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Oxidoreductases in Lipoxin A₄ Metabolic Inactivation

A NOVEL ROLE FOR 15-OXOPROSTAGLANDIN 13-REDUCTASE/LEUKOTRIENE B₄ 12-HYDROXYDEHYDROGENASE IN INFLAMMATION*

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The lipoxins (LX) are autacoids that act within a local inflammatory milieu to dampen neutrophil recruitment and promote resolution. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) and 15-oxoprostaglandin 13-reductase, also termed leukotriene B₄ 12-hydroxydehydrogenase (PGR/LTB₄DH), are two enzymatic activities appreciated for their roles in the metabolism of prostaglandins and LTB₄. Here, we determined whether these oxidoreductases also catalyze the conversion of lipoxin A₄ (LXA₄) and assessed the activities of these LXA₄ metabolites. 15-Oxo-LXA₄ was generated by incubating LXA₄ with 15-PGDH and NAD⁺ for studies of its further conversion. PGR/LTB₄DH catalyzed the NADH-dependent reduction of 15-oxo-LXA₄ to yield 13,14-dihydro-15-oxo-LXA₄. With NADH as a cofactor, 15-PGDH acted as a 15-carbonyl reductase and catalyzed the conversion of 13,14-dihydro-15-oxo-LXA₄ to 13,14-dihydro-LXA₄. Human polymorphonuclear leukocytes (PMN) exposed to native LXA₄, 15-oxo-LXA₄, or 13,14-dihydro-LXA₄ did not produce superoxide anions. At concentrations where LXA₄ and a metabolically stable LXA₄ analog potently inhibited leukotriene B₄-induced superoxide anion generation, the further metabolites were devoid of activity. Neither 15-oxo-LXA₄ nor 13,14-dihydro-LXA₄ effectively competed with ³H-labeled LXA₄ for specific binding to recombinant LXA₄ receptor (ALXR). In addition, introducing recombinant PGR/LTB₄DH into a murine exudative model of inflammation increased PMN number by ~2-fold, suggesting that this enzyme participates in the regulation of PMN trafficking. These results establish the structures of LXA₄ further metabolites and indicate that conversion of LXA₄ to oxo- and dihydro- products represents a mode of LXA₄ inactivation in inflammation. Moreover, they suggest that these eicosanoid oxidoreductases have multifaceted roles controlling the levels of specific eicosanoids involved in the regulation of inflammation.

The lipoxins (LX)¹ are lipid mediators that are generated and act locally at sites of inflammation, where they down-regulate polymorphonuclear leukocyte (PMN) function and promote resolution (1). In humans, three main biosynthetic pathways have been elucidated for LX formation, each involving transcellular biosynthetic utilization of intermediates between distinct cell types that are in close proximity with one another during vascular and inflammatory responses (1). When aspirin is given during inflammation, the aspirin-triggered lipoxins (ATL) are formed via cell-cell interactions involving cells bearing cyclooxygenase II (COX II) that has been acetylated by aspirin and cells that possess 5-lipoxygenase (5-LO) (1). These newly produced ATL may be responsible for some of the beneficial effects of taking aspirin. Results from both *in vitro* and *in vivo* studies indicate that LX and ATL possess potent and selective anti-inflammatory activities (1, 2).

Characteristic of autacoids, LX are rapidly metabolized following biosynthesis and bioaction. Lipoxin A₄ (LXA₄) is converted by specific leukocytes of the monocyte/macrophage lineage to 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄ (3, 4). While 15-hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the dehydrogenation of the C15 hydroxyl group of LXA₄ (C1 = carboxyl carbon, "COOH") to an oxo- group, to form 15-oxo-LXA₄ (4, 5), the enzyme(s) that catalyze subsequent steps in LX metabolism remain to be identified. An oxidoreductase that may catalyze the reduction of the 13,14-double bond in 15-oxo-LXA₄ to 13,14-dihydro-15-oxo-LXA₄ is 15-oxoprostaglandin 13-reductase, also termed leukotriene B₄ 12-hydroxydehydrogenase (PGR/LTB₄DH). Interestingly, this enzyme was cloned independently by several groups (6–8). The porcine kidney enzyme (GenBankTM accession number D49386) was described as an LTB₄DH because of its ability to catalyze the NAD(P)⁺-dependent dehydrogenation of LTB₄ to 12-oxo-LTB₄ (6) and was also identified in human tissues (GenBankTM accession number D49387) (9). The enzyme from porcine lung (GenBankTM

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¹ The abbreviations used are: LX, lipoxins; ALXR, lipoxin A₄ receptor; ATL, aspirin-triggered lipoxin; 13,14-dihydro-LXA₄, 5S,6R,15-trihydroxy-7E,9E,11Z-eicosatrienoic acid; 13,14-dihydro-15-oxo-LXA₄, 5S,6R-dihydroxy-15-oxo-7E,9E,11Z-eicosatrienoic acid; ESI, electrospray ionization; LC/MS/MS, liquid chromatography-tandem mass spectrometry; LTB₄, leukotriene B₄, 5S,12R-dihydroxy-6E,8Z,10Z,14E-eicosatetraenoic acid; LXA₄, lipoxin A₄, 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid; 15-oxo-LXA₄, 5S,6R-dihydroxy-15-oxo-7E,9E,11Z,13E-eicosatetraenoic acid; PG, prostaglandin; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; PGE₁, 11α,15S-dihydroxy-9-oxo-prost-13E-en-1-oi acid; PGR/LTB₄DH, 15-oxoprostaglandin 13-reductase/leukotriene B₄ 12-hydroxydehydrogenase (GenBankTM accession number U87622); PMN, polymorphonuclear leukocyte; RP-HPLC, reversed phase-high pressure liquid chromatography; TNF, tumor necrosis factor; LTA₄, leukotriene A₄.

accession number U87622) was essentially identical to the kidney enzyme (99.7% homology in amino acid sequence), with only a single residue difference, and was isolated and characterized as a 15-oxoprostaglandin 13-reductase in view of its catalytic efficiency for the reduction of the 13,14-carbon-carbon double bond of 15-oxoprostaglandins (7). An additional isoform has also been sequenced from rat liver (GenBankTM accession number U66322) and was termed an LTB₄ 12-hydroxydehydrogenase based on sequence homology to the human kidney enzyme (8). This isoform was initially identified in a screen for cancer chemopreventive markers as a novel cytoprotective enzyme induced by dithiolethiones and was termed dithiolethione-inducible gene-1 (DIG-1) (8). The enzyme converts LTB₄ to 12-oxo- metabolites that are less active than native LTB₄ (8, 9).

In the present work, we employed LC/MS/MS-based analyses to determine whether the isolated recombinant enzymes PGR/LTB₄DH and 15-PGDH catalyze the conversion of LXA₄ to 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄ in a series of reactions. We also assessed the impact of selective metabolic transformation on the inhibitory actions of LXA₄. Dehydrogenation and reduction essentially inactivates LXA₄, giving structures that are less potent and do not effectively compete at the recombinant LXA₄ receptor. These LXA₄ metabolites lose their functional ability to inhibit PMN responses, and introducing this recombinant enzyme at a site of inflammation enhances PMN infiltration.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome *c* (horse heart), β -NADH, NAD⁺, and Wright Giemsa stain were purchased from Sigma. Dulbecco's phosphate-buffered saline with CaCl₂ and MgCl₂ (DPBS++) and without Ca²⁺ or Mg²⁺ (DPBS-), and Dulbecco's modified Eagle's medium were from BioWhittaker (Walkersville, MD). 15-*epi*-16-Phenoxy-LXA₄ was prepared by Dr. N. Petasis (Department of Chemistry, University of Southern California) as in Ref. 5. Leukotriene B₄ (LTB₄) was purchased from Cayman Chemical (Ann Arbor, MI). Male BALB/c mice were 6–8 weeks old and from Harlan Sprague-Dawley. Microcon 10 microconcentrators were obtained from Amicon, Inc. (Beverly, MA). Recombinant murine TNF- α was purchased from Roche Molecular Biochemicals. Tris-HCl and 2-mercaptoethanol were from American Bioanalytical (Natick, MA). Whatman GF/C glass filters were from Fisher. [11,12-³H]LXA₄-methyl ester was prepared by catalytic hydrogenation of 11,12-acetylenic LXA₄-methyl ester as in Ref. 3 and was a gift from Berlex Biosciences (Richmond, CA) that was further isolated by reversed phase-high pressure liquid chromatography (RP-HPLC) as in Ref. 3.

Expression and Purification of Recombinant Oxidoreductases—Human 15-PGDH cDNA was inserted into the pGBT-T19 vector and overexpressed in *Escherichia coli* JM107 (10). Briefly, cells expressing 15-PGDH were grown in 2 liters of Luria-Bertani medium containing 50 μ g/ml ampicillin at 37 °C on a rotary shaker (100 rpm) until the culture reached an A₆₀₀ of 0.1 absorbance units. The cells were then induced with the addition of isopropyl-1-thio- β -D-galactopyranoside (1 mM) and grown overnight. The cell suspension was centrifuged at 8,000 $\times$ *g* for 15 min, and the cell pellets were resuspended in 20 ml of cold potassium phosphate buffer (40 mM, pH 7.0, 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol). At 4 °C, the cell pellets were sonicated and then centrifuged at 10,000 $\times$ *g* for 15 min. The supernatant was applied to a DEAE-Sephacel column (2.5 $\times$ 7.5 cm) and eluted using a 400-ml gradient of 40–250 mM potassium phosphate buffer. 15-PGDH activity in the eluent was assayed spectrophotometrically by measuring NADH formation at 340 nm in a 1-ml reaction mixture consisting of 0.1 M Tris-HCl, pH 9.0, 0.45 mM NAD⁺, and 38 μ M PGE₁. Active fractions were pooled and concentrated by ultracentrifugation. The protein was then applied to a Mono Q column equilibrated with 10 mM imidazole hydrochloride, pH 7.0, 20% glycerol, 0.1 mM dithiothreitol and eluted with a 20-ml gradient of 0.0–0.5 M NaCl. The peak with the highest activity was then rechromatographed over the Mono Q column.

Porcine lung PGR/LTB₄DH (GenBankTM accession number U87622) was overexpressed in *E. coli* JM109 harboring a pGBT-T19 vector into which the coding sequence of PGR/LTB₄DH had been inserted (7). Briefly, cells were grown in 700 ml of Luria-Bertani medium containing 100 μ g/ml ampicillin and 1 mM isopropyl-1-thio- β -D-galactopyranoside

at 37 °C overnight. Cells were pelleted by centrifugation at 4,000 $\times$ *g* for 10 min, resuspended in 20 ml of cold potassium phosphate buffer (40 mM, pH 7.0, 20% glycerol, 1 mM EDTA), and sonicated. The lysate was centrifuged at 100,000 $\times$ *g* for 20 min and the supernatant loaded onto a Cibacron Blue Sepharose column (2.5 $\times$ 10 cm) equilibrated with potassium phosphate buffer (10 mM, pH 7.0, 1 mM EDTA). Protein was eluted with a 400-ml gradient of 0.0–1.0 M KCl in equilibration buffer. Fractions were assayed for PGR/LTB₄DH activity spectrophotometrically based on the conversion of either 15-oxo-PGE₁ or 15-oxo-PGE₂, which form alkaline-dependent chromophores with molar extinction coefficients determined to be 15,160 M⁻¹ cm⁻¹ and 30,300 M⁻¹ cm⁻¹, respectively, under the conditions of the assay. To start the reaction, column eluent was added to the reaction mixture. The mixture had a total volume of 1 ml and contained 0.1 M sodium phosphate, pH 7.4, 1 mM 2-mercaptoethanol, 20 μ g of 15-oxo-PGE₁ or 15-oxo-PGE₂, and 1 mM NADH. The reaction was incubated at 37 °C for 10 min and then stopped with 0.25 ml of 2 M NaOH. The most active fractions were pooled and desalted and concentrated by ultrafiltration. For further purification, the enzyme was applied to a Mono Q column and eluted with a 3-ml gradient of 0.0–0.5 M sodium chloride in Tris-HCl (20 mM, pH 8.0, 1 mM EDTA).

Enzymatic Conversion of Lipoxin A₄—LXA₄ (5 μ g) was incubated with 15-PGDH (2 μ g) and NAD⁺ (1 mM) in 200 μ l of Tris-HCl buffer (50 mM, pH 8.2) for 30 min at 37 °C. An aliquot (1–2 μ l) was taken from each incubation for LC/MS/MS-based analyses. The remaining incubation solution was ultrafiltered with a Microcon 10 microconcentrator to remove the enzyme. 15-Oxo-LXA₄ was then isolated by RP-HPLC, and this procedure was repeated to produce the quantities of 15-oxo-LXA₄ required for further incubations or for activity and binding studies.

15-Oxo-LXA₄ was incubated with PGR/LTB₄DH (2 μ g) in 300 μ l of Tris-HCl buffer (50 mM, pH 7.4, containing 2 mM 2-mercaptoethanol and 2 mM NADH) for 30 min at 37 °C. The product of this reaction, 13,14-dihydro-15-oxo-LXA₄, was identified and confirmed by LC/MS/MS (see "Results"), and PGR/LTB₄DH was then separated from the incubation solution by ultrafiltration. 15-PGDH (2 μ g) was added to this solution and incubated for 30 min at 37 °C to yield 13,14-dihydro-LXA₄. This product was identified by LC/MS/MS and isolated by RP-HPLC for bioassays (*vide infra*).

LC/MS/MS and RP-HPLC—Liquid chromatography-tandem mass spectrometry (LC/MS/MS) results were acquired with an LCQ (Finnigan MAT, San Jose, CA) quadrupole ion trap mass spectrometer system equipped with an electrospray ionization probe. Compounds were suspended in mobile phase and injected into the HPLC component, which consisted of a SpectraSYSTEM P4000 (Thermo Separation Products, San Jose, CA) quaternary gradient pump, a LUNA C18-2 (150 $\times$ 2 mm, 5 μ m) column, and a SpectraSYSTEM UV2000 (Thermo Separation Products, San Jose, CA) ultraviolet/visible (UV-visible) absorbance detector. The column was eluted isocratically with methanol/water/acetic acid (65:34.99:0.01, v/v/v) at 0.2 ml/min into the electrospray probe. Full scan mass spectra (MS) were recorded in the negative ion mode in the range of *m/z* 330–360. For further identification of the analytes, product ion mass spectra (MS/MS) were obtained for *m/z* 353 (13,14-dihydro-LXA₄), *m/z* 351 (LXA₄ and 13,14-dihydro-15-oxo-LXA₄), and *m/z* 349 (15-oxo-LXA₄) (see "Results").

Incubation products were also analyzed employing RP-HPLC with a Hewlett-Packard 1100 Series diode array detector (Hewlett-Packard, Palo Alto, CA) for on-line identification and documentation of characteristic UV chromophores. The system was equipped with a binary pump and a LUNA C18-2 (150 $\times$ 1 mm, 5 μ m) microbore HPLC column, and was eluted isocratically with methanol/water/acetic acid (58:41.99:0.01, v/v/v) at 0.1 ml/min. Static UV measurements of isolated materials were obtained at 1-nm resolution using a Hewlett-Packard 8453 diode array UV-visible spectrophotometer (Hewlett-Packard, Palo Alto, CA).

Human PMN—Human PMN were obtained from fresh venous blood obtained by venipuncture from healthy volunteers who had not taken aspirin or other nonsteroidal anti-inflammatory drugs (for 2 weeks) and who had given written informed consent to a protocol approved by Brigham and Women's Hospital's Human Research Committee. PMN were isolated by dextran sedimentation followed by gradient centrifugation (11) and suspended in DPBS- (3.6 $\times$ 10⁶ cells/ml). As in Ref. 12, superoxide anion levels were measured as the superoxide dismutase-inhibitable reduction of cytochrome *c*, where the increase of the Soret band at 550 nm (ϵ = 21, 100 M⁻¹ cm⁻¹) in the absorbance spectrum is directly proportional to the amount of superoxide anion generated. Briefly, individual incubations were 0.5 ml in total volume (37 °C, pH 7.45) and were done in duplicate for each donor. Cytochrome *c* (0.06 nM) was added to PMN (1–3.6 $\times$ 10⁶ cells/ml) suspensions in

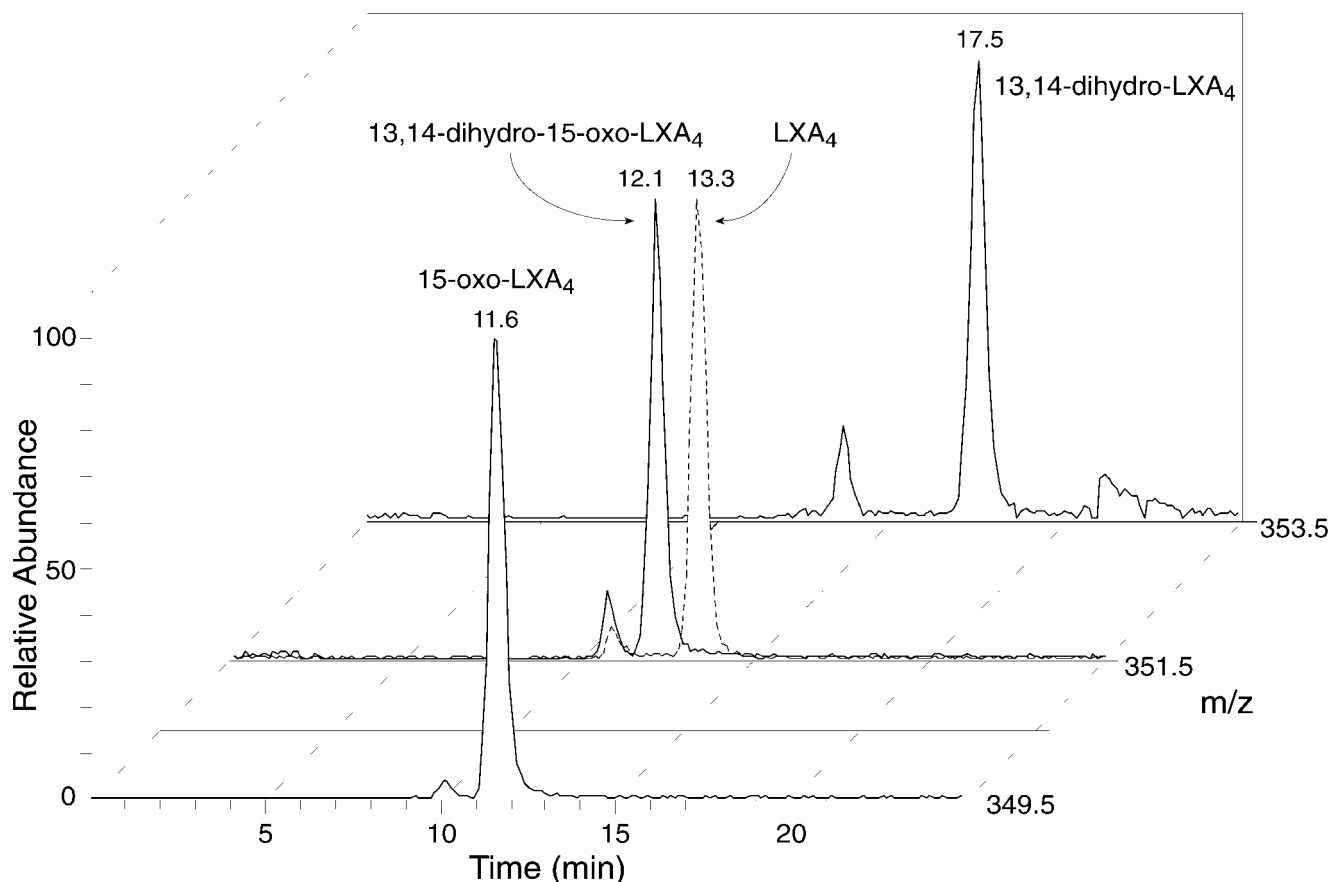


FIG. 1. LC/MS SIM chromatograms of LXA₄-derived dehydration/reduction further metabolites. LXA₄ (5 μ g, $[M -]^- = m/z$ 351.5, retention time = 13.3 min) was incubated in the presence of recombinant 15-PGDH (2 μ g) and NAD⁺ (1 mM) for 30 min (37 °C). The product, 15-oxo-LXA₄ ($[M - H]^- = m/z$ 349.5, retention time = 11.6 min), was 2 atomic mass units lower in mass. 15-Oxo-LXA₄ was then isolated and incubated with recombinant PGR/LTB₄DH (2 μ g) and NADH (4 mM) for 30 min (37 °C), yielding 13,14-dihydro-15-oxo-LXA₄ (m/z 351.5, retention time = 12.1 min), which had a mass 2 atomic mass units higher than 15-oxo-LXA₄. 13,14-Dihydro-15-oxo-LXA₄ was isolated and incubated with 15-PGDH (2 μ g) and NADH (4 mM) for 30 min (37 °C), resulting in conversion to 13,14-dihydro-LXA₄ (m/z 353.5, retention time = 17.5 min). Chromatograms are representative of $n = 5$.

Dulbecco's phosphate-buffered saline containing 1 mM CaCl₂ and 1 mM MgCl₂ (DPBS++). To determine whether the metabolites of LXA₄ induce superoxide anion generation, LTB₄, LXA₄, 15-oxo-LXA₄, or 13,14-dihydro-LXA₄ (100 nM) was added to the cell suspensions, incubated for 10 min, stopped with placement into an ice bath, and then the absorbance spectrum measured. To determine whether the metabolites of LXA₄ inhibit LTB₄-induced superoxide anion generation, cell suspensions were preincubated (5 min) with increasing concentrations of LXA₄ (1–1,000 nM), 15-epi-16-phenoxy-LXA₄, 15-oxo-LXA₄ (0.1–1,000 nM), or 13,14-dihydro-LXA₄ (0.1–100 nM). LTB₄ (100 nM) was then added and incubated for 10 min. The samples were stopped with placement into an ice water bath.

Competitive [³H]LXA₄ Binding—Human LXA₄ receptor (ALXR) cDNA was used as in Ref. 13. [³H]LXA₄ binding was performed with human embryonic kidney (HEK) 293 cells transfected with ALXR. Cells were suspended in DPBS++. Aliquots of HEK293 cells (0.5×10^6 cells/ml) were incubated with ~ 1 nM of [³H]LXA₄ (60,000 cpm, specific activity ~ 10 Ci/mmol) in the absence or presence of increasing concentrations of LXA₄ (0.1–100 nM), 15-oxo-LXA₄ (0.1–100 nM), or 13,14-dihydro-LXA₄ (0.1–100 nM) for 30 min at 4 °C. The bound and unbound radioligands were separated by filtration through Whatman GF/C glass filters. Filters were washed three times with 5 ml of ice-cold Tris-HCl buffer (10 mM, pH 7.6). The radioactivity retained on the filter was determined by scintillation counting. Nonspecific binding was determined in the presence of 3 log order excess of unlabeled LXA₄.

PMN Infiltration into Mouse Air Pouch—Dorsal air pouches were raised in male BALB/c mice (6–8 weeks old) that had been anesthetized with isoflurane by injecting 3 ml of sterile air subcutaneously on days 0 and 3 (as in Ref. 14). On day 6, either PGR/LTB₄DH (5 μ g) in 0.5 ml DPBS– or enzyme that had been denatured by boiling (100 °C, 15 min) was injected locally into the air pouch. Inflammation in the air pouch was induced by local injection of recombinant murine TNF- α (100 ng) dissolved in 0.5 ml of DPBS–. Four hours after administration of

TNF- α , the air pouches were lavaged three times with 3 ml of DPBS–. Aspirates were centrifuged at $800 \times g$ for 15 min at 23 °C. The supernatants were removed, and the cells were suspended in 1 ml of DPBS–. Aliquots of the cell suspension were stained with trypan blue and enumerated by light microscopy. For differential leukocyte counts, 100 μ l of the resuspended aspirate cells were added to 100 μ l of 30% bovine serum albumin and centrifuged onto microscope slides at 2,200 rpm for 4 min using a Cytospin (StatSpin, Norwood, MA). The slides were allowed to air dry, and cells were visualized using Wright Giemsa stain.

RESULTS

Conversion of LXA₄ and Its Metabolites by Eicosanoid Oxidoreductases—LC/MS/MS-based analyses were employed to determine whether 15-PGDH and PGR/LTB₄DH can catalyze, in successive steps, the conversion of LXA₄ to oxo- and dihydro-further metabolites. LXA₄ eluted from the LC system at 13.3 min and had a molecular anion of 351.5 atomic mass units ($[M - H]^- = m/z$ 351.5) (Fig. 1). Consistent with earlier findings (5), LXA₄ was converted to 15-oxo-LXA₄ upon incubation with recombinant human 15-PGDH and NAD⁺. 15-Oxo-LXA₄ was produced to assess the role of oxidoreductases in its conversion to oxo- and dihydro-LXA₄ products, and its MS/MS spectrum is shown for direct comparison to those of the further metabolites (Fig. 2). 15-Oxo-LXA₄ had a retention time of 11.6 min and a molecular anion of 349.5 atomic mass units ($[M - H]^- = m/z$ 349.5) (Fig. 1). The reduction in mass of 2 atomic mass units corresponded to the loss of two hydrogen atoms that occurs with the oxidation of the 15-hydroxyl group to an oxo-group. With prolonged exposure to light, 15-oxo-LXA₄ isomer-

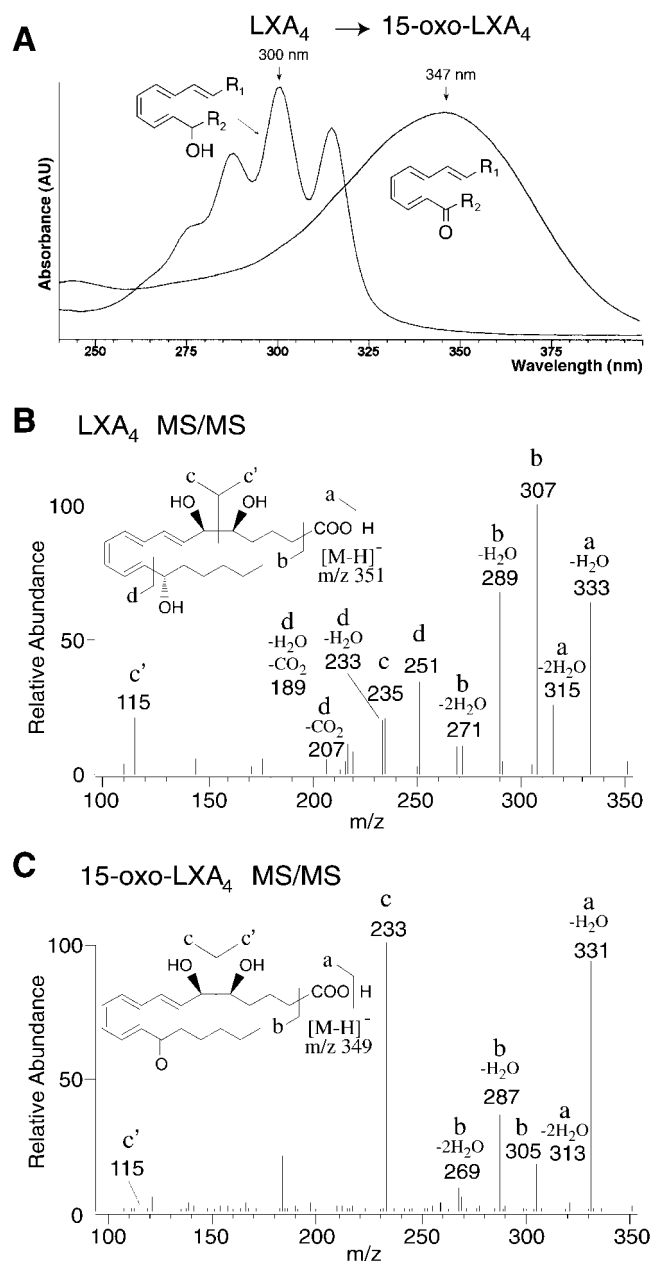


FIG. 2. Conversion of LXA₄ to 15-oxo-LXA₄. LXA₄ was incubated with 15-PGDH as in the legend of Fig. 1 and resulted in the dehydrogenation of the 15-hydroxyl group to an oxo-group. **A**, 15-oxo-LXA₄ was isolated by RP-HPLC, and its absorbance spectrum was determined in methanol ($\lambda_{\text{max}} = 347$ nm) and was red-shifted compared with the tetraene chromophore of LXA₄ ($\lambda_{\text{max}} = 300$ nm, methanol). **B**, ESI-MS/MS spectrum of LXA₄. The molecular anion of LXA₄ ($[M - H]^- = m/z$ 351) was further fragmented to yield diagnostic product ions. **C**, ESI-MS/MS spectrum of 15-oxo-LXA₄. The molecular anion of 15-oxo-LXA₄ ($[M - H]^- = m/z$ 349) fragmented to yield product ions that were 2 atomic mass units lower than their corresponding ions in the LXA₄ MS/MS spectrum (see "Results" for further details). Results are representative of $n = 5$.

izes to its 11-*trans* isomer (retention time 10.1 min). 15-Oxo-LXA₄ was then isolated and incubated with recombinant PGR/LTB₄DH and NADH. The major product of this incubation, 13,14-dihydro-15-oxo-LXA₄, eluted at 12.1 min and m/z 351.5 (Fig. 1). The 2 atomic mass units increase in mass corresponded to the addition of hydrogen across the 13,14-double bond of 15-oxo-LXA₄. 13,14-Dihydro-15-oxo-LXA₄ was isolated and incubated with 15-PGDH, now with NADH as cofactor. The product of this reaction had a retention time of 17.5 min and

a molecular anion of 353.5 atomic mass units ($[M - H]^- = m/z$ 353.5) (Fig. 1). The mass increase of 2 atomic mass units corresponded to the reduction of the 15-oxo-group of 13,14-dihydro-15-oxo-LXA₄ to the 15-hydroxyl group of 13,14-dihydro-LXA₄.

To substantiate their structures, the metabolites of LXA₄ were isolated by RP-HPLC, and their UV absorbance spectra were measured in methanol. The presence of a conjugated system of four double bonds within its structure affords LXA₄ a characteristic tetraene chromophore in its UV absorption spectrum (Fig. 2A). Conversion of the 15-hydroxyl group of LXA₄ by 15-PGDH and NAD⁺ to the 15-oxo-group in 15-oxo-LXA₄ gave a tetraenone chromophore (comprised of a ketone in conjugation with the tetraene). This extension of conjugation lowered the difference in energy between ground and excited electronic states, and consequently the absorption maximum was red-shifted to 347 nm (Fig. 2A).

To provide further evidence for the proposed structures of these LXA₄ metabolites, the product ion mass spectrum (MS/MS) of each compound was recorded and analyzed for product ion fragmentation. The molecular anion of LXA₄ ($[M - H]^- = m/z$ 351) fragments in its MS/MS spectrum (Fig. 2B) via: (i) neutral loss of H₂O and CO₂ to yield the product ions m/z 333 ($[M - H]^- - \text{H}_2\text{O}$), 315 ($[M - H]^- - 2\text{H}_2\text{O}$), 307 ($[M - H]^- - \text{CO}_2$), 289 ($[M - H]^- - \text{H}_2\text{O} - \text{CO}_2$), and 271 ($[M - H]^- - 2\text{H}_2\text{O} - \text{CO}_2$); (ii) cleavage of the 14,15 C-C bond to yield the product ion m/z 251 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3$); (iii) neutral loss of H₂O and CO₂ coupled with the cleavage of the 14,15 C-C bond to yield the product ions m/z 233 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3 - \text{H}_2\text{O}$), 215 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3 - 2\text{H}_2\text{O}$), 207 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3 - \text{CO}_2$), 189 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3 - \text{H}_2\text{O} - \text{CO}_2$), and 171 ($[M - H]^- - \text{CHO}(\text{CH}_2)_4\text{CH}_3 - 2\text{H}_2\text{O} - \text{CO}_2$); and (iv) cleavages of the 5,6 C-C bond to yield the product ions m/z 235 ($[M - H]^- - \text{CHO}(\text{CH}_2)_3\text{COOH}$) and m/z 115 ($\text{CHO}(\text{CH}_2)_3\text{COO}^-$).

The molecular anion of 15-oxo-LXA₄ ($[M - H]^- = m/z$ 349) is 2 atomic mass units lower than that of LXA₄, and consequently its corresponding MS/MS product ion masses are reduced by 2 atomic mass units (Fig. 2C). 15-Oxo-LXA₄ fragments via: (i) neutral loss of H₂O and CO₂ to yield the product ions m/z 331 ($[M - H]^- - \text{H}_2\text{O}$), 313 ($[M - H]^- - 2\text{H}_2\text{O}$), 305 ($[M - H]^- - \text{CO}_2$), 287 ($[M - H]^- - \text{H}_2\text{O} - \text{CO}_2$), 269 ($[M - H]^- - 2\text{H}_2\text{O} - \text{CO}_2$); and (ii) cleavages of the 5,6 C-C bond to yield the product ions m/z 233 ($[M - H]^- - \text{CHO}(\text{CH}_2)_3\text{COOH}$) and m/z 115 ($\text{CHO}(\text{CH}_2)_3\text{COO}^-$). Product ions derived from cleavage of the 14,15 C-C bond were of apparently low relative abundance in the MS/MS spectrum of 15-oxo-LXA₄. The oxo-group at C15 might impair, relative to other cleavages, the mechanism through which these ions are formed.

Incubation of 15-oxo-LXA₄ with PGR/LTB₄DH and NADH yields 13,14-dihydro-15-oxo-LXA₄, which gave an absorbance maximum at 272 nm in its UV spectrum (Fig. 3A). Reduction of the 13,14-double bond interrupted the tetraenone conjugation and resulted in a triene chromophore and an isolated ketone. The molecular anion ($[M - H]^- = m/z$ 351) of 13,14-dihydro-15-oxo-LXA₄ gave fragments in its MS/MS spectrum (Fig. 3B) consistent with: (i) neutral loss of H₂O and CO₂ to yield the product ions m/z 333 ($[M - H]^- - \text{H}_2\text{O}$), 315 ($[M - H]^- - 2\text{H}_2\text{O}$), 307 ($[M - H]^- - \text{CO}_2$), 289 ($[M - H]^- - \text{H}_2\text{O} - \text{CO}_2$), 271 ($[M - H]^- - 2\text{H}_2\text{O} - \text{CO}_2$); and (ii) cleavages of the 5,6 C-C bond to yield the product ions m/z 235 ($[M - H]^- - \text{CHO}(\text{CH}_2)_3\text{COOH}$) and m/z 115 ($\text{CHO}(\text{CH}_2)_3\text{COO}^-$). Since 13,14-dihydro-15-oxo-LXA₄ is 2 atomic mass units higher in mass than 15-oxo-LXA₄, its corresponding product ions are 2 atomic mass units larger, with the exception of fragment *c'* (Figs. 2C and 3B), which is not altered upon conversion. In

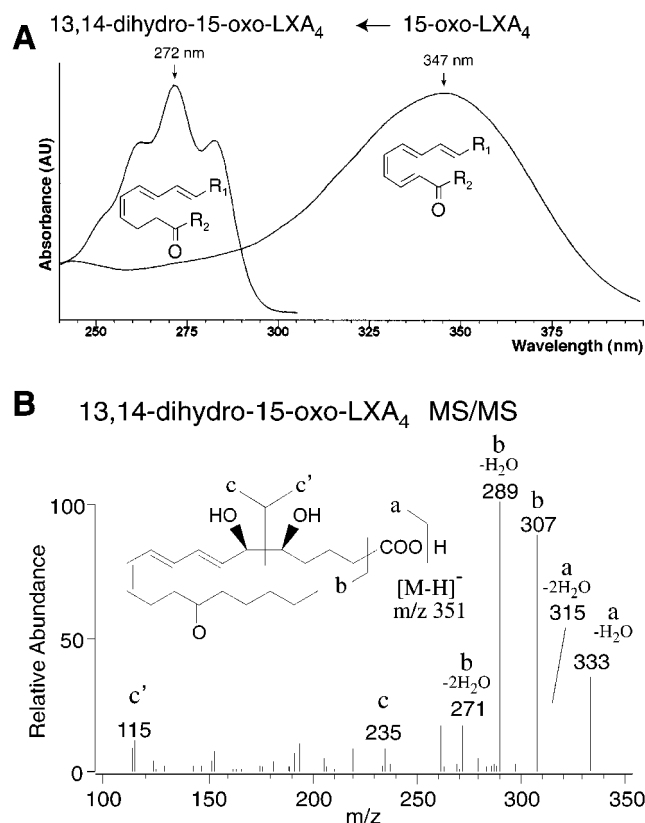


FIG. 3. PGR/LTB₄DH converts 15-oxo-LXA₄ to 13,14-dihydro-15-oxo-LXA₄. 15-Oxo-LXA₄ was incubated with PGR/LTB₄DH as in the legend of Fig. 1 and resulted in the reduction of the 13,14-double bond. **A**, 13,14-dihydro-15-oxo-LXA₄ was isolated by RP-HPLC, and its absorption spectrum was determined in methanol ($\lambda_{\text{max}} = 272$ nm). The absorbance maximum of the triene chromophore of 13,14-dihydro-15-oxo-LXA₄ was blue-shifted relative to the tetraene chromophore of 15-oxo-LXA₄ ($\lambda_{\text{max}} = 347$ nm). **B**, ESI-MS/MS spectrum of 13,14-dihydro-15-oxo-LXA₄. The molecular anion of 13,14-dihydro-15-oxo-LXA₄ ($[M - H]^- = m/z$ 351) was further fragmented, yielding product ions that were 2 atomic mass units higher than corresponding ions in the 15-oxo-LXA₄ MS/MS spectrum (see "Results" for further details). Results are representative of $n = 5$.

addition, the presence of the oxo- group at C15 appeared to discourage fragmentation of the 14,15 C-C bond as product ions from this cleavage were not abundant. Together, these MS/MS product ions and UV spectra are consistent with the proposed metabolite structure.

The ability of PGR/LTB₄DH to catalyze either the oxidation of the C15 hydroxyl group of LXA₄, with NAD⁺ as cofactor, or the direct reduction of the 13,14-carbon-carbon double bond, with NADH as cofactor, was also assessed. In either case, LXA₄ remained intact, and no appreciable conversion of LXA₄ was noted ($n = 3$). In contrast, incubation of isolated 13,14-dihydro-15-oxo-LXA₄ with 15-PGDH and NADH as a cofactor yielded a 13,14-dihydro-LXA₄. This material had a chromophore characteristic of a conjugated triene, namely a triplet band of absorbance, with a wavelength of maximal absorbance at 272 nm (Fig. 4A). Since the triene chromophore remained intact, the 2 atomic mass units increase in mass upon conversion of 13,14-dihydro-15-oxo-LXA₄ ($[M - H]^- = m/z$ 351.5) to 13,14-dihydro-LXA₄ ($[M - H]^- = m/z$ 353.5) confirmed the reduction of the 15-oxo- group to a hydroxyl group, as alteration of the C15 functional group would not impact the chromophore. The MS/MS spectrum also indicated that the 15-oxo- group was reduced, since product ions derived from the 14,15 carbon-carbon bond cleavage were abundant (Fig. 4B). Product ions observed in the MS/MS spectrum of 13,14-dihydro-LXA₄ were:

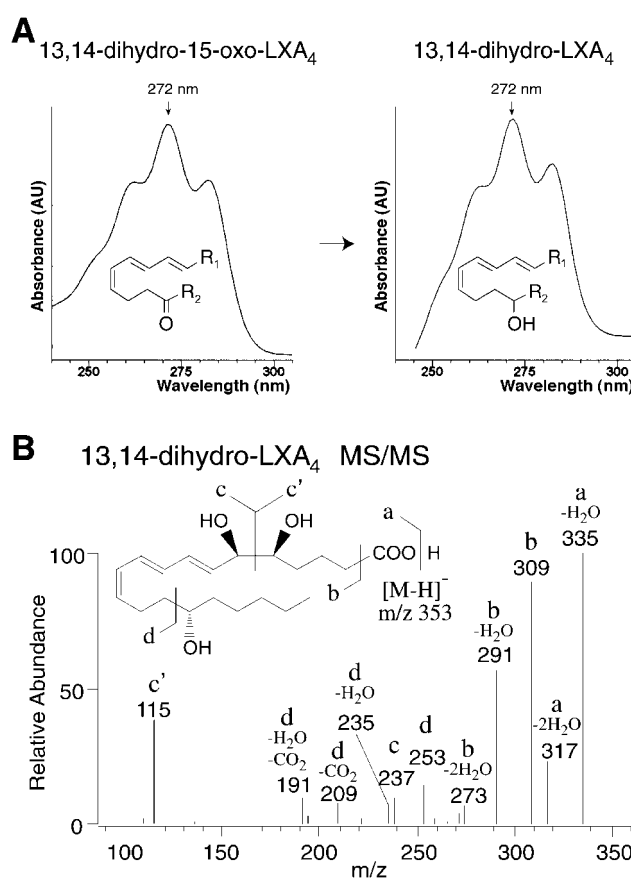


FIG. 4. 15-PGDH converts 13,14-dihydro-15-oxo-LXA₄ to 13,14-dihydro-LXA₄. 13,14-Dihydro-15-oxo-LXA₄ was incubated with 15-PGDH and NADH as in the legend of Fig. 1 and resulted in the reduction of the 15-oxo- group to a hydroxyl group. **A**, 13,14-dihydro-LXA₄ was isolated by RP-HPLC, and its absorption spectrum was determined in methanol ($\lambda_{\text{max}} = 272$ nm), and the wavelength of maximal absorbance was similar to that of 13,14-dihydro-15-oxo-LXA₄ ($\lambda_{\text{max}} = 272$ nm). **B**, ESI-MS/MS spectrum of 13,14-dihydro-LXA₄ (see "Results" for details). Results are representative of $n = 5$.

m/z 335 ($[M - H]^- - H_2O$), 317 ($[M - H]^- - 2H_2O$), 309 ($[M - H]^- - CO_2$), 291 ($[M - H]^- - H_2O - CO_2$), 273 ($[M - H]^- - 2H_2O - CO_2$), m/z 253 ($[M - H]^- - CHO(CH_2)_4CH_3$), m/z 237 ($[M - H]^- - CHO(CH_2)_3COOH$), m/z 235 ($[M - H]^- - CHO(CH_2)_4CH_3 - H_2O$), 209 ($[M - H]^- - CHO(CH_2)_4CH_3 - CO_2$), 191 ($[M - H]^- - CHO(CH_2)_4CH_3 - H_2O - CO_2$), and m/z 115 ($CHO(CH_2)_3COO^-$). These results therefore indicate that 15-PGDH can act as a carbonyl reductase, with NADH as a cofactor, and reduce a C15 oxo- group adjacent to a saturated 13,14 double bond, as in 13,14-dihydro-15-oxo-LXA₄. This functionality of 15-PGDH has not previously been appreciated.

Bioactions of 15-Oxo-LXA₄ and 13,14-Dihydro-LXA₄—To determine whether these LXA₄-derived metabolites are bioactive, isolated compounds were assessed for their ability to regulate superoxide anion generation by isolated human PMN. Incubated alone with PMN, neither LXA₄, 15-oxo-LXA₄, nor 13,14-dihydro-LXA₄ stimulated significant amounts of superoxide anions when directly compared with LTB₄, a potent natural PMN agonist (Fig. 5A). LXA₄ ($EC_{50} = 0.5 \mu M$) and a metabolically stable ATL analog ($EC_{50} = 50$ nM), 15-epi-16-phenoxy-LXA₄ (5), each inhibited LTB₄-induced superoxide anion generation (Fig. 5B). In these incubations, neither 15-oxo-LXA₄ nor 13,14-dihydro-LXA₄ inhibited ($p > 0.05$) superoxide anion formation initiated by LTB₄ (Fig. 5B).

Receptor Competition: 15-Oxo-LXA₄ and 13,14-Dihydro-LXA₄ Do Not Effectively Compete for Specific Binding—Since

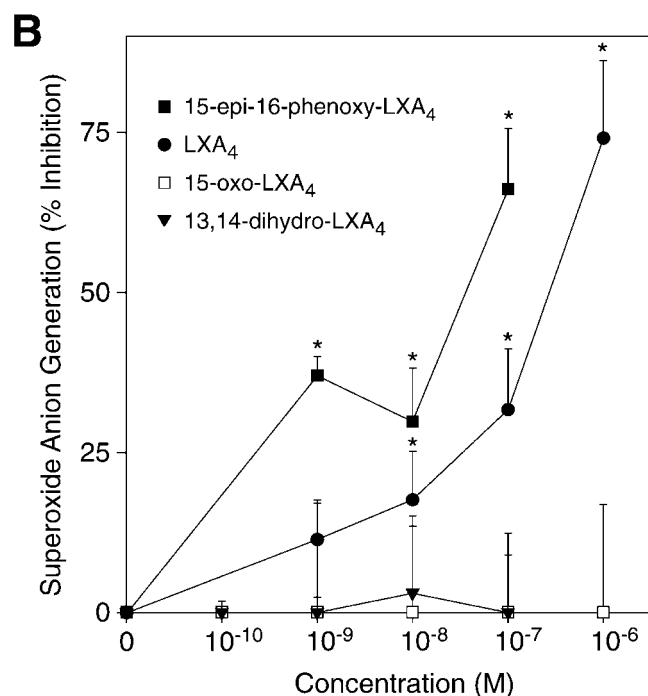
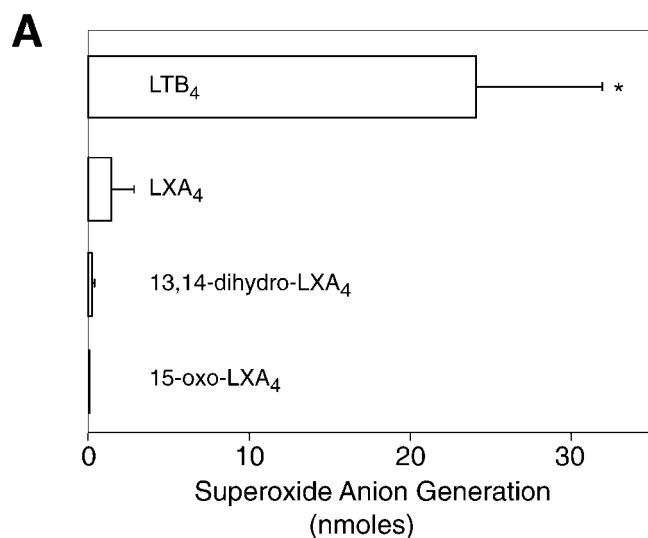


FIG. 5. 15-Oxo-LXA₄ and 13,14-dihydro-LXA₄ neither stimulate nor inhibit the generation of superoxide anions by PMN. A, superoxide anion generation by freshly isolated human PMN was determined (3.6×10^6 cells/ml, 0.5 ml total volume, 10 min, 37 °C) for LTB₄ (100 nM), LXA₄ (100 nM), 15-oxo-LXA₄ (100 nM), and 13,14-dihydro-LXA₄ (100 nM) (see "Experimental Procedures" for details). Values represent the mean $\pm$ S.E. for $n = 4$ separate donors. *, $p < 0.05$ Student's paired t test. B, inhibition of LTB₄-initiated O₂⁻ generation in PMN: comparison among LXA₄ and metabolites. Freshly isolated human PMN were incubated (3.6×10^6 cells/ml, 0.5 ml total volume, 5 min, 37 °C) with increasing amounts of LXA₄ (1–1,000 nM) (●); 15-epi-16-phenoxy-LXA₄ (1–100 nM) (■), a stable analog of LXA₄; 15-oxo-LXA₄ (0.1–1,000 nM) (□); and 13,14-dihydro-LXA₄ (0.1–100 nM) (▼) followed by LTB₄ (100 nM, 10 min, 37 °C). Values represent the mean $\pm$ S.E. for $n = 3$ –5 separate donors. *, $p < 0.05$ Student's paired t test.

12-oxo-LTB₄ has recently been shown to be a competitor with LTB₄ at its receptor (15), we determined whether 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ compete with [³H]LXA₄ for binding to the human LXA₄ receptor, ALXR. Competitive binding with [³H]LXA₄ was carried out with HEK293 cells stably expressing ALXR. 15-Oxo-LXA₄ and 13,14-dihydro-LXA₄ competed only weakly with [³H]LXA₄, for specific binding to the receptor

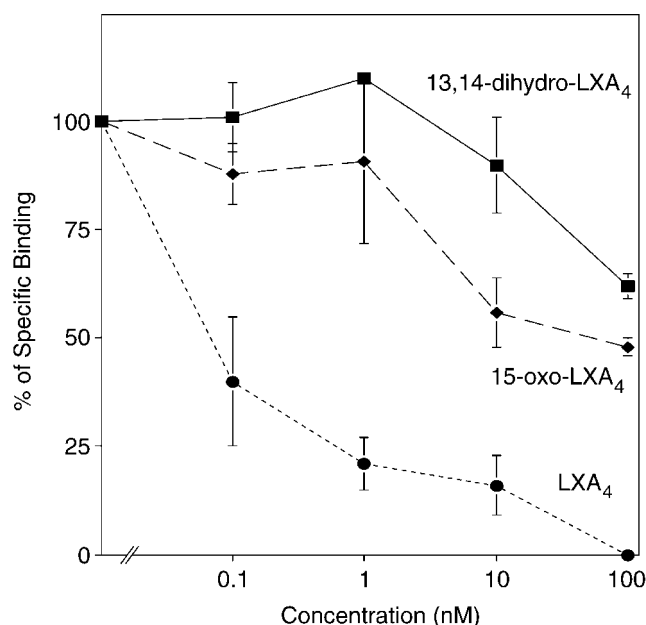


FIG. 6. Receptor competition: 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ do not effectively compete for specific binding. [³H]LXA₄ (1 nM; 60,000 cpm, specific activity ~ 10 Ci/mmol) was incubated with ALXR transfected HEK293 cells (0.5×10^6 cells/ml DPBS++). The cells were then incubated in the absence or presence of increasing concentrations of either LXA₄ (0.1–100 nM) (●), 15-oxo-LXA₄ (0.1–100 nM) (◆), or 13,14-dihydro-LXA₄ (0.1–100 nM) (■) for 30 min at 4 °C. The bound and unbound radioligands were separated by filtration through glass filters, and specific binding was determined. Values represent the mean $\pm$ S.E. for $n = 3$ separate experiments.

($EC_{50} \approx 100$ nM and $EC_{50} > 100$ nM, respectively) compared with unlabeled native LXA₄, which had an EC_{50} for specific binding of 0.1 nM (Fig. 6). These data indicate that 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ bind to ALXR, but with much lower affinity, and provide further evidence for the structural specificity required to activate the LXA₄ receptor.

PGR/LTB₄DH Enhances Neutrophil Recruitment into the Murine Dorsal Air Pouch—Stable analogs of LXA₄ and ATL that resist rapid metabolic conversion potentially inhibit TNF- α -initiated leukocyte trafficking into the 6-day murine dorsal air pouch, an *in vivo* model of inflammation (12, 16). To address whether the presence of the oxidoreductase (PGR/LTB₄DH) at a site of inflammation affects either TNF- α -initiated leukocyte recruitment, or resolution of acute inflammation as monitored by the number of accumulated cells, isolated recombinant PGR/LTB₄DH was introduced into the air pouch just before local injection of TNF- α . At 4 h, mice that received PGR/LTB₄DH and TNF- α had ~ 2 times as many PMN in their pouch exudates *versus* mice that received vehicle and TNF- α (Fig. 7). There were no statistically significant increases in the number of PMN within the pouches of mice that received the denatured enzyme compared with those that were injected with TNF- α alone (not shown). In addition, no statistically significant increases in the number of monocytes, eosinophils, or lymphocytes were observed in the pouch exudates of mice that received either TNF- α alone or TNF- α and the oxidoreductase compared with mice that received vehicle (Fig. 7). These results suggest that activation of this pathway and appearance of the oxidoreductase can regulate PMN trafficking.

DISCUSSION

The results of the present report emphasize that further metabolism of several classes of eicosanoids, including LX, is governed by specific oxidoreductases, a process that is well appreciated in the inactivation of prostaglandins (PG) and leu-

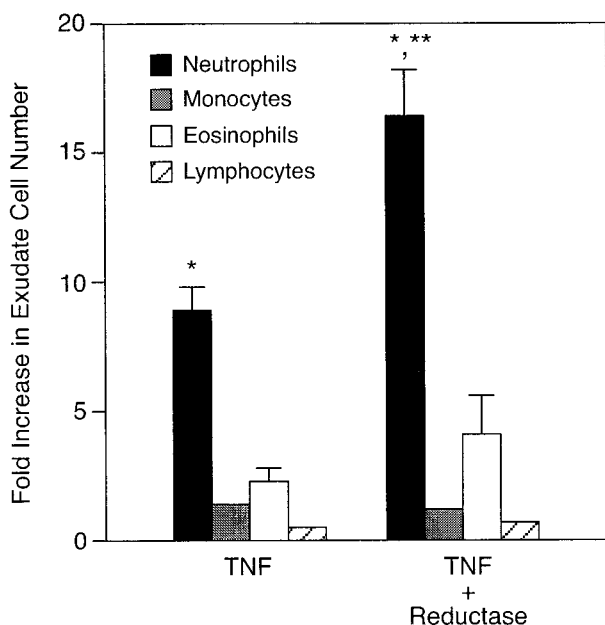


FIG. 7. Recombinant PGR/LTB₄DH enhances neutrophil trafficking into the murine dorsal air pouch at 4 h. Dorsal air pouches were raised in male BALB/c mice (6–8 weeks old). Recombinant PGR/LTB₄DH (5 µg) was injected locally into the air pouch, immediately followed by the injection of TNF-α (100 ng/pouch). The pouches were lavaged 4 h later, and leukocytes were enumerated (see “Experimental Procedures”). The ratios of enumerated leukocyte cell types found in the pouch exudates of mice that received locally administered TNF-α alone or TNF-α and PGR/LTB₄DH *versus* mice that were injected with vehicle were calculated. A statistically significant increase was observed in the number of PMN, but not other cell types (*, $p < 0.05$ Student's two-tailed t test). A statistically significant enhancement in PMN number was also observed in pouch exudates of mice that received PGR/LTB₄DH over mice that received TNF-α only (**, $p < 0.01$ Student's two-tailed t test). Values represent the mean $\pm$ S.E. for $n = 6$ mice.

kotrienes (LT) (9, 15, 17, 18). 15-PGDH catalyzes the oxidation of LXA₄ to 15-oxo-LXA₄, and this reaction likely represents the first step in the further metabolism of LXA₄ by leukocytes at sites of acute inflammation (3, 4, 16). 15-PGDH also catalyzes the conversion of aspirin-triggered 15-epi-LXA₄ to 15-oxo-LXA₄, but at approximately 50% of the rate of conversion of native LXA₄, and 15-epi-LXA₄ has potentially enhanced bioactivity (5). In the present experiments, 15-oxo-LXA₄ was generated to demonstrate its conversion as catalyzed by PGR/LTB₄DH and 15-PGDH with reducing cofactors and was characterized using LC/MS/MS (Figs. 1 and 2). As evidenced by LC/MS/MS analyses (Figs. 1 and 3), PGR/LTB₄DH catalyzed the NADH-dependent conversion of 15-oxo-LXA₄ to 13,14-dihydro-15-oxo-LXA₄. Of interest, this enzyme catalyzed neither the NAD⁺-dependent dehydrogenation of LXA₄ nor the NADH-dependent reduction of its 13,14-carbon-carbon double bond (see “Results”), indicating a limited specificity for either C15 carbonyl- or C12 hydroxyl-containing eicosanoids as substrates. Incubation of 13,14-dihydro-15-oxo-LXA₄ with 15-PGDH and NADH resulted in the reduction of the 15-oxo- group to yield 13,14-dihydro-LXA₄ (Figs. 1 and 4) and revealed an additional catalytic activity for this enzyme. Such duality of function is a recurrent theme among enzymes involved in eicosanoid metabolism. In LT and LX biosynthesis, 5-lipoxygenase catalyzes the insertion of molecular oxygen at C5 of arachidonic acid to give 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid, and the same enzyme possesses leukotriene A₄ (LTA₄) synthase activity (19). These activities were believed earlier to be two distinct enzymes. In addition, LTA₄ hydrolase can catalyze the hydrolysis of the epoxide moiety in LTA₄ to give LTB₄ and possesses an

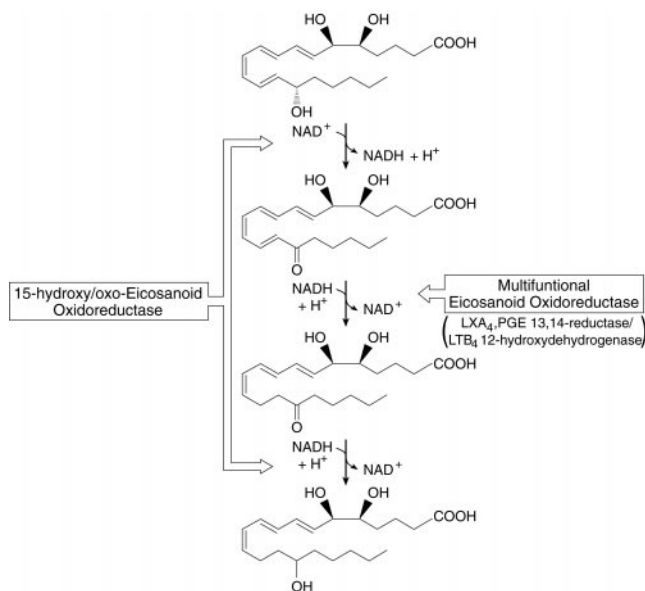


FIG. 8. Lipoxin inactivation. The initial step in LXA₄ inactivation is dehydrogenation of the 15-hydroxyl group, catalyzed by 15-hydroxy/oxo-eicosanoid oxidoreductase (15-PGDH), to yield 15-oxo-LXA₄ with reduced bioactivity. A multifunctional eicosanoid oxidoreductase (PGR/LTB₄DH) catalyzes the reduction of the 13,14 double bond of 15-oxo-LXA₄ to give 13,14-dihydro-15-oxo-LXA₄. This product then serves as a substrate for the 15-hydroxy/oxo-eicosanoid oxidoreductase, which catalyzes the reduction of the C15 oxo- group to give 13,14-dihydro-LXA₄. This product is less bioactive compared with native LXA₄ or its stable analogs that resist conversion.

amino peptidase activity (20). It is therefore likely that 15-PGDH also acts as a 15-carbonyl reductase in the presence of NADH and catalyzes a third step in lipoxin further metabolism (Fig. 8).

15-Oxo-LXA₄ and 13,14-dihydro-LXA₄ were each isolated using RP-HPLC and taken to assess their actions in functional assays with isolated human PMN *in vitro* to determine whether these metabolites have similar bioactivity to native LXA₄. Similar to LXA₄, these metabolites when added alone to PMN did not stimulate superoxide anion generation (Fig. 5A). In sharp contrast, LTB₄-initiated superoxide anion generation was inhibited by LXA₄, in agreement with its ability to inhibit TNF-α (12). The metabolites of LXA₄, 15-oxo-LXA₄ and 13,14-dihydro-LXA₄, were prepared and isolated and were not active in this PMN assay (Fig. 5B). These results indicate that LXA₄ is rendered less active as an inhibitor of PMN upon conversion to oxo- and dihydro- metabolites. These findings are also consistent with earlier results obtained with human monocytes, where these metabolites were also less active (4).

Inhibition of superoxide anion generation in human PMN is mediated via LXA₄ binding to its specific cell surface receptor, ALXR (12). Since the metabolites were less active than LXA₄, it was necessary to determine the extent to which they might bind to ALXR. This was deemed of interest because certain eicosanoids and their metabolites, such as LTB₄ and its dehydrogenation product, 12-oxo-LTB₄, can bind the LTB₄ receptor and serve as partial antagonists (15). If 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ bind but do not activate the receptor, then they may antagonize the binding of LXA₄ and therefore further reduce the activity of this mediator. Consistent with the loss of activity, 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ were weak competitors with [³H]LXA₄ for binding with essentially 2–3 orders of magnitude less activity (Fig. 6). Of interest, aspirin-triggered 15-epi-LXA₄, which has increased biological potency compared with LXA₄ (1), displaces [³H]LXA₄ from the LXA₄ receptor on human PMN with equal potency to the native compound (21),

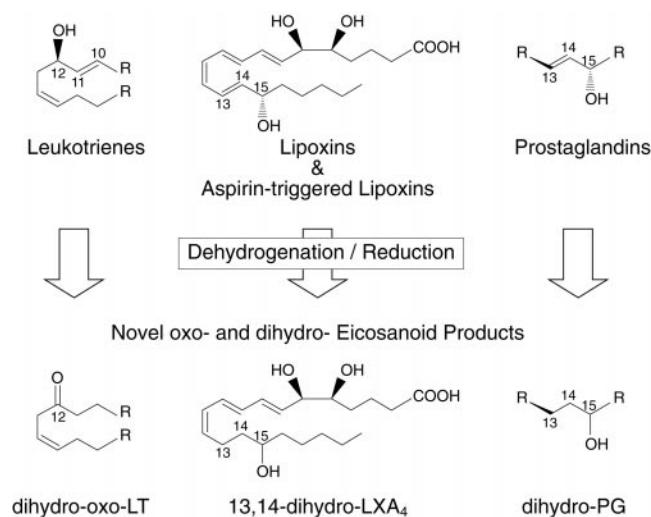


FIG. 9. **Class-specific oxidoreductase metabolites.** Separate eicosanoid classes are converted to oxo- and dihydro-containing compounds by recombinant eicosanoid oxidoreductases. Eicosanoid oxidoreductase plays a pivotal role in the conversion of leukotrienes, lipoxins, and prostaglandins to dihydro- and oxo- metabolites. The recombinant enzyme catalyzes the dehydrogenation of LTB₄ to 12-oxo-LTB₄ and the reduction of the 13,14 double bond of 15-oxo-PG and 15-oxo-LXA₄, resulting in the alteration of bioactivity for each eicosanoid class.

while 15-deoxy-LXA₄ does not compete for specific binding in the same concentration range (22), suggesting the need for a 15-hydroxyl group for binding. In addition, changes in the three-dimensional structural geometry of LXA₄ brought about by, for example, reduction of its planar 13,14-carbon-carbon double bond to the tetrahedral geometry of the 13,14-carbon-carbon single bond in 13,14-dihydro-LXA₄ might provide a basis to explain the loss in receptor binding and concomitant loss of activity.

Sequential dehydrogenation and reduction reactions that yield oxo and dihydro- products, where key enzymes display the ability to cross specific eicosanoid classes (*i.e.* PGE, LTB₄, and LXA₄), comprise routes of lipid mediator further metabolism (Fig. 9). Similar to LX, PG and LT undergo metabolic conversion via dehydrogenation and reduction to products with varied activities (17, 23). Results of studies addressing the activities of PGE₁ indicate that further metabolic conversions are modes of both inactivation and activation (24, 25). For example, 13,14-dihydro-PGE₁ inhibits platelet deposition onto de-endothelialized human veins with potency equal to that of PGE₁, while 15-oxo-PGE₁ and 13,14-dihydro-15-oxo-PGE₁ are orders of magnitude less potent (24). Each of the LTB₄ metabolites, 12-oxo-LTB₄, 10,11-dihydro-12-oxo-LTB₄, and 10,11-dihydro-LTB₄, stimulates human neutrophils with far lower potency relative to the parent compound (26). Yet *in vitro*, human PMN in the absence of other stimuli or inflammatory cytokines convert LTB₄ to predominantly ω -oxidation products 20-hydroxy-LTB₄ and 20-carboxy-LTB₄ (18). Metabolic conversion therefore represents an additional and physiologically relevant means to control duration of eicosanoid actions and their functions.

This level of control is of particular interest in view of leukocyte traffic at sites of inflammation (16), and since 15-PGDH and PGR/LTB₄DH can catalyze the conversion of more than one class of eicosanoid, it follows that the cell type(s) trafficking these enzymes into a site of inflammation and the inflammatory milieu can regulate the biological half-lives of lipid mediators. Taking into account that LX, LT, and PG can be gener-

ated within the inflammatory milieu and that in certain settings these mediators possess opposing bioactivities, further metabolism, and not just rate of formation, may contribute significantly to the overall course of events that occur in inflammation and its resolution. As a line of evidence to support this concept, PGR/LTB₄DH was locally administered in the murine air pouch model (16) of TNF- α -driven inflammation (Fig. 7). PGR/LTB₄DH gave an almost 2-fold increase in the number of PMN present within the inflammatory exudates. Since (i) dexamethasone, an anti-inflammatory steroid, has recently been found to suppress 15-PGDH expression as induced by an inflammatory stimulus (27), and (ii) increased levels of both COX-II derived PGE₂ and LXA₄ accelerate resolution of edema and are found in the exudates of allergen-evoked pleurisy in rats (28), it follows that PGR/LTB₄DH is likely to catalyze the conversion of these mediators at temporally distinct steps and therefore prolong the duration of inflammation, for example via inactivation of the endogenous inhibitory signals. Furthermore, although the specific mediators that are metabolized have not yet been identified *in vivo*, the present results indicate that an increase in the level of PGR/LTB₄DH within a site of inflammation gives rise to increases in PMN trafficking.

In conclusion, we have used recombinant enzymes to sequentially generate 15-oxo-LXA₄, 13,14-dihydro-15-oxo-LXA₄, and 13,14-dihydro-LXA₄ from LXA₄ and have characterized the bioactivities and structures of these compounds using LC/MS/MS. Our results indicate that PGR/LTB₄DH can catalyze the NADH-dependent reduction of 15-oxo-LXA₄ to give 13,14-dihydro-15-oxo-LXA₄ and that 15-PGDH can act as a 15-carbonyl reductase to give 13,14-dihydro-LXA₄ (Fig. 8B), suggesting that these enzymes are multifunctional with respect to eicosanoid class. In addition, 15-oxo-LXA₄ and 13,14-dihydro-LXA₄ had reduced activity with respect to native LXA₄'s ability to inhibit PMN or act at the LXA₄ receptor, suggesting that further metabolism of LXA₄ to oxo and dihydro- products is a mode of inactivation and that the functional impact of these enzymes on inflammation is dependent on their appearance at sites of exudate formation and resolution.

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