 Stable Analogs Are Potent Mimetics That Stimulate Human Monocytes and THP-1 Cells via a G-protein-linked Lipoxin A_4 Receptor

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