Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers.

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Research Article

Neutrophil (PMN) migration across intestinal epithelial barriers, such as occurs in many disease states, results in modifications in epithelial barrier. Here, we investigated the impact of lipoxin A4 (LXA4), an eicosanoid with counterregulatory inflammatory roles, on PMN migration across cultured monolayers of the human intestinal epithelial cell line T84. Transepithelial migration of PMN was assessed in the apical-to-basolateral direction and in the basolateral-to-apical direction. In the apical-to-basolateral direction, preexposure of PMN to LXA4 (10 nM, 15 min) stimulated an 87 +/- 5% increase in transepithelial migration of PMN as determined by a PMN myeloperoxidase assay. The LXA4-elicited effect on transmigration was present throughout the 2-h assay period and was not secondary to LXA4 effects on epithelial monolayer integrity as judged by measurement of transepithelial resistance. PMN migration in the basolateral-to-apical direction was modulated by LXA4 with a comparable time- and concentration-dependence to that in the apical-to-basolateral direction. However, qualitative differences in how LXA4 modulates transmigration in the two opposing directions were observed. In the basolateral-to-apical direction, preexposure of PMN to LXA4 (10 nM, 15 min) diminished PMN transepithelial migration by 33 +/- 4%. Structure-function studies revealed that LXA4 and 11-trans-LXA4 (50% of LXA4 effect), but not LXB4, inhibited basolateral-to-apical PMN transmigration. The action of LXA4 was not sensitive to inhibitors of cyclooxygenase or specific leukotriene biosynthesis, but was sensitive to […]

Lipoxin A_4 Modulates Transmigration of Human Neutrophils across Intestinal Epithelial Monolayers

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Abstract

Neutrophil (PMN) migration across intestinal epithelial barriers, such as occurs in many disease states, results in modifications in epithelial barrier. Here, we investigated the impact of lipoxin A_4 (LXA_4), an eicosanoid with counterregulatory inflammatory roles, on PMN migration across cultured monolayers of the human intestinal epithelial cell line T_{84}. Transepithelial migration of PMN was assessed in the apical-to-basolateral direction and in the basolateral-to-apical direction. In the apical-to-basolateral direction, preexposure of PMN to LXA_4 (10 nM, 15 min) stimulated an 87±5% increase in transepithelial migration of PMN as determined by a PMN myeloperoxidase assay. The LXA_4-elicited effect on transmigration was present throughout the 2-h assay period and was not secondary to LXA_4 effects on epithelial monolayer integrity as judged by measurement of transepithelial resistance. PMN migration in the basolateral-to-apical direction was modulated by LXA_4 with a comparable time- and concentration-dependence to that in the apical-to-basolateral direction. However, qualitative differences in how LXA_4 modulates transmigration in the two opposing directions were observed. In the basolateral-to-apical direction, preexposure of PMN to LXA_4 (10 nM, 15 min) diminished PMN transepithelial migration by 33±4%. Structure-function studies revealed that LXA_4 and 11-*trans*-LXA_4 (50% of LXA_4 effect), but not LXB_4, inhibited basolateral-to-apical PMN transmigration. The action of LXA_4 was not sensitive to inhibitors of cyclooxygenase or specific leukotriene biosynthesis, but was sensitive to staurosporine, a protein kinase C inhibitor. These results suggest that migration of PMN across epithelia in the physiological direction may be qualitatively different following PMN exposure to eicosanoids. We propose that such retention of PMN at this specific anatomic location may serve an important role in mucosal defense. (*J. Clin. Invest.* 1993. 92:75–82.) Key words: inflammation • intestinal disease • eicosanoid • arachidonic acid

Introduction

PMN migration across intestinal epithelia is the hallmark of active intestinal inflammation and occurs in such disease states as ulcerative colitis, Crohn's disease and infectious enterocolitis (1, 2). Transepithelial migration of PMN is particularly evident in the intestinal crypt and eventuates in crypt abscess formation, a characteristic of severe inflammatory disease (1, 2). In patients with inflammatory bowel disease, the degree of PMN transepithelial migration, assessed quantitatively, has been shown to correlate with patients symptoms, as well as with the degree of intestinal epithelial barrier dysfunction (3). Studies of human mucosa in such diseases suggest that PMN transepithelial migration predates focal breakdown of the epithelial surface (1, 2), and that defective epithelial barrier function also predates structural discontinuities in the mucosa (4).

An early and crucial step in the acute inflammatory response is attachment of neutrophils (PMN) to endothelial surfaces and subsequent emigration from the vasculature (1, 2). However, during inflammatory episodes in organs lined by columnar epithelia, subsequent PMN migration across the epithelial surface occurs (1, 2). While substantial progress has been made in the understanding of PMN-endothelial interactions (5–9), relatively limited information is available concerning PMN-epithelial interactions (10–15). We have previously modelled the event of PMN transepithelial migration using human PMN and cultured, human-derived, intestinal epithelial monolayers derived from the cell line T_{84} (reviewed in reference 16). Such monolayers are composed of columnar epithelial cells with features similar to those of natural crypt epithelia (17, 18), the site at which the bulk of PMN transepithelial migration occurs in a variety of active inflammatory intestinal diseases (1, 2).

Previous studies using this epithelium as a model for examination of PMN-intestinal epithelial interactions indicate that PMN transmigration elicits a reversible decrease in transepithelial resistance caused by impalement of intercellular tight junctions (11), and requires the PMN β_2-integrin CD11b/CD18 (14, 15). While we have shown that PMN transmigration is quantitatively more substantial in the physiological (basolateral-to-apical) direction (14), qualitative differences in transepithelial migration, relating to the direction of transmigration, have generally not been observed (11, 12, 14) (with the exception of IFN-γ–modulated PMN transepithelial migration [19]).

Lipoxins are lipoxygenase-derived, biologically active eicosanoids produced by PMN, platelets, eosinophils, and macrophages (20, 21). These compounds have been shown to elicit selective counterregulatory responses in human PMN in vitro, including the inhibition of leukotriene B_4 (LTB_4) and FMLP-stimulated chemotactic responses (22, 23), blocking of Ca^{2+} mobilization (24), and inhibition of LTD_4-induced adhesion to mesangial cells (25). In vivo, lipoxins are potent inflammatory mediators which act to inhibit leukocyte migration (26), decrease LTD_4-induced vasoconstriction (27) and modulate LTD_4-induced airway obstruction (28).

Here we examine the impact of PMN exposure to lipoxin A_4 (LXA_4)[1] on subsequent transepithelial migration of human

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1. *Abbreviations used in this paper:* LXA_4, lipoxin A_4; MPO, myeloperoxidase; PKC, protein kinase C; PMN CE, PMN cell equivalents.

PMN across monolayers of a cryptlike human intestinal epithelial cell line. Previous exposure of PMN to nanomolar concentrations of LXA_4 substantially alters subsequent PMN transepithelial migration. The action of LXA_4 was found to be specific for PMN, showed a similar time and dose dependency, and the effect was dependent on the direction of PMN transepithelial migration.

Methods

Lipoxins. Synthetic LXA_4, LXB_4, and 11-*trans*-LXA_4 were obtained from Cascade Biochem Ltd. (Berkshire, UK). Concentrations were determined from extinction coefficients as described previously (28). All eicosanoid stock solutions were stored at −70°C in methanol (American Scientific Products Div. American Hospital Supply Corp., McGaw, IL). Eicosanoids were diluted in modified HBSS to a concentration of 1 μM before all experiments. PMN or T_{84} monolayers were exposed to lipoxins at indicated concentrations and allowed to incubate at 37°C for the indicated period of time. Vehicle controls consisted of dilutions of the solvent (ethanol) equivalent to the highest concentration of lipoxin used in any given experiment (0.01%).

Cell culture. Approximately 350 epithelial monolayers were used for these studies. T_{84} intestinal epithelial cells (passages 70–95) were grown and maintained as confluent monolayers on collagen-coated permeable supports as previously described in detail (11, 12). Monolayers were grown on 0.33 cm^2 ring-supported polycarbonate filters (Costar Corp., Cambridge, MA) and used 6–14 d after plating as described previously (16). Transepithelial resistance to passive ion flow was measured as previously described (14–16). Inverted monolayers, used to study transmigration of PMN in the basolateral-to-apical direction, were constructed as described before (14, 16).

Transmigration assay. The PMN transepithelial migration assay has been previously detailed (11, 12, 14, 15). Briefly, human PMN were isolated from normal human volunteers and suspended in modified HBSS (without Ca^{2+} and Mg^{2+}, with 10 mM Hepes, pH 7.4, Sigma Immunochemicals, St. Louis, MO) at a concentration of 5×10^7/ml. Before addition of PMN, T_{84} monolayers were extensively rinsed in HBSS to remove residual serum components. Transmigration assays were performed by the addition of PMN (40 μl) to HBSS (160 μl) in the upper chambers after chemoattractant (1 μM FMLP in HBSS) was added to the opposing (lower) chambers. Unless otherwise indicated, PMN were not washed free of LXA_4 before addition to monolayers, and therefore, a fivefold dilution of lipoxin was present during the transmigration assay. For apical-to-basolateral transmigration experiments, PMN (2×10^6) were added at time 0. We have previously shown that transmigration in the basolateral-to-apical direction, while qualitatively similar, is substantially more efficient than in the apical-to-basolateral direction (14). Therefore, fivefold fewer PMN (4×10^5) were added when transmigration proceeded in the basolateral-to-apical direction so that baseline transmigration signals be approximately equivalent in both directions (14). Transmigration was allowed to proceed for 120 min, unless otherwise noted. All PMN transepithelial migration experiments were performed in a 37°C room to ensure that epithelial monolayers, solutions, plasticware, etc., were maintained at uniform 37°C temperature.

When used, inhibitors to cyclooxygenase (indomethacin; Sigma Immunochemicals), leukotriene biosynthesis (MK886; a kind gift from Merck Frosst, Quebec, Canada), G-proteins (pertussis toxin, Calbiochem, San Diego, CA), or protein kinase C (H7, Sigma Immunochemicals; and staurosporine, Sigma Immunochemicals) were preincubated with PMN at indicated concentrations for 15 min at 37°C. Inhibitors were washed free from PMN by two washes with HBSS. PMN were subsequently exposured to LXA_4 (10 nM) and PMN transepithelial migration was assessed as described above in the apical-to-basolateral direction.

Transmigration was quantitated by assaying for the PMN azurophilic granule marker myeloperoxidase (MPO) as described previously (14). After each transmigration assay, nonadherent PMN were extensively washed from the surface of the monolayer, and PMN cell equivalents (PMN CE), estimated from a standard curve, were assessed as the number of PMNs associated with the monolayer, the number that had completely traversed the monolayer (i.e., across the monolayer into the reservoir bath), as well as the total number of transmigrating PMN (the sum of monolayer and reservoir-associated PMN).

Data presentation. Individual experiments were performed using large numbers of uniform groups of monolayers and PMN from individual blood donors on individual days. PMN isolation was restricted to five different blood donors (repetitive donations) over the course of these studies. Myeloperoxidase assay data were compared by two-factor ANOVA or by comparison of means using Student's *t* test. PMN transmigration results are represented as PMN CE derived from a daily standard PMN dilution curve. Monolayer-associated PMN are represented as the number of PMN CE per monolayer and reservoir-associated PMN (i.e., PMN that had completely traversed the monolayer into the lower chamber) are represented as the number of PMN CE per milliliter (total volume of 1 ml). Values are expressed as the mean and SEM of *n* experiments.

Results

LXA_4 exposure to T_{84} epithelial monolayers does not alter subsequent FMLP-induced PMN transmigration. PMN's can be induced to transmigrate across T_{84} epithelial monolayers in response to a transepithelial gradient of the chemotactic peptide FMLP (1 μM) (11, 14). To determine whether LXA_4 exposure to T_{84} intestinal epithelial cells influenced subsequent PMN transmigration, epithelial cell monolayers were incubated with LXA_4 at a concentration 10 nM for 15 min at 37°C (conditions which elicit significant effects when PMN's are preexposed to LXA_4, see below), with and without removal of LXA_4 from monolayers, followed by addition of untreated PMNs under chemotactic conditions. In these experiments, PMN transmigration across T_{84} monolayers exposed to LXA_4 did not differ from vehicle control (14.8 ± 1.4 vs $15.7 \pm 1.8 \times 10^4$ PMN CE/ml for control and LXA_4-exposed monolayers, $n = 6$ each, NS). Removal of LXA_4 from monolayers by washing three times with HBSS before addition of PMN had no apparent effect on the total number of transmigrating PMN ($14.3 \pm 1.1 \times 10^4$ PMN CE/ml, $n = 6$, NS compared to either control or LXA_4-exposed monolayers).

In addition, exposure of intestinal epithelial cells to LXA_4 did not significantly influence the integrity of T_{84} epithelial monolayers. To examine this, transepithelial resistance to passive ion flow was assessed before and after addition of 10 nM LXA_4 to T_{84} intestinal epithelial monolayers for 2 h (simulated conditions for entire transmigration assay period). During this period, transepithelial resistance did not significantly decrease after addition of LXA_4 (baseline resistance $1,255 \pm 56$ $\Omega \cdot cm^2$ and $1,089 \pm 108$ $\Omega \cdot cm^2$ after 2 h, $n = 8$, NS). These results suggest that monolayer integrity, as assessed by transepithelial resistance, was not affected by LXA_4 treatment and that epithelial preexposure to LXA_4 has no subsequent effect on FMLP-induced PMN transmigration.

LXA_4 does not stimulate transmigration. To investigate whether LXA_4 could serve to stimulate PMN transmigration in this assay system, dilutions of LXA_4 in the range of 0.01–10 nM were placed in the lower chamber of transmigration wells. Untreated PMN's were added to the upper chamber and assessed for chemotactic capacity toward LXA_4 in the apical-to-

basolateral direction. LXA_4 was no more effective than HBSS in promoting PMN transmigration; compared to FMLP (1 μM), PMN migration toward LXA_4 resulted in a total of 6±2.1, 9±3.6, 6±2.4, and 12±1.4% of FMLP-induced PMN transmigration for 0.01, 0.1, 1.0, and 10 nM LXA_4, respectively. In the absence of a chemotactic gradient (HBSS), 10±2.9% of FMLP-induced transmigration occurred. These results indicate that LXA_4, in the concentrations tested, did not stimulate PMN transepithelial migration.

Preexposure of PMN to LXA_4 enhances FMLP-induced PMN transmigration in the apical-to-basolateral direction. To determine if PMN exposure to LXA_4 alters subsequent FMLP-induced transmigration, PMN were incubated with 10 nM LXA_4 for 15 min, then added directly to the apical surface of T_{84} epithelial monolayers, and subsequently assessed for their ability to traverse T_{84} epithelial monolayers using a myeloperoxidase assay (14). PMN preexposure to LXA_4 resulted in significantly increased PMN transepithelial migration in the apical-to-basolateral direction (Fig. 1). Increased PMN migration was evident in both monolayer-associated PMN numbers (2.98±0.57 vs 6.93±1.77 $\times$ 10^4 PMN CE/monolayer for vehicle control and LXA_4-exposed PMN, respectively, $P < .001$), as well as the number of PMN that completely traversed the epithelial monolayer (6.61±0.50 vs 11.02±2.91 $\times$ 10^4 PMN CE/ml for vehicle control and LXA_4-exposed, respectively, $P < 0.01$), resulting in a nearly twofold increase in the total number of transmigrating PMN (9.58±1.05 $\times$ 10^4 PMN CE/ml for vehicle control and 17.95±2.15 $\times$ 10^4 PMN CE/ml for LXA_4 preexposed PMN, $P < 0.01$). As reported previously (14), examination of 1 μm T_{84} epithelial monolayer sections revealed that PMNs are only rarely associated with the apical epithelial surface and the majority of monolayer-associated PMN are found subjunctionally, indicative of transmigration. Therefore, monolayer-associated PMN in this apical-to-basolateral assay are considered transmigrated across the tight junction, the rate-limiting barrier in PMN transepithelial migration (14).

To further characterize this transmigratory event, PMNs were preexposed to LXA_4 (10 nM) for various periods of time and subsequently assessed for their ability to transmigrate across T_{84} epithelial monolayers in the apical-to-basolateral direction (Fig. 2). Results in Fig. 2 *A* show that preexposure of PMNs to LXA_4 resulted in increased total PMN transmigration after a LXA_4 preexposure period of 5–30 min. (compared to vehicle controls, for PMNs preexposed to 10 nM LXA_4, transmigration increased by 50, 68, and 51% at 5, 15, and 30 min exposure times, respectively, all $P < 0.025$). Transmigration had returned to vehicle control values by 45 and 60 min of PMN preexposure to LXA_4. Preexposure of PMN to LXA_4 was found to be a necessary prerequisite for LXA_4 action on stimulating PMN transmigration. Indeed, exposure of PMNs to 10 nM LXA_4 immediately before their addition to epithelial monolayers (i.e., 0 min preexposure) resulted in no effect on subsequent FMLP-induced PMN migration (Fig. 2 *A*,

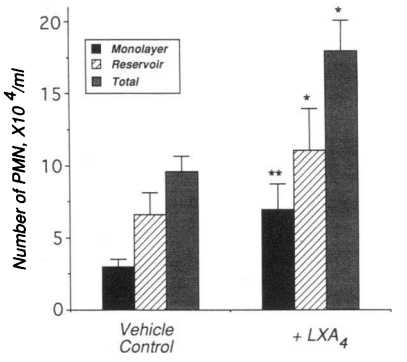

Figure 1. LXA_4 enhances PMN transepithelial migration in the apical-to-basolateral direction. PMN (5×10^7/ml) were preincubated with 10 nM LXA_4 for 15 min at 37°C and layered on the apical surface of washed T_{84} epithelial monolayers at a density of 2×10^6/monolayer. PMN were driven to transmigrate basolaterally under the influence of a 1-μM gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme MPO from washed monolayers, lower reservoirs and total MPO activity after 120 min, relative to a known standard number of PMN. Since tight junctions are the rate limiting barrier to passive paracellular permeation, transmigration is defined as movement of PMN across the tight junction. Since monolayer-associated PMN were largely below the tight junction (see Results), total transmigration in the apical-to-basolateral direction equals the sum of PMN in the opposite reservoir plus monolayer PMN. Data are pooled from nine individual monolayers in each condition, and results are expressed as the mean and SEM. Asterix indicates statistically significant compared to vehicle control (*$P < 0.01$; **$P < 0.001$).

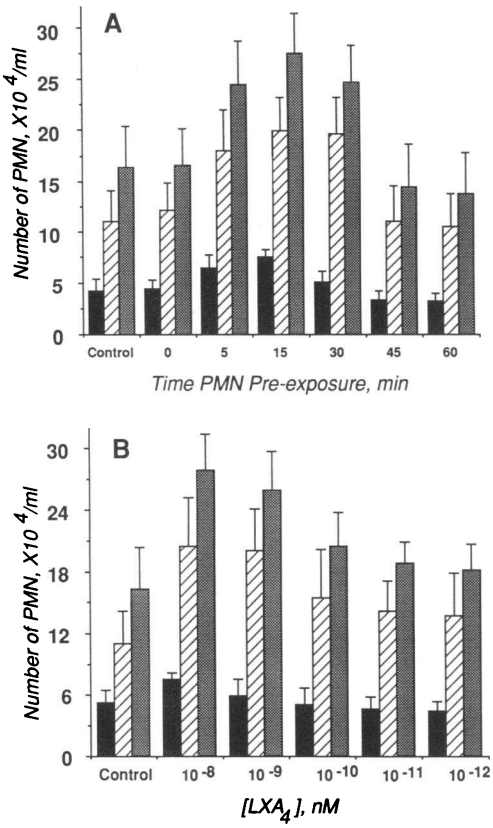

Figure 2. Preexposure time and concentration dependence of lipoxin A_4 on enhanced PMN transepithelial migration in the apical-to-basolateral direction. PMN (5×10^7/ml) were preincubated with 10 nM LXA_4 for various periods of time in the range of 0–60 min at 37°C (*A*) or preincubated with various indicated concentrations of LXA_4 for 15 min at 37°C (*B*) and layered on the apical surface of washed T_{84} epithelial monolayers at a density of 2×10^6/monolayer. PMN were driven to transmigrate basolaterally under the influence of a 1 μM gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme MPO from washed monolayers, lower reservoirs, and total MPO activity after 120 min, relative to a known standard number of PMN. Data are pooled from 7–10 individual monolayers in each condition, and results are expressed as the mean and SEM. ■, Monolayer; ▨, reservoir; ■, total.

16.34±4.07 vs 16.61±3.56 × 10^4 total PMN CE/ml for vehicle control and LXA_4 preexposure for 0 min, respectively, NS). The LXA_4 preexposure time-dependent enhancement of subsequent neutrophil transmigration was largely caused by reservoir-associated PMN (11.06±3.05 vs 18.02±3.35, 19.96±3.18, 19.64±3.54 × 10^4 PMN CE/ml for vehicle control and PMN LXA_4 preexposure times of 5, 15, and 30 min, respectively, two-factor ANOVA $P < 0.01$). However, a significant increase in the number of monolayer-associated PMN occurred at 15 min of LXA_4 preexposure (4.17±1.17 for vehicle control vs 7.55±0.71 × 10^4 PMN CE/monolayer for PMNs exposed to 10 nM LXA_4, $P < 0.01$).

The concentration dependence of LXA_4 preexposure to PMN and subsequent PMN transepithelial migration in the apical-to-basolateral direction is shown in Fig. 2 *B*. Preexposure of PMN to LXA_4 concentrations in the range of 1.0 pM–10 nM for 15 min at 37°C elicited increased PMN transmigration at doses of 0.1, 1.0, and 10 nM final concentrations. Similar to the time course data presented above, LXA_4-elicited stimulation of PMN transmigration was manifest as an increase in the number of PMN in lower reservoirs (11.06±3.05 vs 20.38±4.83, 19.96±4.83, 15.43±4.65, 14.11±3.01, and 13.71±4.14 × 10^4 PMN CE/ml for vehicle control, and PMN LXA_4 preexposure doses of 10, 1, 0.1, 0.01, and 0.001 nM, respectively, for 15 min, 37°C, two-factor ANOVA, $P <$ 0.025).

To determine whether the stimulatory action of LXA_4 was present throughout the incubation period, PMNs were preexposed to LXA_4 (10 nM, 15 min), layered on the apical surface of T_{84} monolayers and driven to transmigrate basolaterally. Monolayers were harvested at various time points during transmigration and assayed for PMN by myeloperoxidase content. As shown in Fig. 3, the stimulatory effect of LXA_4 on PMN migration in the apical-to-basolateral direction was present by

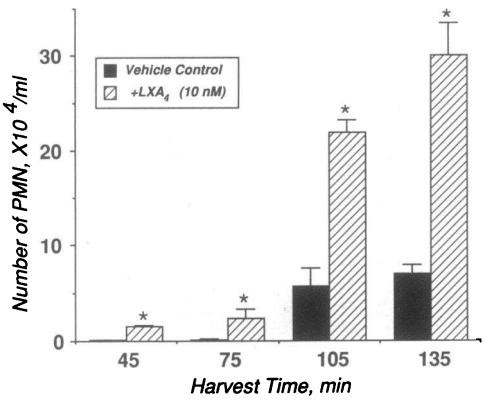

Figure 3. Lipoxin A_4 enhancement of PMN transepithelial migration in the apical-to-basolateral direction is present throughout the assay period. PMN (5 × 10^7/ml) were preincubated with 10 nM LXA_4 for 15 min at 37°C and layered on the apical surface of washed T_{84} epithelial monolayers at a density of 2 × 10^6/monolayer. PMN were driven to transmigrate basolaterally under the influence of a 1-μM gradient of FMLP. Monolayers were harvested at the indicated periods of time. Shown here are the results obtained from assaying the PMN-specific enzyme MPO, relative to a known standard number of PMN. Total MPO activity (including reservoir- and monolayer-associated MPO activity) is indicated. Data are pooled from six individual monolayers in each condition, and results are expressed as the mean and SEM. Asterix indicates statistically significant compared to vehicle control ($P < 0.001$).

45 min after addition of PMN (0.06±0.04 vs 1.51±0.12 × 10^4 PMN CE/ml for vehicle control and PMN exposed to LXA_4, respectively, $P < 0.05$), and was maintained throughout the 135-min experimental period (two-factor ANOVA, $P < 0.01$).

To assess whether preexposure of PMN to LXA_4 is reversible, PMN were incubated with 10 nM LXA_4 for 15 min, washed twice in Ca^{2+} and Mg^{2+}-free HBSS, and assessed for their ability to migrate across monolayers of T_{84} epithelial cells in the apical-to-basolateral direction. Here, PMNs exposed to LXA_4 did not differ from control in their ability to migrate across T_{84} epithelial monolayers (14.1±0.4 vs 14.5±0.3 × 10^4 PMN CE/ml for control and LXA_4 exposure followed by washout, respectively, $n = 6$ each, NS). In the presence of LXA_4, a total of 18.6±1.0 × 10^4 PMN CE/ml migrated ($n = 6$, $P < 0.025$ compared to control and washout control), suggesting that LXA_4-induced enhancement of PMN migration in the apical-to-basolateral direction requires the presence of LXA_4.

Finally, we determined whether PMN-conditioned LXA_4 and epithelial-conditioned LXA_4 maintained its ability to enhance PMN transmigration in the apical-to-basolateral direction. Samples of LXA_4 (10 nM) were incubated with either PMN or T_{84} epithelial cells for 15 or 45 min. Supernatants were harvested and subsequently exposed to PMN for 15 min and added to the apical surface of T_{84} monolayers under transmigratory conditions (1 μM FMLP transepithelial gradient) for 2 h at 37°C. Compared to PMN preexposed to HBSS (11.3±2.2 × 10^4 total PMN CE/ml), PMN preexposed to PMN-conditioned LXA_4 (15 min) resulted in a total PMN migration 18.0±2.1 × 10^4 PMN CE/ml ($n = 3$, $P < 0.05$ compared to control). PMN preexposed to epithelial-conditioned LXA_4 (15 min) resulted in a total PMN migration 16.8±3.3 × 10^4 PMN CE/ml ($n = 3$, $P < 0.05$ compared to control). Supernatants from PMN-conditioned LXA_4 (45 min) were not effective in enhancing PMN transmigration in the apical-to-basolateral direction (12.36±3.1 × 10^4 total PMN CE/ml compared to HBSS control of 14.3±3.2 × 10^4 total PMN CE/ml, $n = 3$, P = NS). These results suggest that enhancement of PMN migration in the apical-to-basolateral direction involves a step that is subsequent to PMN preincubation with LXA_4.

PMN preexposure to LXA_4 decreases FMLP-induced PMN transmigration in the basolateral-to-apical direction. We have recently reported that quantitative as well as qualitative differences can exist in PMN transepithelial migration depending on the direction of migration (19). To investigate the effect of LXA_4 on the polarity of transmigration, we prepared inverted monolayers (which permit basolateral-to-apically directed transmigration) to assess whether PMN preexposure to LXA_4 exhibited similar effects as shown above on subsequent PMN transmigration. Results in Fig. 4 show that preexposure of PMN to LXA_4 (10 nM) for 15 min markedly decreased PMN transmigration in the basolateral-to-apical direction. Unlike the results found in the apical-to-basolateral direction, transmigration of PMNs in this physiologically relevant direction was significantly decreased compared to vehicle controls (28.02±3.08 vs 18.77±1.48 × 10^4 PMN/ml for control and PMNs preexposed to 10 nM LXA_4 for 15 min, respectively, $P < 0.01$, Fig. 4). Transmigration in the basolateral-to-apical direction resulted in no significant difference in the number of monolayer-associated PMNs after preexposure to LXA_4 (Fig. 4, 2.01±0.20 vs 2.00±0.31 for control and PMN exposed to LXA_4, respectively, P = NS). This polarized action of LXA_4 was confirmed by performing parallel apical-to-basolateral and

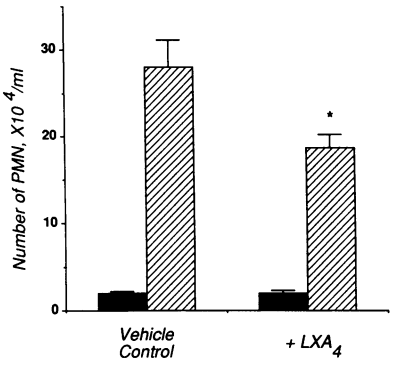

Figure 4. Lipoxin A_4 decreases PMN transepithelial migration in the physiologically relevant basolateral-to-apical direction. PMN (1×10^7/ml) were preincubated with 10 nM LXA_4 for 15 min at 37°C and layered on the basolateral surface of washed T_{84} epithelial monolayers (i.e., inverted monolayers) at a density of 4×10^5/monolayer. PMN were driven to transmigrate apically under the influence of a 1-μM gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme MPO from lower reservoirs and washed monolayers after 120 min, relative to a known standard number of PMN. Since tight junctions are the rate limiting barrier to passive paracellular permeation, transmigration is defined as movement of PMN across the tight junction. Since monolayer-associated PMN were largely below the tight junction (see Results), total transmigration in the basolateral-to-apical direction equates with PMN in the opposite reservoir only. Data are pooled from nine individual monolayers in each condition, and results are expressed as the mean and SEM. Asterix indicates statistically significant compared to vehicle control ($P < 0.01$). ■, Monolayer; ▨, reservoir.

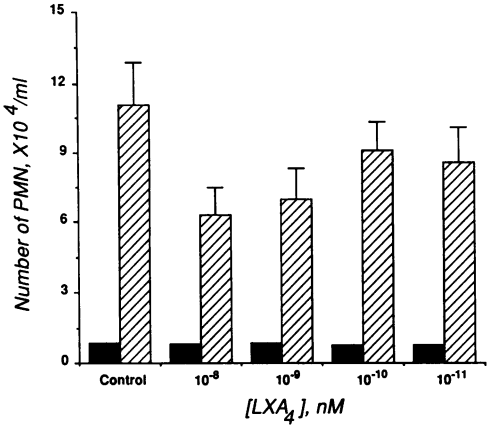

Figure 5. Concentration dependence of lipoxin A_4 on decreased PMN transepithelial migration in the basolateral-to-apical direction. PMN (1×10^7/ml) were preincubated with various indicated concentrations of LXA_4 for 15 min at 37°C and layered on the basolateral surface of washed T_{84} epithelial monolayers at a density of 4×10^5/monolayer. PMN were driven to transmigrate basolaterally under the influence of a 1 μM gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme MPO from lower reservoirs and washed monolayers after 120 min, relative to a known standard number of PMN. Data are pooled from 9–12 individual monolayers in each condition, and results are expressed as the mean and SEM. ■, Monolayer; ▨, reservoir.

basolateral-to-apical transmigration experiments using T_{84} cells from the same plating and same passage and using PMN from the same donors on three separate occasions.

A time course of LXA_4 preexposure to PMNs was next performed for basolateral-to-apical directed migration. The results revealed that, similar to the apical-to-basolateral direction, decreased transepithelial migration was present at 15 min of PMN preexposure to 10 nM LXA_4 (11.07 ± 1.83 for control vs $6.29 \pm 1.21 \times 10^4$ PMN/ml, $P < 0.01$). No differences in the number of monolayer-associated PMN were present at any period of LXA_4 exposure (data not shown). Dose-response experiments (all 15 min preexposure, Fig. 5) revealed that preexposure of PMN to 10 and 1 nM LXA_4 resulted in a significantly reduced number of transmigrating PMN in the basolateral-to-apical direction ($11.07 \pm 1.83 \times 10^4$ PMN/ml for control samples vs 6.29 ± 1.21 and $6.99 \pm 1.33 \times 10^4$ PMN/ml after preexposure to 10 and 1 nM LXA_4, respectively, both $P < 0.025$). Again, this diminished transmigratory response in the basolateral-to-apical direction was associated with reservoir-associated PMN only, with no apparent effect on the number of monolayer-associated PMN.

We also determined whether PMN-conditioned LXA_4 or epithelial-conditioned LXA_4 were effective in decreasing PMN transmigration in the basolateral-to-apical direction. Similar to our results in the apical-to-basolateral direction (see above), PMN preexposed to either PMN-conditioned LXA_4 (8.07 ± 1.63 vs buffer control $13.18 \pm 1.91 \times 10^4$ PMN/ml, $n = 4$, $P < 0.025$) or epithelial-conditioned LXA_4 (9.01 ± 1.76 compared to buffer control of $14.23 \pm 2.06 \times 10^4$ PMN/ml, $n = 4$, $P < 0.04$) maintained activity that decreased PMN transepithelial migration in the basolateral-to-apical direction.

Preexposure of PMN to structurally related lipoxins. To investigate the specificity of LXA_4 causing decreased transmigration in the physiological direction, we also examined the effects of PMN exposure LXB_4 and 11-*trans*-LXA_4 (Fig. 6). PMN's were preincubated with these compounds using conditions as described above (10 nM, 15 min, 37°C) and layered on T_{84} monolayers (physiologically oriented) under chemotactic conditions (1 μM FMLP in lower reservoir). Preexposure of PMNs to 10 nM LXB_4, 11-*trans*-LXA_4 produced a $7 \pm 4\%$ (P = NS compared to vehicle control) and $16 \pm 6\%$ ($P < 0.05$) inhibition of PMN transmigration, respectively, while LXA_4

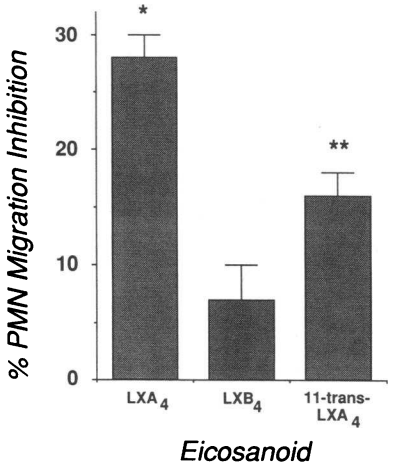

Figure 6. Comparison of lipoxin A_4 structurally related compounds on decreased PMN transepithelial migration in the basolateral-to-apical direction. PMN (1×10^7/ml) were preincubated with 10 nM LXA_4, LXB_4, or 11-*trans*-LXA_4 for 15 min at 37°C and layered on the basolateral surface of washed T_{84} epithelial monolayers (i.e., inverted monolayers) at a density of 4×10^5/monolayer. PMN were driven to transmigrate apically under the influence of a 1 μM gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme MPO after 120 min, relative to a known standard number of PMN. Total MPO activity (including reservoir- and monolayer-associated MPO activity) is expressed as the percent PMN migration inhibition. Data are pooled from seven individual monolayers in each condition, and results are expressed as the mean and SEM. Asterix indicates statistically significant compared to vehicle control (*$P < 0.01$; **$P < 0.05$).

inhibited migration by 28±4% ($P < 0.01$). These observations suggest structural specificity for LXA_4.

Effect of inhibitors on LXA_4-elicited enhancement of PMN transepithelial migration in the apical-to-basolateral direction. To determine whether LXA_4-induced modulation of PMN transmigration could be pharmacologically altered, a series of experiments were done in which PMN were exposed to specific inhibitors, washed free of inhibitor and subsequently assayed for the LXA_4 effect on PMN transepithelial migration in the apical-to-basolateral direction.

Preexposure of PMN to indomethacin (50 μM, 15 min, 37°C), a cyclooxygenase inhibitor (30), did not effect baseline PMN transmigration in the presence of a transepithelial gradient of FMLP (109±13% vehicle control, $n = 6$, P = NS compared to untreated control). Likewise, preexposure of PMN to indomethacin followed by exposure to LXA_4 (10 nM) did not alter the LXA_4-elicted increase in FMLP-driven PMN transmigration in the apical-to-basolateral direction (61±11% increase vs 54±7% increase over control for LXA_4-treated PMN with and without indomethacin, respectively, P = NS). In addition, PMN pretreatment with the compound MK886 (10 ng/ml), a specific inhibitor of leukotriene generation (31), did not alter baseline FMLP-driven PMN transmigration and did not effect the LXA_4-elicited increase in PMN transepithelial migration (data not shown).

Staurosporine, a potent inhibitor of protein kinase C (PKC) (32), was assessed for its ability to inhibit the LXA_4 effect. Interestingly, staurosporine alone (10 nM final concentration) was found to inhibit PMN transepithelial migration (93±5% inhibition vs vehicle control, $n = 3$, $P < 0.001$). These data were also confirmed using the PKC inhibitor H7 (33) (100 μM final concentration, 91±4% inhibition compared to vehicle control, $n = 3$, $P < 0.001$). Likewise, the LXA_4-elicited (10 nM) increment in transmigration was sensitive to staurosporine (89±7% inhibition compared to vehicle control, $n = 3$, $P < 0.001$). Preexposure of PMN to pertussis toxin (34) (2 μg/ml) also inhibited baseline FMLP-driven transmigration (91±8% inhibition compared to vehicle control, $n = 6$, $P < 0.001$). The LXA_4-elicited (10 nM) increase in PMN transmigration (46±3% increase compared to control, $P < 0.01$) was also sensitive to PMN preexposure to pertussis toxin (87±4% inhibition of control, $n = 6$, $P < 0.001$).

We next assessed the possibility of differential sensitivity to staurosporine for baseline and LXA_4-stimulated increases in FMLP-driven PMN transmigration. Staurosporine inhibited baseline PMN transepithelial migration in a dose-dependent manner (94±4%, 96±9%, 67±11%, 54±8%, 35±11%, and 11±6% inhibition compared to vehicle controls for concentrations of 100, 10, 1, 0.1, 0.01, and 0.001 nM staurosporine, respectively, $P < 0.01$ by ANOVA). From this dose response, we selected a concentration that was approximately half-maximal in inhibiting PMN transmigration (0.1 nM, see above). PMN were then preexposed to staurosporine (0.1 nM, 15 min, 37°C), washed free of inhibitor, and subsequently assessed for the LXA_4 effect on PMN transepithelial migration. Here, the LXA_4-elicited increase in transepithelial migration of PMN was observed to be sensitive to PKC inhibition, since the relative inhibition by staurosporine was equivalent with and without LXA_4 (54±6% and 49±7% decrease in total PMN transmigration for staurosporine-treated PMN in the presence and absence of LXA_4, respectively, P = NS; both decreased compared to staurosporine-untreated controls, $n = 6$, $P < 0.025$). These results indicate that LXA_4-elicited increases in PMN transmigration in the apical-to-basolateral direction are not sensitive to inhibition of the cyclooxygenase pathway or the specific inhibition of leukotriene generation, but is sensitive to inhibitors of PKC.

Discussion

During inflammatory processes, PMN are recruited from the blood by signals derived at inflammatory sites. At sites of acute inflammation, PMN function may be regulated by a variety of inflammatory signals, including both protein- and lipid-derived signals. PMN function at organ-specific sites, including the intestine, is thought to contribute to epithelial dysfunction during disease. Here we report for the first time that the arachidonic acid-derived eicosanoid LXA_4 modulates PMN migration across a model human intestinal epithelium. In addition, we report that LXA_4 exerts an effect on transmigration in a polarized fashion.

LXA_4 enhances PMN transepithelial migration in the apical-to-basolateral direction. For technical reasons, previous studies of PMN transepithelial migration have focused on "nonphysiologically" oriented monolayers, in which leukocyte migration is in the apical-to-basolateral direction (11, 13, 14, 19, 35). PMNs preexposed to LXA_4 and driven to transmigrate across epithelial monolayers oriented nonphysiologically resulted in enhanced PMN migration (Figs. 1 and 2). The action of LXA_4 was found to be specific for PMN but not epithelial cells, since enhanced PMN migration in this direction was dose- and time-dependent, and no measurable effects on PMN transepithelial migration were observed when epithelial monolayers were preexposed to LXA_4 under conditions that promoted enhanced PMN migration. These results are consistent with previous studies which report that LXA_4, in similar concentrations used here, was capable of activating PMN in vitro (36). In our model system, LXA_4 enhanced PMN transmigration in a manner independent to that of FMLP, since in all conditions PMN migration was driven toward a gradient of FMLP, suggesting that the proportion of PMN migration exceeding that of FMLP controls is dependent on a LXA_4-mediated event. Moreover, these results suggest that the action of LXA_4 may be synergistic with FMLP, since LXA_4 by itself does not promote PMN migration in the absence of FMLP (see Results).

We have also found that preexposure of PMN to LXA_4 modulates migration of PMN in a polarized manner. That is, an opposite effect was observed depending on the direction of PMN migration. The observed effect of LXA_4 inhibition of PMN transmigration in the physiologically oriented (basolateral-to-apical) direction was dependent on concentration, as well as the duration of preexposure (Figs. 4 and 5). These effects were found to be selective for LXA_4, since no effect was observed with the positional isomer LXB_4 (Fig. 6). As described for leukocyte movement across endothelia (38), PMN migration across epithelial monolayers is likely a multistep process requiring engagement and disengagement of several receptor-ligand complexes between PMN and epithelial cell. The specific events involved in PMN transepithelial migration are poorly understood at the present time, but in part requires the PMN β2 integrin CD11b/CD18 and are independent of intercellular adhesion molecule-1 (14). In addition, PMN transepithelial migration can be regulated by exposure of T_{84} epithelial

monolayers to the lymphokine IFN-γ (19). In light of the polarized nature of this epithelium (17, 18, 37), it would not be surprising that the sequence by which PMN encounters epithelial ligands directly regulates PMN transmigration. Moreover, PMN migration across endothelia requires a sequential series of activation and deactivation steps on the PMN surface, of which lipid-derived activating factors may play an important role (reviewed in reference 38). Whether LXA_4 could act as a lipid-derived factor for expression of a crucial ligand in the regulation of PMN transmigration across epithelia is not known. Evidence to support this hypothesis is provided by a recent study characterizing lipoxin binding sites on human PMN (39). With a reported K_d of 0.5 nM and ~ 1,800 binding sites per cell, the range of LXA_4 concentrations used in the present study (0.01–10 nM) should provide maximal activation of subsequent signal transduction steps, most of which remain to be elucidated, but appears to involve a G protein–associated activation step (39) and possibly a signaling step through PKC as determined by inhibition using staurosporine (see Results).

Recent in vivo studies have shown that LXA_4 is an important inflammatory mediator at several distict anatomic sites, including the lung (28), kidney (40), blood vessel (41), and hamster cheek pouch (26). The data reported here suggest that LXA_4-elicited alterations exert effects at the level of PMN and subsequently modulate PMN-epithelial interactions. Previous studies have shown that production of lipoxygenase products of arachidonic acid correlates with intestinal inflammation. Specifically, an enhanced conversion of arachidonate to 5-, 12- and 15-hydroxy-eicosatetraenoic acid (HETE) has been shown in ulcerative colitis homogenates (42) as well as increased biosynthesis of LTB_4 in Crohn's disease (43). The present results suggest that lipoxins, and specifically by their action on PMN, may play a role in intestinal disease.

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