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*J Immunol* 2012; 189:4960-4969; Prepublished online 8 October 2012; doi: 10.4049/jimmunol.1201922
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Hepoxilin A3 Facilitates Neutrophilic Breach of Lipoxigenase-Expressing Airway Epithelial Barriers

David L. Tamang,†‡, Waheed Pirzai,† Gregory P. Pribe,‡ David C. Traficante,‡ Gerald B. Pier,‡ John R. Falck,§,** Christophe Morisseau,‖ Bruce D. Hammock,‖ Beth A. McCormick,‡ Karsten Gronert,** and Bryan P. Hurley*†,

A feature shared by many inflammatory lung diseases is excessive neutrophilic infiltration. Neutrophil homing to airspaces involves multiple factors produced by several distinct cell types. Hepoxilin A3 is a neutrophil chemoattractant produced by pathogen-infected epithelial cells that is hypothesized to facilitate neutrophil breach of mucosal barriers. Using a Transwell model of lung epithelial barriers infected with Pseudomonas aeruginosa, we explored the role of hepoxilin A3 in neutrophil transepithelial migration. Pharmacological inhibitors of the enzymatic pathways necessary to generate hepoxilin A3, including phospholipase A2 and 12-lipoxygenase, potently interfere with migration. Both transformed and primary human lung epithelial cells infected with P. aeruginosa generate hepoxilin A3 precursor arachidonic acid. All four known lipoxigenase enzymes capable of synthesizing hepoxilin A3 are expressed in lung epithelial cell lines, primary small airway epithelial cells, and human bronchial epithelial cells. Lung epithelial cells produce increased hepoxilin A3 and lipid-derived 12-HETE, 12-hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid; HXA3, hepoxilin A3;

Abbreviations used in this article: ARA, arachidonic acid; BAL, bronchoalveolar lavage; CDC, cinnamyl 3,4-dihydroxy-o-cyanocinnamate; DAG, diacyl glycerol; 12-HETE, 12-hydroxyeicosatetraenoic acid; HXA3, hepoxilin A3; LC, liquid chromatography; LDH, lactate dehydrogenase; LO, lipoxigenase; LTB4, leukotriene B4; MPO, myeloperoxidase; MS/MS, tandem mass spectrometry; PAF, platelet activating factor; PLA2, phospholipase A2; PMN, polymorphonuclear cell; SAEC, small airway epithelial cell; sEH, soluble epoxide hydrolase.

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they release caustic molecules that result in gross tissue damage (3, 5). Neutrophils are among the first responders in innate immunity, rapidly deployed to sites of infection to confront a variety of pathogens (5–7). However, without appropriate resolution, neutrophils can exacerbate pathology during infectious and idiopathic inflammatory processes; such is the case in both acute and chronic lung diseases, including pneumonia, cystic fibrosis, and severe bouts of asthma (3, 6–8).

To reach the airspace during infection, neutrophils must exit the circulatory system of the lung, navigate through the extracellular milieu, and ultimately cross the mucosal epithelial barrier. Evidence points to a tiered signaling hierarchy that mediates neutrophil adhesion, initial migration, deep tissue homing, and entry into organ spaces (9, 10). In this study, we examine the process by which neutrophils migrate across lung epithelial barriers. Our group previously identified the eicosanoid hepoxilin A3 (HXA3) as a potent chemoattractant that drives neutrophil migration across gut and lung epithelial barriers (11–15). HXA3 likely interacts directly with neutrophil receptors and induces chemotaxis without granule secretion or superoxide production (15–17).

A diverse array of biological functions is exhibited by the group of lipid mediators known as eicosanoids, particularly during inflammatory processes (18). Eicosanoids, such as HXA3, are generated through the liberation of arachidonic acid (ARA) from membrane phospholipids catalyzed by phospholipase A2 (PLA2) (16). Lipoxigenases (LOs) represent an enzyme family that converts ARA into a discrete subset of bioactive lipid eicosanoids (19). The 12-LO enzymatic activity (as defined by the site of oxygen addition to ARA) is a prerequisite for HXA3 synthesis and is known to be exhibited by at least three LO proteins identified in humans (16, 20, 21). The catalytic efficiency varies be-

The Journal of Immunology, 2012, 189: 4960–4969.
between each enzyme and potentially between tissues where enzymes are expressed. Three human genes that encode enzymes with 12-LO activity include elox12, elox15, and elox12B, which encode 12-LO, 15-LO, and 12(R)-LO, respectively (19, 22–25).

In the current study, we examined the prevalence of pathogen-induced, HXA3-mediated polymorphonuclear cell (PMN) trans-epithelial migration in a variety of lung models. We used multiple human lung epithelial carcinoma cell lines, normal bronchial epithelial cells transformed by adenovirus, primary lung epithelial cells, and in vivo mouse model of lung infection to explore the role of HXA3 in mediating PMN transepithelial migration. We analyzed lung epithelial cell models for expression of 12-LOs and evaluated the efficacy with which multiple distinct strategies to target HXA3 impact pathogen-induced PMN transepithelial migration. We also explored whether mice produce 12-LO products in the lung when infected with pathogen. Our studies provide further evidence for the hypothesis that HXA3 mediates PMN transepithelial migration in response to infection and likely contributes to PMN infiltration of the airspace.

Materials and Methods

Bacterial strains

*P. aeruginosa* strain PAO1 and nonpathogenic *Escherichia coli* K12 strain MC1000 were grown aerobically in Luria–Bertani broth overnight at 37°C. For infection of epithelial cells, overnight cultures were washed once in HBSS and resuspended at a concentration of 6 × 10⁶ bacteria/ml of HBSS.

Cell culture

The source and description of lung epithelial cell lines maintained in various media with antibiotics are described in Table I. Polyanized monolayers were grown on the underside of 0.33-cm² collagen-coated Transwell filters to study PMN migration in the physiological basolateral to apical direction, as previously described (11–14). Epithelial barriers derived from each cell line were restrictive to the movement of small proteins, as determined by measurement of HRP flux across each epithelial barrier (12).

PMN isolation

PMNs (polymorphonuclear leukocytes or neutrophils) were isolated from human blood treated with acid citrate/dextrose (Massachusetts General Hospital Institutional Review Board protocol #1999-P-007782). The buffy coat was recovered by centrifugation. Plasma and mononuclear cells were removed by aspiration, and the majority of the RBCs were removed using 2% gelatin sedimentation. Residual RBCs were removed by lysis in cold NH₄Cl lysis buffer. After lysis, cells were washed, counted, and resuspended in HBSS(−) at a concentration of 5 × 10⁶ cells/ml (11–14).

PMN transepithelial migration assay

Transwell inserts containing lung epithelial cell monolayers seeded on the underside were exposed to 25 μl 6 × 10⁶ bacteria/ml for 1 h (11–14). Postinfection, PMNs (1 × 10⁶) were added to the top (basolateral) chamber and incubated for 2 h at 37°C. PMNs that fully migrated across the cell monolayer and reached the bottom (apical) chamber were quantified by the myeloperoxidase (MPO) assay. Uninfected lung epithelial monolayers or monolayers infected with nonpathogenic *E. coli* strain MC1000 serve as negative controls for PMN transmigration. Establishment of a concentration gradient of PMN chemoattractant FMLF (100 nM added to the apical chamber of uninfected monolayers at the same time that PMNs are added to the basolateral chamber) serves as a positive control for the ability of freshly isolated PMNs to migrate (26).

Cell viability/barrier-integrity assays

The amount of lactate dehydrogenase (LDH) released into the supernatant with and without infection of PAO1 in the presence or absence of each of the inhibitors used was quantified using the LDH-based In Vitro Toxicology Assay Kit (Sigma, St. Louis MO). Barrier integrity of lung epithelial monolayers grown on Transwells was assayed by the HRP flux assay, as described previously (12).

Inhibitors

Lung epithelial cells were pretreated for 1–2 h with each inhibitor, followed by washing prior to infection and/or addition of PMNs for each assay. PLA₂ inhibitor (ONO-RS-082), LO inhibitor (cinnaamyl 3,4-dihydroxy-c-cyanocinnamate [CDC]), diacetyl glycerol (DAG) lipase inhibitor (RHC-80267), and cyclooxygenase inhibitor (NS-398) were purchased from Enzo Life Sciences (Farmingdale, NY). For experiments involving stable HXA3 analogs (PN-II-218-36 and MV-I-237-20), they were added at various concentrations to the apical well of the infected Transwells at the same time that the PMNs were added to the basolateral well; PMNs that migrated across the monolayers in the presence or absence of apically applied analogs were quantified by MPO. Stable HXA3, ether analogs were synthesized in the Falk laboratory (P. Narendra, N. Rami Reddy, V.L. Manthant, and J.R. Falk, manuscript in preparation). The inhibitors presented in this study did not affect cell viability either in the presence or absence of bacterial infection as assessed by both the LDH-release assay and the barrier-integrity assay (HRP flux). None of the compounds had any major effect on the amount of bacteria adhering to the lung epithelial monolayers.

**PGE₂ enzyme immunoassay**

Lung epithelial cells were grown to confluence in 24-well plates and used 5–7 d after seeding. Lung epithelial cells were pretreated with inhibitors for 1 to 2 h, washed, and infected with 6 × 10⁶ bacteria/ml for 1 h at 37°C. Each well was washed three times in HBSS, followed by incubation at 37°C for 2 h. Supernatants were collected, and the amount of PGE₂ in each well was quantified using the PGE₂ Express EIA Kit from Cayman Chemical (Ann Arbor, MI) (11).

**ARA-release assay**

Lung epithelial cells were grown to confluence in 24-well plates and used 5–7 d after seeding (14). Cells were washed three times with PBS(−), treated with media containing 0.2 μC/ml ³H[ARA], and incubated for 18–24 h. Cells were then washed three times to remove unincorporated ³H[ARA] and treated with 0.5 ml bacteria (6 × 10⁷ bacteria/ml). Following infection, cells were incubated at 37°C for up to 6 h. Supernatants (100 μl) were collected at 2, 4, and 6 h, and radioactivity was measured by scintillation counting. After collection of supernatant, cells were lysed with 500 μl/well 1% SDS, 1% Triton X-100 and sampled (250 μl) for measurement by scintillation counting. Data are displayed as the percentage cpm of the total, measured in the supernatant at each time point (% ARA release = [released/released + cell associated] × 100). Lung epithelial cell viability was maintained during the 6-h infection, as determined by the LDH-release assay.

**RT-PCR**

Lung epithelial cells were grown in six-well plates to confluence. Cells were lysed, and RNA was purified using the Aurum total RNA mini kit (Bio-Rad). The RNA concentration was standardized to 0.1 μg/ml. Before use, samples were treated with RQ1 DNase (Promega), according to the manufacturer’s protocols. cDNA was generated from 2.5 μl clean RNA using the iScript kit (Invitrogen). cDNA (2 μl) was amplified using the iTag PCR kit (Bio-Rad) and primers specific to the genes gapdh, elox12, elox15, elox12B, and elox13 (Table II). Primers were synthesized at the Massachusetts General Hospital DNA Core facility. The amplified product was run on a 1.5% agarose gel containing 100 μg/ml ethidium bromide (Bio-Rad) and imaged under a UV light. The expected product sizes listed in Table II were confirmed using EZ Load 100 bp PCR Molecular Ruler (Bio-Rad).

**Extraction of lipids from supernatants**

Lung epithelial cells were seeded on 25-cm² flasks (small airway epithelial cells, H292) or 162-cm² flasks (H292 and BEAS-2B) and grown to confluence. Confluent cell monolayers were treated or not with 6 × 10⁶ bacteria/ml HBSS for 1 h at 37°C, washed three times with HBSS, and incubated for an additional 2 h at 37°C. Supernatants were collected, acidified to pH 4, and, in experiments involving eicosanoid quantification, mixed with lipid extraction standards [leukotriene B₄ ([LTB₄]-d₄/15(S)-HETE-d₄, Cayman Chemical] (27, 28). Acidified supernatants were poured through a Supelco Discovery DSC-18 SPE Column and eluted with methanol. The lipid fraction suspended in methanol was dried under a stream of nitrogen to 100 μl methanol and stored at −80°C until processed further (11).

**Measurement of lipid-associated PMN chemoattractant activity**

Each extracted lipid sample for H292 lung epithelial cells, with or without PAO1 infection (prepared in triplicate and stored at −80°C), was dried...
under a stream of nitrogen and resuspended in 1.7 ml HBSS. One milliliter of resuspended extracted lipid supernatants (referred to as straight) was added to the apical well of a Transwell containing H292 lung epithelial barrier. A volume of 0.6 ml resuspended lipids was diluted in 1.2 ml HBSS (referred to as “diluted 1:3”). One milliliter of diluted 1:3 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. The remaining 0.8 ml diluted 1:3 resuspended lipids was further diluted by adding 0.8 ml HBSS (referred to as “diluted 1:6”). One milliliter of diluted 1:6 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. The remaining 0.6 ml diluted 1:6 resuspended lipids was further diluted by adding 0.6 ml HBSS (referred to as “diluted 1:12”). One milliliter of diluted 1:12 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. Lipids extracted from BEAS-2B and SAEC supernatants that were treated or not with PAO1 (prepared in triplicate and stored at −80˚C) were resuspended in 1 ml HBSS after drying the remaining methanol with a stream of nitrogen. Resuspended lipid extracts were then added to the apical well of a Transwell containing H292 lung epithelial barrier. PMNs (1 × 10⁶) were added to the top (basolateral) chamber in a volume of 120 µl for all diluted resuspended lipid samples that were added to the bottom (apical) chamber. Transwells were then incubated at 37˚C for 2 h, and PMNs that fully migrated across the cell monolayer and reached the bottom (apical) chamber were quantified by the MPO assay (11).

Assessment of soluble epoxide hydrolase sensitivity
Lipid extracts from the supernatant of lung epithelial cells in the presence or absence of PAO1 infection (prepared in triplicate and stored at −80˚C) were resuspended in 1 ml HBSS after removing methanol by drying under a stream of nitrogen. Each sample was split into two 0.5-ml fractions. To one of the two fractions, 3.5 µl soluble epoxide hydrolase (sEH; 10 mg/ml) was added (29, 30). Fractions with and without sEH were mixed at 100 rpm for 2 h at 30˚C. After incubation, 0.7 ml HBSS was added to each fraction and mixed, and 1 ml mixed fraction was added to the apical well of a Transwell containing H292 lung epithelial barrier. PMNs (1 × 10⁶) were added to the top (basolateral) chamber in a volume of 120 µl for all diluted resuspended lipid samples, with or without sEH treatment, that were added to the bottom (apical) chamber. Transwells were then incubated at 37˚C for 2 h, and PMNs that fully migrated across the cell monolayer and reached the bottom (apical) chamber were quantified by the MPO assay. HXA3 and 12-hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid (12-HETE) were quantified after lipid extraction from either human lung epithelial cell supernatants or bronchoalveolar lavage (BAL) fluid of mice by liquid chromatography (LC)/tandem mass spectrometry (MS/MS)-based lipidomics (11). In brief, extracted samples were analyzed by a triple quadruple linear ion trap LC/MS/MS system (Waters Acquity UPLC UFLC 2690) equipped with a LUNA C18-2 mini-bore column using a mobile phase gradient of water/acetonitrile/acetic acid (72:28:0.01, v:v:v) and isopropanol/acetonitrile (60:40, v:v) with a 0.50-ml/min flow rate (31). MS/MS analyses were carried out in negative ion mode, and prominent fatty acid metabolites were quantified by multiple reaction monitoring using established transitions for HXA3 (335→127 m/z), 12-HETE (319→179 m/z), LTB₄-d₄ (339→197 m/z), and 15-HETE-d₄ (327→182 m/z). Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical). Structures were confirmed for selected autocoids by MS/MS analyses using enhanced product ion mode, with appropriate selection of the parent ion in quadrupole 1.

Mouse model of acute pneumonia
C3H/HeN mice (6–8 wk old), purchased from Harlan Laboratories, were injected i.p. with anesthesia (ketamine + xylazine) prior to intranasal challenge with 4 × 10⁷ CFU P. aeruginosa (PA01) or PBS control (10 µl into each nostril) (32, 33). Five mice/treatment were used for each individual experiment. After 18 h, mice were euthanized by CO₂ asphyxiation in accordance with an Institutional Animal Care and Use Committee-approved protocol.

Collection of BAL fluid
BAL was performed at 18 h postinfection using 1 ml 0.5 mM EDTA in PBS without calcium or magnesium and repeated three times. Fluid was centrifuged (220 × g) for 5 min, and harvested cells were washed with ammonium chloride lysis buffer to remove RBCs. Total cell counts were determined by hemocytometer following dilution in trypan blue-containing buffer (32, 33). MPO activity of BAL fluid cells was assessed as previously described (12). For measurement of eicosanoids 12-HETE and HXA3, supernatants from centrifuged BAL fluid were subjected to the lipid-extraction protocol with standards and quantified by LC/MS/MS, as described above.

Statistics
Data displayed for each figure are presented as a representative experiment with a mean (SD) of at least three independent data points/condition. Each experiment was repeated multiple times yielding similar results. Statistical analysis was performed using a two-tailed unpaired Student t test for each internally controlled experiment; p values < 0.05 were considered significant.

Results
A diversity of human lung epithelial cell models was examined to determine whether they were capable of forming barriers on Transwells and whether they facilitate pathogen-induced PMN transepithelial migration. The lung epithelial models include carcinoma cell lines, as well as transformed normal bronchial epithelial cells (Table I). All cell lines form functional barriers, as confirmed by the restriction of HRP movement across each monolayer of A549, Calu-3, H292, and BEAS-2B cells grown on Transwells (data not shown) (12). To investigate whether P. aeruginosa (PA01) is capable of instigating PMN transepithelial migration across each of these barriers, the apical surface of each monolayer was treated with HBSS or infected with either pathogenic PA01 or nonpathogenic E. coli (MC1000). After 1 h, non-adherent bacteria were washed away, and PMNs were added to the basolateral side (12, 26). The number of PMNs that migrated from the basolateral to the apical well after incubation for 2 h was determined by quantification of MPO activity. We found a consistent trend in PMN transepithelial migration among all of the lung epithelial barriers tested (Fig. 1A). Few PMNs migrated across uninfected monolayers or monolayers infected with MC1000. In contrast, infection with PA01 resulted in a robust and highly significant increase in the numbers of PMNs that migrated across lung epithelial barriers. We also observed that a significant number of PMNs migrated across uninfected monolayers derived from each of the cell lines in response to an imposed chemotactic gradient of FMLF. This served as a positive control for the ability of isolated PMNs to migrate toward a chemotactic gradient in each assay with each distinct cell line monolayer (26). These data are consistent with observations previously reported for A549 and Calu-3 cells, suggesting that PA01-induced PMN transepithelial migration is a highly reproducible phenomenon observed using a range of distinct lung epithelial cell barrier models (12, 14).

Inhibitors of 12-LOs and PLA₂ were previously observed to potently interfere with PAO1-induced PMN migration across A549 barriers (11, 12, 14). To expand the investigation of this phenomenon in alternative lung epithelial cell models, we established monolayers of a distinct carcinoma lung epithelial cell line (H292) and a transformed normal bronchial epithelial cell line (BEAS-2B) (Table I). Prior to treatment with buffer or infection with bacteria, monolayers were treated for 1–2 h with pharmacological inhibitors of eicosanoid metabolic enzymes. ONO-RS-082 and CDC inhibit PLA₂ and 12-LOs, respectively. Both enzymatic activities are crucial for the generation of the PMN chemotractant HXA3. The cyclooxygenase inhibitor NS-398 interferes with synthesis of a group of eicosanoids unrelated to the synthesis of HXA3 known as PGs, which are bioactive but do not serve as PMN chemoattractants (34, 35). The DAG lipase inhibitor RHC-80267 was previously shown not to impact PAO1-induced epithelial migration across A549 monolayers (14). DAG lipase is an alternative means of generating the eicosanoid pre-
cursors ARA from DAG, rather than from membrane phospholipids, as is the case with PLA2 (36).

Pretreatment of H292 and BEAS-2B lung epithelial monolayers with either a 12-lipoxygenase inhibitor (CDC) (37) or a PLA2 inhibitor (ONO-RS-082) (38) potently blocked PAO1-induced PMN transepithelial migration, indicating a key role for these epithelial enzymatic activities in facilitating pathogen-induced PMN transepithelial migration (Fig. 1B, 1C). Neither inhibitor interfered with migration across uninfected monolayers in response to an imposed FMLF gradient, indicating that the general ability of PMNs to move across epithelial monolayers is not impacted by pretreatment with either inhibitor. These studies are consistent with previous observations using A549 lung epithelial monolayers (12, 14). Also consistent with previous studies involving A549 monolayers, the DAG lipase inhibitor had no significant affect on PAO1-induced PMN migration across H292 and BEAS-2B monolayers (Fig. 1B, 1C) (14). The inhibitor of the cyclooxygenase pathway (NS-398), an eicosanoid metabolic pathway unrelated to HXA3 synthesis, exerted no significant impact on PAO1-induced PMN transepithelial migration (Fig. 1B, 1C). We previously demonstrated that PAO1 infection of lung epithelial cells results in a significant increase in the release of PGs, specifically PGE2 (11). Importantly, the concentration of NS-398 used in the PMN-migration experiments (Fig. 1B, 1C) was sufficient to prevent PAO1-induced PGE2 release (Fig. 1D), confirming the effectiveness of the pharmacological inhibitor in our assay. Pretreatment of H292 cells with the 12-LO inhibitor CDC had no effect on PGE2 release, as would be expected. However, at an equivalent concentration, CDC is capable of interfering with PAO1-induced HXA3 release, as previously reported (12). Migration in response to HBSS alone or infection with MC1000 was minimal for all conditions tested (Fig. 1B, 1C).

The rate-limiting step in eicosanoid synthesis is often attributed to the generation of ARA (39, 40). We previously demonstrated that infection of A549 lung epithelial cells with PAO1 resulted in a significant release of ARA (11, 14). We examined ARA release from a distinct transformed human lung epithelium cell line (H292), as well as from primary SAECs, after 2, 4, and 6 h of stimulation with PAO1 or MC1000 (Fig. 2A, 2B). The results indicated a significant increase in ARA release by both H292 and SAEC epithelium treated with pathogenic PAO1 over time relative to untreated epithelial cells or epithelium treated with nonpatho-

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** PMN migration across *P. aeruginosa*-treated lung epithelial barriers is diminished following inhibition of the HXA3 synthetic pathway. (A) Four distinct lung epithelial cell lines (Table I: A549, Calu-3, H292, and BEAS-2B) grown on Transwells to form epithelial barriers were treated with either *P. aeruginosa* strain PAO1 or K12 *E. coli* strain MC1000. Monolayers incubated in HBSS alone served as a negative control. A chemoattractant gradient of FMLF (100 nM) was established to serve as a positive control for PMN movement. *Statistically significant difference compared with negative controls (HBSS and MC1000). (B and C) Transwells were pretreated with pharmacological inhibitors CDC (12-LO inhibitor; 50 μM), ONO-RS-082 (PLA2 inhibitor; 5 μM), RHC-80267 (DAG lipase inhibitor; 50 μM), or NS-398 (COX inhibitor; 50 μM) or vehicle control (1:1000 DMSO) prior to infection. The number of PMNs that migrated across epithelial barriers H292 (B) and BEAS-2B (C) was assessed in response to infection with PAO1, MC1000, or uninfected controls (HBSS/FMLF), with or without pretreatment of each pharmacological inhibitor. *Statistically significant difference within a treatment group comparing inhibitor with vehicle control. (D) The amount of PGE2 released (ng/ml) by H292 cells in the presence or absence of PAO1 infection, with or without pharmacological inhibitor pretreatment, was quantified by enzyme immunoassay. Each data point represents the average of at least three separate wells. Each experiment was performed on at least three separate occasions, yielding similar results. *p < 0.05, uninfected (HBSS) versus PAO1 infected within a treatment group, two-tailed unpaired Student t test.

<table>
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<th>Cell Line</th>
<th>Description</th>
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<th>Media</th>
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<td>Lonza® (CC-2647)</td>
<td>SAGM® (BulletKit)</td>
</tr>
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*American Type Tissue Culture, Manassas, VA.
Lonza Walkersville, Walkersville, MD.
Invitrogen, Carlsbad, CA.

Table I. Cell lines used in this study

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**TABLE I.** Cell lines used in this study
genic MC1000 (Fig. 2A, 2B). These results are consistent with the hypothesis that PAO1 infection induces increased cellular generation of eicosanoids.

Our studies suggest that 12-LO activity is critical for PAO1-induced PMN transepithelial migration. There are three human LOs that exhibit 12-LO activity: 12-LO (gene *alox12*), 12(R)-LO (gene *alox12B*), and 15-LO (gene *alox15*) (19, 21, 25). The 12-LOs convert ARA to HXA₃ via the short-lived intermediate 12-HpETE (20). In the case of 12(R)-LO, an additional enzyme eLOX₃ (gene *elox3*), serving as a hepoxilin synthase, is required to convert 12-HpETE to HXA₃ (19, 22). A full panel of lung epithelial models described in Table I, including multiple cell lines, primary normal human bronchial epithelial cells, and primary SAECs, were evaluated for expression of all known LO genes involved in direct synthesis of HXA₃. Specific primers to amplify *alox12*, *alox15*, *alox12B*, and *elox3* were designed and used to analyze gene expression by RT-PCR (Table II). We found that all four 12-LO genes were expressed in every lung epithelial model investigated, suggesting that lung epithelial cells express the genes necessary to synthesize the PMN chemoattractant HXA₃ (Fig. 2C).

Our hypothesis predicts that lung epithelial cells synthesize and release HXA₃ in response to infection with PAO1, leading to directed PMN migration across infected epithelial barriers. Because HXA₃ is an eicosanoid, its chemotactic potential is expected to be enriched in the lipid-extracted fraction of supernatants from lung epithelial cells infected with PAO1. To determine whether H292 lung epithelial cells produce increased lipid-associated chemotactic activity in response to PAO1 infection, H292 cells were treated with PAO1, after which supernatants were collected, acidified, and passed through a C18 column, followed by elution with methanol. Methanol fractions were dried under a stream of nitrogen and resuspended in HBSS for assessment of PMN chemotactic potential by establishing a gradient of the fraction at various dilutions across epithelial monolayers grown on Transwells (Fig. 3A, 3B). PAO1 clearly induced significantly more lipid-associated chemotactic activity compared with either uninfected H292 cells (Fig. 3A) or H292 cells infected with the nonpathogenic *E. coli* strain MC1000 (Fig. 3B). The observation that PAO1 is capable of significantly increasing PMN chemotactic activity in the lipid fraction of lung epithelial cell supernatant was not restricted to the H292 cell line. The normal bronchial transformed cell line BEAS-2B (Fig. 3C), as well as the primary SAECs (Fig. 3D), also released significantly greater lipid-associated PMN chemotactic activity when cells were infected with PAO1, as assessed by quantifying the number of PMNs migrating across an H292 barrier in response to gradients of supernatant-derived lipid-enriched fractions from infected and uninfected BEAS-2B and SAEC cells.

Our previous studies demonstrated that the lung epithelial cell line A549 releases the PMN chemotactic eicosanoid HXA₃ in response to infection with PAO1 (12). We observed that PAO1 infection of H292 cells results in a >5-fold increase in the amount of HXA₃ released, as quantified by LC/MS/MS (2.6 ± 1.9 pg [uninfected] versus 14.4 ± 0.9 pg [PAO1 infected]). This observation is consistent with our finding of increased lipid-associated chemotactic activity following PAO1 exposure (Fig. 3A). We used the enzyme sEH to determine whether HXA₃ contributes to the increased lipid-associated chemotactic activity described above (29). A unique structural feature of HXA₃ among PMN lipid chemotactic factors is that it possesses an epoxide group (16). The enzyme sEH is capable of hydrolyzing the epoxide group and converting hepoxilins into trioxilins, which lack PMN chemotactic activity (41). Lipid-associated fractions from conditioned supernatant of H292 cells treated or not with PAO1 were subjected to treatment with sEH prior to analysis of chemotactic potential. As expected, lipid-extracted conditioned supernatants from PAO1-treated H292 cells exhibited substantial chemotactic activity, whereas very little chemotactic activity was observed from lipid-extracted conditioned supernatants from uninfected H292 cells (Fig. 3E). The amount of chemotactic activity derived from lipid-extracted conditioned supernatants of PAO1-infected H292 cells was significantly reduced when extracts were pretreated with sEH, suggesting that the chemotactic factor responsible for directing PMNs to move across epithelial monolayers was sensitive to sEH treatment (Fig. 3E). Such results are consistent with the hypothesis that HXA₃ is a lipid-associated chemotactic factor that is responsible for PAO1-induced PMN transepithelial migration (12).

Thus far, our evidence suggests that lung epithelial cells produce HXA₃ in response to infection with *P. aeruginosa*, and this molecule serves as a PMN chemoattractant that is hypothesized to guide PMNs across epithelial monolayers. We next used structural analogs of HXA₃, whereby the epoxide was replaced with ether, which is designed to be stable yet nonfunctional. Elimination of the epoxide abolishes chemotactic activity based on observations that trioxilins, HXA₃ metabolites lacking the epoxide, no longer possess PMN chemotactic activity (15). Further, it is believed that the epoxide group contributes to the relative instability of HXA₃, and removal generates a more stable molecule (16). One of the analogs (PN-II-218-36) was designed to competitively inhibit

**FIGURE 2.** ARA release following *P. aeruginosa* infection and LO gene expression in lung epithelial cell models. The lung epithelial cell line H292 (A) or primary lung epithelial cells (SAECs) (B) (Table I) were incubated overnight with [³H]ARA, followed by washing and treatment with HBSS alone, infection with *P. aeruginosa* strain PAO1, or infection with *K. pneumoniae* strain MC1000. Supernatants were sampled at 2, 4, and 6 h post-treatment/infection. The percentage of ARA release represents the amount released into the supernatant as a percentage of the total (ARA released + cell-associated ARA). Each symbol represents an average of at least three separate occasions, yielding similar results. (C) RNA was extracted from a panel of human lung epithelial cells (Table I) to assess gene expression by RT-PCR, shown in this figure in duplicate sample wells. The known human 12-LOs (alox12, alo15, and alo12B) and the hepoxilin synthase (elox3) were analyzed individually using gene-specific primers (Table II). *p < 0.05, statistically significant increase compared with HBSS control for the same time point, two-tailed unpaired Student t test.
HXA₃ chemotactic activity, because it is structurally identical to HXA₃ with the exception that the epoxide is replaced with ether. In light of the well-known ω-hydroxylation of HXA₃, a second compound (MV-I-237-20) bearing an additional hydroxyl group on the C(20)-position was also prepared to address this contingency (42, 43). A gradient of HXA₃ added exclusively to the apical well of lung epithelial monolayers drove >30% of PMNs added to the basolateral well across the monolayer (Fig. 4A), consistent with previous reports (12, 13). At the same concentration, neither HXA₃ analog exhibits any PMN chemotactic activity (Fig. 4A). When the compound PN-II-218-36 was added to the apical side of PAO1-infected lung epithelial monolayers, PMN transepithelial migration was impeded in a dose-dependent manner, with complete inhibition at the addition of 156 µg/ml PN-II-218-36 (Fig. 4B, black bars). The structurally less similar analog MV-I-237-20 was not as effective at interfering with PMN transepithelial migration (Fig. 4B, gray bars). The effect of PN-II-218-36 appears to be selective to PAO1-induced migration, because PMNs migrating toward an fMLF gradient were significantly less impacted by the presence of analog PN-II-218-36 (Fig. 4C).

P. aeruginosa-induced acute pneumonia models have been widely used to investigate various aspects of PMN recruitment into the airspace (44–53). Our in vitro system models migration of PMNs across the airway epithelial barrier, an event representing the final step in PMN emigration from circulation to the airspace during acute infectious pneumonia. Because we hypothesize that HXA₃ is the key chemoattractant for directing PMNs across the lung epithelial barrier during infection with PAO1, we next investigated whether HXA₃ is produced in the airspace during in vivo infection. C3H/HeN mice were challenged with 4 × 10⁸ CFU PAO1 or mock infection (PBS) (10 ml into each nostril) and sacrificed 18 h postinfection. BAL fluid was collected to determine the number of cells and the amount of MPO activity present. In addition, the quantity of 12-LO–synthesized eicosanoids, including 12-HETE and HXA₃, was measured in the BAL fluid. There was a significant increase in the number of cells and MPO activity in PAO1-infected mice, suggesting substantial PMN recruitment to the airspace, consistent with previous studies (Fig. 5A, 5B) (32, 33). This observation correlated with a highly significant increase in 12-LO eicosanoids—PMN chemoattractant HXA₃ and the more stable 12-LO metabolite 12-HETE—in the BAL fluid of infected mice (Fig. 5C, 5D).

**Discussion**

PMN infiltration of the airway mucosa and accumulation in the airspace, if excessive and prolonged, can be severely detrimental to

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**Table II. Primers used in this study**

<table>
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<tr>
<th>Gene</th>
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<th>Reverse (5’–3’)</th>
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<td>eLox3</td>
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**FIGURE 3.** Lipid-based chemotactic activity is increased in supernatants of various lung monolayers treated with *P. aeruginosa* and is sensitive to sEH treatment. The amount of PMN chemotactic activity was quantified in serial dilutions of lipids extracted from the supernatants of H292 cells infected with *P. aeruginosa* strain PAO1 and compared with serial dilutions of lipid-extracted supernatants from H292 cells in the absence of infection (HBSS) (A) or infected with K12 *E. coli* strain MC1000 (B). The amount of PMN chemotactic activity was quantified in lipids extracted from the supernatants of BEAS-2B (C) and primary lung epithelial cells (SAEC) (D) in the presence and absence of PAO1 infection. *Statistically significant difference compared with negative controls (HBSS and MC1000). (E) Lipids from the supernatants of uninfected (HBSS) and PAO1-infected H292 cells were extracted and split into two groups: a control group and a group subjected to treatment with sEH. Both groups were evaluated for PMN chemotactic activity. Each symbol represents the average of at least three separate wells. Experiments were performed on at least two separate occasions, yielding similar results. *p < 0.05, sEH treatment versus no treatment, two-tailed unpaired Student t test.
in the absence of analog, two-tailed unpaired Student similar results. Experiments were performed on at least two separate occasions, yielding PAO1. The data represent the average of at least three separate wells. To either an established gradient of fMLF (100 nM) or infection with II-218-36 (110 μM) and PN-II-218-36 were added, at a range of doses, to the apical side at the same time that PMNs were added to the basolateral side. (B) To determine whether either analog impacts P. aeruginosa strain PAO1-induced PMN transepithelial migration, structural analogs MV-I-237-20 and PN-II-218-36 were added, at a range of doses, to the apical side at the same time that PMNs were added to the basolateral side. (C) The ability of PN-II-218-36 (110 μg) to influence transepithelial PMN migration in response to either an established gradient of fMLF (100 nM) or infection with PAO1. The data represent the average of at least three separate wells. Experiments were performed on at least two separate occasions, yielding similar results. *p < 0.05, versus PAO1-induced transepithelial migration in the absence of analog, two-tailed unpaired Student t test.

health during airway inflammatory disease states (5–7). Although recruitment of PMNs to mucosal surfaces is critical during infection to provide patogen clearance, pathology from such infections often reflects indiscriminant and potent actions of PMNs on host tissue (52). Moreover, in the case of airway inflammation not directly associated with microbial threats, any potential benefits derived from the presence of PMNs in the airspace would appear to be outweighed by their propensity to cause host tissue damage. Therefore, a thorough understanding of the mechanism by which PMNs exit the microvasculature, as well as specifically how they cross mucosal epithelial barriers during disease, may lead to the development of therapies geared toward reducing the numbers of PMNs that breach the mucosal barrier and collect in the airspace (6, 10).

A plethora of host-derived chemoattractants engages PMNs in an effort to relay them to the airspace (54). These include protein CXC chemokines (CXCL1–3, CXCL5, and CXCL8), CXC-like extracellular matrix breakdown product N-acetyl Pro-Gly-Pro, protein fragment complement component C5a, and lipid mediators LTB₄ and platelet activating factor (PAF) (55–58). Despite the wealth of knowledge regarding these chemoattractants and their receptors, a clear understanding of their respective roles in PMN recruitment following infection in vivo is unclear. CXC chemokines are released basolaterally from infected mucosal epithelial cells where they imprint underlying extracellular matrix, enabling directed PMN movement through subepithelial spaces (10, 26, 59, 60). Evidence from in vivo modeling notes a requirement for CXC chemokines, such as CXCL5 and CXC-like N-acetyl Pro-Gly-Pro, in the recruitment of PMNs to the airspace, likely by directing PMNs across the endothelial barrier and through the tissue space leading up to the epithelial barrier (55–58, 60, 61). Chemoattractants LTB₄, PAF, and C5a are found in the infected lung, but a discrete role in the orchestration of PMN movement from blood to airspace has not been elucidated (62, 63). The bacterial tripeptide fMLF triggers antibacterial responses once primed PMNs arrive at the site of infection but is not likely involved in recruitment (26, 57).

Using a multifaceted in vitro model, we previously identified the eicosanoid HXA₃ as the key chemotactic signal that mediates directed migration of PMNs across epithelial barriers (12, 15, 60). Thus, identification of HXA₃ as a mediator of PMN transepithelial migration reveals an additional, but uniquely important, chemoattractant that is hypothesized to facilitate PMN recruitment to the airspace during infection. Our studies provide further support, both in vitro and in vivo, for the involvement of HXA₃ in facilitating transepithelial migration of PMNs in the lung.

As described above, we demonstrated that the phenomena of P. aeruginosa-induced PMN migration across lung epithelial barriers is shared among a range of distinct lung epithelial cell barrier models. P. aeruginosa-induced PMN transepithelial migration represents both a PLA₂- and a 12-LO–dependent event, as evidenced by failure of PMNs to migrate in response to epithelial infection following pretreatment with specific inhibitors of each of these enzymatic pathways. PLA₂ and 12-LO enzymatic pathways are responsible for mediating the synthesis of HXA₃ (16, 60). It is unclear which specific PLA₂ or 12-LO isoform is critical for lung epithelial cells to synthesize HXA₃ in response to P. aeruginosa infection. Our previous studies suggested that the calcium-dependent PLA₂ isoform cytosolic PLA₂-α, generally thought to be the major PLA₂ isoform responsible for eicosanoid generation, is not involved in HXA₃ synthesis, despite being activated in lung epithelial cells upon infection with P. aeruginosa and serving a key role in the generation of the eicosanoid PGE₂ (11).

Because 12-LO activity was described to be present in more than one enzyme, multiple enzymatic sources for HXA₃ generation can be considered (19, 64). In the context of human LOs, there are three known enzymes with 12-LO activity: 12-LO, 12(R)-LO, and 15-LO encoded by the genes alox12, alox12B, and alox15, respectively (19, 21, 25). 12(R)-LO also requires the heparinolytic synthetic enzyme eLOX3, encoded by the gene elox3, to generate HXA₃ (22). We observed in this study that all lung epithelial models, including primary SAECs and primary bronchial epithelial cells, express all four known human LO genes that encode enzymes with the capacity for HXA₃ synthesis. Although these different LO enzymes catalyze HXA₃ formation at different rates, each of these expressed genes has the potential to encode enzymes involved in the production of HXA₃ by lung epithelial cells in response to treatment with P. aeruginosa. Dissecting the LO family in an inflammatory context to determine which LO enzyme(s) are critical contributors to the generation of HXA₃ represents an important future pursuit (19, 21, 64).

We demonstrated previously that A549 lung epithelial cells produce HXA₃ in response to P. aeruginosa and that gradients of HXA₃ established across A549 barriers facilitate PMN trans-
epithelial migration (12, 13). We found in the current investigation that infection of the H292 cell line also resulted in the release of HXA3 and that gradients of HXA3 across H292 monolayers drive PMN transepithelial migration. In addition to multiple lung epithelial cell lines, we observed that primary lung epithelial cells release the universal eicosanoid precursor ARA in response to infection with *P. aeruginosa* in a time-dependent manner. Increased release of ARA is supportive of the hypothesis that enhanced production of eicosanoids, such as HXA3, occurs in response to pathogen infection. Because our hypothesis assumes that HXA3 is released in response to infection and this PMN chemoattractant drives transepithelial migration, we predicted that the lipid-enriched fraction of the supernatant from *P. aeruginosa*-infected lung epithelial cells would possess abundant chemotactic activity. This was indeed the case because multiple lung epithelial cell lines, including primary SAECs, exhibited a significant increase in lipid fraction-associated PMN chemotactic activity that is sensitive to sEH; this activity is consistent with an increased presence of functional HXA3 structural analogs in the supernatants of infected lung epithelial cells. Addition of inhibitory analogs might interfere with the ability of native HXA3 to interact with PMNs and promote chemotaxis. Consistent with the notion is the observation that HXA3 structural analogs were more effective at interfering with *P. aeruginosa*-induced PMN transepithelial migration than with PMN migration in response to a gradient of the peptide chemoattractant fMLF. Little is known about the receptor with which HXA3 interacts on PMNs to evoke a chemotactic response. Future studies using native HXA3 and the inhibitory analogs described above will greatly assist in addressing this important issue.

Investigators have explored many aspects of PMN airspace recruitment using *P. aeruginosa*-infected mice. Such studies have established roles for key adhesion molecules, cytokines, chemoattractants, TLRs, signaling components, and transcription factors in the multifaceted process of emigration of PMNs from bloodstream to airway (44–53). Multiple studies have now convincingly established HXA3 as a PMN chemoattractant (12, 13, 15, 17, 60). We showed that airway epithelial cells secrete HXA3 in response to infection and hypothesized that HXA3 is the key chemoattractant required to facilitate PMN breach of the mucosal barrier. To extend our in vitro observations into the context of an in vivo model, we examined whether an increase in the concentration of HXA3 occurs in the airspace of mice in association with PMN accumulation subsequent to infection. Using a well-established model of acute bacterial pneumonia in mice, we observed that PMNs accumulate in the airspace following intranasal exposure to *P. aeruginosa*, consistent with previous observations. Further, we observed a significant increase in the presence of eicosanoids derived from the 12-LO enzymatic pathway, including 12-HETE and HXA3. Therefore, our results suggest that, in addition to an increase in the number of PMNs in the airspace in response to *P. aeruginosa* infection, there is an increase in the concentration of HXA3 measured in the BAL fluid. Although the increased presence of HXA3 located within the airspace and associated with greater numbers of transmigrated PMNs suggests a potential role for HXA3 in driving PMNs across the mucosa into the airspace, such an observation is insufficient to demonstrate a cause-and-effect relationship. However, the novel finding that in vivo infection of the lung with *P. aeruginosa* results in release of HXA3 in the airspace, in combination with the substantial evidence from in vitro modeling demonstrating the role of HXA3 in driving PMN transepithelial migration, encourages further investigation of this potentially important inflammatory mechanism (11–15, 17, 60).
Future studies will examine whether reducing HXA₃ synthesis and/or activity in vivo will have an impact on the numbers of PMNs that accumulate in the airspace following infection.

In conclusion, our study provides further evidence for the role of HXA₃ in PMN recruitment to the airspace, particularly with respect to its chemotactic activity in guiding PMNs across the mucosal barrier. Based on our studies, interfering with the synthesis and/or function of the eicosanoid chemoattractant HXA₃ represents a potentially compelling mechanisms target for developing and exploring a novel class of pharmaceutical compounds with significant untapped therapeutic potential for alleviating PMN-mediated mucosal surface injury during lung disease. Eicosanoid subsets, including leukotrienes and PGs, have been exploited as therapeutic targets to alleviate overzealous inflammation. Multiple leukotriene inhibitors are available for treatment of allergies and asthma, including the 5-LO inhibitor zileuton (66). Ibuprofen, a cyclooxygenase inhibitor, is an anti-inflammatory therapy used for many ailments, including cystic fibrosis (67). Few, if any, studies have explored the therapeutic benefits of 12-LO inhibition or HXA₃ neutralization in lung disease. There are many circumstances in which controlling PMN infiltration into the airspace could alleviate inflammatory damage and improve lung function. Such a targeted therapeutic strategy could prove effective for disease processes as diverse as pneumonia, cystic fibrosis, and asthma. Further exploration of the role of HXA₃ in PMN recruitment is warranted to realize this novel potential therapeutic benefit.

Acknowledgments

We thank Vindhira Mani and Kyle Seaman for technical assistance with LC/MS/MS analyses. We also thank Dr. Ronald E. Kleinman, Physician in Chief of Massachusetts General Hospital for Children, and Dr. W. Allan Walker, Director of the Mucosal Immunology Laboratory at Massachusetts General Hospital, for continued support.

Disclosures

The authors have no financial conflicts of interest.

References


