

Lipoxin A₄ and B₄ Are Potent Stimuli for Human Monocyte Migration and Adhesion: Selective Inactivation by Dehydrogenation and Reduction

By Jane F. Maddox and Charles N. Serhan

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

Summary

Monocyte recruitment and adherence are important events in inflammatory and vascular diseases. Here, we evaluated the actions of lipoxin A₄ (LXA₄) and LXB₄, a series of lipoxygenase products from arachidonic acid generated by cell-cell interactions, on human monocytes. LXA₄ and LXB₄ (10⁻⁷ M) each increased monocyte migration in chamber chemotaxis assays and, in migration under agarose, exhibited chemotactic indices similar to those of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine at 10⁻¹⁰-10⁻⁸ M and to the chemokine macrophage inflammatory protein-1α (MIP-1α) at 10⁻⁸-10⁻⁷ M with a rank order of potency: Monocyte chemotactic protein-1α > LXA₄ ≈ LXB₄ ≈ MIP-1α. Lipoxins also stimulated monocyte adherence to laminin. In addition, human monocytes rapidly transformed LXA₄ and LXB₄ to several metabolites. LXB₄ (>80%) was converted within 30 s to new products, in a trend similar to that of LXA₄. The novel monocyte-derived LXB₄ products were identified as 5-oxo-6,7-dihydro-LXB₄ and 6,7-dihydro-LXB₄, indicating a role for site-selective dehydrogenation and reduction. Unlike monocytes, intact polymorphonuclear leukocytes (PMN) did not metabolize LXA₄ in significant quantities, and only ~12% of exogenous LXB₄ was ω-oxidized to 20-OH-LXB₄ and 20-COOH-LXB₄ by PMN. To determine if lipoxin conversion altered bioactivity, we evaluated the actions of these metabolites on monocytes. Each of the novel products of LXA₄ and LXB₄ from monocytes, namely oxo- and dihydrolipoxins, were essentially inactive in stimulating monocyte adherence. In contrast, the ω-oxidation products of LXB₄ isolated from PMN were equipotent with LXB₄ for monocyte adherence. Dehydrogenation of LXA₄ in monocytes appears to be carried out by a 15-hydroxyprostaglandin dehydrogenase, which is present in human monocytes as determined by reverse transcription PCR and Western blots. Together, these results provide the first evidence that LXA₄ and LXB₄ are both potent stimulants for migration and adherence of human monocytes. Moreover, they underscore the importance of the major route of lipoxin metabolism in leukocytes, namely, the rapid dehydrogenation and inactivation carried out by monocytes.

Activation of peripheral blood leukocytes, leading to adherence to damaged vessel walls and extravasation into surrounding tissue, is an important event in many inflammatory and vascular diseases (1, 2). Leukotriene B₄ (LTB₄)¹, a

lipoxygenase product of arachidonic acid, is a potent activator of PMN chemotaxis and adherence (3). Another class of lipoxygenase products from cell-cell interactions, the lipoxins, display selective activities on human leukocytes that have been shown to be both stimulatory and inhibitory, depending on the cell type involved. In human PMN, lipoxin A₄ (LXA₄) (10⁻⁹-10⁻⁷ M) induces chemokinesis but inhibits chemotaxis toward LTB₄ and FMLP (4). LXA₄ (10⁻⁸ M) also inhibits FMLP-stimulated PMN transmigration across intestinal epithelium (5). In human eosinophils, LXA₄ also inhibits chemotaxis toward both FMLP and platelet activating factor (6). Lipoxin B₄ (LXB₄) (nanomolar range) is reported to stimulate colony formation and differ-

¹Abbreviations used in this paper: BCECF-AM, [2'-7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl]-ester; CR, carbonyl reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HETE, hydroxy-eicosatetraenoic acid; LTB₄, leukotriene B₄; LXA₄, lipoxin A₄; LXB₄, lipoxin B₄; MCP-1, monocyte chemotactic protein-1; MIP-1α, macrophage inflammatory protein-1α; NAD⁺, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; RP-HPLC, reverse phase HPLC; RT, reverse transcription.

entiation toward macrophage lineage in mononuclear leukocyte populations (7). These findings indicate that LXA₄ and LXB₄ each display selective actions on human leukocyte subtypes.

The major routes of further metabolism, which regulate eicosanoid bioactivity in human tissues, include β -oxidation, ω -oxidation, and dehydrogenation (for review see reference 8). Our laboratory has demonstrated that human peripheral blood monocytes transform LXA₄ to 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄ (9), which suggested that lipoxins, or their metabolites, may have bioactions in monocytes and that the transformation may alter lipoxin bioactivity to enhance or decrease their potency. The present results document previously unrecognized bioactivities of both LXA₄ and LXB₄ in human monocytes and establish the importance of lipoxin further metabolism in these cells.

Materials and Methods

Cell Isolation. Human monocytes were isolated using a modification of the method of Denholm and Wolber (10). Briefly, whole blood collected in acid citrate dextrose from healthy volunteers was centrifuged (200 g) at 25°C for 15 min for removal of platelet-rich plasma. The cells were then layered over Ficoll-Hypaque (LSM; Organon Teknika, Durham, NC) and centrifuged (500 g) at 25°C for 35 min. The mononuclear cell layer was collected, washed once, and resuspended in PBS²⁻ containing 0.1% BSA. 8 ml of a Percoll: 10 \times HBSS (10:1.65) mixture was added to 4 ml mononuclear cells in 10 \times 1.5-cm round-bottom silanized polypropylene tubes and centrifuged (370 g) at 25°C for 30 min. Monocytes were collected from the upper 5 mm of the gradient and washed before counting and viability assessment. Human PMN were isolated by the Bøyum method (11) from peripheral blood obtained from healthy volunteers. Cells were identified by Wright-Giemsa staining. The monocyte population was >90% with <10% contaminating lymphocytes, and PMN were >95% pure. Both monocytes and PMN contained platelets at approximately a 2:1 ratio of platelets to leukocytes. Viability of cells in all reported experiments was >95% as determined by their ability to exclude trypan blue.

Chemotaxis. Chamber chemotaxis was evaluated using a microchamber technique according to the method of Falk et al. (12). Briefly, monocytes were isolated as above and resuspended at 5 \times 10⁶/ml in PBS²⁺. Chemoattractant solution or vehicle (PBS²⁺ containing 0.05% ethanol) was added to the lower wells of a 48-well chemotaxis chamber (Neuro Probe, Cabin John, MD). A polycarbonate membrane with 5- μ m diameter pores (Poretics Corp., Livermore, CA) was layered on top of the chemoattractant wells, and monocytes were added to the top wells. After incubation for 90 min at 37°C, the membrane was removed, scraped of cells from the upper surface, and stained with modified Wright-Giemsa stain. Cells that had migrated through the membrane in four high-power fields were counted.

Chemotaxis under agarose was performed using conditions from the methods of Al-Sumidaie et al. (13). Briefly, agarose migration plates were prepared using 0.75% agarose (Type IV; Sigma Chemical Co., St. Louis, MO) dissolved in HBSS²⁺ (pH 7.0) with 10% human serum, which was added to 60-mm diameter tissue culture dishes. Chemotactic agents and HBSS²⁺ (10 μ l)

were added to each of the inner and outer wells, respectively, and the plates were incubated at 37°C in humidified air with 5% CO₂ for 45 min. Fluid was aspirated from all wells and replaced with fresh chemoattractant or HBSS²⁺, and cells (10⁶ in 10 μ l) were added to the middle wells of each set. The plates were then incubated at 37°C in humidified air with 5% CO₂ for 18–20 h, after which the cells were fixed with glutaraldehyde and stained with modified Wright-Giemsa stain. Cell migration was quantitated by measuring the linear distance the cells had moved from the margin of the wells toward the chemoattractant (*D*, directed migration) and away from it (*C*, random migration) as well as toward (*A*) and away (*B*) from vehicle control wells. The chemotactic index (*D/C*) and migration index (*D/[(A + B)/2]*) were calculated for each chemoattractant as in Colgan et al. (14).

Laminin Adhesion. Isolated monocytes were suspended in PBS²⁻ (4 \times 10⁶/ml) and [2'-7'-bis-(carboxyethyl)-5(6')-carboxy-fluorescein acetoxymethyl]-ester (BCECF-AM; Calbiochem Corp., La Jolla, CA) was added (1.0 μ M) and incubated with cells for 20 min at 37°C. Cells were washed and resuspended (3.3 \times 10⁶/ml) in PBS²⁺ containing 0.1% BSA. 90- μ l aliquots 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. 10 μ l of agonist or vehicle was added to each well, and plates were incubated at 37°C for 20 min. After incubation, wells were aspirated and washed once with PBS²⁺ containing 0.1% BSA. Cells adhering to the wells were solubilized with 0.025 M NaOH containing 0.1% SDS (100 μ l), followed by fluorescence quantitation on a plate fluorimeter.

Products of Lipoxin Metabolism: Isolation and Identification. For generation and isolation of the oxo- and dihydro- metabolites of lipoxins, monocytes were resuspended at 125 \times 10⁶ cells/5 ml PBS²⁺ and incubated with 500 ng LXA₄ and 100,000 dpm [11, 12-³H]LXA₄, as in Serhan et al. (9), or 1.0 μ g LXB₄ at 37°C. ω -Oxidation products of LXA₄ and LXB₄ were obtained from PMN resuspended at 150 \times 10⁶ cells/3 ml PBS²⁺ and incubated with 2.0 μ g LXA₄ or LXB₄ at 37°C. Incubations were stopped at 0.5, 1, 5, and/or 20 min with cold methanol containing PGB₂ as an internal standard. Products were extracted and chromatographed as described in Serhan et al. (9). To obtain quantities of compounds sufficient for structural identification and biological assays, multiple incubations (*n* = 4) were extracted, stored at -20°C, and pooled before reverse phase (RP)-HPLC separation. The HPLC system consisted of a gradient dual pump (LKB-Pharmacia, Piscataway, NJ) equipped with an Altex Ultrasphere-ODS (4.6 mm \times 25 cm) column (Alltech Assoc. Inc., Deerfield, IL), flow rate 1 ml/min, eluted (T₀-20 min) with methanol/H₂O/acetic acid (65:35:0.01, vol/vol/vol) and methanol/acetic acid (99.99:0.01, vol/vol) in a linear gradient (20–45 min) that was used to quantitate and recover lipoxin-derived metabolites. On-line spectra were recorded using a diode array detector (1040M series II; Hewlett-Packard Co., Palo Alto, CA) equipped with HPLC^{3D} ChemStation software (Hewlett-Packard Co.). Gas chromatography/mass spectroscopy was performed as in Serhan et al. (9) using a mass selective detector quadrupole (model 5971A; Hewlett-Packard Co.) and gas chromatograph (model 5890; Hewlett-Packard Co.) using a HP-Ultra 2 column (cross-linked 5% phenyl methyl silicone gum phase; 25 cm \times 0.2 mm \times 0.33 μ m). Before analysis, products were treated with diazomethane and converted to their trimethylsilyl derivatives using bis(trimethylsilyl)trifluoroacetamide.

Monocyte-derived LXA₄ metabolites 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄ were isolated

and identified as in Serhan et al. (9) and gave physical criteria consistent with reported data. The monocyte-derived LXB₄ products were identified using the criteria of Ho and Wong (15) for 6,7-dihydro-LXB₄ that was identified from potatoes. This product had an HPLC retention time of 2.5 min and showed a UV spectrum with triplet maxima at 258, 268, and 279 nm (± 4 nm, recorded on line). The mass spectrum of the Me₃Si derivative of the methyl ester gave prominent ions at m/z 203 [base peak, Me₃SiO=CH-CH(CH₂)₃-COOMe] and 173 [Me₃SiO=CH(CH₂)₄CH₃]. The 5-oxo-6,7-dihydro-LXB₄ also displayed triplet absorbance at UV λ_{\max} of 259, 269, and 280 nm, and had a RP-HPLC retention time of 35.6 min. Its methyl ester Me₃Si derivative was identified with prominent ions at m/z 208 [Me₃SiO=CH(CH₂)₆(CH₂)₂], 173 [Me₃SiO=CH(CH₂)₄CH₃], 129 [base peak, O=C(CH₂)₃COOMe] and 118.

PMN-derived products of LXB₄ were identified as in Mizukami et al. (16, 17). Briefly, the UV chromophores of the ω -oxidation products, 20-OH-LXB₄ and 20-COOH-LXB₄, were essentially identical to LXB₄, showing UV λ_{\max} at 287, 301, and 315 nm (± 4 nm). The RP-HPLC retention time of 20-OH-LXB₄ was 3.6 min, and the mass spectrum of the Me₃Si methyl ester derivative was characterized by major ions as reported in Mizukami et al. (16). 20-COOH-LXB₄ eluted from this HPLC system at 2.9 min and its Me₃Si methyl ester derivative showed major ions in its mass spectrum in agreement with those recently reported by Mizukami (17), namely m/z 203 (base peak), 217, 409 (M⁺-217), 423 (M⁺-203), and 626 (M⁺).

Reverse Transcriptase PCR. Total RNA was isolated from monocytes using differential extraction of RNA with phenol at an acidic pH (18), and preparations with a RNA/protein ratio >2 were used for poly(A)⁺ RNA selection. Poly(A)⁺ RNA was selected by affinity chromatography on oligo(dT)-cellulose (18). Poly(A)⁺ RNA (1 μ g) from monocytes was reverse transcribed to cDNA in 20 μ l total volume using avian leukemia virus reverse transcriptase (RT), RNasin® (10 U), MgCl₂ (5 mM), oligo(dT) primer (0.5 μ g), dNTPs (1 mM), and buffer with a RT kit (Promega Corp., Madison, WI) and incubated for 1 h at 42°C. The reaction was stopped by heating to 100°C for 10 min, immediately followed by storage on ice. Specific primers were designed for 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and carbonyl reductase (CR) using Sequence Analysis Software Package (Genetics Computer Group, University of Wisconsin, Madison, WI) and published sequences of the two genes (19, 20). The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as published in Hla and Maciag (21). PCR amplifications were performed using cDNA (5 μ l) with either 15-PGDH-, GAPDH-, or CR-specific primers (Integrated DNA Technologies, Inc., Coralville, IA). The sequences of the sense and antisense primers for PGDH were: PGDH sense, 5'-CAC GTG AAC GGC AAA GTG-3'; PGDH antisense, 5'-GCA TTA TTG ACC AAA ATG TCC A-3'. The sequences for the GAPDH primers, which were used as a control, were: GAPDH sense, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; and GAPDH antisense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. CR primers were: CR sense, 5'-CCT GAG CCA GGT CTG TTC TC-3'; and CR antisense, 5'-GGG TGT GGG ATC AGC AAC-3'. Sequence amplification was performed in a total volume of 25 μ l, using 0.5 μ M each of sense and antisense primers, dNTPs (0.2 mM), MgCl₂ (1.5 mM), Taq polymerase (1.5 U) (Promega Corp.) and 10 \times buffer (2.5 μ l) included with the enzyme. The reactions were amplified by 35 repetitive cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Amplified DNA was

electrophoresed in 2% agarose gels containing ethidium bromide, and bands were photographed.

SDS-PAGE and Immunoblotting. Monocytes were suspended in PBS²⁻ (50 \times 10⁶ cells/ml) and sonicated in an ice bath for three bursts of 30 s each (Branson Sonifier 250; Branson Sonic Power Co., Danbury, CT). The 100,000 g supernatant was used in SDS-PAGE and immunoblots for 15-PGDH. Human placenta (1 g) was cut into pieces \sim 1 mm³, 3 ml PBS²⁻ was added to the tissue, and 1.5-ml aliquots were homogenized 4 \times 2 min at 0°C at the highest setting of a tissue homogenizer (Talboys Instrument Corp., Montrose, PA). Supernatants used for SDS-PAGE and immunoblotting were prepared by centrifuging the homogenate at 10,000 g for 20 min at 4°C.

Proteins were fractionated by SDS-PAGE according to the method of Laemmli (22). Electrophoretic transfer of proteins was performed as in Towbin et al. (23), except polyvinylidene difluoride (DuPont-NEN, Boston, MA) was used as the transfer membrane. 15-PGDH was visualized using rabbit anti-PGDH antiserum as 1° antibody (a generous gift from Dr. H.-H. Tai, University of Kentucky, Lexington, KY) and a 2° peroxidase-conjugated goat anti-rabbit IgG (Sigma Chemical Co.), followed by treatment with chemiluminescent reagents (DuPont-NEN) and exposure on radiographic film (RX; Fuji Photo Film Co., Elmsford, NY).

Statistical Analysis. Data were analyzed using paired or unpaired Student's *t* tests, and significance was determined at $p < 0.05$.

Results

Chemotaxis. LXA₄ and LXB₄ both proved to be potent chemoattractants for monocytes (Fig. 1). In chamber chemotaxis assays with monocytes, FMLP is maximally active at concentrations of 10⁻⁸-10⁻⁷ M (24) and in the present experiments, it increased migration by 4.5 times over cells exposed to vehicle alone. Both LXA₄ and LXB₄

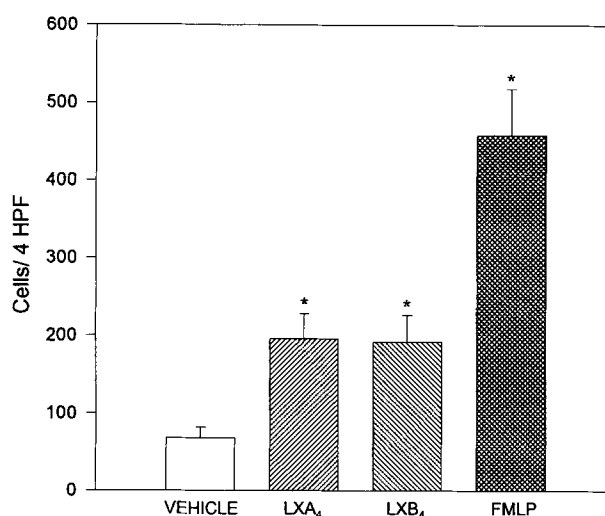


Figure 1. LXA₄ and LXB₄ stimulate human monocyte chemotaxis. Chemotaxis was quantitated in chambers using agonists at equimolar concentrations (10⁻⁷ M). Values represent mean numbers (\pm SE) of monocytes that migrated through 5- μ m polycarbonate filters in four standard high-power fields after incubation for 90 min at 37°C for three separate experiments performed in triplicate. *Denotes values significantly higher than vehicle ($p < 0.05$).

gave statistically significant monocyte chemotaxis at 10^{-7} M that was approximately three times greater than vehicle controls. Next, migration under agarose was used to examine concentration dependence of the lipoxin response and distinguish chemotaxis from chemokinesis. The use of migration under agarose allowed assessment of directed versus spontaneous migration and also required fewer cells than the chamber system, which permitted direct comparison of concentration dependency for both LXA₄ and LXB₄ with cells from the same donor. The chemotactic response to LXA₄ and LXB₄ was concentration dependent from 10^{-10} – 10^{-6} M (Fig. 2). The chemotactic indices (as defined in Colgan et al. [14]) for LXA₄ and LXB₄ were comparable to that of FMLP from 10^{-10} – 10^{-8} M. Only at 10^{-7} M was the chemotactic index of FMLP greater than LXA₄ and LXB₄, and at 10^{-6} M FMLP, this index dropped precipitously, whereas with LXA₄ and LXB₄, chemotactic response continued to increase. Monocyte migration for LXA₄ was also directly compared with human recombinant chemokines, monocyte chemoattractant protein (MCP-1) and macrophage inflammatory protein (MIP-1 α) (R&D Systems, Inc., Minneapolis, MN) (Table 1). The relative potency of LXA₄ was equal to MIP-1 α and was ~ 1 log less than MCP-1. These data reveal that LXA₄ and LXB₄ are potent activators of monocyte migration at submicromolar concentrations, comparable to equimolar concentrations of the chemotactic peptide, FMLP, and the monocyte chemokines.

Adherence. Since chemotaxis is one of the steps involved in diapedesis of leukocytes from blood vessels into tissue, it was also of interest to examine the ability of li-

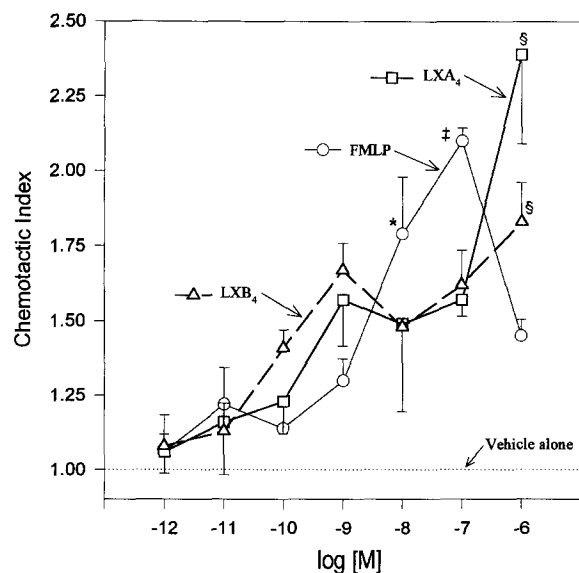


Figure 2. Monocyte migration induced by LXA₄ and LXB₄ is concentration dependent. Chemotaxis toward lipoxins, FMLP, or vehicle (PBS²⁺ plus 0.35% ethanol) was quantitated under agarose as described in Materials and Methods. Values are the results from an experiment performed in quadruplicate and are representative of four separate experiments. *Denotes values significantly higher than LXA₄ ($p < 0.05$); †values significantly higher than LXA₄ and LXB₄ ($p < 0.05$); ‡values significantly higher than FMLP ($p < 0.05$).

Table 1. Monocyte Migration: Comparison of LXA₄ with Chemokines

Concentration	Migration index*		
	MCP-1	LXA ₄	MIP-1 α
10^{-7} M	$2.93 \pm 0.24^{\ddagger}$	2.61 ± 0.46	2.74 ± 0.23
10^{-8} M	$2.56 \pm 0.56^{\ddagger}$	2.48 ± 0.48	2.61 ± 0.12

*Monocyte migration was measured under agarose as described in Materials and Methods. Data represent means \pm SE of four separate experiments, and all values were significantly higher than vehicle control ($p < 0.05$).

†Denotes values significantly higher than LXA₄ at equal concentration as determined by paired t test ($p < 0.05$).

poxins to stimulate another of the components of this process, namely, adherence of monocytes to a matrix. Monocyte adherence to endothelial cells is well established, and significant adhesion to subendothelial extracellular matrix proteins such as laminin has also been shown (25). To focus solely on the impact of lipoxins on monocytes, we used laminin-coated wells for adherence assays. In these experiments, LXA₄ and LXB₄ were again compared with well-established activators of monocyte adherence, namely, FMLP and LTB₄ (24). At 10^{-7} M, all four agonists significantly increased adherence above vehicle alone and, specifically, LXA₄ increased monocyte adherence by $41.3 \pm 2.1\%$ and LXB₄ by $52.4 \pm 15.5\%$ above controls (Fig. 3). In direct comparison, LTB₄ and FMLP increased monocyte adherence by $23.5 \pm 8.9\%$ and $58.6 \pm 19.1\%$, respectively, at equimolar concentrations. Since these were previously

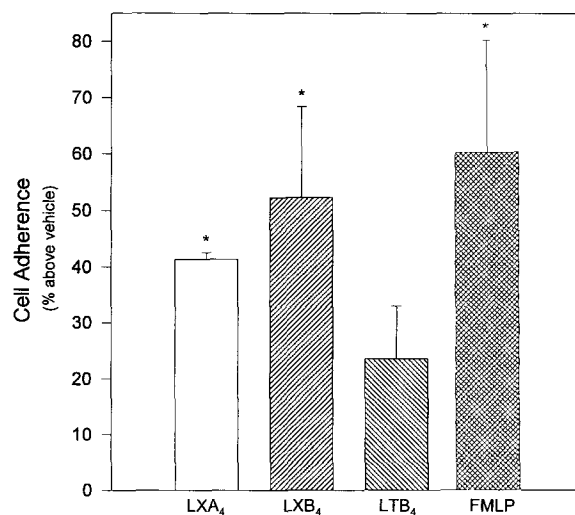


Figure 3. LXA₄ and LXB₄ stimulate human monocyte adherence to laminin-coated plates. Cells were labeled with BCECF-AM before incubation (3×10^5 cells in 100 μ l) with each compound for 20 min at 37°C. Values represent means \pm SE for three separate experiments performed in quadruplicate and are expressed as the percentage above vehicle. Adherence with exposure to vehicle alone was $39.9 \pm 7.7\%$ (mean \pm SE). All agonist values were significantly higher than vehicle ($p < 0.05$). *Denotes values significantly higher than LTB₄ ($p < 0.05$).

unrecognized bioactions of LXA₄ and LXB₄, we next established a time course of monocyte adhesion, as well as concentration dependence for both LXA₄ and LXB₄. The stimulatory action of the lipoxins proved to be concentration dependent (Fig. 4), with the highest activity tested at 10⁻⁶ M for both compounds. Minimal monocyte adhesion activity was observed at 10⁻¹⁰ M for both LXA₄ or LXB₄. The time course of lipoxin-stimulated monocyte adhesion showed a rapid increase that was apparent within the first 5 min and plateaued between 10 and 20 min (Fig. 4, inset). This is similar to the time course previously demonstrated for both LTB₄ and FMLP induced monocyte adherence (24). These results establish that LXA₄ and LXB₄ can rapidly and significantly stimulate monocyte adherence to laminin which, at 10⁻⁷ M, is not significantly different from that induced by FMLP, and is greater than the LTB₄ response (*p* < 0.05).

Transformation of Lipoxins by Leukocytes. Because intact human monocytes transform LXA₄ (9), it was of interest to examine the fate of LXB₄ incubated with monocytes and determine whether further metabolism regulated lipoxin bioactivity. Also, Mizukami et al. (16) recently reported ω-oxidation of LXB₄ by isolated microsomes from human PMN. Therefore, we directly compared transformation of LXA₄ and LXB₄ by intact human PMN and monocytes. Incubation of monocytes with either LXA₄ or LXB₄ resulted in rapid loss of the native compounds (Fig. 5). Within the first 30 s there was ~80% conversion of both LXA₄ and LXB₄ to several new products. In contrast, with intact PMN, LXA₄ was not appreciably further metabo-

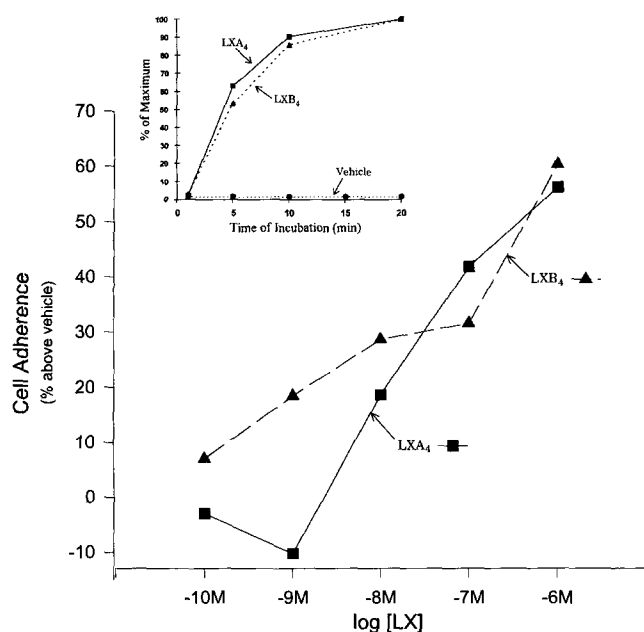


Figure 4. Concentration dependence of monocyte adhesion. Monocytes were incubated with LXA₄ and LXB₄ as in Fig. 3. Values are from an experiment performed in quadruplicate and are representative of four separate donors. (Inset) Time course of lipoxin-stimulated adhesion. Cell incubations performed as in Fig. 3 with lipoxin (10⁻⁷ M) were terminated at the indicated time points.

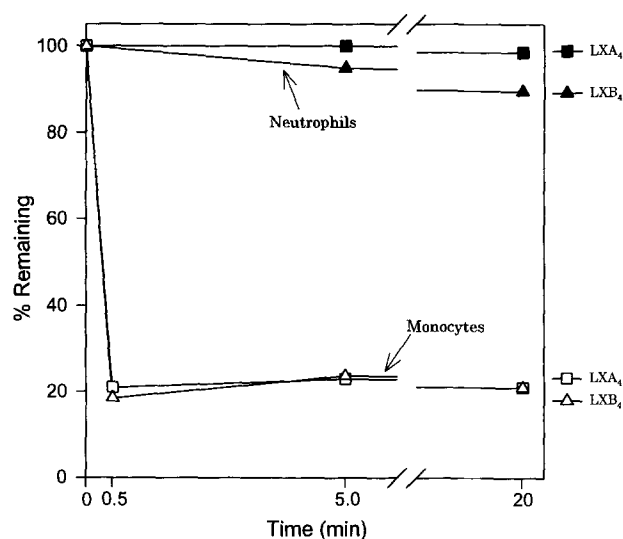


Figure 5. Time course of conversion of LXA₄ and LXB₄ by human monocytes versus neutrophils. Lipoxins were added to cell incubations as described in Materials and Methods. At the indicated time intervals, aliquots (0.5 ml) were removed, placed in 2 vol cold methanol with internal standard (PGB₂), extracted, and chromatographed by RP-HPLC. Values are the means from cells obtained from two separate donors and are expressed as the percentage of remaining lipoxin.

lized, and in incubations with LXB₄ and intact PMN, <15% conversion of the native LXB₄ was observed (Fig. 5).

Next, for evaluation of their biological significance, each of the lipoxin-derived products from human leukocytes was isolated as described in Materials and Methods. The LXA₄-derived compounds included 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄, as reported in Serhan et al. (9). Products of LXB₄ from both monocytes and PMN were separated by RP-HPLC and collected for gas chromatography/mass spectroscopy analysis and bioassay (see Materials and Methods). LXB₄-derived metabolites isolated and identified from monocyte incubations were 5-oxo-6,7-dihydro-LXB₄ and 6,7-dihydro-LXB₄. We were not able to isolate a sufficient quantity of putative 5-oxo-LXB₄ from intact monocytes for physical analysis and bioassays because of its apparent chemical instability. Together, these results indicate that monocytes rapidly convert both LXA₄ and LXB₄ (Fig. 5) by dehydrogenation and reduction of a double bond, illustrated in Fig. 6. Products from PMN incubations with LXB₄ were identified as 20-OH-LXB₄ and 20-COOH-LXB₄, which indicates transformation via only ω-oxidation by intact PMN (Fig. 6). When PMN were exposed to FMLP (10⁻⁷ M) at the time of LXB₄ addition, conversion to ω-oxidation products increased to ~50% of added LXB₄ (*n* = 2). In contrast, addition of the Ca²⁺ ionophore A23187 (2.5 μM) completely inhibited transformation of LXB₄ by PMN (*n* = 3), presumably due to competition for ω-oxidation at the enzyme level with endogenously generated LTB₄, indicating that LTB₄ is the preferred substrate in PMN. This interpretation is consistent with the *K_m* values reported by Mizukami et al. (16). Thus, the major route to lipoxin

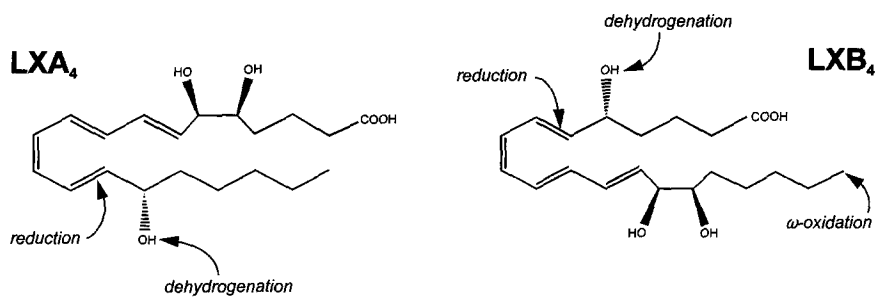


Figure 6. Sites for conversion of LXA₄ and LXB₄ by human monocytes and PMN. (Left) Arrows denote sites of dehydrogenation and reduction of LXA₄ by monocytes. (Right) Arrows denote sites in LXB₄ for dehydrogenation and reduction (by monocytes) and ω -oxidation (by PMN).

transformation in human leukocytes is by dehydrogenation and reduction in monocytes.

Bioactivity of Lipoxin-derived Metabolites. Each of the further metabolites of lipoxins was taken for direct comparison of its bioactivity with that of the parent compound. Monocyte adhesion to laminin was the assay selected for these comparisons because it required modest amounts of the isolated compounds of interest. Monocyte-derived LXA₄ products (15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄) did not stimulate monocyte adherence (Fig. 7 A). Also, LXB₄ products from monocytes (5-oxo-6,7-dihydro-LXB₄ and 6,7-dihydro-

LXB₄) did not carry biologic activity (Fig. 7 B). In contrast, the ω -oxidation products isolated from PMN (20-OH-LXB₄ and 20-COOH-LXB₄) were similar to the activity of native LXB₄ in stimulating monocyte adherence to laminin (Fig. 7 B). Together, these results indicate that LXA₄ and LXB₄ are inactivated by monocytes. Also, the finding that the ω -oxidation products were essentially equipotent with LXB₄ indicates that the monocyte response is stereoselective.

Monocyte 15-PGDH. Conversion of LXA₄ by monocytes was consistent with the actions of nicotinamide adenine dinucleotide (NAD⁺)-dependent 15-PGDH. In this regard, LXA₄ competes (K_i $8.2 \pm 2.6 \mu\text{M}$) with 15-S-hydroxyeicosatetraenoic acid (HETE), an established substrate of 15-PGDH (26), for dehydrogenase activity in monocyte/macrophage-differentiated HL-60 cells (9), which display 15-PGDH activity (27). Based on the presence of an alcohol at carbon 15 in both lipoxins and prostaglandins, we considered 15-PGDH or carbonyl reductase (a nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent PG dehydrogenase) as candidates involved in the conversion of lipoxins by monocytes. To test this, we evaluated the presence of these enzymes in monocytes via RT-PCR and protein immunoblotting techniques. The results of RT-PCR showed a distinct band using the 15-PGDH primers, indicating that 15-PGDH RNA is present in monocytes, as was the control enzyme, GAPDH (Fig. 8 A). Carbonyl reductase primers produced less consistent re-

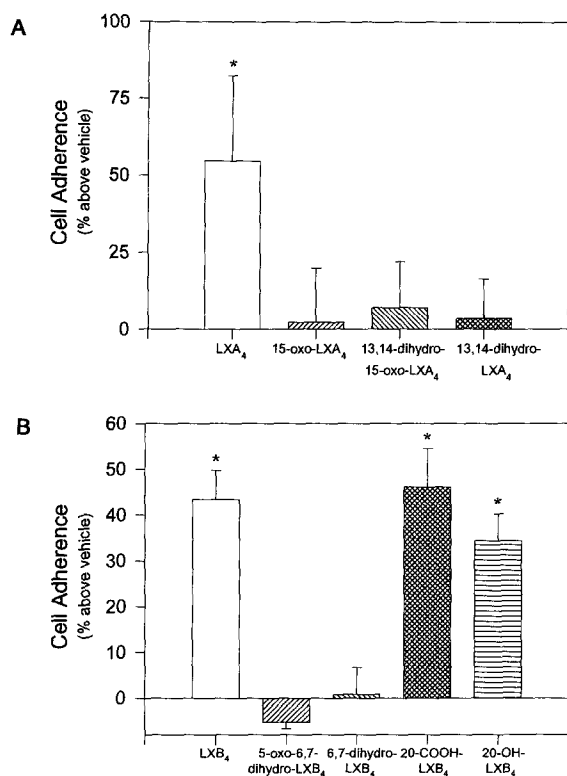


Figure 7. LXA₄ and LXB₄ are inactivated by further metabolism in human monocytes. Biologically derived lipoxin metabolites were isolated as in Materials and Methods, and each compound (10^{-7} M) was incubated with cells for 20 min as in Fig. 3. Values are means (\pm SE) from four separate experiments, each performed in quadruplicate. (A) LXA₄-derived metabolites from monocytes. Adherence of cells exposed to vehicle alone was $40.5 \pm 15.1\%$ (mean \pm SE). (B) LXB₄-derived metabolites from monocytes (5-oxo-6,7-dihydro-LXB₄ and 6,7-dihydro-LXB₄) and PMN (20-OH-LXB₄ and 20-COOH-LXB₄). Adherence of cells exposed to vehicle alone was $20.6 \pm 11.7\%$ (mean \pm SE). *Denotes values significantly higher than vehicle alone ($p < 0.05$).

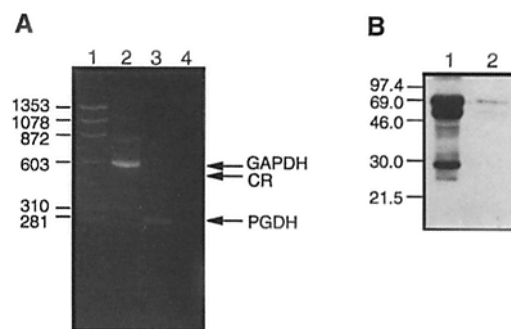


Figure 8. (A) Agarose gel of RT-PCR products from human monocyte mRNA. Lane 1: Molecular mass marker (ϕ X174 HaeIII digest); lane 2: GAPDH primers; lane 3: 15-PGDH primers; lane 4: CR primers (no band visible). (B) Western blot analysis of 15-PGDH in human placenta and monocytes. Lane 1: 50 μg cytosolic placental protein; lane 2: 100 μg cytosolic protein from monocytes. Molecular mass markers (kD) are on the left. The 15-PGDH protein band is 29 kD. The higher molecular mass bands have been noted previously and are consistent with those presumed to be aggregates of the 15-PGDH protein (27).

sults, with no cDNA product visible from some donors (as shown in Fig. 8 A), or a very faint band from other donors. Because 15-PGDH primers resulted in consistent, distinct bands via RT-PCR, we examined the presence of the 15-PGDH protein in monocyte cytosol by Western blot. A protein immunoblot, using human placenta as positive control, shows a faint band at 29 kD from monocyte cytosol (Fig. 8 B). In addition, stronger bands were visible at ~58–60 kD (also seen in the placenta), which have been previously documented in 15-PGDH immunoblots and are attributed to incomplete dissociation of the protein into subunits (27). Taken together, the RT-PCR and immunoblot demonstrate the presence of both 15-PGDH mRNA and immunoreactive enzyme in human monocytes.

Discussion

Results obtained in these studies demonstrate for the first time that both LXA₄ and LXB₄ are potent agonists for human monocyte migration and adhesion. LXA₄ and LXB₄, at submicromolar concentrations, stimulate migration of monocytes in a dose range similar to the chemoattractant peptide FMLP and the monocyte chemokines MCP-1 and MIP-1 α (Figs. 1 and 2, Table 1). Both LXA₄ and LXB₄ also stimulate adherence of monocytes to laminin-coated plastic in a concentration-dependent manner and were equipotent with FMLP and LTB₄ (Figs. 3 and 4). Both LXA₄ and LXB₄ were subject to rapid further metabolism by human monocytes and were only minimally transformed by intact PMN (Fig. 5). Direct comparison of both LXA₄- and LXB₄-derived monocyte products in monocyte adherence revealed that the novel products of both lipoxins did not carry biologic activity (Fig. 7). These results are in sharp contrast to those observed with the quantitatively minor ω -oxidation products of LXB₄ isolated from PMN, namely, 20-OH-LXB₄ and 20-COOH-LXB₄, which were equipotent with the parent molecule (Fig. 7 B).

Activation of monocyte migration and adherence is relevant to numerous inflammatory and vascular diseases, including vital initiating events in atherosclerosis (1, 2). These are also the first steps in normal differentiation and maturation of circulating monocytes to tissue macrophages (1). Because our results show that LXA₄ and LXB₄ stimulate both chemotaxis and adherence of monocytes, it follows that lipoxins may play a role in physiologic monocyte trafficking and/or pathologic processes in which monocytes are involved. This is underscored by the finding that monocytes inactivate both LXA₄ and LXB₄ (Fig. 8).

It is of interest to point out that lipoxins activate human monocytes and inhibit human PMN activity stimulated by other agonists. LXA₄ inhibits FMLP-stimulated transmigration of PMN through epithelium (5) and FMLP- or LTB₄-induced PMN chemotaxis (4). In addition, LXA₄ and LXB₄ inhibit LTB₄- and LTC₄-stimulated adherence of PMN to endothelium (28) and LTD₄-stimulated PMN adherence to mesangial cells (29). The differential action of lipoxins with PMN versus monocytes is intriguing. Monocytes and PMN can respond in opposing manners to the

same stimulus; for example, phorbol ester stimulates PMN adhesion but inhibits monocyte adhesion to endothelial cells (30). Therefore, it is possible that lipoxins are acting via some shared intracellular second messengers in both monocytes and PMN, but are eliciting antithetical responses from the cells. Recently, we identified a previously denoted orphan seven-transmembrane receptor as a high-affinity receptor for LXA₄ present in PMN and differentiated HL-60 cells that has 67% sequence homology with the FMLP receptor (31). The presence of this receptor mRNA has also been identified in human monocytes (32). These results, together with those presented here, suggest that LXA₄ is likely acting via the same receptor in monocytes and PMN. Mobilization of intracellular Ca²⁺ by lipoxins was also examined, and we found that, at submicromolar concentrations, LXA₄ stimulates increases in intracellular Ca²⁺ (monitored by Fura-2) in both monocytes and THP-1 cells, but in parallel experiments LXB₄ did not induce a signal of comparable magnitude (Romano, M., and C. Serhan, manuscript in preparation). This suggests that the post-receptor interactions in each cell type may be distinct. Along these lines, LXB₄ does not bind to the same receptor as LXA₄ (31), and likely has a unique receptor or site of action in monocytes.

In general, eicosanoids can be rapidly transformed to increase or decrease their activity in target tissues (33–35). LXA₄ is metabolized in monocytes by dehydrogenation and reduction (Fig. 6), and the proposed sequence of transformation is LXA₄→15-oxo-LXA₄→13,14-dihydro-15-oxo-LXA₄→13,14-dihydro-LXA₄ (9). Based on results presented here (see Materials and Methods), it is likely that LXB₄ is transformed via a similar pathway (Fig. 8). Therefore, the hypothetical scheme for LXB₄ metabolism in monocytes can proceed as follows: LXB₄→5-oxo-LXB₄→5-oxo-6,7-dihydro-LXB₄→6,7-dihydro-LXB₄.

An increase or decrease in the bioactivity of individual eicosanoid further metabolites can be cell type and/or tissue specific. The LTB₄ metabolites 20-OH-LTB₄ and 20-COOH-LTB₄, for example, are known to be markedly less potent chemoattractants for PMN than LTB₄ (33), and dehydrogenation of prostanoids is generally thought to be an inactivation step (for reviews see references 8 and 35). In contrast, the recently identified 5-oxo-HETE, a dehydrogenase product of 5-HETE isolated from PMN, has been shown to possess potent stimulatory properties for PMN (34). LXA₄ and LXB₄ ω -oxidation products were isolated from microsomes from human PMN (16, 36, 37) and rat liver cells (17), but no evaluation of biological activity of these metabolites was presented. These are likely the lipoxin products of the P450 enzyme, which acts on LTB₄ as a preferred substrate (16). These results are the first to compare bioactivities of the novel monocyte lipoxin-derived metabolites and the ω -oxidation products of lipoxins with the parent compounds. The results clearly show that conversion of LXA₄ and LXB₄ in monocytes by dehydrogenation and reduction of double bonds results in inactivation. In contrast, ω -oxidation of LXB₄ to 20-OH-LXB₄ and 20-COOH-LXB₄ causes no apparent change in potency in

stimulating monocyte adhesion to laminin. These results give clues to the key structural elements of the lipoxin molecules involved in recognition and activation of monocytes; namely, changes at the ω end (carbon 20) appear to have no effect, but alteration of the hydroxyl groups at C5 in LXB₄ or C15 in LXA₄, or reduction of the adjacent double bond (Fig. 8), results in a loss of activity. These results suggest that the segments of the molecules containing the tetraene structures are key in defining bioactivity in the monocyte.

Several key enzymes have been identified that are involved in the metabolism of eicosanoids via each of the main pathways, including β -oxidation, ω -oxidation, dehydrogenation, and/or reduction. LTB₄, for example, is converted by ω -oxidation in PMN by a cytochrome P450 enzyme (38). Dehydrogenases are important enzymes catalyzing the transformation of PGE₂ to its major metabolite, 15-keto-PGE₂, in many different tissues (35) and in converting 5-HETE to 5-oxo-HETE in human PMN (39). NAD⁺-dependent 15-PGDH is one enzyme responsible for this further metabolism of prostanoids and has been cloned from human placental tissue (19). An NADP⁺-dependent prostaglandin dehydrogenase activity is also present in placental tissue, attributed to the somewhat nonspecific activity of carbonyl reductase, which has also been cloned (20). Both lipoxins carry C15 alcohol groups (Fig. 6), and in PMA-differentiated HL-60 cells, which express 15-PGDH (27), LXA₄ blocked ~50% of 15-HETE conversion to 15-oxo-HETE at equimolar concentrations (9). Therefore, we hypothesized that one or both of these dehydrogenase enzymes could catalyze the formation of oxo-lipoxins. Results showed distinct presence of 15-PGDH mRNA in monocytes (Fig. 8 A, lane 3). Western blots also

established that 15-PGDH protein is transcribed in monocytes and, therefore, is likely to contribute to the metabolism of LXA₄ by monocytes. This finding does not preclude the presence of additional, and/or potentially novel, enzymes that can carry out similar transformations. The C5 alcohol group of LXB₄ is less likely to be susceptible to 15-PGDH activity and, though it is possible that the unique dehydrogenase that converts 5-HETE to 5-oxo-HETE is responsible (39), there is a lack of extensive conversion of LXB₄ to 5-oxo-LXB₄ by intact PMN (Figs. 5 and 6), where this eicosanoid dehydrogenase activity was observed.

The results of this report are the first to describe potent actions for LXA₄ and LXB₄ on human monocyte migration and adherence, and rapid metabolic transformation by monocytes to novel products that do not carry these activities. Monocytes routinely migrate into normal tissue for differentiation into resident macrophages, whereas PMN generally extravasate in response to injury or infection, and in these scenarios, PMN are immediately responsive, whereas monocytes migrate in at a later stage. These distinctive behavior patterns of monocytes versus PMN suggest that these cell types respond uniquely to the same stimuli. This difference in temporal response would be an ideal setting for the production of lipoxins by PMN interacting with platelets and endothelium at sites of inflammatory or vascular injury. This early response phase (40–42) could lead to recruitment of monocytes to the same site, but at a later time point. Thus, our results suggest that lipoxins generated by PMN–platelet interactions in vascular and/or inflammatory diseases could recruit monocytes, which then are able to self-limit the reaction by rapid further metabolism and downregulation of LXA₄ and LXB₄ bioactivity.

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Address correspondence to C. N. Serhan, Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesia Research Laboratories, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115.

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