

# Neutrophil-mediated Changes in Vascular Permeability Are Inhibited by Topical Application of Aspirin-triggered 15-epi-lipoxin A<sub>4</sub> and Novel Lipoxin B<sub>4</sub> Stable Analogues

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## Abstract

Neutrophil (PMN) activation is critical in inflammation and reperfusion injury, suggesting that PMN-directed therapies may be of clinical use. Here, leukotriene B<sub>4</sub> (LTB<sub>4</sub>)-induced PMN influx in ear skin was equivalent between 5-lipoxygenase knockout and wild-type mice. To explore actions of lipoxin (LX) in PMN-mediated tissue injury, we prepared several novel LX stable analogues, including analogues of LXA<sub>4</sub> and aspirin-triggered 15-epi-LXA<sub>4</sub> as well as LXB<sub>4</sub>, and examined their impact in PMN infiltration and vascular permeability. Each applied topically to mouse ears inhibited dramatically PMN-mediated increases in vascular permeability (IC<sub>50</sub> range of 13–26 nmol) with a rank order of 15(*R/S*)-methyl-LXA<sub>4</sub> > 16-para-fluoro-phenoxy-LXA<sub>4</sub> ~ 5(*S*)-methyl-LXB<sub>4</sub> ≥ 16-phenoxy-LXA<sub>4</sub> > 5(*R*)-methyl-LXB<sub>4</sub>. These LX mimetics were as potent as an LTB<sub>4</sub> receptor antagonist, yet results from microphysiometry with mouse leukocytes indicated that they do not act as LTB<sub>4</sub> receptor level antagonists. In addition, within 24 h of delivery, > 90% were cleared from ear biopsies. Neither IL-8, FMLP, C5a, LTD<sub>4</sub>, nor platelet-activating factor act topically to promote PMN influx. When applied with LTB<sub>4</sub>, PGE<sub>2</sub> enhanced sharply both infiltration and vascular permeability, which were inhibited by a fluorinated stable analogue of aspirin-triggered LX. These results indicate that mimetics of LXs and aspirin-triggered 15-epi-LXA<sub>4</sub> are topically active in this model and are potent inhibitors of both PMN infiltration and PMN-mediated vascular injury. (*J. Clin. Invest.* 1998. 101:819–826.) Key words: leukocytes • lipid mediators • vascular permeability • antiinflammatory receptors • aspirin

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## Introduction

Neutrophil-dependent endothelial cell injury leads to changes in vascular permeability, edema, and further release of chemoattractants (1). The hallmark of reperfusion injury is considered to be diminished barrier function of vascular endothelium. Leukotriene B<sub>4</sub> (LTB<sub>4</sub>)<sup>1</sup> is among the most potent neutrophil stimuli and thus participates in tissue injury via recruiting PMNs in pathophysiologic scenarios (2). Lipoxins (LXs) are trihydroxytetraene-containing eicosanoids that are, among other *in vivo* sites, also generated within vascular lumen primarily by platelet-leukocyte interactions by pathways that are activated during multicellular responses such as inflammation, atherosclerosis, and thrombosis (for a review, see reference 3). Recently, aspirin was found to trigger the biosynthesis of a new group of compounds termed 15-epi-LX or aspirin-triggered lipoxins (ATLs) that may contribute to some of the beneficial actions ascribed to aspirin (3). Thus, these two LX branches involving cell-cell interactions within the eicosanoid cascade appear to produce "endogenous stop signals," while the 5-lipoxygenase (5-LO) pathway generates LTs that are primarily proinflammatory mediators.

LXs inhibit human PMN responses, including (a) FMLP and LTB<sub>4</sub>-induced chemotaxis (4), (b) adhesion and transmigration with endothelial cells (5), and (c) FMLP-induced transmigration through epithelial cells (6). These actions of LXA<sub>4</sub> were also demonstrated recently using an acute inflammation model, where PMN infiltration was inhibited dramatically by stable analogues of both LXA<sub>4</sub> and aspirin-triggered 15-epi-LXA<sub>4</sub>. These inhibitory actions were likely mediated by specific LXA<sub>4</sub> receptors on mouse PMNs, since the bioactive LXA<sub>4</sub> analogues compete with [<sup>3</sup>H]LXA<sub>4</sub> binding to LXA<sub>4</sub> receptors (7).

LXB<sub>4</sub> is a positional isomer of LXA<sub>4</sub>, carrying alcohol groups at carbon 5S, 14R, and 15S positions, instead of the C-5S, 6R, and 15S positions present in LXA<sub>4</sub>. Although LXA<sub>4</sub> and LXB<sub>4</sub> show similar bioactivities in some systems (5), in many others they each show distinct actions ([8]; see reference 3 for a recent review). Here, we report that aspirin-triggered LXA<sub>4</sub> and novel fluorinated LXA<sub>4</sub> as well as LXB<sub>4</sub> stable analogues inhibit PMN-directed actions *in vivo*.

## Methods

**Materials.** BALB/c mice (6–8 wk old) and 5-LO (+/+) mice (129/SV IMR strains, JR2448) and (–/–) mice (129/SV-ALOX5<sup>tm1Fun</sup>, JR2485) were from The Jackson Laboratory (Bar Harbor, ME). Syn-

1. **Abbreviations used in this paper:** ATL, aspirin-triggered LX; LC/MS/MS, liquid chromatography-tandem mass spectrometry; LO, lipoxygenase; LT, leukotriene; LX, lipoxin; MPO, myeloperoxidase; PAF, platelet-activating factor.

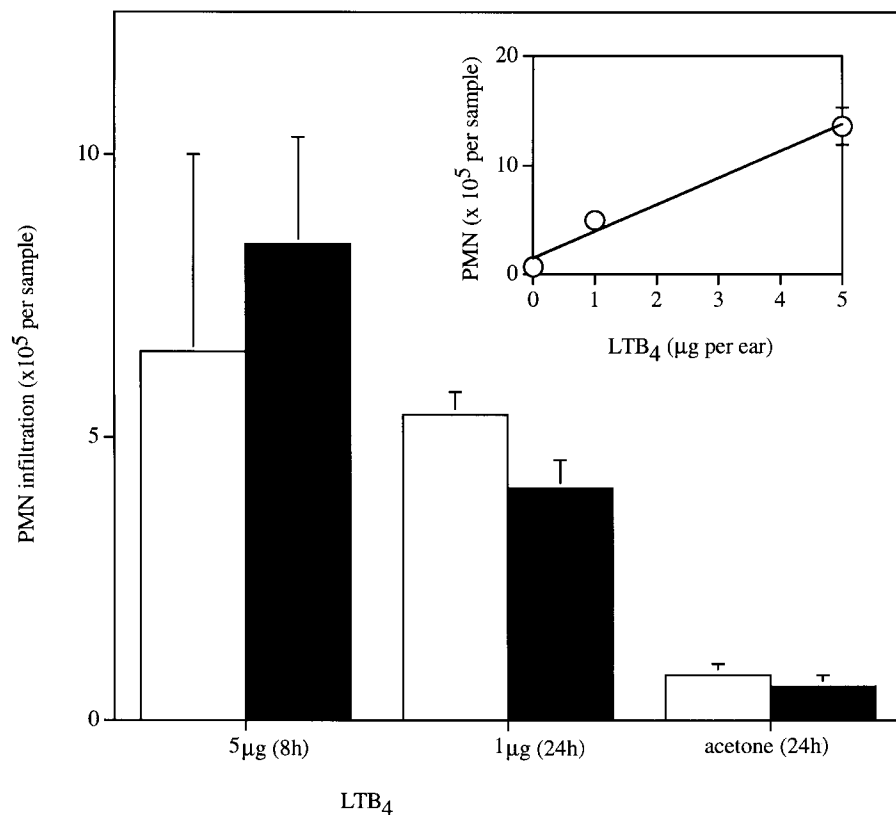
thetic LTB<sub>4</sub> and platelet-activating factor (PAF) were from Cascade Biochem Ltd. (Reading, Berkshire, UK). Evans blue, FMLP, and C5a were from Sigma Chemical Co. (St. Louis, MO). Synthetic LX analogues, 15(*R/S*)-methyl-LXA<sub>4</sub> [5(*S*),6(*R*),15(*R/S*)-trihydroxy-15-methyl-7,9,13-trans-11-cis-eicosatetraenoic acid methyl ester], 16-phenoxy-LXA<sub>4</sub> [15(*S*)-16-phenoxy-17,18,19,20-tetranol-LXA<sub>4</sub> methyl ester], 16-para-fluoro-phenoxy-LXA<sub>4</sub>, 15-epi-16-para-fluoro-phenoxy-LXA<sub>4</sub>, 5(*S*)-methyl-LXB<sub>4</sub> [5(*S*),14(*R*),15(*S*)-trihydroxy-5(*R*)-methyl-6,8,12-trans-10-cis-eicosatetraenoic acid], and 5(*R*)-methyl-LXA<sub>4</sub> [5(*R*), 14(*R*),15(*S*)-trihydroxy-5(*S*)-methyl-6,8,12-trans-10-cis-eicosatetraenoic acid] (used as methyl ester unless indicated), were designed from knowledge of both 15-epi-LXA<sub>4</sub> [5(*S*),6(*R*),15(*R*)-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid] and LXB<sub>4</sub> [5(*S*),14(*R*),15(*S*)-trihydroxy-6,8,12-trans-10-cis-eicosatetraenoic acid] structure and bioactivities, respectively (see reference 3). After total organic synthesis, each was isolated by reverse phase HPLC, and their identities were confirmed by nuclear magnetic resonance as by Serhan et al. (9) in the case of LXA<sub>4</sub> analogues, and Maddox et al. (10) for the LXB<sub>4</sub> analogues. The LTB<sub>4</sub> antagonist U-75302 (11) was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Recombinant human IL-8 was from R & D Systems (Minneapolis, MN).

**Mouse ear inflammation.** BALB/c mice (6–8 wk old) were used. The inner side of the right ear was treated with acetone (i.e., control), and the inner side of the left ear was treated topically with the compounds to be tested prepared in acetone. After 5–7 min, LTB<sub>4</sub> (1 μg in acetone) was applied to both ears. At 24 h, punch biopsy samples (6 mm diameter; Acu-Punch®; Acuderm, Inc., Ft. Lauderdale, FL) were obtained, and myeloperoxidase (MPO) activity and PMN infiltration were quantified as described previously (7). Percent inhibition of PMN infiltration was calculated after background levels of MPO activity present in mouse ear skin were subtracted. For the purpose of quantifying vascular permeability, 0.2 ml of Evans blue (0.5% in PBS<sup>2-</sup>) was injected intravenously immediately after the topical ap-

plication of test compounds. After 24 h, punch biopsies (4 mm diameter; Acu-Punch®) were obtained, and Evans blue was extracted in formamide (55°C for 1 h) and quantified by measuring absorbances at 610 nm with subtraction of reference absorbance at 450 nm. The mean absorbance was 0.13±0.01 (*n* = 40) for ears exposed to LTB<sub>4</sub> and 0.06±0.01 (*n* = 4) for ears exposed to vehicle alone. The difference between these values represented complete vascular permeability, and percent inhibition was calculated from the difference between the right (control) and left (experimental) ears. Statistical analysis was performed using Student's *t* test.

**LX analogues within ear tissue.** Each eicosanoid prepared in acetone was applied topically. The right ear was biopsied as soon as the ear surface appeared dry, placed in ice-cold methanol (400 μl containing 1 μg of PGB<sub>2</sub> for internal standard), and kept at -20°C until extracted. The left ear was processed in the same fashion at 24 h. Next, each sample was homogenized gently with a glass homogenizer, washed twice with methanol (200 μl), once with ethanol (200 μl), and extracted using Extract-Clean solid-phase extraction cartridges (500 mg C<sub>18</sub>; Alltech Associates Inc., Deerfield, IL). Materials that eluted in the methyl formate fractions were concentrated under a stream of N<sub>2</sub> and injected into a liquid chromatography–tandem mass spectrometer (LC/MS/MS) (LCQ; Finnigan Corp., San Jose, CA) using settings reported previously (10). Each compound was quantified using PGB<sub>2</sub> as an internal standard.

**Microphysiometer: mouse leukocyte response.** Blood was collected by heart puncture into acid citrate dextrose (as anticoagulant), and red cells were lysed by NH<sub>4</sub>Cl (12). The remaining leukocytes were enumerated, and differential counts were PMN 28%, lymphocyte 68.5%, monocyte 3%, and eosinophil 0.5% (which were within normal range for mouse peripheral blood). These mouse peripheral blood leukocytes (3.75 × 10<sup>5</sup> cells in each chamber) were tested as described recently (13) using microphysiometry (Cytosensor®; Molecular Devices Corp., Sunnyvale, CA).



**Figure 1.** PMN infiltration induced by topical application of LTB<sub>4</sub>; 5-LO knockout mice vs. wild-type. Ears of 5-LO (+/+) or 5-LO (-/-) mice were treated with indicated amounts of LTB<sub>4</sub> suspended in acetone. Punch biopsies were obtained at indicated time intervals, and MPO activity and PMN infiltration were determined. White bars, 5-LO (+/+); black bars, 5-LO (-/-). Results are mean±SEM of *n* = 3. (Inset) Concentration-dependent PMN infiltration to the ear of BALB/c mouse at 24 h. Results are mean±SEM of *n* = 3–5.

## Results

***LTB<sub>4</sub>-induced PMN infiltration in 5-LO knockout mice.*** To assess the role of 5-LO products in recruitment of PMNs to skin, we evaluated the chemotactic capacity for LTB<sub>4</sub> in 5-LO knockout mice. Topical application of LTB<sub>4</sub> (5 μg) induced significant PMN infiltration to mouse ear that reached maximum at 24 h (Fig. 1, and cf. reference 7). Both 5-LO (-/-) and (+/+) mice showed essentially equivalent levels of PMN infiltration into the ears, indicating that LTB<sub>4</sub> receptor signaling was intact in 5-LO (-/-) mice and suggesting that inhibitors of 5-LO might be of limited use in this tissue. After 24 h exposure to topical LTB<sub>4</sub>, ~ 5 × 10<sup>5</sup> PMNs infiltrated per 6-mm punch biopsy, which was equivalent to the levels induced by five times more LTB<sub>4</sub> within 8 h, and thus this amount of topically applied LTB<sub>4</sub> was selected for further experiments (Fig. 1, inset).

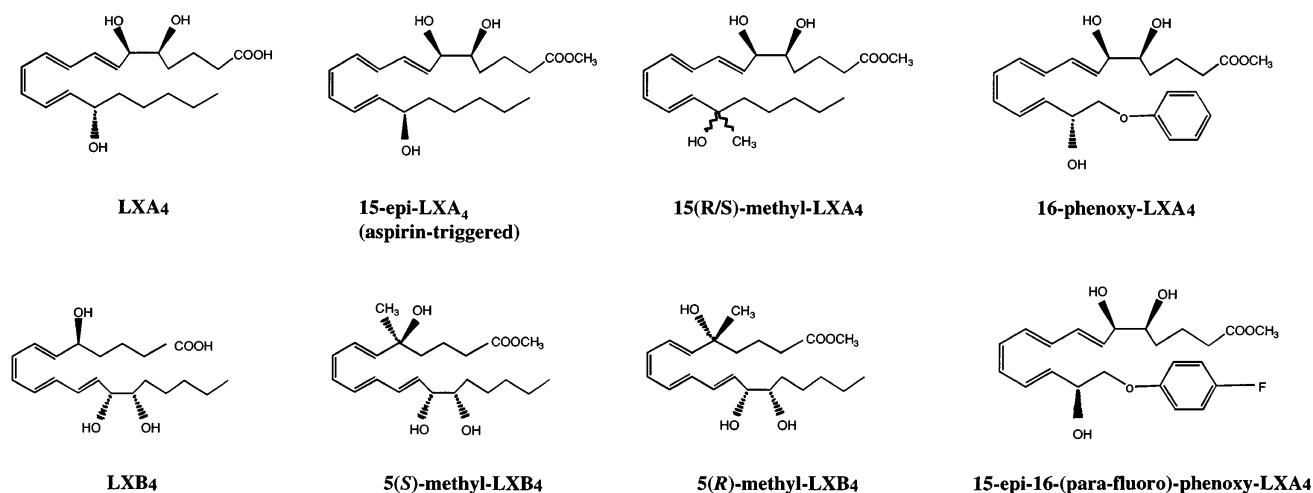
***LXA<sub>4</sub> stable analogues inhibit both PMN infiltration and vascular permeability changes.*** To explore other approaches, we evaluated LX stable analogues that were designed as mimetics of the endogenous antiinflammatory actions noted for LXA<sub>4</sub> (9) and recently for LXB<sub>4</sub> (10) in vitro. Here, we examined two LXA<sub>4</sub> stable analogues and tested their ability to inhibit both PMN infiltration and changes in vascular permeability in vivo. 15(*R/S*)-methyl-LXA<sub>4</sub>, which has a methyl group at C-15 position (racemate 15*R/S*), is an analogue of both the aspirin-triggered 15-epi-LXA<sub>4</sub> and native LXA<sub>4</sub>; 16-phenoxy-LXA<sub>4</sub>, which has a phenoxy group at C-16 position, is an analogue of native LXA<sub>4</sub> that prevents enzymatic inactivation with recombinant enzyme in vitro ([9]; and see structures in Fig. 2). Both analogues act at LXA<sub>4</sub> receptors (7).

When applied topically to mouse ears, these two stable analogues inhibited both PMN infiltration and vascular permeability changes in a concentration-dependent fashion (Fig. 3, *A* and *B*). At 130 nmol per ear, the degree of inhibition of PMN infiltration was > 90% for both analogues, with apparent IC<sub>50</sub>s noted at a range of ~ 13–26 nmol per ear for each analogue. In

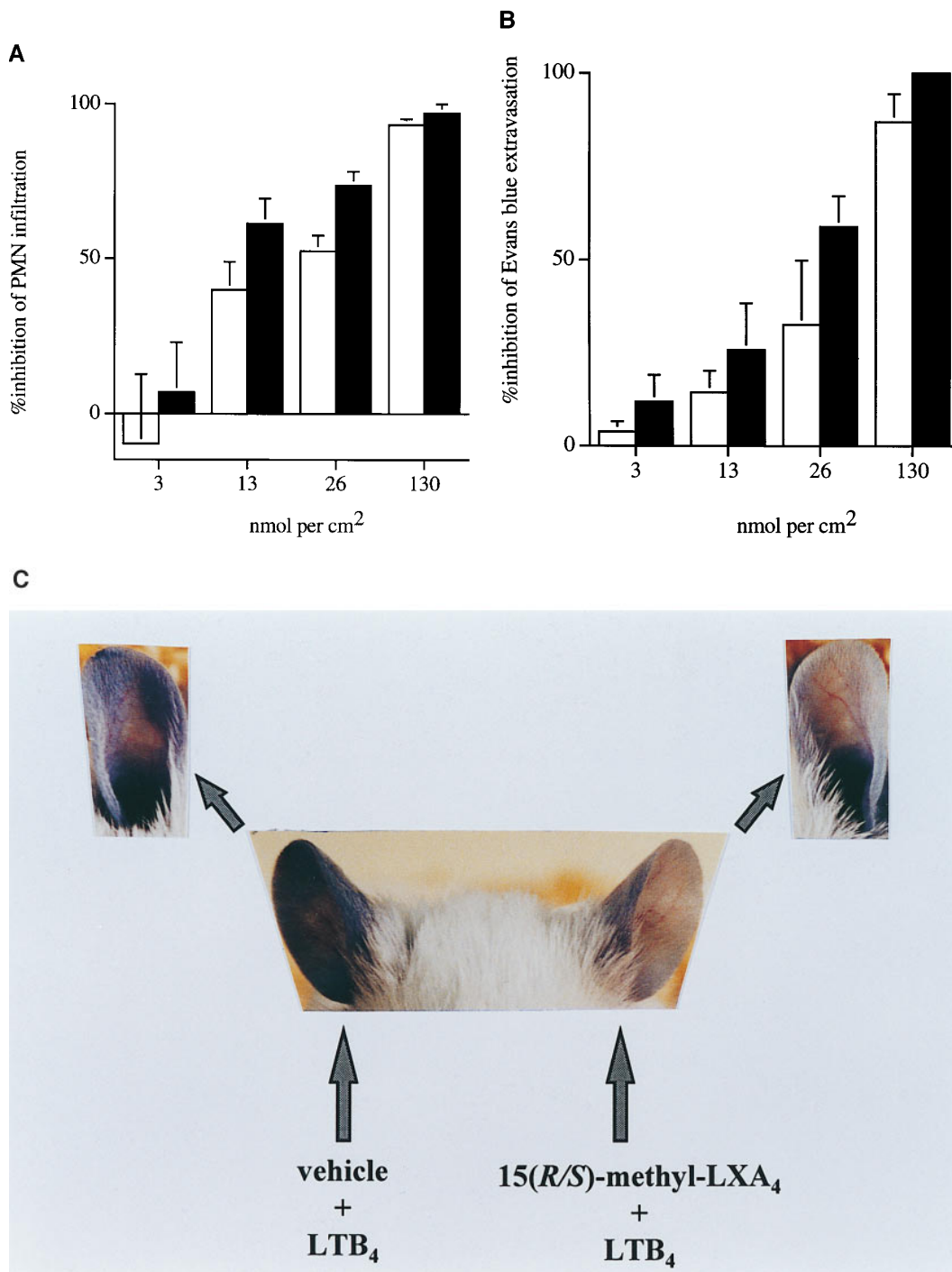
the same concentration range, these two LXA<sub>4</sub> stable analogues also inhibited the vascular permeability, namely, extravasation of Evans blue. At 130 nmol per ear, the inhibition of vascular permeability change was > 98% for 15(*R/S*)-methyl-LXA<sub>4</sub>, and ~ 87% for 16-phenoxy-LXA<sub>4</sub>, respectively. Moreover, their impact was striking and visible (see Fig. 3 *C*). The inhibition of vascular permeability changes paralleled inhibition of PMN infiltration with both analogues (Fig. 4), further implicating PMN involvement in these vascular permeability events and tissue damage (1, 2).

***Comparison of LX stable analogues with LTB<sub>4</sub> receptor antagonist.*** We next compared three LXA<sub>4</sub> analogues to native LXA<sub>4</sub> and the actions of the LTB<sub>4</sub> receptor antagonist U-75302. In addition, we evaluated the impact of recently designed novel LXB<sub>4</sub> analogues that resist enzymatic inactivation in vitro (10) to determine whether they also possess antiinflammatory actions (for structures, see Fig. 2). When applied topically at 26 nmol per ear, the stable analogues were three to four times more potent than native LXA<sub>4</sub>. Among the five lipoxin stable analogues tested, 15(*R/S*)-methyl-LXA<sub>4</sub> was the most potent (> 70% inhibition), and its inhibitory actions on PMN infiltration and vascular permeability changes were significantly greater than topically applied native LXA<sub>4</sub> (*P* < 0.05). A 16-para-fluoro derivative of 16-phenoxy-LXA<sub>4</sub> was prepared for these experiments to assess whether fluorination of the phenoxy ring could enhance potency. Results in Fig. 4, *A* and *B*, indicate that 16-para-fluoro-phenoxy-LXA<sub>4</sub> was also potent and retained the activity at levels comparable to 16-phenoxy-LXA<sub>4</sub>.

Both of the two new LXB<sub>4</sub> analogues inhibited PMN infiltration and vascular permeability. The *S* enantiomer, 5(*S*)-methyl-LXB<sub>4</sub>, was significantly more potent than 5(*R*)-methyl-LXB<sub>4</sub>, indicating a preferred stereoselectivity for inhibition. The rank order of inhibitory potency was 15(*R/S*)-methyl-LXA<sub>4</sub> > 16-para-fluoro-phenoxy-LXA<sub>4</sub> ~ 5(*S*)-methyl-LXB<sub>4</sub> > 16-phenoxy-LXA<sub>4</sub> > 5(*R*)-methyl-LXA<sub>4</sub> for both PMN infil-



**Figure 2.** Structures of LX stable analogues used in the experiments. In 15(*R/S*)-methyl-LXA<sub>4</sub>, the hydrogen at C-15 position was replaced by a methyl group as a racemate, and thus carries properties of both 15-epi-LXA<sub>4</sub> and native LXA<sub>4</sub>. 16-phenoxy-LXA<sub>4</sub> has a phenoxy group at C-16, and 16-(para-fluoro)-phenoxy-LXA<sub>4</sub> was fluorinated in the para position. LXB<sub>4</sub> is a positional isomer of LXA<sub>4</sub>, and in 5(*S*)- and 5(*R*)-methyl-LXB<sub>4</sub>, hydrogen at C-5 position was replaced by a methyl group at the *S* and *R* configuration, respectively. Note that these analogues were used in their methyl ester form for the experiments.

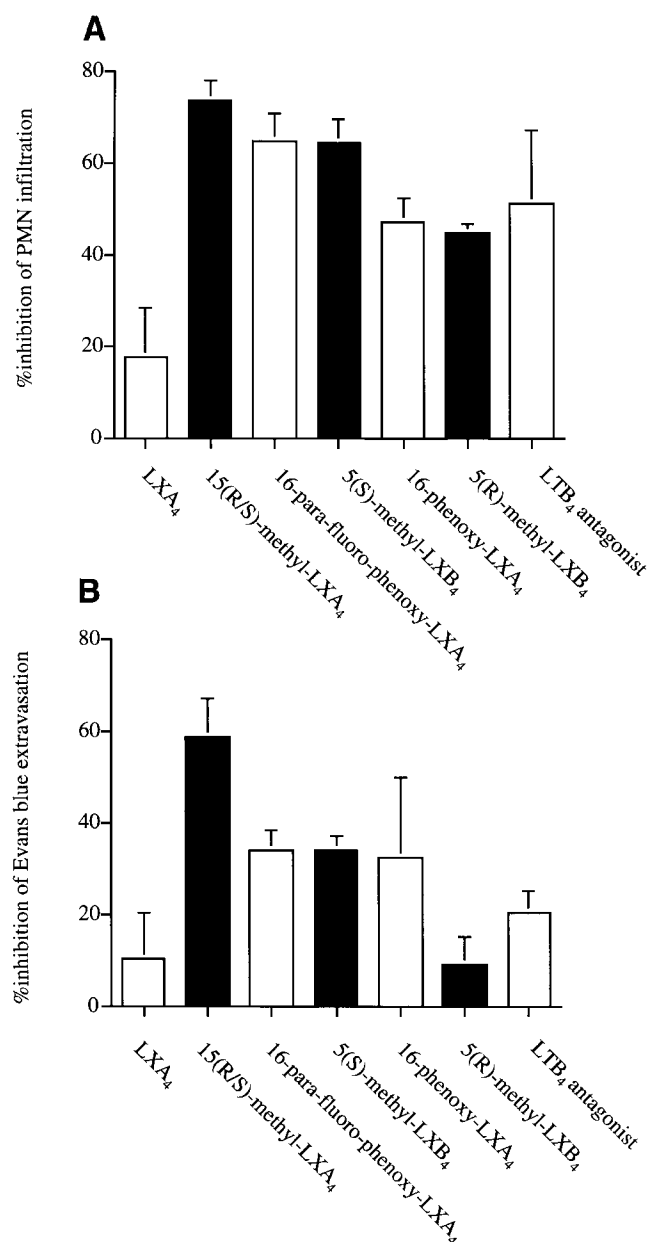


**Figure 3.** LXA<sub>4</sub> analogues inhibit vascular permeability change and PMN infiltration in the mouse ear. Mouse ears were treated topically with vehicle (acetone) or indicated amounts of LXA<sub>4</sub> analogues, and then inflammation was induced by topical application of LTB<sub>4</sub> (1 μg). Evans blue (0.5%; 200 μl) was injected intravenously to the tail vein immediately after treatment of the ears. Punch biopsies were obtained after 24 h, and MPO activity (A) and Evans blue (B) were quantified; see Methods. *Black bars*, 15(R/S)-methyl-LXA<sub>4</sub>; *white bars*, 16-phenoxyl-LXA<sub>4</sub>. Results are mean ± SEM of *n* = 3–5 except for 15(R/S)-methyl-LXA<sub>4</sub> at 130 nmol per ear in B (*n* = 2). (C) Photograph of mouse ears illustrating LTB<sub>4</sub>-stimulated vascular permeability and inhibition by LX analogue. Right ear was treated with acetone, and left ear was treated simultaneously with 15(R/S)-methyl-LXA<sub>4</sub> (130 nmol). LTB<sub>4</sub> was applied to both ears as in A. Photographs were taken after 7 h of treatment.

tration and vascular permeability changes. Together, these results provide further evidence that these LX analogues inhibit vascular permeability changes via blocking of PMN infiltration. Of interest, each of these LX analogues proved to be as potent or more potent than topical application of equivalent amounts of the LTB<sub>4</sub> receptor antagonist U-75302 (Fig. 4, A and B).

**Clearance of LX analogues applied topically.** LX are rapidly inactivated by conversion to oxo- and dihydro-containing products (for a review, see reference 3). Each of the LX analogues tested here resisted rapid conversion by recombinant

dehydrogenase in vitro (9, 10). To address whether these analogues were cleared and/or present in an extractable form within the ear after topical application, we biopsied ears at the time of application and after a time interval (i.e., 24 h), when high level inhibition was found for each LX analogue (Table I and Fig. 4). The eicosanoids recovered after extraction of ear biopsies (i.e., immediately after application and at 24 h) were identified and quantitated using LC/MS/MS. LTB<sub>4</sub> was monitored for purposes of direct comparison, and representative selected ion chromatograms of PGB<sub>2</sub> (internal standard) and LTB<sub>4</sub> obtained after extraction from biopsies are shown in Fig.



**Figure 4.** LXA<sub>4</sub> as well as LXB<sub>4</sub> stable analogues inhibit PMN infiltration and vascular permeability change: the rank order of potency. Experiments were performed as in Fig. 3, using 26 nmol of compounds per ear, and compared with native LXA<sub>4</sub> and LTB<sub>4</sub> receptor antagonist U-75302. (A) Percent inhibition of PMN infiltration; (B) percent inhibition of vascular permeability change (Evans blue extravasation). Results are mean ± SEM of *n* = 3.

5. Their retention times matched those obtained with standards, and the insets give their respective molecular ion-1 (M-1) or [M-H]<sup>-</sup> used for the identification of PGB<sub>2</sub> (left) and LTB<sub>4</sub> (right). Two of the more active LXA<sub>4</sub> analogues and an LXB<sub>4</sub> analogue were examined. After initial applications to the ears, only ~ 10–20% of the applied compounds were present within the target area of the biopsies (Table I). At 24 h after application, this value was reduced to < 10%, indicating that > 95% of the added LX analogues were not recoverable in extractable forms and thus were likely cleared by the ear tissue.

**Table I.** Tissue Recovery/Clearance of LX Analogues

Topically applied eicosanoid*	Material in initial ear biopsy <sup>‡</sup>		Tissue-extractable eicosanoid after 24 h <sup>§</sup>		Amount cleared <sup>  </sup>
	ng	%	ng	%	
LTB <sub>4</sub>	4369 ± 980	12.5	471 ± 120	10.8	~ 89.2
15-(R/S)-methyl-LXA <sub>4</sub> -me	3466 ± 30	17.3	122 ± 33	3.5	~ 96.5
16-phenoxy-LXA <sub>4</sub> -me	2560 ± 180	12.8	144 ± 3	5.6	~ 94.4
5(S)-methyl-LXB <sub>4</sub> -me	2919 ± 170	14.6	14 ± 1	0.5	~ 99.5

Values represent the means of three separate mice ± SEM. \*Eicosanoids were applied in 20 μl of 1 μg/μl acetone to ears of three mice for each compound. <sup>‡</sup>A 6-mm-diameter punch biopsy was taken from the ear immediately after the ear surface appeared dry (i.e., < 60 s). The ear biopsies were placed in 400 μl cold methanol containing 1 μg of PGB<sub>2</sub>, representing initial values. Eicosanoids were extracted, identified, and quantified using LC/MS/MS (see Methods). Recovery of each compound was normalized to the internal standard (PGB<sub>2</sub>). The percentage of the applied compound within the area of the punch is given in the second column. <sup>§</sup>Biopsy was obtained at 24 h after application. The percent remaining is shown in the second column. <sup>||</sup>Percent cleared or lost from the biopsied sample area. *me*, methyl ester.

*Do LXA<sub>4</sub> analogues antagonize at the LTB<sub>4</sub> receptor?* Although native LXA<sub>4</sub> does not compete for the specific binding of [<sup>3</sup>H]LTB<sub>4</sub> (4°C) to human PMN (14), it was not known whether LX analogues would antagonize the actions of LTB<sub>4</sub> by blocking at mouse leukocyte LTB<sub>4</sub> receptors. To test potential antagonism at the receptor level, we used a Cytosensor<sup>®</sup> microphysiometer. In general, when cell surface receptors are engaged with specific ligands, intracellular events are initiated that lead to rapid acidification of the extracellular microenvironment, which is monitored by the Cytosensor<sup>®</sup>. This rapid change in pH enables assessment of receptor-level antagonism (15) and was used here because of the small number of mouse leukocytes that could be obtained from individual mice. This precluded competition binding experiments with mouse leukocytes and labeled LTB<sub>4</sub> because of low sensitivity. When mouse peripheral leukocytes were exposed to LTB<sub>4</sub> (100 nM), acidification rates increased by ~ 40% from baseline, and this increase was inhibited significantly by the LTB<sub>4</sub> receptor antagonist U-75302 (Fig. 6 and *inset*). In contrast, the most potent LX analogue among the panel tested, namely 15-(R/S)-methyl-LXA<sub>4</sub>, did not alter LTB<sub>4</sub>-induced changes in acidification rates.

*Can ATL analogues block other inflammatory stimuli?* To evaluate the antiinflammatory actions of LX analogues on neutrophil-mediated vascular injury induced by agents other than LTB<sub>4</sub>, we examined a panel of known inflammatory mediators to test the specificity and/or generality of the actions of LX analogues (Table II). PGE<sub>2</sub> augmented dramatically the LTB<sub>4</sub>-induced PMN infiltration and vascular permeability change, although the effects of this PG by itself were minimal. PMA, a tumor promoter and topical irritant that bypasses surface receptors, caused concentration-dependent changes (not shown) in both PMN infiltration and vascular permeability (see Table II), and 100 ng PMA per ear was chosen for further evaluation. Well-appreciated agents such as FMLP, C5a, IL-8, PAF, or LTD<sub>4</sub> did not cause significant changes in these parameters at amounts applied as high as 1–25 μg compared with

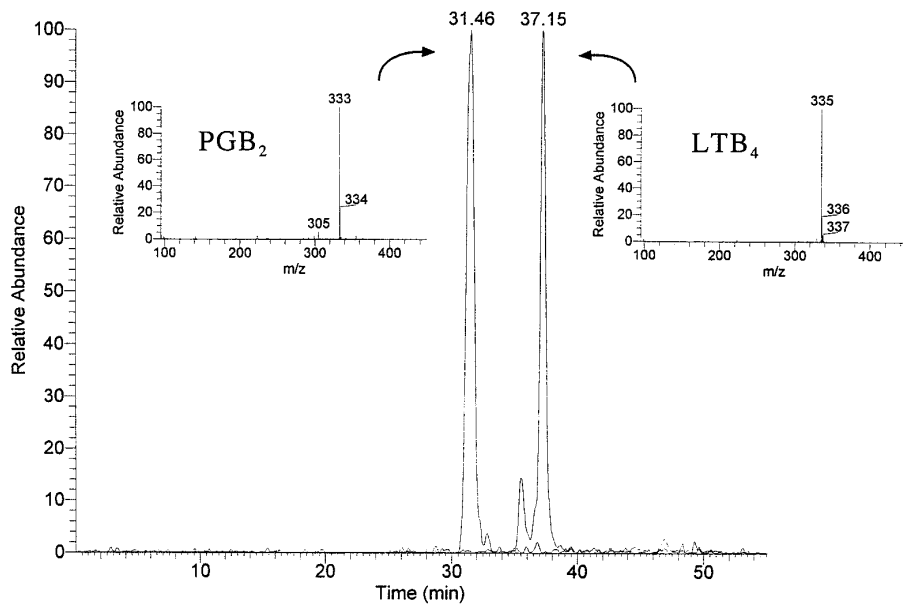


Figure 5. LC/MS chromatograms of ear biopsy tissue. Representative selected ion chromatograms of compound extracted from ear biopsy samples at initial application of LTB<sub>4</sub> after addition of internal standard (PGB<sub>2</sub>) and extraction (see Table I). Inset (right), LTB<sub>4</sub>, MS M-1 m/z 335. Inset (left), PGB<sub>2</sub> M-1 m/z 333.

LTB<sub>4</sub> (Table II). These findings did not permit their further evaluation in this model.

We next evaluated the impact of 15-epi-LXA<sub>4</sub> using a stable analogue, 15-epi-16-para-fluoro-phenoxy-LXA<sub>4</sub> (for structure, see Fig. 2). This LX analogue not only inhibited LTB<sub>4</sub> but also PGE<sub>2</sub>-enhanced inflammation. Of interest, the LX analogue also inhibited PMA-induced PMN influx, with little impact at 24 h on PMA-induced vascular leakage (Table II). Thus, LXA<sub>4</sub> stable analogues clearly inhibit native mediators as topically active agents but only partially blocked PMA actions that were restricted to inhibition of PMN influx. This ob-

servation with PMA and this LX analogue indicates that a major component of PMA-induced vascular leakage is not mediated by PMN-dependent mechanisms and likely involves direct vascular sites of actions for PMA that are not blocked by LX analogues.

## Discussion

The results presented here are the first to demonstrate inhibition of PMN-induced vascular permeability changes by LX an-

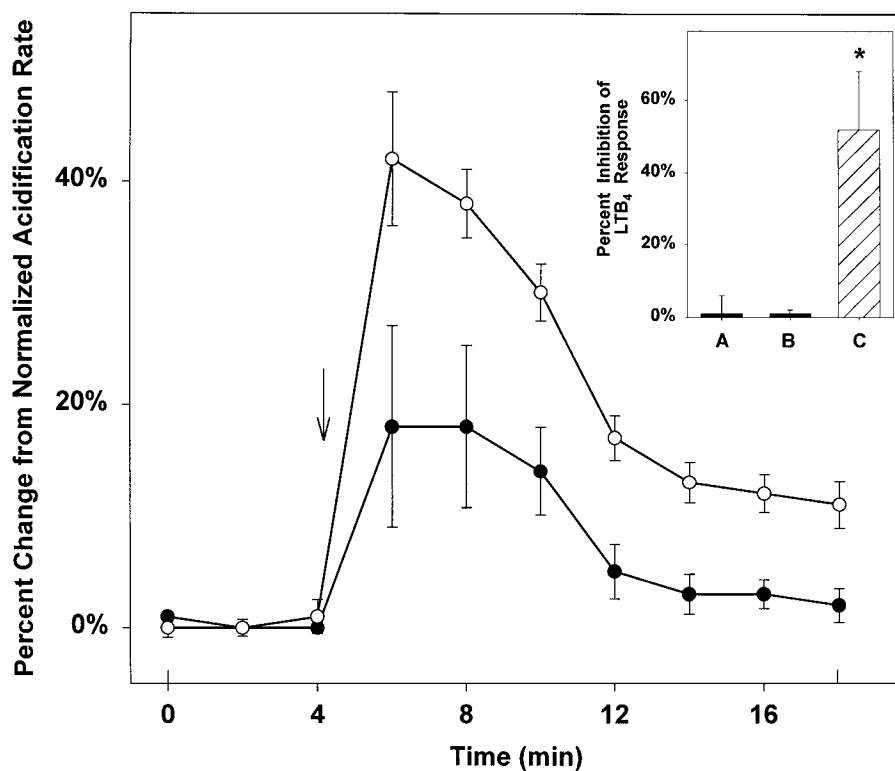


Figure 6. LXA<sub>4</sub> mimetics do not act as LTB<sub>4</sub> receptor antagonists. Mouse peripheral leukocytes were obtained and examined with a microphysiometer. Each chamber contained  $3.75 \times 10^5$  leukocytes. Cells were perfused with vehicle (○) or LTB<sub>4</sub> receptor antagonist U-75302 ( $10^{-6}$  M for 15 min, 37°C) before exposure to LTB<sub>4</sub> ( $10^{-7}$  M). Arrow, Time of LTB<sub>4</sub> addition. Inset, Percent inhibition of LTB<sub>4</sub> response by A,  $10^{-7}$  M of 15(R/S)-methyl-LXA<sub>4</sub>; B,  $10^{-6}$  M; and C, LTB<sub>4</sub> antagonist U-75302 ( $10^{-6}$  M). \* $P < 0.05$  vs. vehicle treatment. Results are mean  $\pm$  SEM with cells from three individual mice.

Table II. Mouse Ear Acute Inflammation Caused by Various Agents: an Antiinflammatory Action of Aspirin-triggered 15-*epi*-LXA<sub>4</sub> Analogue

Agents	Amount/ear	PMN infiltration	Percent inhibition by analogue	Vascular permeability change	Percent inhibition by analogue
	$\mu\text{g}$	% of LTB <sub>4</sub>		% of LTB <sub>4</sub>	
LTB <sub>4</sub>	1.0	100	84±1	100	60±11
PGE <sub>2</sub>	1.0	7±4	—	19±11*	—
LTB <sub>4</sub> + PGE <sub>2</sub>	1.0	159±26	90±7	230±72	61±7
FMLP	6.5	0±0	—	0±0	—
C5a	3.5	0±0	—	0±0	—
IL-8	25	0±0	—	0±0	—
PAF	16	0±0	—	0±0	—
LTD <sub>4</sub>	1.5	0±0	—	0±0	—
PMA	100 <sup>§§</sup>	180±14	42±8	207±19	3±3

Equimolar ( $\sim 3$  nmol per cm<sup>2</sup> ear) of each eicosanoid in acetone was applied topically to the mouse ears. The amounts of other potential proinflammatory agents applied to the ear are indicated. PMN infiltration and vascular permeability change were determined after 24 h as in Methods, and expressed as a percentage of LTB<sub>4</sub>-induced change. To evaluate the antiinflammatory action of aspirin-triggered 15-*epi*-LXA<sub>4</sub> with several stimuli, the stable analogue 15-*epi*-16-*para*-fluoro-phenoxy-LXA<sub>4</sub> ( $\sim 52$  nmol) (for structure, see Fig. 2) was applied topically to the left ears. The right ears were treated with vehicle alone, and both ears were treated with the agents. After 24 h, the PMN infiltration (MPO) and vascular permeability changes (Evans blue) were determined, and the percent inhibition was calculated as in Methods. Results represent the mean±SEM of three different mice. \**n* = 6; †*n* = 4; § amount in nanograms; —, Not determined.

alogues and the first evaluation and direct comparison of both novel LXB<sub>4</sub> stable analogues and a fluorinated LX analogue derivative. The dose response for two bioactive LXA<sub>4</sub> analogues (Fig. 3) and the rank order of potency for five analogues were essentially parallel in their ability to inhibit both PMN infiltration and vascular permeability changes (Fig. 4). These results suggest that these LX analogues exert anti-inflammatory actions by preventing PMN infiltration and subsequent events that lead to tissue injury. It is of particular interest that the most potent of the analogues, 15(*R/S*)-methyl-LXA<sub>4</sub>, had no direct effect on LTB<sub>4</sub>-triggered proton efflux, whereas the LTB<sub>4</sub> antagonist U-75302, also used for comparison in topical application experiments (Fig. 4), was clearly inhibitory (Fig. 6). These findings (Figs. 1–6), taken together with previous findings indicating that LXA<sub>4</sub> does not compete for specific binding of [<sup>3</sup>H]LTB<sub>4</sub> (4°C) to human neutrophils expressing LTB<sub>4</sub> receptors (14), and that LXA<sub>4</sub> interacts with its own specific site of action (3, 4), it appears that anti-PMN actions of LX analogues reported here are mediated by events independent of LTB<sub>4</sub> receptor level competition, which are likely to involve LX-specific receptors. Thus, these findings are consistent with our recent results with LXA<sub>4</sub> analogues (7) and the notion that activation of LX-specific receptors may represent novel therapeutic targets.

Of particular interest, other well-known proinflammatory mediators such as FMLP, IL-8, PAF, LTD<sub>4</sub>, and C5a were not topically active in this model and thus were unable to promote either vascular leakage or PMN influx, and essentially no visible alterations were noted (see Table II and Fig. 3). Although not topically active in this model, LXA<sub>4</sub> inhibits PAF, LTD<sub>4</sub>, LTB<sub>4</sub>, and FMLP-directed cellular actions in vitro ([4, 6, 9]; for a review, see reference 3). Of particular interest, PGE<sub>2</sub> was topically active when applied together with LTB<sub>4</sub>, and in this setting visibly enhanced this inflammatory response. On its own, PGE<sub>2</sub> gave only a marginal response (Table II), a finding consistent with results from the hamster cheek pouch microcirculation,

where PGE<sub>2</sub> potentiates local LTB<sub>4</sub>-induced extravasation of plasma and leukocyte emigration (16). In these experiments, the fluorinated 15-*epi*-LXA<sub>4</sub> analogue also proved to be a potent inhibitor of the responses induced by the combined actions of both PG and LT (Table II), thus providing further evidence for the unique actions of LX, ATL, and their analogues. PMA induced both PMN influx and vascular leak, but only the PMN influx component was inhibitable by the LXA<sub>4</sub> analogue. Dexamethasone (20  $\mu\text{g}/\text{ear}$ ) inhibited both PMA-induced responses (not shown; *n* = 4). Together, these findings indicate that analogues of aspirin-triggered LXA<sub>4</sub> are potent inhibitors of PMN-induced vascular leakage and suggest that LX and dexamethasone act via distinct antiinflammatory mechanisms.

LXB<sub>4</sub> is as potent as LXA<sub>4</sub> in inhibiting PMN transmigration across monolayers of endothelial cells and inhibiting LTB<sub>4</sub>-induced adhesion to endothelial cells in vitro (5). Results in Fig. 4 clearly establish that the novel LXB<sub>4</sub> analogues also display antiinflammatory actions in vivo. In this regard, 5(*S*)-methyl-LXB<sub>4</sub>, which carries the stereochemistry of native LXB<sub>4</sub>, was significantly more potent than 5(*R*)-methyl-containing LXB<sub>4</sub> analogue for both inhibition of PMN infiltration and vascular permeability change, indicating that stereochemistry of the C-5 position is critical for topically delivered bioactivity. These results also provide further evidence for selective sites of action for LXB<sub>4</sub>. Its receptor and/or site of action remain to be identified.

The stable LX analogues tested were designed to resist rapid inactivation and proved to resist conversion by cells and isolated recombinant enzymes in vitro (9, 10). Therefore, it was of interest to determine whether the LX analogues remained within the ear tissues after topical application or if they were effectively cleared. Our results indicate that at 24 h after topical application > 95% of the LX analogues were not present in extractable or recoverable forms from the biopsies, which suggests that they were either cleared from the biopsied

areas or were present within the tissue in a form that was not extractable using the current methodology. We also found no evidence using LC/MS/MS work station-based analyses of anticipated local metabolites of these compounds persisting within the 24-h biopsies (data not shown). Along these lines, it is noteworthy that, in addition to local clearance, an alternate explanation may lie with the possibility that LX analogues could have been subject to local metabolism and/or covalent modification that results in their binding to tissue matrix components during the 24-h in vivo interval. Whether such matrix forms of LX analogues exist and whether the LX analogues are in an inactive or bioactive configuration are of interest. Nevertheless, it is clear from these results that LX analogues are not retained in their native form within the local microenvironment (i.e., ear biopsies), and this may be another useful property of these LX analogues.

Taken together, results of this study indicate that stable analogues of aspirin-triggered 15-epi-LXA<sub>4</sub>, LXA<sub>4</sub>, and LXB<sub>4</sub> serve as potent, topically active agents that inhibit PMN recruitment and PMN-mediated changes in vascular permeability. Moreover, they provide additional new tools for investigating the actions of LX.

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