15-Epi-lipoxin A₄ Inhibits Myeloperoxidase Signaling and Enhances Resolution of Acute Lung Injury

Driss El Kebir^{1*}, Levente József^{1*}, Wanling Pan¹, Lili Wang¹, Nicos A. Petasis², Charles N. Serhan³, and János G. Filep¹

¹Research Center, Maisonneuve-Rosemont Hospital and Department of Pathology and Cell Biology, University of Montréal, Montréal, Quebec, Canada; ²Department of Chemistry, University of Southern California, Los Angeles, California; and ³Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

Rationale: Apoptosis is essential for removal of neutrophils from inflamed tissues and efficient resolution of inflammation. Myeloperoxidase (MPO), abundantly expressed in neutrophils, not only generates cytotoxic oxidants but also signals through the β_2 integrin Mac-1 to rescue neutrophils from constitutive apoptosis, thereby prolonging inflammation.

Objectives: Because aspirin-triggered 15-epi-lipoxin A₄ (15-epi-LXA₄) modulates Mac-1 expression, we investigated the impact of 15-epi-LXA₄ on MPO suppression of neutrophil apoptosis and MPO-mediated neutrophil-dependent acute lung injury.

Methods: Human neutrophils were cultured with MPO with or without 15-epi-LXA₄ to investigate development of apoptosis. Acute lung injury was produced by intratracheal injection of carrageenan plus MPO or intraperitoneal injection of live *Escherichia coli* in mice, and the animals were treated with 15-epi-LXA₄ at the peak of inflammation.

Measurements and Main Results: 15-Epi-LXA₄ through down-regulation of Mac-1 expression promoted apoptosis of human neutrophils by attenuating MPO-induced activation of extracellular signal-regulated kinase and Akt-mediated phosphorylation of Bad and by reducing expression of the antiapoptotic protein Mcl-1, thereby aggravating mitochondrial dysfunction. The proapoptotic effect of 15-epi-LXA₄ was dominant over MPO-mediated effects even when it was added at 4 hours post MPO. In mice, treatment with 15-epi-LXA₄ accelerated the resolution of established carrageenan plus MPO-evoked as well as *E. coli*-induced neutrophil-dependent pulmonary inflammation through redirecting neutrophils to caspase-mediated cell death and facilitating their removal by macrophages.

Conclusions: These results demonstrate that aspirin-triggered 15-epi-LXA₄ enhances resolution of inflammation by overriding the powerful antiapoptosis signal from MPO, thereby demonstrating a hitherto unrecognized mechanism by which aspirin promotes resolution of inflammation.

Keywords: acute lung injury; neutrophil; apoptosis; lipoxins; resolution of inflammation

Neutrophils play a central role in innate immunity and are rapidly recruited to sites of infection and injury. However, their many defense mechanisms that destroy invading microorgan-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Myeloperoxidase (MPO) signals through the β_2 -integrin Mac-1 to suppress neutrophil apoptosis, thereby prolonging inflammation. Aspirin-triggered 15-epi-lipoxin A₄ (15-epi-LXA₄) modulates Mac-1 expression, but it is not known whether it could affect MPO signaling.

What This Study Adds to the Field

Our results demonstrate that aspirin-triggered 15-epi-LXA₄ overrides MPO suppression of neutrophil apoptosis by blocking β_2 integrin-mediated outside-in signaling and enhances resolution of MPO-sustained acute lung injury. These observations identify a new mechanism by which aspirin promotes resolution of inflammation in which neutrophils play a central role.

isms are potentially deleterious to tissues (1, 2). Mature neutrophils undergo constitutive apoptosis that renders them unresponsive to proinflammatory stimuli and allows removal by scavenger macrophages (3–5). Neutrophil survival and apoptosis is profoundly influenced by signals from the inflammatory microenvironment, including bacterial constituents, proinflammatory cytokines, and proapoptotic stimuli, such as Fas ligand or tumor necrosis factor (5). Delayed neutrophil apoptosis has been detected in patients with inflammatory diseases, including acute respiratory distress syndrome (6), sepsis (7), and acute coronary artery disease (8). Thus, facilitating neutrophil apoptosis is critical for minimizing damage to the surrounding tissue and for the resolution of inflammation.

One of the principal enzymes released from neutrophils is myeloperoxidase (MPO). MPO catalyzes the formation of hypochlorous acid, a potent oxidant that has been implicated in killing bacteria and tissue destruction through induction of necrosis and apoptosis (2, 9–11). MPO-derived secondary oxidants and nitration of protein tyrosine residues also modulate signaling pathways, including mitogen-activated protein kinases (12), transcription factors (13), and NO synthase (14). MPO binds to the β_2 integrin CD11b/CD18 (Mac-1) (15, 16), induces neutrophil activation (15), signals to rescue neutrophils from constitutive apoptosis *in vitro*, and prolongs neutrophil-mediated acute lung injury (ALI) in mice (17). *MPO*^{−/−} mice exhibit greatly reduced renal neutrophil accumulation after ischemia-reperfusion (18) and lung injury associated with *Escherichia coli* septicemia (19).

The antiinflammatory lipids lipoxin A₄ (LXA₄) and 15-epi-LXA₄ are typically generated by transcellular biosynthesis. In particular, acetylation at Ser⁵³⁰ by aspirin (20) or S-nitrosylation at Cys⁵²⁶ by atorvastatin (21) redirects the activity of cyclo-

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*These authors contributed equally to this work.

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Present address for L.J. is Yale University School of Medicine, Boyer Center for Molecular Medicine, New Haven, CT.

Correspondence and requests for reprints should be addressed to Dr. János G. Filep, M.D., Research Center, Maisonneuve-Rosemont Hospital, University of Montreal, 5415 blvd de l'Assomption, Montreal, QC, Canada H1T 2M4. E-mail: janos.g.filep@umontreal.ca

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oxygenase-2 to catalyze the conversion of arachidonate to 15R-HETE that can be converted by neutrophils and other cells to 15-epi-LXA₄. LXA₄ and 15-epi-LXA₄ inhibit neutrophil activation and trafficking across the vascular endothelium (22). Because these actions are, in part, mediated through down-regulation of Mac-1 expression (23–25), we investigated whether aspirin-triggered 15-epi-LXA₄ can influence MPO suppression of neutrophil apoptosis *in vitro* and the resolution of neutrophil-dependent inflammation *in vivo*. Here, we demonstrate that 15-epi-LXA₄ overrides the powerful antiapoptosis signal from MPO, redirects neutrophil to apoptosis, and accelerates resolution of MPO-mediated acute airway inflammation in mice through a caspase-mediated proapoptotic effect. These findings indicate a new mechanism by which aspirin promotes resolution of inflammation.

Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

MPO and Lipoxins

MPO purified from human leukocytes was obtained from Athens Research and Technology (Athens, GA) (purity > 97% on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, RZ value of 0.82, no detectable eosinophil peroxidase contamination). 15-Epi-LXA₄ was from Calbiochem (La Jolla, CA). 15-Epi-16-*p*-fluorophenoxy-LXA₄ (ATLa), a metabolically stable analog of 15-epi-LXA₄, and the biologically inactive analog 15-deoxy-LXA₄ were prepared by total organic synthesis (27). Structures were confirmed by reversed-phase high performance liquid chromatography (HPLC), nuclear magnetic resonance, and mass spectral analysis.

Neutrophil Isolation and Culture

Neutrophils were isolated (28) from the venous blood of healthy volunteers who had denied taking any medication for at least 2 weeks. Neutrophils (5×10^6 cells per ml, purity >96%, viability >98%, apoptotic <3%) were cultured in Hanks' balanced salt solution supplemented with 10% autologous serum on a rotator. The Clinical Research Committee at the Maisonneuve-Rosemont Hospital approved the experimental protocols. Neutrophils were either pretreated for 20 minutes with 15-epi-LXA₄, ATLa, or 15-deoxy-LXA₄ (0.03–2 μ M) and then challenged with MPO (10–160 nM) at 37°C or were first challenged with MPO and then treated with 15-epi-LXA₄ at 60 minutes and 4 hours post MPO. In some experiments, MPO was pretreated with 4-aminobenzoic acid hydrazide (20 μ M) to inhibit its enzymatic activity (17) before addition to neutrophils. At the designated time points, cells were processed as described below.

Assessment of Apoptosis

Apoptosis was assessed with flow cytometry using fluorescein isothiocyanate–conjugated annexin-V (BD Biosciences, Mountain View, CA) in combination with propidium iodide (Molecular Probes, Eugene, OR) and the percentage of cells with hypoploid DNA (29). DNA cleavage was assayed by detection of cytoplasmic histone-associated DNA fragments (Cell Death ELISA; Roche, Laval, QC, Canada) and gel electrophoresis (17).

Caspase-3 Activation

Activated caspase-3 in neutrophils was detected with flow cytometry using fluorescein isothiocyanate–labeled DEVD-fmk (Calbiochem) (17).

Detection of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

Neutrophils (5×10^5 cells) were incubated for 15 minutes with the lipophilic fluorochrome chloromethyl-X-rosamine (CMXRos, 200 nM, Molecular Probes) and the fluorescence was analyzed in a FACScan flow cytometer (29).

Western Blot Analysis

Proteins from 10^7 neutrophils were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene

fluoride (PVDF) membranes, blocked with 5% nonfat milk, and probed with antibodies to phosphorylated extracellular signal-regulated kinase (ERK) 1/2, Akt, p38 mitogen-activated protein kinase (MAPK), Bad(Ser¹¹²), and Bad(Ser¹³⁶) (all from Cell Signaling Technologies, Danvers, MA), Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or β -actin (Sigma, Oakville, ON, Canada) (29).

Mac-1 Expression

Surface expression of Mac-1 on blood or isolated neutrophils was assessed using R-phycoerythrin–conjugated anti-CD11b (Becton Dickinson) antibody with a FACScan flow cytometer (BD Biosciences, Mountain View, CA) (25).

MPO Release

Conditioned media were analyzed for MPO by a specific ELISA (Assay Design, Ann Arbor, MI). The intraassay and interassay coefficients of variation were less than 8%.

In Vivo ALI

Female BALB/c mice (aged 8–12 wk, Charles River Laboratories, St-Constant, QC, Canada) were housed in pathogen-free conditions. The Animal Care Committee of the Maisonneuve-Rosemont Hospital approved the protocols. Mice were injected intratracheally with 0.1 ml of 0.25% λ -carrageenan (Fluka-Sigma-Aldrich, Buchs, Switzerland) plus 10 μ l of 16 μ M MPO (17), followed 24 hours later by intravenous injection of 200 μ g/kg 15-epi-LXA₄ in 100 μ l saline or appropriately diluted ethanol as a vehicle control. At the same time, some mice were also injected intraperitoneally with the pan-caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk, Calbiochem, 10 μ g/kg in 0.2 ml), followed by two additional doses of zVAD-fmk 4 and 8 hours later (30). Additional groups of mice were challenged intraperitoneally with 2×10^8 live *E. coli* (American Type Culture Collection, ATCC 25922) or 1×10^9 live bacteria for survival studies (19). At 1 hour postinfection, mice were treated with 15-epi-LXA₄ or vehicle as just described.

Assessment of Inflammation

At the indicated times, mice were killed and the lungs were lavaged and bronchoalveolar lavage (BAL) fluid protein, IL-6 levels, total and differential leukocyte counts, and neutrophil viability and apoptosis were determined (17). BAL fluid cells, cytospinned into poly-L-lysine-coated slides, were stained with hematoxylin and eosin and assayed for macrophages containing apoptotic bodies. In separate groups of mice, the lungs were removed without lavage, fixed in 4% formaldehyde, and processed for standard histological evaluation (17). Lung dry-to-wet weight ratio was determined after placement of tissues in a drying oven at 56°C for 3 days. Tissue MPO activity was measured using o-dianisidine as a substrate and human MPO (Sigma) as a standard (31). BAL fluid IL-6 levels were measured by using a mouse IL-6 ELISA kit (BD Biosciences). The intra-assay and inter-assay coefficients of variation were less than 6%.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Statistical comparisons were made by analysis of variance using ranks (Kruskal-Wallis test) followed by Dunn multiple contrast hypothesis tests to identify differences between various treatments or by the Mann-Whitney U test (two-tailed). Correlations were analyzed by the Spearman test. *P* values less than 0.05 were considered statistically significant. Kaplan-Meier survival curves were compared using the log-rank test.

RESULTS

Aspirin-triggered 15-epi-LXA₄ Overcomes MPO Delay of Apoptosis in Human Neutrophils

Confirming our previous results (17), MPO prolonged neutrophil life span by delaying intrinsic apoptosis (Figure 1A). MPO partially prevented disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) that precedes development of apoptotic morphology in neutrophils undergoing constitutive programmed

Figure 1. Aspirin-triggered 15-epi-LXA₄ prevents myeloperoxidase (MPO) suppression of neutrophil apoptosis. Human neutrophils (5×10^6 cells/ml) were cultured for 20 minutes with 15-epi-LXA₄ and then with MPO for 24 hours. Viability and mitochondrial transmembrane potential ($\Delta\Psi_m$) were assessed by propidium iodide and CMXRos staining, respectively; apoptosis was assessed by annexin-V–fluorescein isothiocyanate binding and analysis of nuclear DNA content. (A) MPO suppression of intrinsic apoptosis. Data are means $\pm$ SEM of five experiments with different blood donors. * $P < 0.05$, ** $P < 0.01$ versus untreated. (B) Effects of 15-epi-LXA₄ on MPO (160 nM) suppression of apoptosis. Untreated neutrophils (C, control) are shown for comparison. Data are means $\pm$ SEM ($n = 6$). * $P < 0.05$, ** $P < 0.01$ versus MPO-treated. (C) Effects of 15-epi-LXA₄, 15-epi-16-*p*-fluorophenoxy-LXA₄ (ATLa), and 15-deoxy-LXA₄ (all at 2 μM) on MPO or catalytically inactive MPO (cat-inactMPO, 160 nM) suppression of apoptosis ($n = 4$ –6). ** $P < 0.01$ versus untreated (control), † $P < 0.05$ versus MPO. (D) DNA fragmentation in neutrophils maintained in culture for 24 hours. Values of selected DNA standards (left lane) are shown on the left margin. (E) 15-Epi-LXA₄ does not inhibit peroxidase activity. MPO (160 nM) was incubated for 10 minutes with or without 15-epi-LXA₄ (2 μM), and peroxidase activity was measured using o-dianisidine as a substrate ($n = 5$). (F–H) Impact of MPO and 15-epi-LXA₄ on MAP kinases, Bad, and Mcl-1. Neutrophils were lysed after isolation (t₀) or after culture with MPO (160 nM) with or without 15-epi-LXA₄ (2 μM) (F, G) for 30 minutes or (H) for 2 hours. Proteins were subjected to immunoblotting with antibodies to phosphorylated kinases, Bad, Mcl-1, or β -actin. Blots are representative of three separate experiments.

cell death (29, 32). Because the apoptosis-delaying action of MPO is mediated through the β_2 integrin Mac-1 (17) and 15-epi-LXA₄ down-regulates Mac-1 expression on human neutrophils (23–25), we compared 15-epi-LXA₄ and its metabolically stable analog ATLa with MPO for their effects on apoptosis and cellular signaling. Although neither 15-epi-LXA₄ nor ATLa, up to a concentration of 2 μM , affected neutrophil survival (data not shown), they markedly attenuated the MPO actions on $\Delta\Psi_m$ and apoptosis (Figures 1B and 1C). Apparent maximum inhibition was achieved at approximately 2 μM with 15-epi-LXA₄ and ATLa being virtually equally potent inhibitors (Figure 1C). The biologically inactive analog 15-deoxy-LXA₄ had no detectable effects. Electrophoretic analysis confirmed the ability of 15-epi-LXA₄ to prevent MPO suppression of DNA fragmentation (Figure 1D). 15-Epi-LXA₄ did not

inhibit the catalytic activity of MPO (Figure 1E). Pretreatment of MPO with 4-aminobenzoic acid hydrazide resulted in greater than 95% loss of peroxidase activity (17). Like MPO, catalytically inactive MPO suppressed apoptosis and this was also reversed by 15-epi-LXA₄ (Figure 1C). Binding of MPO to Mac-1 results in rapid phosphorylation of ERK 1/2 and Akt (17) that generate survival signals for neutrophils. We further probed the effects of 15-epi-LXA₄ on these signaling pathways. 15-Epi-LXA₄ alone slightly increased phosphorylation of p38 MAPK, but not Akt and ERK 1/2 (Figure 1F). By contrast, 15-epi-LXA₄ reduced MPO-induced phosphorylation of ERK 1/2 and Akt (Figure 1F) and their downstream target Bad (Figure 1G). Expression of Mcl-1, a key regulator of apoptosis in neutrophils (33, 34), decreased rapidly within 2 hours of culture, an effect that was prevented by MPO (Figure 1H).

Figure 2. Treatment with 15-epi-LXA₄ reverses neutrophil responses to myeloperoxidase (MPO). (A) Human neutrophils (5×10^6 cells/ml) were first challenged with MPO (160 nM), then treated with 15-epi-LXA₄ (2 μ M) at 60 minutes post MPO. Cell viability and apoptosis were assessed after 24 hours of culture ($n = 4$). * $P < 0.05$ versus untreated, † $P < 0.05$ versus MPO-treated. (B) Cell lysates, prepared 30 minutes after addition of 15-epi-LXA₄, were immunoblotted for phospho-Akt and phospho-extracellular signal-regulated kinase 1/2. Blots are representative of three independent experiments.

15-epi-LXA₄ inhibited MPO-mediated preservation of Mcl-1 (Figure 1H) and produced a small inhibition of p38 MAPK phosphorylation by MPO (Figure 1F).

When neutrophils were first challenged with MPO and then treated with 15-epi-LXA₄ at 60 minutes post MPO, the proapoptotic action of 15-epi-LXA₄ was dominant over MPO-mediated effects (Figure 2A). Treatment with 15-epi-LXA₄ attenuated MPO-induced phosphorylation of Akt and ERK 1/2 (Figure 2B). Such an inhibitory action was still detectable when 15-epi-LXA₄ was added 4 hours post MPO (data not shown), further highlighting the therapeutic potential of this antiinflammatory lipid.

MPO up-regulates neutrophil CD11b expression and induces MPO release (15). We found that 15-epi-LXA₄ inhibited MPO-stimulated CD11b expression on isolated neutrophils as well as on whole blood neutrophils (Figure 3A) and attenuated MPO

release (Figure 3B) in a concentration-dependent fashion. We observed a negative correlation between neutrophil CD11b expression levels assayed at 30 minutes post MPO and development of apoptosis assessed at 24-hour culture (Figure 3C), further corroborating the importance of CD11b-mediated “outside-in” signaling in determining the fate of neutrophils.

15-Epi-LXA₄ Promotes Neutrophil Apoptosis and Enhances Inflammatory Resolution

Having shown the ability of 15-epi-LXA₄ to override the apoptosis-delaying action of MPO *in vitro*, we investigated the effects of 15-epi-LXA₄ on the resolution of inflammation in mice. In the carrageenan model, low concentration of intratracheal carrageenan instillation evokes neutrophil-mediated ALI that resolves within 5 days without treatment (35). Co-administration of MPO prolongs carrageenan-induced inflammation parallel with suppression of neutrophil apoptosis (17). Treatment with 15-epi-LXA₄ accelerated the resolution of established inflammation when administered intravenously at near the peak of inflammation (24 h) evoked by carrageenan plus MPO (Figure 4). We observed, over a period of 4 days of administering 15-epi-LXA₄, decreases in BAL fluid total leukocyte and neutrophil numbers with increases in the number of monocytes/macrophages (Figure 4A). 15-Epi-LXA₄ reduced tissue MPO content, an index of neutrophil accumulation (Figure 4B). Antiinflammatory actions of 15-epi-LXA₄ were also evident, as 15-epi-LXA₄ attenuated edema formation (Figures 4C and 4D) and the release of IL-6 into the bronchoalveolar space (Figure 4E). Furthermore, 15-epi-LXA₄ augmented the amount of cytoplasmic histone-associated DNA fragments in BAL cells (Figure 4F) and caspase-3 activity in BAL neutrophils (Figure 4G). Lungs of mice treated with 15-epi-LXA₄ exhibited markedly reduced inflammatory infiltrate and lung injury (Figure 5A). Decreases in BAL fluid neutrophil number (Figure 6A) occurred parallel with increases in the percentage of annexin-V-positive cells (Figure 6B) and the percentage of macrophages containing apoptotic bodies (Figures 5B and 5C).

Next, we investigated whether the enhanced resolution was mediated by 15-epi-LXA₄ induction of caspase-3-dependent neutrophil apoptosis *in vivo*. Intraperitoneal injection of the pan-caspase inhibitor zVAD-fmk prevented the 15-epi-LXA₄-induced reduction of neutrophil accumulation (Figure 6A), increase in neutrophil apoptosis (Figure 6B), and suppression of edema formation (Figure 6C) in carrageenan plus MPO-induced lung inflammation. Of note, zVAD-fmk enhanced

Figure 3. 15-Epi-LXA₄ attenuates myeloperoxidase (MPO)-induced up-regulation of Mac-1 expression and MPO release. Human neutrophils (5×10^6 cells/ml) or whole blood were incubated for 20 minutes with 15-epi-LXA₄ and then challenged with MPO (160 nM) for 30 minutes. (A) Neutrophil Mac-1 is expressed as percentage of CD11b staining on unstimulated (control) neutrophils after correction with staining with an isotype-matched irrelevant antibody. * $P < 0.05$, ** $P < 0.01$ versus MPO. (B) MPO levels in the medium were measured with an ELISA. Data are means $\pm$ SEM ($n = 4-7$). ** $P < 0.01$ versus untreated. † $P < 0.05$, †† $P < 0.01$ versus MPO (160 nM). (C) Neutrophil CD11b expression inversely correlates with development of apoptosis. Neutrophils were pretreated for 20 minutes with 15-epi-LXA₄ (2 μ M) and then challenged with MPO (160 nM). Surface expression of CD11b and apoptosis (annexin-V staining) were assessed at 30 minutes and at 24 hours post MPO, respectively.

release (Figure 3B) in a concentration-dependent fashion. We observed a negative correlation between neutrophil CD11b expression levels assayed at 30 minutes post MPO and development of apoptosis assessed at 24-hour culture (Figure 3C), further corroborating the importance of CD11b-mediated “outside-in” signaling in determining the fate of neutrophils.

Figure 4. 15-Epi-LXA₄ induces neutrophil apoptosis and enhances resolution of lung inflammation. Twenty-four hours after intratracheal instillation of 100 μ l 0.25% carrageenan plus myeloperoxidase (MPO) (160 nmol) (Day 1), mice were treated intravenously with vehicle or 15-epi-LXA₄ (200 μ g/kg). Mice were killed at the indicated times and (A) bronchoalveolar lavage (BAL) fluid total leukocyte, neutrophil, and monocyte/macrophage numbers, (B) lung tissue MPO activity, (C) BAL fluid protein concentration, (D) lung dry-to-wet weight ratio, (E) BAL fluid IL-6 level, (F) BAL cell cytoplasmic histone-associated DNA fragments, and (G) neutrophil caspase-3 activity were determined. Data are means $\pm$ SEM (n = 6–8 mice per group). *P < 0.05, **P < 0.01. RFU = relative fluorescence units.

tion, (D) lung dry-to-wet weight ratio, (E) BAL fluid IL-6 level, (F) BAL cell cytoplasmic histone-associated DNA fragments, and (G) neutrophil caspase-3 activity were determined. Data are means $\pm$ SEM (n = 6–8 mice per group). *P < 0.05, **P < 0.01. RFU = relative fluorescence units.

inflammatory cell accumulation (Figure 6A) and edema formation (Figure 6C) in the airways.

The proresolution action of 15-epi-LXA₄ was further confirmed in the *E. coli* septicemia-associated lung injury model, which was shown to involve endogenous MPO (19). In this model, treatment with 15-epi-LXA₄ reduced tissue MPO content (Figure 7A) and BAL fluid neutrophil numbers (Figure 7B) with concomitant increases in the number of apoptotic neutrophils (Figure 7C) and caspase-3 activity in BAL neutrophils (Figure 7D). 15-Epi-LXA₄ also reduced *E. coli*-induced edema formation (Figure 7E) and tissue injury (Figure 7F). We observed greater survival in 15-epi-LXA₄-treated animals. Approximately 60% of mice died within 7 hours after *E. coli* (10⁹) challenge, whereas all 15-epi-LXA₄-treated mice were alive at this time (Figure 7G).

DISCUSSION

Our results indicate that aspirin-triggered 15-epi-LXA₄ and its metabolically stable analog ATL_a can effectively override MPO suppression of apoptosis of human neutrophils *in vitro* by blocking β_2 -integrin-mediated outside-in signaling. Furthermore, systemic administration of 15-epi-LXA₄ induces apoptosis in neutrophils *in situ* and facilitates resolution of MPO-sustained neutrophil-dependent pulmonary inflammation *in vivo*.

Accumulating evidence indicates that MPO may contribute to tissue damage through mechanisms independent of catalyzing formation of reactive oxygen and nitrogen species. Indeed, once released from leukocytes, MPO binds to and signals through Mac-1 (15, 16) to activate neutrophils (15) and to delay intrinsic apoptosis (17). Thus, MPO evokes neutrophil responses similar to those triggered by engagement of Mac-1 with its endothelial cell counter-ligand ICAM-1 during endothelial transmigration (36, 37). MPO up-regulates surface expression of CD11b and evokes MPO release from neutrophils (15), implicating an autocrine and paracrine mechanism for perpetuation of the inflammatory response. Our data suggest that by inhibiting MPO-induced CD11b expression and MPO release, 15-epi-LXA₄ could interrupt this loop.

Inhibition of neutrophil activation and trafficking are important components of the antiinflammatory activities of 15-epi-LXA₄ (reviewed in Reference 22). Down-regulation of neutrophil Mac-1 expression by 15-epi-LXA₄ and consequent inhibition of neutrophil adhesion to the endothelium has been well documented (23–25), although the underlying molecular mechanisms are incompletely understood. We previously reported that serum amyloid A through binding to the formyl-peptide receptor-like 1/lipoxin receptor generates an antiapoptosis signal that can be overcome by 15-epi-LXA₄ in human neutrophils *in vitro* (29). We have now shown that 15-epi-LXA₄ can also override a powerful Mac-1-mediated outside-in survival signal, thus redirecting neutrophils to programmed cell death. 15-Epi-LXA₄ stimulated limited phosphorylation of p38 MAPK, but this was insufficient to affect the fate of neutrophils (29). By contrast, 15-epi-LXA₄ down-regulation of Mac-1 expression attenuated neutrophil responses to MPO, including ERK and Akt-mediated phosphorylation of the proapoptotic protein Bad. Nonphosphorylated Bad associates with Mcl-1 and prevents its antiapoptotic actions (38). Furthermore, 15-epi-LXA₄ attenuated MPO-mediated up-regulation of Mcl-1 expression. Reduced Bad phosphorylation and loss of expression of Mcl-1 are probably the critical events in redirecting neutrophils to apoptosis by 15-epi-LXA₄. These would aggravate mitochondrial dysfunction, ultimately leading to caspase-3-mediated cell death. This interpretation is further supported by the observations that inhibition of prosurvival molecules, such as ERK and Bcl-xL promotes, whereas inhibition of the proapoptotic molecule Bax prevents, resolution of carrageenan-induced pleurisy in rats (39). Lipoxins through attenuation of Akt phosphorylation could also inhibit PDGF-induced cell proliferation (40). Our results point toward neutrophil mitochondria as potential targets for the prevention and/or treatment of ALI. Indeed, inhibition of mitochondrial complex I with metformin (41) or promotion of collapse of mitochondrial transmembrane potential with 15-epi-LXA₄ (the present study) result in diminished severity of lung injury in spite of differences in the underlying molecular mechanisms. It remains to be investigated whether other lipoxin receptor ligands, such as

Figure 5. 15-Epi-LXA₄ enhances resolution of lung inflammation. Twenty-four hours after intratracheal instillation of 100 μ L 0.25% carrageenan plus myeloperoxidase (MPO) (160 nmol) (Day 1), mice were treated intravenously with vehicle or 15-epi-LXA₄ (200 μ g/kg). Mice were killed at the indicated times, and lungs were prepared for histological analysis or analysis of bronchoalveolar lavage (BAL) fluid monocytes/macrophages. (A) Lung tissue sections from naive mice (Day 0), mice with inflammation evoked by carrageenan plus MPO (Day 1), or mice treated with 15-epi-LXA₄ or vehicle for 24 hours (Day 2). Hematoxylin and eosin stain; scale bars: 100 μ m. (B, C) 15-Epi-LXA₄ enhances the number of macrophages containing apoptotic bodies (arrows). Asterisks indicate intact neutrophils. (C) Percentage of BAL fluid macrophages containing apoptotic bodies. Results are means $\pm$ SEM (n = 6 mice per group). ***P < 0.001.

annexin A1 and humanin, could also override MPO signaling in neutrophils. Of note, annexin A1 was found to accelerate constitutive neutrophil apoptosis (42).

We have shown that 15-epi-LXA₄ administered at the peak of inflammation promoted resolution of carrageenan plus MPO-induced and *E. coli* septicemia-associated ALI. 15-Epi-LXA₄ redirected neutrophils to apoptosis *in vivo*, reduced accumulation of neutrophils in the bronchoalveolar space, and attenuated lung injury. In addition, 15-epi-LXA₄ also inhibited vascular

permeability and release of the proinflammatory cytokine IL-6, further supporting its proresolution role. The concentrations of 15-epi-LXA₄ in our study were lower than those used in other *in vivo* models (43, 44), but were higher than those reported for cultured cells (reviewed in Reference 22). These might reflect interactions of 15-epi-LXA₄ with serum components, such as albumin. Nevertheless, it is impressive that this lipophilic compound overcomes interactions with plasma components to regulate neutrophils *in situ*. In spite of rapid metabolism in

Figure 6. Caspase inhibition prevents 15-epi-LXA₄-induced resolution of inflammation. Twenty-four hours after intratracheal instillation of carrageenan plus myeloperoxidase, mice were treated with 15-epi-LXA₄ (200 μ g/kg, intravenously) and/or the pan-caspase inhibitor zVAD-fmk (10 μ g/kg, intraperitoneally three times at 4-hour intervals). Mice were

killed at 24 hours post 15-epi-LXA₄ and (A) bronchoalveolar lavage (BAL) fluid neutrophil and monocyte/macrophage numbers, (B) the percentage of annexin-V-positive (apoptotic) neutrophils, and (C) BAL fluid protein concentration were determined. Results are means $\pm$ SEM (n = 7–8 mice per group). **P < 0.01 versus vehicle. †P < 0.05, ††P < 0.01 versus 15-epi-LXA₄-treated.

Figure 7. 15-Epi-LXA₄ attenuates lung inflammation and prolongs survival of *Escherichia coli*-infected mice. One hour after intraperitoneal injection of 2×10^8 live *E. coli*, BALB/c mice were treated intravenously with vehicle or 15-epi-LXA₄ (200 μ g/kg). Mice were killed at 6 hours post *E. coli* and (A) lung tissue myeloperoxidase activity, (B) BAL fluid neutrophil count, (C) the percentage of annexin-V-positive (apoptotic) neutrophils, (D) neutrophil caspase-3 activity, and (E) lung dry-to-wet weight ratio were determined. Data are means $\pm$ SEM ($n = 6$ mice per group). * $P < 0.05$, ** $P < 0.01$. RFU = relative fluorescence units. (F) Lung tissue sections from naive mice (control), mice challenged with *E. coli* and treated with 15-epi-LXA₄ or vehicle. Hematoxylin and eosin stain; scale bars: 100 μ m. (G) Kaplan-Meier survival plots for mice challenged intraperitoneally with 10^9 live *E. coli* and treated with 15-epi-LXA₄ or vehicle ($n = 10$ per group). $P = 0.0136$ by the log-rank test.

blood (half-life ~ 3 –4 h) (42), 15-epi-LXA₄ effectively redirected neutrophils to apoptosis and accelerated resolution of inflammation when given as a single intravenous injection. These observations would indicate that once 15-epi-LXA₄ overcame pro-survival signals, neutrophil apoptosis would progress even in the absence of 15-epi-LXA₄.

The mouse models were chosen for their clinical relevance and because of the self-resolving nature of carrageenan-induced inflammation (35) that can be prolonged by coadministration of MPO (17) and contribution of endogenous MPO to *E. coli* septicemia-associated lung injury (19). Our results indicate that spontaneous resolution as well as enhanced resolution of inflammation by 15-epi-LXA₄ was intimately linked to caspase-dependent apoptosis of neutrophils that have already emigrated into the airways. 15-epi-LXA₄ induced apoptosis in large proportions of BAL fluid neutrophils as assessed by annexin-V labeling, detection of intracellular caspase-3 activity, and cytoplasmic histone-associated DNA fragments. Furthermore, the effects of 15-epi-LXA₄ were prevented by the pan-caspase inhibitor zVAD-fmk. It is unlikely that the pro-resolution action of 15-epi-LXA₄ *in vivo* was due to reduced generation of MPO-derived reactive oxygen and nitrogen species, because 15-epi-LXA₄ did not inhibit the catalytic activity of MPO and reversed the antiapoptosis action of catalytically inactive MPO *in vitro*. *MPO*^{−/−} mice exhibit reduced renal neutrophil accumulation and injury after ischemia-reperfusion (18) and reduced lung injury and greater survival after intraperitoneal *E. coli* injection compared with wild-type mice (19). Although absence of MPO-derived oxidant production during *E. coli* septicemia in *MPO*^{−/−}

mice could reduce lung injury and mortality, it is not known whether MPO deficiency could also affect the longevity of neutrophils. Reduced neutrophil life span would protect against tissue damage in these models of inflammation.

15-Epi-LXA₄ inhibits cytokine production (28), sequesters chemokines (45), and facilitates phagocytosis of apoptotic neutrophils by macrophages (46, 47), contributing to its pro-resolution properties. We have observed enhanced accumulation of monocytes/macrophages in the airways at 3 and 5 days post 15-epi-LXA₄ treatment. 15-Epi-LXA₄ is believed to promote recruitment of monocytes in a nonphlogistic fashion, consistent with facilitation of tissue repair (5, 22, 48). The increased number of macrophages with apoptotic bodies, generated from dying cells, indicates enhanced clearance by 15-epi-LXA₄ of apoptotic neutrophils and other cells. Ingestion of apoptotic cells induces the synthesis and release of mediators with pro-resolution properties, such as IL-10 and TGF- β 1 (5, 48).

Our *in vitro* and *in vivo* work showing that 15-epi-LXA₄ or ATL₄ overcame the powerful antiapoptosis signal from MPO lends additional support to the notion that neutrophil apoptosis is a critical determinant of the outcome of the inflammatory process and is a potential target for therapeutic interventions. Thus, suppression of neutrophil apoptosis may ensue prolonged inflammation (5, 48), whereas induction of neutrophil apoptosis would enhance resolution of inflammation (30, 39, 49). Here we showed that neutrophil apoptosis could be enhanced with the antiinflammatory lipid 15-epi-LXA₄ in both exogenous and endogenous MPO-mediated mouse models of ALI. This action of 15-epi-LXA₄ resembles that of cyclin-dependent kinase

inhibitors (30). However, unlike 15-epi-LXA₄, these latter compounds evoked apoptosis in monocytes/macrophages in a carrageenan-induced pleurisy model.

In summary, our study identifies a novel inflammatory resolution mechanism for aspirin-triggered 15-epi-LXA₄. Our data show that 15-epi-LXA₄ overrides MPO suppression of neutrophil apoptosis *in vitro* and accelerates resolution of neutrophil-dependent inflammation by promoting caspase-dependent apoptosis *in vivo*.

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