Cutting Edge: Lipoxin (LX) A₄ and Aspirin-Triggered 15-Epi-LXA₄ Block Allergen-Induced Eosinophil Trafficking

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Tissue eosinophilia prevention represents one of the primary targets to new anti-allergic therapies. As lipoxin A₄ (LXA₄) and aspirin-triggered 15-epi-LXA₄ (ATL) are emerging as endogenous “stop signals” produced in distinct pathologies including some eosinophil-related pulmonary disorders, we evaluated the impact of in situ LXA₄/ATL metabolically stable analogues on allergen-induced eosinophilic pleurisy in sensitized rats. LXA₄/ATL analogues dramatically blocked allergic pleural eosinophil influx, while concurrently increasing circulating eosinophilia, inhibiting the earlier edema and neutrophilia associated with allergic reaction. The mechanisms underlying this LXA₄/ATL-driven allergic eosinophilia blockade was independent of mast cell degranulation and involved LXA₄/ATL inhibition of both IL-5 and eotaxin generation, as well as platelet activating factor action. These findings reveal LXA₄/ATL as a novel class of endogenous anti-allergic mediators, capable of preventing local eosinophilia. The Journal of Immunology, 2000, 164: 2267–2271.

A n appreciation of the vast biosynthetic and functional capacity of eosinophils places these cells as having a critical role in the pathogenesis of allergic conditions. This notion is inferred from clinical and experimental studies 1) showing eosinophilia as a prominent sign of allergic disorders; 2) correlating eosinophil-derived granular proteins and lipid mediators with tissue hyperreactivity and damage; and 3) associating the remission of allergic symptoms with the resolution of eosinophilia (1–3). Thus, new therapeutic approaches for the treatment of allergic diseases could be aided by the development of anti-eosinophilic tools. At present, the most effective pharmacological approach for severe eosinophilic reactions, such as asthma, comprises glucocorticoid therapy (4, 5). However, steroids display a wide range of unwanted side effects. Endogenous down-regulators generated during allergic reactions could provide insight to potential therapies that may avoid the unwanted actions related with steroid treatment.

Lipoxins represent a relatively new class of arachidonate products (6, 7) generated in airway-related tissues of patients with asthma and other lung diseases, suggesting these mediators as naturally occurring molecules associated with eosinophil-related pathologies (8–11). Notably, both lipoxin A₄ (LXA₄) and its natural analogue 15-epi-LXA₄ (ATL, the LXA₄ 15-epimer triggered in the presence of aspirin) display strong abilities to modulate leukocyte functions. Specifically concerning eosinophils, it was shown that eosinophils can generate LXA₄ (12) and LXA₄ inhibits platelet activating factor (PAF)- or FMLP-induced eosinophil chemotaxis in vitro (13). The actions of LXA₄ and ATL in models of allergen-induced eosinophilic reaction in vivo have yet to be addressed. Here, to evaluate the anti-allergic impact of LXA₄/ATL on eosinophilic response, we used two distinct metabolically stable analogues—15(R/S)-methyl-LXA₄ (ATL₁) and 15-epi-16-p-fluoro-phenoxyl-LXA₄ (ATL₂)—in an allergic pleurisy model in actively sensitized rats.

Materials and Methods

Allergic pleurisy in actively sensitized rats

Wistar rats (150–200 g) of both sexes were obtained from the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Active sensitization was achieved by a s.c. injection (0.2 ml) of a mixture containing OVA (50 μg) (Sigma, St. Louis, MO) and Al(OH)₃ (5 mg) in 0.9% NaCl solution (saline). Intraperitoneal (i.p.l.) injection of allergen—OVA (12 μg/cavity) dissolved in sterile saline—was done 14 days postsensitization. Control groups consist of nonsensitized rats (receiving only saline) challenged with allergen. All i.p.l. injections were performed in a final volume of 0.1 ml. At
different time points, the rats were killed under CO2 atmosphere and the pleural cavity was rinsed with 3 ml of heparinized saline (10 IU/ml). The pleural effluent was collected and its volume measured with a graduated syringe.

**Pleurisy triggered by PAF**

Naive rats were i.p. stimulated with PAF (1 μg/cavity) (1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine; Bachem, Bubendorf, Switzerland). PAF was diluted in sterile saline containing 0.01% BSA. Control animals were injected with the same volume of vehicle. Six or 24 h post-stimulation, the pleural fluid was collected, as described above, for analyses.

**Evaluation of edema and leukocyte alterations**

Analyses of pleural edema and mast cell enumeration were performed 6 h postallergen by quantifying protein content of pleural supernatant using the Biuret technique (14) and evaluating pleural effluent samples stained with toluidine blue dye in Neubauer chambers (15). At 6 or 24 h of allergic stimulation, total leukocyte counts from samples of pleural effluent and peripheral blood were determined in a Coulter Counter ZM (Coulter Electronic, Palo Alto, CA) after RBC lysis. Differential leukocyte analysis was performed under an oil immersion objective on cytto centrifuged, and blood smears were stained with May-Grünwald-Giemsa dye.

**Histamine, eotaxin, and IL-5 measurements**

Histamine stored in the unpurified cellular suspension recovered from the pleural cavity was spectrophotometrically determined in the supernatant after cell lysis and protein precipitation with 0.4 N perchloric acid as described before (15). For eotaxin and IL-5 analyses, the pleural exudates were collected with 1 ml of PBS containing 10 mM EDTA. The pleural fluid samples were centrifuged at 2500 rpm for 10 min at 4°C, and the eotaxin or IL-5 levels of the supernatants were analyzed by ELISA using an anti-mouse eotaxin Ab or a mouse IL-5 Quantikine kit (R&D Systems, Minneapolis, MN).

**Treatments**

Two different LX4 synthetic analogues were used: 15(R/S)-methyl-LX4 (55, 6R, 15S, 3R, 9S, 13R-trihydroxy-15-methyl-7, 9, 13-trans-11- cis-eicosatetraenoic acid) and 15-epi-16-p-fluorophenoxo-LX4 (prepared by Prof. Nicos A. Petasis, Department of Chemistry, University of Southern California), here termed ATL1 and ATL2, respectively. Both ATLs (50–500 ng/cavity), as well as the native LX4 (500–1000 ng/cavity), were i.p. injected 5 min before allergic or PAF stimulation. In control groups, their vehicles (1% ethanol in sterile saline) replaced the ATLs and LX4.

**Analysis of intracytoplasmic Ca2+ in eosinophils**

Eosinophils were isolated from the peritoneal cavity of normal rats using Percoll density gradients as previously reported (16). Eosinophil suspensions (1 × 10^7/ml) of 85–95% purity and 96% viability (trypan blue exclusion), were loaded with 1 μM fura-2 AM (Molecular Probes, Eugene, OR) in Ca2+/Mg2+-free PBS with BSA for 30 min at 37°C. After two washes, eosinophils at 5 × 10^5/ml (1.2 ml) were dispensed into quartz cuvettes with constant stirring at 37°C and equilibrated with 1 mM CaCl2 for 15 min before addition of agonist. Changes in fluorescence were measured in a Shimadzu RF1501 spectrofluorophotometer (Kyoto, Japan). Calculation of cytosolic-free Ca2+ was derived from fluorescence spectra (excitation at 340 nm and 380 nm; emission at 510 nm) in accordance with established methodology (17). During the experiments, PAF (10−7 M) or eotaxin (10−8 M) (R&D Systems) were added 60 s after commencing recording, and ATL1 was incubated for 15 min before addition of agonists.

**Statistical analysis**

Statistical analysis involving two groups was done with Student’s t test, whereas ANOVA and Newman-Keuls-Student’s test compared more than two groups. Values of p ≤ 0.05 was considered significant.

**Results and Discussion**

Allergen challenge (OVA, 12 μg/cavity) into actively sensitized rats caused a marked pleural eosinophil infiltration within 24 h (Fig. 1A) and coincided with a selective blood eosinophilia (Fig. 1B). This eosinophilic reaction was preceded by a neutrophilic reaction (6 h), consisting of intense increase in the blood and pleural neutrophil counts (Fig. 1). Although the mechanism underlying the recruitment of eosinophils to the allergic inflamed tissues remains to be elucidated, recent evidence indicate a multistep process comprised of at least two combined components (1–3): an IL-5-driven systemic step (18), and concurrent in situ events controlled by local elaborated lipid mediators (PAF and LTB4) and specific chemokines (e.g., eotaxin) (19). Even though a single model cannot mimic all features of allergic human disease, the in vivo model used here clearly mimics both key systemic and local components of the allergen-evoked eosinophilic reaction.

When administered in situ to rat pleural cavities, the LX4/ATL stable analogues inhibited in a concentration-dependent fashion the allergen-induced pleural eosinophil infiltration apparent within 24 h (Fig. 2). At 500 ng/cavity, the degree of inhibition of pleural eosinophilia was >90% for both analogues, with an IC50 of ~250 ng/cavity for each. Comparable inhibitory effect was obtained with dexamethasone at a dose 20-fold higher than LX4/ATL doses used herein, as reported (20). Consistent with previous observations (21), treatment with either ATL1 or ATL2 did not elicit any inflammatory or toxic effects, with no detectable perturbation of microvascular permeability, mast cell, eosinophil, or other cell populations (not shown). Consistent with earlier findings, the impact of native LX4 (500–1000 ng/cavity) were notably weaker than its metabolically stable analogues (not shown) with a rank order of ATL1 > ATL2 > LX4. This phenomenon is due to a rapid inactivation of LX4 in tissues, while its stable analogues—designed to retain the bioactivity and resist to degradation—display more potent effects (22, 23).

In situ inhibitory actions of LX4/ATL stable analogues on allergic inflammation were not restricted to eosinophils. LX4/ATL blocked the earlier pleural edema (96% for ATL1 and 94% for ATL2; p < 0.001). Moreover, they inhibited pleural neutrophilia noted 6 h after allergen challenge (Fig. 3A, upper panel), confirming their well-known impact on neutrophil responses both in vitro and in vivo (24–29). These data indicate that common migratory

**FIGURE 1.** Pleural (A) and blood (B) kinetics of eosinophil (circles) or neutrophil (squares) alterations induced by allergic challenge of nonsensitized (open symbols) or actively sensitized (filled symbols) rats. Both groups were challenged with allergen (OVA, 12 μg/cavity, i.pl.). Each value represents the mean ± SEM from at least eight animals. *, Significantly different from the nonsensitized group (p < 0.01).
steps shared by both granulocytes could be the target(s) of LXA4/ATL inhibition. However, at 500 ng/cavity, ATL1 or ATL2 significantly increased the 24-h-related circulating eosinophilia induced by allergen (Fig. 2B), while impairing the development of allergen-induced 6 h neutrophilia in blood (Fig. 3A, lower panel). Because ATLs did not interfere with blood neutrophil or eosinophil counts of nonsensitized rats (not shown), these findings suggest distinct inhibitory mechanisms for neutrophilia vs eosinophilia. Moreover, a local regulatory event of eosinophil trafficking from microvessels to the pleural cavity was blocked by LXA4/ATL (vide infra), keeping eosinophils in peripheral circulation.

Although allergic pleural eosinophilia depends on an early mast cell activation (15), no significant differences in the allergen-induced mast cell degranulation were detected between nontreated and LXA4/ATL-treated groups (Fig. 3B). Because these data ruled out the mast cell as a potential cellular target of LXA4/ATL-dependent inhibition of allergic reaction, we further investigated potential direct impacts on eosinophils. Here, the effect of LXA4/ATL on Ca\(^{2+}\) mobilization in purified rat eosinophils loaded with fura-2 AM was assessed. ATL1 neither modified PAF- nor eotaxin-induced rise in cytosolic-free Ca\(^{2+}\) (Fig. 4A) nor evoked Ca\(^{2+}\) mobilization per se (not shown). Intracellular Ca\(^{2+}\) concentrations were 104 ± 6 14 nM (mean ± SEM) for untreated PAF-stimulated cells vs 112 ± 6 18 nM for ATL1-treated PAF-stimulated cells and 68 ± 12 nM for untreated eotaxin-stimulated cells vs 73 ± 15 nM for ATL1-treated eotaxin-stimulated cells. Although these data suggest that LXA4/ATL do not down-regulate the Ca\(^{2+}\)-driven locomotory functions of eosinophils, we cannot ignore the possibility that LXA4/ATL are affecting some other regulatory steps of motility downstream from Ca\(^{2+}\) influx in rat eosinophils.

In situ ATL1 or ATL2 (500 ng/cavity) abolished the allergen-induced eotaxin formation that precedes local eosinophil infiltration into pleural spaces of rats (Fig. 4B), while LXA4 (1000 ng/cavity) did not. Inasmuch as ATL1 or ATL2 (500 ng/cavity) only partially inhibited the pleural IL-5 generation induced by allergen (101 ± 9 pg/cavity in nontreated vs 51.6 ± 3.6 and 50.7 ± 5.8 pg/cavity in ATL1- or ATL2-treated animals, respectively). Considering the critical role of eotaxin in several allergic eosinophilic models (19), including some developed in rats (30), our data suggest that the local generation of eotaxin represent one critical target for the in situ LXA4/ATL anti-eosinophilic action. This first report of LXA4/ATL inhibition of eotaxin secretion is consistent with recent findings suggesting LXA4/ATL as endogenous regulators of chemokine production (29, 31, 32).

**FIGURE 2.** LXA4/ATL blocks allergen-induced eosinophilic pleurisy in actively sensitized rats. In situ pretreatment with ATL1 or ATL2 altered allergen-induced pleural (A) and blood (B) eosinophilia detected 24 h postchallenge in actively sensitized rats. All animals received allergen (OVA, 12 μg/cavity, i.pl.) and the treatments were performed as described in Materials and Methods. Results are expressed as the mean ± SEM from five animals. *, p < 0.001 compared with nonsensitized group. **, p < 0.01 compared with allergen-challenged sensitized group.

**FIGURE 3.** Effect of LXA4/ATL on allergen-induced early responses in actively sensitized rats. A. In situ pretreatment with ATL1 (500 ng/cavity), ATL2 (500 ng/cavity) inhibited allergen-induced pleural (upper panel), and blood (lower panel) neutrophilia detected 6 h postchallenge in actively sensitized rats. Results were expressed as the mean ± SEM from five animals. *, p < 0.001 compared with nonsensitized group. **, p < 0.01 compared with allergen-challenged sensitized group. B. Lack of effect of in situ LXA4 (1000 ng/cavity) or ATL (500 ng/cavity) on allergen-induced pleural histamine release (inset) and mast cell degranulation in actively sensitized rats. The pleural histamine and mast cell analyses were performed at 6 h postchallenge. Each value represents the mean ± SEM from five animals. *, Significantly different from the nonsensitized group (p < 0.01).
to PAF (10−2 M) are recovered from airway tissues of patients with asthma and nonsensitized group were below the detection limit (not shown). Solid lines indicate pretreatment in actively sensitized rats. Eotaxin levels in the allergen-challenged groups of rats pretreated with ATL1 (500 ng/cavity) or ATL2 (500 ng/cavity) blocked allergen-induced eotaxin release detected 6 h postchallenge in actively sensitized rats. Eotaxin levels in the allergen-challenged nonsensitized group were below the detection limit (not shown). Solid lines represent the mean of each group. *p < 0.001 compared with control. **p < 0.001 compared with allergen-challenged sensitized group. C. In situ pretreatment with ATL1 (500 ng/cavity) or ATL2 (500 ng/cavity) blocked PAF-induced pleural eosinophil accumulation detected within 24 h. Results are expressed as the mean ± SEM from five animals. *p < 0.001 compared with control group. **p < 0.001 compared with PAF-stimulated group.

When administered in situ, ATL1 or ATL2 (500 ng/cavity) dramatically reduced the pleural eosinophilia observed 24 h after PAF stimulation (Fig. 4C). It is known that i.pl. injection of PAF also produced a neutrophilic reaction within 6 h (33, 34), that was also abolished by both ATLs (92% for ATL1 and 96% for ATL2; p < 0.001). Because PAF is clearly involved in the development of allergic pleural eosinophilia in rats (35), our results indicate that part of beneficial effect of LXA4/ATL in allergic reactions results from inhibition of PAF-driven events.

The notions that in situ LXA4 could display beneficial action on allergic diseases came from clinical results showing that lipoxins are recovered from airway tissues of patients with asthma and other lung disorders (8–11), and LXA4 inhalation by asthmatic subjects reduces LTC4-induced airway obstruction (36). The present results are first to uncover a novel mechanism of prevention for allergen-induced eosinophil trafficking in vivo by in situ LXA4 and ATL. These eosinophil-directed actions of LXA4/ATL appear to be modulated by inhibition of in situ expression of IL-5 and eotaxin, as well as local PAF action. Impairment of allergen-induced eotaxin production in vivo by endogenously generated lipid mediators appears to be a unique property of lipoxins. Because eosinophils are implicated as the major effectors of allergic disorders, our results position LXA4/ATL stable analogues as candidates for novel alternative anti-allergic therapies.

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