Pathogen-induced chemokine secretion from model intestinal epithelium is inhibited by lipoxin A4 analogs.

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Research Article

Enteric pathogens induce intestinal epithelium to secrete chemokines that direct movement of polymorphonuclear leukocytes. Mechanisms that might downregulate secretion of these proinflammatory chemokines and thus contain intestinal inflammation have not yet been elucidated. The antiinflammatory activities exhibited by the arachidonate metabolite lipoxin A4 (LXA4) suggests that this eicosanoid, which is biosynthesized in vivo at sites of inflammation, might play such a role. We investigated whether chemokine secretion could be regulated by stable analogs of LXA4. Monolayers of T84 intestinal epithelial cells were infected with Salmonella typhimurium, which elicits secretion of distinct apical (pathogen-elicited epithelial chemoattractant) and basolateral (IL-8) chemokines. Stable analogs of LXA4 inhibited S. typhimurium-induced (but not phorbol ester-induced) secretion of both IL-8 and pathogen-elicited epithelial chemoattractant. LXA4 stable analogs did not alter bacterial adherence to nor internalization by epithelia, indicating that LXA4 stable analogs did not block all signals that Salmonella typhimurium activates in intestinal epithelia, but likely led to attenuation of signals that mediate chemokine secretion. Inhibition of S. typhimurium-induced IL-8 secretion by LXA4 analogs was concentration- (IC50 approximately 1 nM) and time-dependent (maximal inhibition approximately 1 h). As a result of these effects, LXA4 stable analogs inhibited the ability of bacteria-infected epithelia to direct polymorphonuclear leukocyte movement. These data suggest that LXA4 and its stable analogs may be useful in downregulating active inflammation at mucosal surfaces.

Pathogen-induced Chemokine Secretion from Model Intestinal Epithelium is Inhibited by Lipoxin A_4 Analogs

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Abstract

Enteric pathogens induce intestinal epithelium to secrete chemokines that direct movement of polymorphonuclear leukocytes. Mechanisms that might downregulate secretion of these proinflammatory chemokines and thus contain intestinal inflammation have not yet been elucidated. The antiinflammatory activities exhibited by the arachidonate metabolite lipoxin A_4 (LXA_4) suggests that this eicosanoid, which is biosynthesized in vivo at sites of inflammation, might play such a role. We investigated whether chemokine secretion could be regulated by stable analogs of LXA_4. Monolayers of T84 intestinal epithelial cells were infected with *Salmonella typhimurium*, which elicits secretion of distinct apical (pathogen-elicited epithelial chemoattractant) and basolateral (IL-8) chemokines. Stable analogs of LXA_4 inhibited *S. typhimurium*–induced (but not phorbol ester-induced) secretion of both IL-8 and pathogen-elicited epithelial chemoattractant. LXA_4 stable analogs did not alter bacterial adherence to nor internalization by epithelia, indicating that LXA_4 stable analogs did not block all signals that *Salmonella typhimurium* activates in intestinal epithelia, but likely led to attenuation of signals that mediate chemokine secretion. Inhibition of *S. typhimurium*–induced IL-8 secretion by LXA_4 analogs was concentration- ($IC_{50} \sim 1$ nM) and time-dependent (maximal inhibition $\sim$ 1 h). As a result of these effects, LXA_4 stable analogs inhibited the ability of bacteria-infected epithelia to direct polymorphonuclear leukocyte movement. These data suggest that LXA_4 and its stable analogs may be useful in downregulating active inflammation at mucosal surfaces. (*J. Clin. Invest.* 1998. 101:1860–1869.) Key words: IL-8 • *Salmonella* • neutrophil • transmigration • inflammation • chemokine

Introduction

The intestinal epithelium, in addition to being an entry point for many invasive pathogens, is also an active participant in the inflammatory response due to immune and other causes. Epithelial cell signaling pathways that are activated by some invasive bacteria such as *Salmonella typhimurium* mediate uptake of these pathogens into these usually nonphagocytic cells (1, 2). After internalization of the bacteria, the intestinal epithelium secretes chemokines (3, 4) that play a role in recruiting PMN from the peripheral circulation and directing them to transmigrate across the epithelium to the intestinal lumen (5). Epithelial modulation of PMN movement is thought to be mediated by polarized secretion of distinct chemokines. IL-8 is secreted basolaterally by intestinal epithelial cell lines and human colon in response to proinflammatory cytokines or invasive bacteria (6). This secretion leads to gradients of IL-8 that are chemotactic for neutrophils being formed in the subepithelial extracellular matrix (7). These gradients of IL-8 have been shown to be largely responsible for PMN movement through the extracellular matrices of model epithelia (7). The chemokines that direct neutrophil transmigration across the intestinal epithelial monolayer are only now being described. These chemokines must be preferentially secreted apically in order to establish gradients that would direct PMN to migrate across the epithelium to the apical surface. The first such chemokine, pathogen-elicited epithelial chemoattractant (PEEC; 8),[1] has recently been described. As the ability of bacteria to induce PEEC secretion in model intestinal epithelia (and subsequently to induce the epithelium to direct PMN to transmigrate) appears to correlate well with a bacteria's ability to cause enteritis in humans (5, 8), secretion of PEEC, like secretion of IL-8, appears to be an important proinflammatory event.

Epithelial recruitment of PMN in response to detection of luminal pathogens may play a role in host defense against some pathogens. In contrast, chronic inflammatory diseases are characterized by acute flares defined by neutrophil–epithelial interactions that are responsible for the majority of clinical symptoms, but occur in the absence of any known pathogen (9). The acute flares of such chronic inflammatory states (i.e., Crohn's disease, ulcerative colitis) might thus result from aberrant activation of epithelial proinflammatory pathways. If so, such flares and the symptoms that result from them could perhaps be alleviated by downregulating epithelial secretion of chemokines. That intestinal inflammation is normally self-limiting suggests that mechanisms downregulating inflammation exist in intestinal epithelia, although they are only now beginning to be described. A class of molecules that is a suitable candidate for involvement in activating antiinflammatory sig-

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1. *Abbreviations used in this paper:* EPEC, enteropathogenic *Escherichia coli;* LO, lipoxygenase; LX, lipoxin; PEEC, pathogen-elicited epithelial chemoattractant.

naling pathways, and hence downregulating intestinal inflammation, is the lipoxins (LX). Lipoxins are produced from arachidonate by the combined actions of 5-lipoxygenase (LO) and 12-LO or 15-LO (for review see reference 10). While the biological behavior of lipoxins, when tested in various experimental systems, includes vasoregulatory and immunoregulatory actions, the general profile of lipoxin behavior appears to be emerging as antiinflammatory, particularly in respect to neutrophil motility. One lipoxin in particular, LXA_4, has an array of antiinflammatory actions, including inhibition of leukocyte diapedesis in the microcirculation of the hamster cheek pouch (11), depressing contraction of the guinea pig ileum (12), and inhibition of PMN migration across model endothelium (13) and epithelium (14). Synthetic analogs of LXA_4 exhibit greater antiinflammatory activity than does native LXA_4, probably because they are more resistant to degradation than the native eicosanoid (15). One stable analog of LXA_4, 15(R/S)-methyl-LXA_4, also closely resembles 15-epi-LXA_4 that is synthesized in vivo in the presence of aspirin (16), and may be responsible for some of the therapeutic effects of this commonly used nonsteroidal antiinflammatory agent.

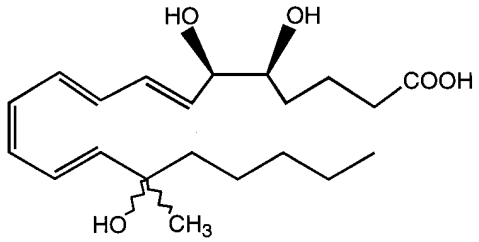
LX inhibition of PMN transepithelial migration requires that both the PMN and the epithelial cells be exposed to the lipoxins (14). It remained to be determined whether LX in the absence of leukocytes had a direct effect on epithelial cells. Here, using monolayers of T84 cells, *S. typhimurium*, and human neutrophils to model pathogen-induced intestinal inflammation, we report that epithelia respond functionally to LXA_4 analogs by exhibiting attenuated basal and pathogen-induced epithelial cell chemokine/chemoattractant secretion. This novel action of LXA_4 and its stable analogs resulted in attenuated PMN migration across model epithelia.

Methods

Materials. Lipoxin A_4 and lipoxin B_4 were obtained from Cascade Biochem (Reading, Berkshire, United Kingdom). Lipoxin A_4 analogs (15[R/S]-methyl-LXA_4-methyl ester, 16-phenoxy-LXA_4-methyl ester, 15-deoxy-LXA_4-methyl ester) and 15-epi-LXA_4-methyl ester were prepared by total organic synthesis, and their structures were confirmed by HPLC, NMR, and mass spectral analysis (15). Daily working stocks of lipoxin (100 μM) concentrations were verified by UV spectroscopy using a molar extinction coefficient of 50,000 cm^{-1} M^{-1}, $\lambda_{max}^{MeOh} = 301$ nm. These solutions were stored at −70°C in 99% ethanol. *S. typhimurium* (x_{3306}) was cultured as previously described (3). Enteropathogenic *Escherichia. coli* (EPEC) received from G. Hecht (University of Illinois, Chicago, IL) was cultured as previously described (17).

Salmonella typhimurium-induced IL-8 secretion. Unless otherwise indicated in figure legend, the following procedure was used. Confluent monolayers of T84 cells, grown on 0.33-cm^2 collagen-coated permeable supports (resistance > 600 Ω × cm^2), were washed three times with HBSS and placed into 300 ml of HBSS that contained 100 nM LX or vehicle (always 0.1% ethanol). The time from which LX-containing HBSS was prepared and T84 cells being placed into it was never > 3 min. 60 min later, monolayers were placed in empty wells, and 25 μl of *S. typhimurium*–containing HBSS (1.6×10^{10} bacteria/ml) was placed on the apical surface of each monolayer. This inoculum has been previously shown to correspond to 30 associated bacteria per T84 cell (3). 45 min later, the monolayers were returned to the same LX-containing HBSS in which they had been incubated before adding *S. typhimurium*. Antibiotics were not used (except in separate experiments measuring bacterial internalization, as described below). 5 h after adding the bacteria, T84 cell supernatants were removed and assayed for IL-8. IL-8 was measured by ELISA as previously described (3) except for a few minor modifications: 96-well plates (Linbro/Titretek; ICN Biomedicals, Costa Mesa, CA) were coated overnight with goat α-human IL-8 (R & D Systems, Inc., Minneapolis, MN), and the detecting antibody used was rabbit α-human IL-8 (Endogen, Inc., Woburn, MA). When Caco-2 BBE cells were used, the entire experiment was performed in culture media (DMEM supplemented with 10% FCS) because we did not observe detectable IL-8 secretion in response to *S. typhimurium* in these cells when the experiment was performed in HBSS.

Salmonella typhimurium internalization assay. S. typhimurium adherence to and internalization by T84 cell monolayers was measured as previously described (3). Replication of internalized bacteria was assessed by adding a 4-h incubation at 37°C after noninternalized bacteria were killed by a 1-h incubation with gentamicin (500 mg/ml). Relative in vitro growth of *S. typhimurium* was measured by inoculating 10 ml of Luria broth (±100 nM 15 R/S-methyl-LXA_4) with 0.01 ml of a stationary phase bacterial culture and measuring OD at 405 nm 5 h after inoculation.



15(R/S)-methyl-LXA_4

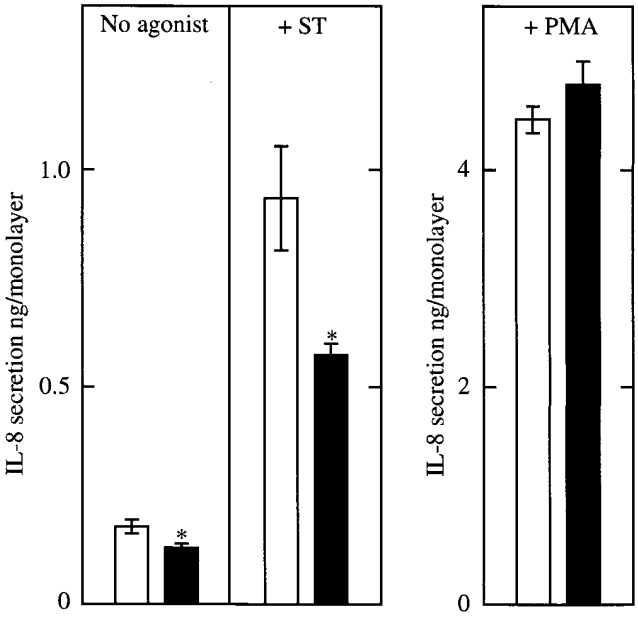


Figure 1. Lipoxin A_4 stable analog attenuates IL-8 secretion from model intestinal epithelia. Monolayers of T84 cells were incubated in 100 nM 15(R/S)-methyl-LXA_4 (shown above) or vehicle (0.1% ethanol) for 1 h before being treated with buffer (*no agonist*), *S. typhimurium* (*ST*), or PMA (1 ng/ml). 5 h later, basolateral supernatants were assayed for IL-8. *Open bars,* control (vehicle). *Solid bars*, +100 nM 15(R/S)-methyl-LXA_4. Data are the means±SEM of six monolayers/condition from one experiment, and are representative of four separate experiments.

PEEC isolation. Confluent 5-cm^2 monolayers of T84 cells were washed three times with HBSS and placed into 1.5 ml of HBSS that contained 1 nM 15(R/S)-methyl-LXA_4 or vehicle (0.1% ethanol). 60 min later, 1 ml of HBSS containing *S. typhimurium* (1.6×10^{10} bacteria/ml) and 1 nM 15(R/S)-methyl-LXA_4 or vehicle was placed on the apical surface of each monolayer. 45 min later, the monolayers were washed three times with HBSS to remove nonadherent bacteria, and were placed into 300-μl fresh HBSS. 4 h later, T84 cell apical supernatants were removed, and PEEC was isolated, partially purified by collecting the filtrate over a 1-kD cutoff filter, and concentrated 50-fold (8). We verified that statistically significant amounts of 15(R/S)-methyl-LXA_4 were not present in these PEEC isolates by measuring the LX concentrations of solutions before and after they passed over the 1-kD cutoff filters.

Assay for PEEC concentration by measuring its ability to drive PMN transepithelial migration. Virgin (i.e., not exposed to *S. typhimurium* or LX) confluent T84 inverted monolayers were washed three times in HBSS before 300 μl of PEEC (in HBSS) isolated from vehicle (control) or LX-treated *S. typhimurium*–infected T84 monolayers were placed in their apical (bottom) reservoir. 2×10^6 PMN were then placed in the basolateral reservoir and allowed 2 h (at 37°C) to transmigrate. The number of PMN that had migrated to the basolateral reservoir was measured enzymatically as previously described (18).

Assay for PEEC concentration by measuring its ability to induce cytoplasmic [Ca^{++}] changes in PMN. PMN were loaded with the calcium indicator Indo-1 and cytoplasmic [Ca^{++}] measured as previously described (19, 20) using a Hitachi F-4500 spectrofluorometer. PMNs (2×10^6) were suspended in 960 μl of HBSS and stimulated with 40 μl of 50-fold concentrated PEEC (final PEEC concentration = 2× that found in apical supernatants).

Measurement of IL-8 mRNA. T84 monolayers (5 cm^2) were treated with indicated lipoxin or vehicle (0.1% ethanol) and infected with *S. typhimurium* as described above. At the indicated time after adding the bacteria, RNA was extracted. IL-8 mRNA and actin mRNA as a control were then analyzed by Northern blot as previously described (21). Film was scanned, densitometry was measured via National Institutes of Health image, and relative IL-8 was quantitated by normalizing to actin mRNA levels.

Salmonella typhimurium-induced PMN transepithelial migration and matrix imprinting. Inverted monolayers were treated with 100 nM 15(R/S)-methyl-LXA_4 for 1 h and infected with *S. typhimurium* as described above. *S. typhimurium*–induced PMN transepithelial migration and matrix imprinting were then measured as previously described (7).

Data analysis. Because absolute values of IL-8 and PEEC secretion vary with T84 cell passage number, the effects of lipoxins on T84 monolayer secretion of these chemokines are displayed as either the

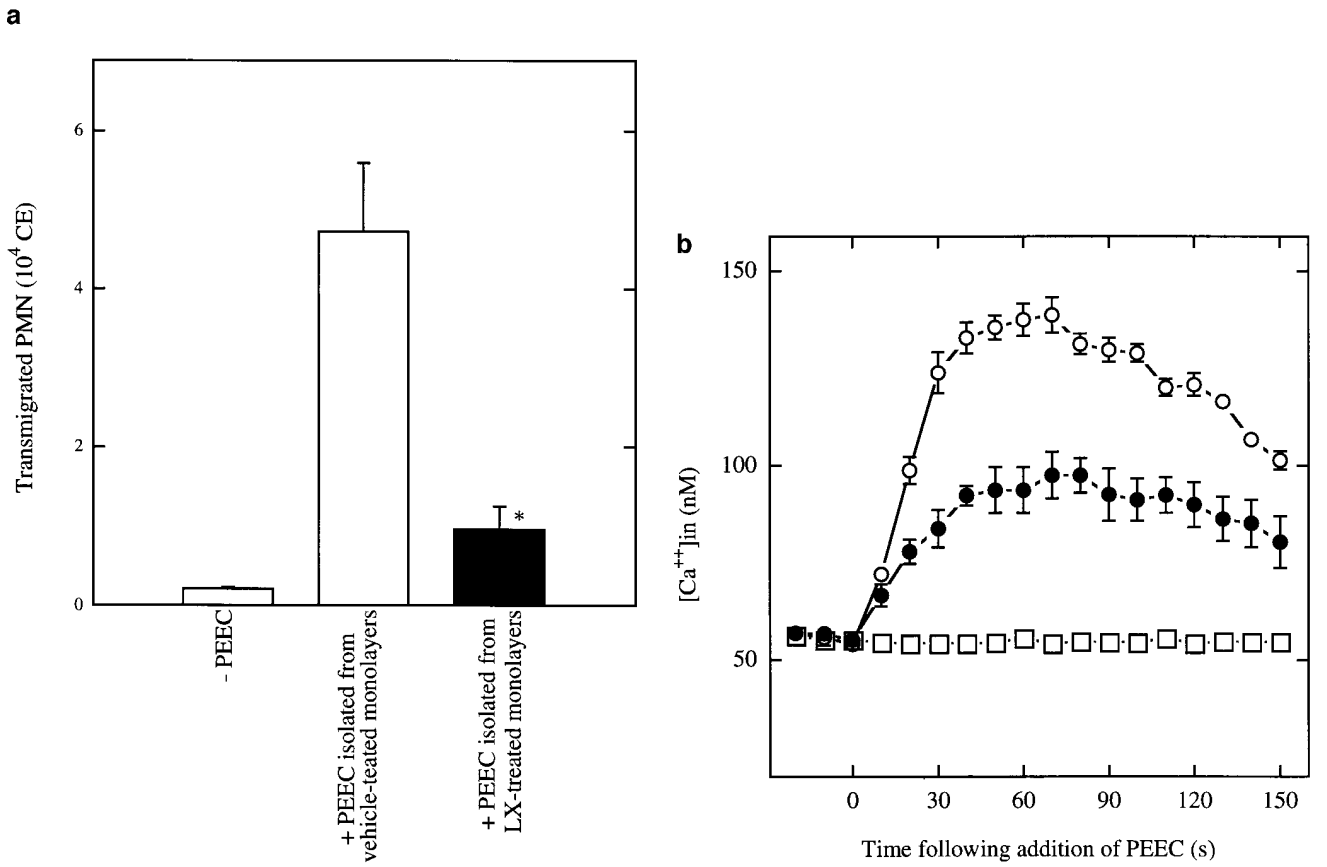


Figure 2. Lipoxin A_4 stable analog inhibits *S. typhimurium*–induced PEEC secretion. Inverted monolayers of T84 cells were incubated in 1 nM 15(R/S)-methyl-LXA_4 or vehicle (0.1% ethanol) for 1 h before adding *S. typhimurium*. 1 h later, nonadherent *S. typhimurium* was washed off, and PEEC was isolated from the apical supernatants that were collected over the following 2 h. (*a*) PEEC, concentrated 50-fold from apical supernatants, was placed in the apical reservoir of T84 monolayers, and its ability to induce PMN when placed in the basolateral reservoir to transmigrate was measured. (*b*) Indo-1–loaded PMN (2×10^6/ml) were suspended in HBSS while their cytoplasmic [Ca^{++}] was measured continuously by spectofluorimetry. PEEC was added at a final concentration equal to twice that found in the apical supernatants of *S. typhimurium*–infected T84 cells. □, No PEEC; ○, +PEEC isolated from *S. typhimurium*–infected control monolayers; ●, +PEEC isolated from *S. typhimurium*–infected LX-treated monolayers. PEEC isolates were pooled from three separate experiments, each using five monolayers per condition. These data are the means of a triplicate analysis±SEM of the pooled isolates.

results from a representative experiment or as a percent of control (vehicle) values. Additionally, there is variability in the way PMN from different donors transmigrate in response to PEEC, explaining why absolute numbers of transmigrated PMN vary between experiments. Statistical significance was assessed using Student's *t* test. $*P < 0.05$ when compared with control.

Results

To investigate whether lipoxins (LX) could affect epithelial chemokine secretion, model intestinal epithelia (i.e., confluent monolayers of T84 cells) were exposed to vehicle (0.1% ethanol) or a stable analog of LXA_4, and then infected with a pathogenic strain of *S. typhimurium*. 5 h after exposure to the bacteria, basolateral supernatants of the monolayers were assayed for IL-8, as this chemokine is known to be preferentially secreted to that surface. Noninfected monolayers exhibited a relatively low level of IL-8 secretion that was mildly inhibited (5–20% inhibition in several different experiments) by 15(R/S)-methyl-LXA_4 (Fig. 1), while *S. typhimurium* infection led to much greater IL-8 secretion that was inhibited $\sim$ 50% by this stable LXA_4 analog (Fig. 1). Importantly, IL-8 secretion induced by PMA, a known potent but nonphysiologic activator of IL-8 secretion, was not inhibited by 15(R/S)-methyl-LXA_4 (Fig. 1), suggesting that LXA_4 analogs did not affect the ability of T84 cells to secrete IL-8, but rather may have downregulated some portion of the signaling pathways that get activated by *S. typhimurium.* However, LXA_4 analogs did not inhibit all signals that get activated by *S. typhimurium* in T84 cells since the ability of T84 monolayers to internalize this bacterium (a process requiring many host-generated signals; 1, 2) was not affected by 100 nM 15-R/S-methyl LXA_4 (control monolayers internalized 0.30±0.05% [$n = 5$] of *S. typhimurium* inoculum compared with 0.31±0.04% [$n = 5$] for LX-treated), nor did 100 nM 15-R/S-methyl LXA_4 affect replication of *S. typhimurium* in vitro (data not shown) or inside T84 cells (the number of intracellular bacteria increased over a 4-h period by a factor of 6.2±0.5 vs. 6.4±0.6 for control and LX-treated, respectively). Similarly, LXA_4 analogs did not affect *S. typhimurium* adherence to T84 monolayers (1.60±0.22% [$n = 5$] of inoculum adhered to control monolayers compared with 1.59±0.10% [$n = 5$] for LX-treated). Because there are some differences in the responses of different intestinal epithelial cell lines to inflammatory stimuli (6, 22), we measured whether LXA_4 analogs could also attenuate *S. typhimurium*–induced IL-8 secretion in other model systems. The colon-derived cell line HT29-Cl19a exhibited about 10-fold less IL-8 secretion in response to *S. typhimurium* than did T84 cells. This small response was inhibited by 100 nM 15 R/S-methyl-LXA_4 to a level below the quantitation limit of our assay ($\geq$ 60% inhibition, $P < 0.02$ based on comparison OD values). Ileum-derived $Caco2_{BBE}$ cells exhibited about 15-fold less IL-8 secretion than did T84 cells. In this model system, *S. typhimurium*–induced IL-8 secretion showed a trend toward being attenuated by 15 R/S-methyl-LXA_4, although we have not as yet observed statistically significant differences (59±71% inhibition) compared with the control. Thus, although there were large differences in the absolute amounts of IL-8 secreted by different model systems, LX attenuation of IL-8 secretion was not restricted to a specific intestinal epithelial model.

Apical supernatants of *S. typhimurium*–infected T84 cells were assayed for PEEC, as release of this recently characterized proinflammatory chemoattractant has been demonstrated only at that surface (8). The relative concentration of PEEC isolated from apical supernatants was assessed by two means. First, the ability of PEEC partially purified from control (vehicle) and LX-treated *S. typhimurium*–infected monolayers to drive PMN across virgin (i.e., not having been exposed to *S. typhimurium* or LX) epithelial monolayers was measured. LXA_4 analog pretreatment of *S. typhimurium*–infected monolayers led to PEEC isolates that drove significantly fewer PMN to transmigrate (Fig. 2 *a*), indicating that LXA_4 analog–treated monolayers secreted less PEEC than control monolayers in response to *S. typhimurium*. Second, we measured the ability of partially purified PEEC isolates to induce cytoplasmic [Ca^{++}] changes in PMN, as this is a known activity of this chemoattractant (8). PEEC isolated from LXA_4 analog–treated *S. typhimurium*–infected monolayers induced smaller changes in cytoplasmic [Ca^{++}] in PMN than in PEEC isolated from control *S. typhimurium*–infected monolayers (Fig. 2 *b*), strengthening the suggestion that LXA_4 analog treatment of epithelial cells resulted in attenuated PEEC secretion by *S. typhimurium*–infected monolayers. Because LXA_4 has been shown directly to inhibit chemoattractant-induced calcium changes in PMN (23, 24) as well as PMN transepithelial migration (14), it is important to point out that no residual LXA_4 analogs likely remained in partially purified PEEC isolates for two reasons: first, the monolayers from which PEEC was isolated were washed extensively with LXA_4 analog-free buffer before col-

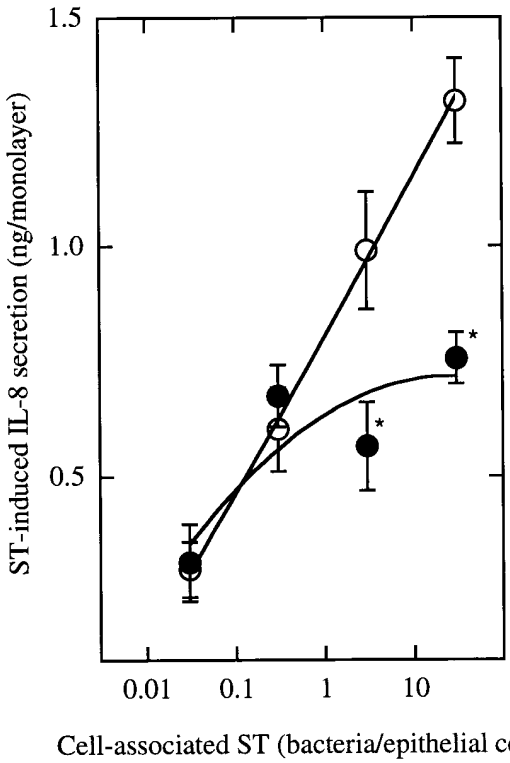


Figure 3. Lipoxin A_4 stable analog reduces epithelial secretion of IL-8 induced by large *S. typhimurium* (ST) inoculums. Monolayers of T84 cells were incubated in 100 nM 15(R/S)-methyl-LXA_4 (●) or vehicle (○) for 1 h before adding *S. typhimurium*. Data are the means±SEM of six monolayers/condition from one experiment, and are representative of three separate experiments.

lection of PEEC. Second, one step of the PEEC isolation procedure is collecting the filtrate from a 1,000-D nominal cutoff filter; a filter that, as expected and verified in separate experiments, does not retain statistically significant amounts of 15(R/S)-methyl-LXA_4 (mol wt = 380.5).

We next sought evidence to characterize further the structural, concentration, and temporal requirements of LXA_4 inhibition of *S. typhimurium*–induced IL-8 secretion. We first investigated whether LXA_4 analogs might shift the dose response to *S. typhimurium,* or whether LXA_4 analogs attenuated the maximal response to *S. typhimurium*. Although LXA_4 analogs mildly inhibited basal IL-8 secretion by T84 monolayers (Fig. 1), LXA_4 analogs did not significantly inhibit *S. typhimurium*–induced (i.e., basal subtracted) IL-8 secretion in response to relatively low infectious doses of *S. typhimurium* (Fig. 3). Rather, LXA_4 analogs attenuated this pathogen-elicited inflammatory event when the number of monolayer-associated *S. typhimurium* was one or more bacteria per epithelial cell (Fig. 3). To gain insight into the structural requirements of LX inhibition of *S. typhimurium*–induced IL-8 secretion, we investigated whether native LXA_4, aspirin-triggered 15 epi-LXA_4, stable LXA_4 analogs, or lipoxin B_4 (LXB_4; displayed in Fig. 5 *a*) could inhibit this proinflammatory event. *S. typhimurium*–induced IL-8 secretion was inhibited by LXA_4, 15-epi-LXA_4, and by two stable analogs of LXA_4 (15[R/S]-methyl-LXA_4, 16-phenoxy-LXA_4) that have previously been shown to mimic the bioactivity of LXA_4 (15, 25, 26; Fig. 4). *S. typhimurium*–induced IL-8 secretion was not significantly inhibited by 15-deoxy-LXA_4, a stable LXA_4 analog that does not exhibit the bioactivity of the native compound (15), nor was it inhibited by LXB_4 (Fig. 4), a lipoxin whose bioactivity differs in some cases from LXA_4 (14, 27, 28), and which is not a specific ligand for the LXA_4 receptor (29). The greatest level of inhibition (45± 3.5%) was exhibited by 15(R/S)-methyl-LXA_4, which is both a stable analog of LXA_4 and is also structurally similar to 15-epi-LXA_4 (see Fig. 4 *a*). Inhibition of *S. typhimurium*–induced IL-8 secretion by 15(R/S)-methyl-LXA_4 was concentration-dependent (Fig. 5), exhibiting an IC_{50} ranging from 0.5 to 3 nM in several different experiments, and saturable at $\sim$ 10 nM.

To characterize the temporal requirements of LXA_4 analog inhibition of *S. typhimurium*–induced IL-8 secretion, T84 monolayers were incubated with 15(R/S)-methyl-LXA_4 for the indicated time intervals before colonization by *S. typhimurium*. Although LXA_4 analogs inhibited basal IL-8 release (Fig. 1), an event presumably already in progress when the monolayers were exposed to LXA_4 analogs, LXA_4 analog inhi-

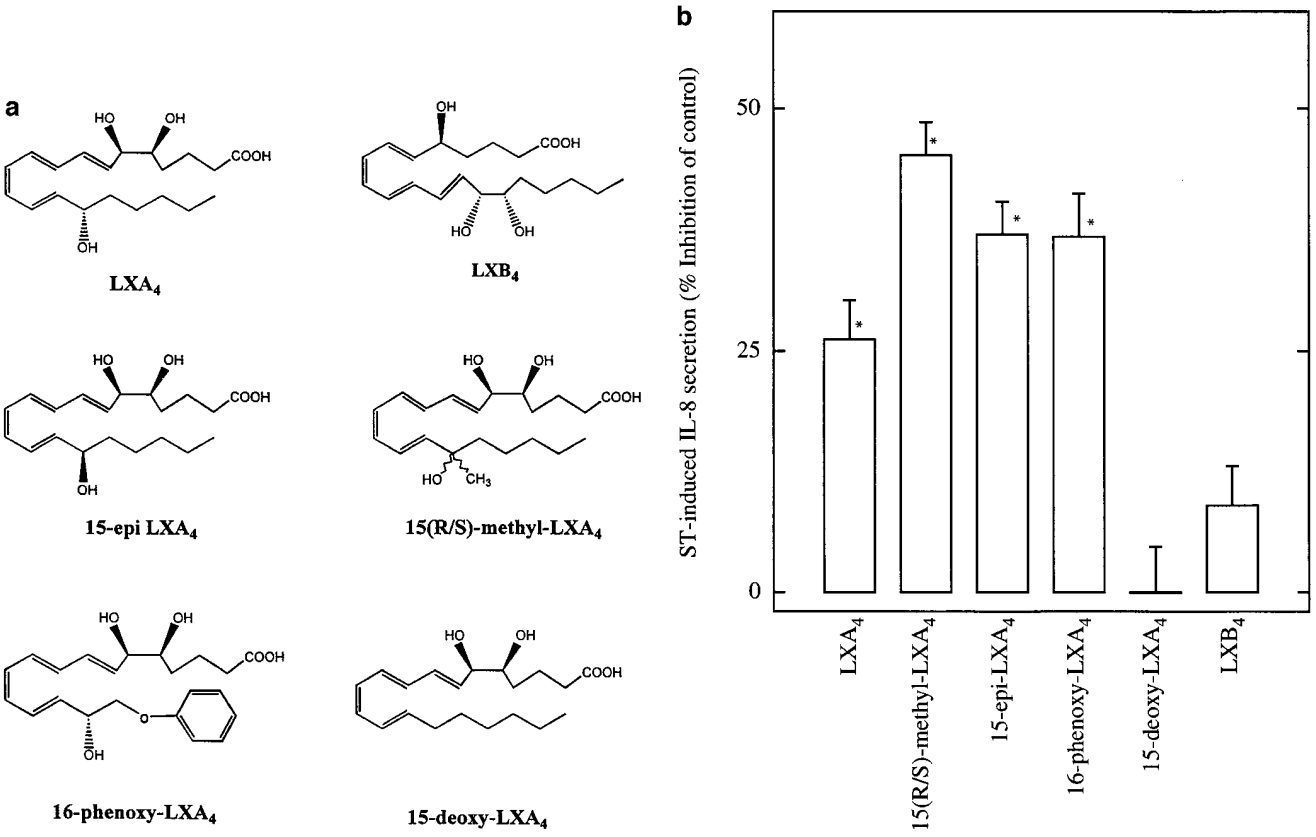


Figure 4. LXA_4, 15-epi-LXA_4, and two stable LXA_4 analogs stereoselectively block *S. typhimurium* (*ST*)–induced IL-8 secretion by model intestinal epithelia. (*a*) Structure of lipoxins and lipoxin analogs used in these experiments, shown as carboxylic acids, although analogs were synthesized and used as methyl esters. Aspirin-triggered 15-epi-LXA_4 differs from native LXA_4 by carrying its C-15 alcohol group in the R configuration. 15(R/S)-methyl-LXA_4 differs from the native eicosanoid in that it carries a methyl group as a racemate at C-15. 15-deoxy-LXA_4 carries no C-15 alcohol group while 16-phenoxy-LXA_4 carries a bulky substituent near the critical C-15 position. LXB_4 differs from LXA_4 at several positions. (*b*) Monolayers of T84 cells were incubated in 100 nM of the indicated compound or vehicle (0.1% ethanol) for 1 h before adding *S. typhimurium*. 5 h later, basolateral supernatants were assayed for IL-8. Data are the means±SEM of three separate experiments, each performed in at least quadruplicate. Results are normalized to vehicle control.

bition of *S. typhimurium*–induced (i.e., basal subtracted) IL-8 secretion required that the T84 cells be exposed to LXA_4 analogs for 1 h or more before infection. A typical experiment using six monolayers per condition found that cells treated with 100 nM 15 R/S-methyl-LXA_4 for 30 min secreted 1.14±0.15 ng IL-8 per monolayer compared with 1.16±0.11 ng IL-8/monolayer for vehicle-treated cells, while a 1-h treatment with this LXA_4 analog led to *S. typhimurium*–induced IL-8 secretion being 0.64±0.05 ng/monolayer compared with 1.10±0.04 ng/monolayer for control (vehicle-treated) cells. 2-h treatment with this LXA_4 analog caused *S. typhimurium*–induced IL-8 secretion to be 0.65±0.04 ng/monolayer compared with 1.20±0.18 ng/monolayer for the control. We then asked if monolayers that were exposed to LXA_4 analogs for 1 h but were washed with LXA_4 analog-free buffer before infection by *S. typhimurium* would still exhibit attenuated IL-8 secretion in response to this bacterium. As shown in Fig. 6 *a* (compared with Fig. 6 *b*), LX inhibition was only partially maintained under these conditions. However, if as in Fig. 6 *c*, LXA_4 analog-treated monolayers were washed with LX-free buffer after infection by *S. typhimurium*, but still, as in Fig. 6 *a*, allowing IL-8 secretion to occur in the absence of LX, nearly full inhibition was maintained (Fig. 6, *c* and *d*). These results indicate that LX need be present only during the early signaling events that lead to chemokine secretion.

To ascertain whether LXA_4 analogs exhibited a general downregulatory effect on IL-8 secretion, or was specific to *S. typhimurium,* we measured whether LXA_4 analogs could also inhibit IL-8 secretion induced by other physiological agonists. The noninvasive bacteria EPEC also elicits IL-8 secretion, albeit 5–10-fold less than *S. typhimurium,* presumably by a somewhat different mechanism (17). We found that EPEC-induced IL-8 secretion could also be inhibited by LXA_4 analogs (0.14±0.06 vs. 0.06±0.03 ng/monolayer for vehicle-treated and 100 nM 15-R/S-methyl-LXA_4–treated, respectively; $P < 0.05$). Furthermore, the ability of LX to inhibit stimulus-induced IL-8 secretion was not limited to bacteria, as IL-8 secretion induced by TNFα could also be attenuated by an LXA_4 analog (monolayers treated for 1 h with vehicle or 100 nM 15 R/S-methyl-LXA_4 before stimulation with 10 ng/ml TNFα secreted 0.42±0.04 vs. 0.26±0.03 ng/monolayer, respectively; $P < 0.05$). Interestingly, IL-8 secretion induced by saturating concentra-

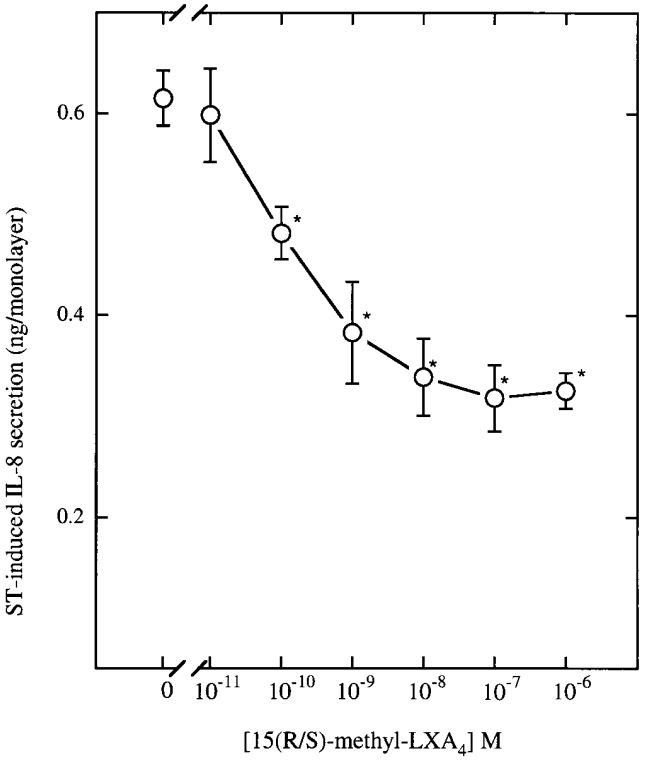


Figure 5. Concentration-dependence of inhibition of *S. typhimurium*–induced IL-8 secretion by stable LXA_4 analog. Monolayers of T84 cells were incubated with indicated concentrations of 15(R/S)-methyl-LXA_4 for 1 h before adding *S. typhimurium*. 5 h later, basolateral supernatants were assayed for IL-8. Data are the means±SEM of six monolayers/condition from one experiment, and are representative of four separate experiments.

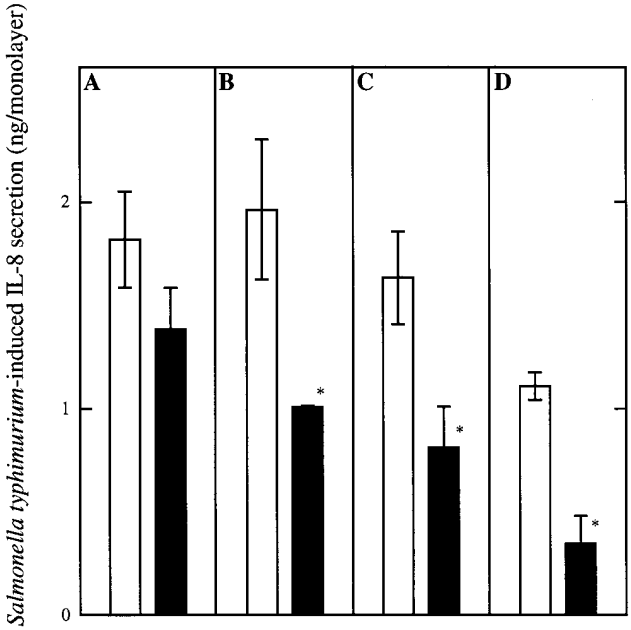


Figure 6. Incubating T84 monolayers in LX-free buffer after *S. typhimurium* colonization does not affect LX-inhibition of *S. typhimurium*–induced IL-8 secretion. Monolayers of T84 cells were incubated with vehicle (*open bars*) or 100 nM 15(R/S)-methyl-LXA_4 (*solid bars*) for 1 h before adding *S. typhimurium*. 5 h after adding *S. typhimurium*, basolateral supernatants were assayed for IL-8. Note that the differences in control values between different panels, particularly the lower control value in *D*, are due to supernatants being collected over a shorter time interval as washing out LX also washed out any IL-8 that had been secreted before that point. Data are the means±SEM of six monolayers/condition from one experiment, and are representative of three separate experiments. (*A*) Monolayers were washed 3× with LX-free HBSS before adding bacteria. After adding *S. typhimurium*, monolayers were placed, and chemokine secretion was allowed to occur into fresh HBSS that contained no LX. (*B*) Monolayers were never washed after adding LX. Rather, monolayers were placed, and chemokine secretion was allowed to occur into the LX-containing HBSS in which the cells had incubated before adding *S. typhimurium*. (*C*) Monolayers were washed free of LX 1 h after adding bacteria. After being washed, monolayers were placed, and chemokine secretion was allowed to occur into fresh HBSS that contained no LX. (*D*) Monolayers were washed free of LX 2 h after adding bacteria. After being washed, monolayers were placed, and chemokine secretion was allowed to occur into fresh HBSS that contained no LX.

tions of TNFα (> 25 ng/ml) was not significantly affected by LXA_4 analogs (data not shown). To begin to elucidate the mechanism by which LX attenuate IL-8 secretion, we measured whether LX affected levels of IL-8 mRNA. Basal IL-8 mRNA was not detectable by Northern blot analysis. 2 h after exposure to *S. typhimurium,* IL-8 mRNA increased to detectable levels and continued to increase during the next 3 h. IL-8 mRNA levels were ~ 50% lower in LXA_4 analog-treated T84 monolayers compared with vehicle-treated controls (Fig. 7), correlating with our measurements of IL-8 secretion. These results indicate that LXA_4 analog attenuation of chemokine secretion is mediated at the mRNA level.

Having established that LXA_4 analogs could partially inhibit epithelial secretion of these proinflammatory chemokines that drive PMN movement, we next investigated whether this inhibition was sufficient to lead to attenuated PMN movement in response to *S. typhimurium*. *S. typhimurium*–infected polarized epithelial monolayers drive PMN placed near their basolateral surface to move across the epithelium to the apical surface, but do not drive PMN placed near their apical surface to migrate to the basolateral surface, thereby mimicking *S. typhimurium* infection in vivo where PMN are driven to the intestinal lumen (3). IL-8 has been shown to be largely responsible for directing PMN through the basolateral extracellular matrix (lamina propria) of model epithelia in response to *S. typhimurium* (7). As shown in Fig. 8 *a*, LXA_4 and an LXA_4 analog inhibited *S. typhimurium*–induced movement of PMN through T84 matrices, indicating that one consequence of LX inhibition of IL-8 secretion was reduced PMN movement to a subepithelial compartment. Once PMN have migrated through the subepithelial matrix, their migration across the epithelium in response to *S. typhimurium* is driven, at least in large part, by PEEC (8). *S. typhimurium*–induced PMN transepithelial migration was attenuated by nanomolar concentrations of 15(R/S)-methyl-LXA_4 (Fig. 8 *b*), indicating that, analogously to the above, LXA_4 inhibition of PEEC secretion resulted in clear attenuation of PMN transepithelial migration.

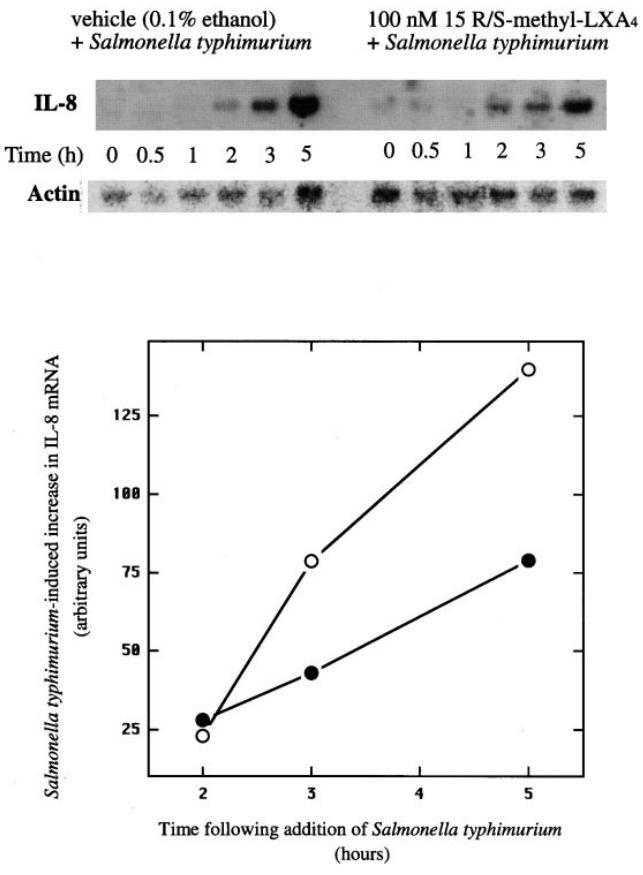


Figure 7. IL-8 mRNA levels in *S. typhimurium*–infected T84 monolayers are lower in cells treated with a LXA_4 stable analog. T84 monolayers (5 cm^2) were treated with 100 nM 15 R/S-methyl-LXA_4 for 1 h before adding *S. typhimurium.* At the indicated time point after adding the bacteria, RNA was extracted, and IL-8 mRNA (and in a second hybridization to the same membrane actin mRNA) was analyzed by Northern blot. IL-8 mRNA was quantitated by densitometry and normalized relative to actin mRNA levels. This experiment was performed twice, and similar results were obtained both times. (○) Vehicle-treated control; (●) +100 nM 15(R/S)-methyl-LXA_4.

Discussion

Biosynthesis of LX in vivo appears to be one means by which inflammation is self-limited (10). While some of the antiinflammatory effects of LX can be explained by LX acting directly on the neutrophil, LX may also act directly on other cell types, thus providing an additional mechanism by which LX can modulate inflammation (e.g., LXA_4 stable analogs have recently been shown to act directly on vascular endothelium; 30). Since epithelial cells play an important role in mediating inflammation at mucosal surfaces, we speculated that epithelium could be an additional site of antiinflammatory action of this class of eicosanoids. Consistent with this hypothesis, we have shown here that LX inhibited both basal and pathogen-elicited secretion of proinflammatory chemokines by model intestinal epithelia. This suppression was unlikely the result of a general inhibition of LX on the function of epithelial cells, as neither epithelial uptake of bacteria nor PMA-induced IL-8 secretion was affected by LX. Reduced PMN movement across model epithelia in response to infection by the enteric pathogen *S. typhimurium* was an important consequence of LX inhibition of epithelial chemokine secretion. Such transepithelial migration of PMN appears to play a role in producing symptoms associated with intestinal inflammation (31). However, before this report there was no defined means (other than generally cytotoxic approaches such as global inhibition of protein synthesis) of inhibiting secretion of the proinflammatory chemokines that direct PMN movement. Hence, the demonstration that LX can downregulate epithelial secretion of pro-inflammatory chemokines suggests a potential therapeutic strategy for active intestinal inflammation.

It seems likely that the observed actions of lipoxins on epithelial chemokine secretion were mediated by the LXA_4 receptor: a seven-transmembrane α-helix G-protein–linked receptor that mediates the actions of LXA_4, 15-epi-LXA_4, and stable LXA_4 analogs on PMN and monocytes (25, 29). Consistent with this notion, it has recently been established that intestinal epithelial cell lines express this receptor (32), and the observed IC_{50} values were similarly supportive of LXA_4 receptor–mediated actions. Although native LXA_4 competes somewhat more effectively for the LXA_4 receptor than do the 15(R/S)-methyl-LXA_4 or 16-phenoxy-LXA_4 analogs (26), given the stability of these LXA_4 analogs it is not surprising that they provide greater attenuation of *S. typhimurium*–induced epithelial IL-8 secretion than the native eicosanoid. 15-deoxy-

LXA_4, which lacks a carbon 15 alcohol, did not exhibit antiinflammatory behavior in the model system used here or in others tested previously (15), consistent with both the earlier suggestion that the trihydroxytetraene structure is required for bioactivity, and the likely role of the LXA_4 receptor in mediating the antiinflammatory actions of LXA_4 analogs.

Ligation of the LXA_4 receptor was found to attenuate chemokine secretion in an agonist-specific fashion. For example, while IL-8 secretion induced by pathogens *S. typhimurium* or EPEC were reduced, IL-8 secretion induced by the strong protein kinase C agonist PMA was not affected by lipoxins. Hence, in contrast to protein kinase C activation, signaling cascades initiated by pathogens likely selectively interface with LXA_4 receptor-mediated signaling cascades (neither the pathogen-activated or the LXA_4 receptor–signaling cascades are currently understood). Furthermore, given the suggestion that *S. typhimurium* may induce an increase in cytoplasmic $[Ca^{++}]$ upon attachment to eukaryotic cells (33), we also examined the influence of thapsigargin (which causes a release of Ca^{++} from intracellular stores) on the responses observed. Thapsigargin also stimulated IL-8 secretion in the absence of pathogens, however, such chemokine secretion was again not affected by LXA_4 stable analogs (data not shown). Lastly, we observed that LX-mediated attenuation of *S. typhimurium*–induced chemokine secretion was best detected when T84 cells were exposed to LX for 1 h or more before adding bacteria. Keeping in mind that *S. typhimurium*–induced chemokine secretion takes several hours to become maximal, possible explanations for the temporal requirements of LX action include the following: (*a*) the LXA_4 receptor-mediated signaling cascade involves protein synthesis; (*b*) LX induces epithelial synthesis of another eicosanoid or other mediating product that attenuates chemokine secretion; or (*c*) LX -induced signals are transduced by a somewhat slow mechanism.

Most nonsteroidal antiinflammatory agents act by inhibiting enzymes that make, or receptors that recognize, proinflammatory eicosanoids. In contrast, LXA_4 stable analogs appear to attenuate epithelial cell secretion of proinflammatory chemokines by activating receptor-mediated pathways that naturally downregulate inflammation. Furthermore, LXA_4 receptor–mediated antiinflammatory effects may more tightly focus on counterregulation of inflammation than do other

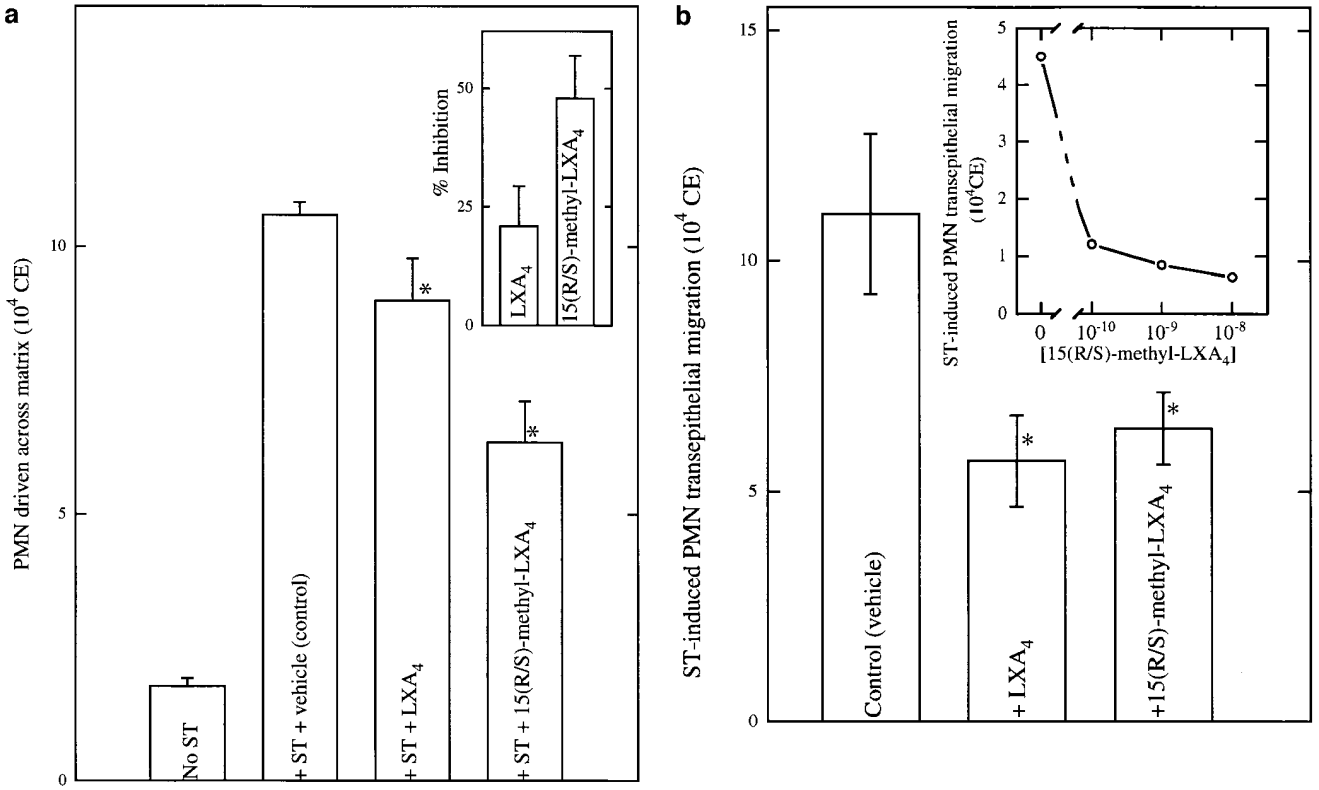


Figure 8. LXA_4 stable analog treatment of epithelial monolayer leads to attenuated PMN migration. (*A*) Inverted monolayers of T84 cells were incubated with 100 nM 15(R/S)-methyl-LXA_4 or vehicle (0.1% ethanol) for 1 h before adding *S. typhimurium*. 3 h after adding *S. typhimurium*, T84 cells were removed from their underlying matrices by treatment with EGTA. The abilities of the matrices (which remain attached to the collagen-coated filters on which the cells were grown) to drive PMN movement was assessed by placing PMN upon them and measuring how many were driven through the matrix in the physiologically relevant direction. Data are the means±SEM of four monolayers/condition from one experiment, and are representative of three separate experiments. *Inset* shows this data expressed as percent inhibition of control response. (*B*) Inverted monolayers of T84 cells were incubated with 15(R/S)-methyl-LXA_4 (1 nM, indicated concentration in *inset*) or vehicle (0.1% ethanol) for 1 h before adding *S. typhimurium*. 1 h after adding *S. typhimurium*, monolayers were washed three times in HBSS to remove nonadherent bacteria (and presumably LX), and were placed into fresh HBSS that contained no LX. PMN were then placed in the apical reservoir, and PMN transepithelial migration to the basolateral reservoir was measured. Data in main panel are the means±SEM of four monolayers/condition of a single experiment, and are representative of three separate experiments. Data in *inset* are the means of a representative experiment performed in triplicate.

nonsteroidal antiinflammatory compounds. For example, it is known that aspirin, via its effects on prostaglandin H synthase-1, diminishes prostaglandin synthesis, and it is recognized that prostaglandins are necessary for maintening epithelial barrier function in the mucosa of the alimentary tract (34). Thus, depletion of mucosal prostaglandins leads to surface erosions, resulting in direct tissue exposure to the complex and noxious content of the gut lumen. Interestingly, it has recently been recognized that aspirin-mediated acetylation of prostaglandin H synthase-2 results in production of a unique agonist for the LXA_4 receptor 15-epi-LXA_4 (16). 15-epi-LXA_4 displays the bioactivities of other active lipoxin stable analogs (15) including, as shown here, inhibition of agonist-induced release of epithelial proinflammatory cytokines. Thus, it is likely that aspirin exposure results in counterbalancing injurious (diminished prostaglandin synthesis) and antiinflammatory (including production of a stable LXA_4 receptor ligand) effects on intestinal mucosa. If so, direct activation of the LXA_4 receptor would seemingly permit a more targeted antiinflammatory approach to diseases of the intestinal mucosa characterized by active inflammation.

Although proinflammatory chemokines are known to drive the immune inflammatory response, methods of inhibiting their release have not been established. Here, we establish one such method in a physiologically relevant context; i.e., inhibition of chemokine release using nanomolar concentrations of analogs of an eicosanoid that is produced by humans. Furthermore, LX attenuation of chemokine secretion sufficient to lead to attenuated epithelial orchestration of PMN movement described here was observed in response to a known gastroenterological pathogen that induces neutrophil movement as part of its virulence mechanism (5). Thus, direct inhibition of epithelial proinflammatory signaling cascades by LX represents a potential means of therapeutic intervention in such disease states. The overall effect of LX on inflammation may even be greater than that measured on chemokine secretion alone, since LX also directly affects PMN by downregulating their response to chemoattractants (19, 20). Adding further to the therapeutic potential of LX stable analogs is the possibility that downregulating chemokine secretion in this manner may be part of the mechanism by which inflammation is normally locally contained.

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