Lovastatin Decreases Acute Mucosal Inflammation Via 15-epi-Lipoxin A₄

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Abstract

The wide spread use of statins for hypercholesterolemia has uncovered pleiotropic anti-inflammatory properties that were unexpected based on the drugs’ original design, yet mechanisms for these protective actions remains uncertain. Here, lovastatin triggered biosynthesis of the anti-inflammatory and pro-resolving mediator 15-epi-lipoxin A₄ (15-epi-LXA₄). During interactions between human neutrophils and airway epithelial cells, the statin induced increase in 15-epi-LXA₄ was associated with increased 14,15-epoxyeicosatrienoic acid (14,15-EET) generation. When added to activated neutrophils, 14,15-EET enhanced 15-epi-LXA₄ biosynthesis. In a murine model of airway mucosal injury and inflammation, lovastatin increased 15-epi-LXA₄ formation in vivo and markedly decreased acute lung inflammation. Administration of 15-epi-LXA₄ also inhibited lung inflammation in an additive manner with lovastatin. Together, these results indicate that statin-triggered 15-epi-LXA₄ generation during human leukocyte-airway epithelial cell interactions is an endogenous mechanism for statin-mediated tissue protection at mucosal surfaces that may also be relevant in statins’ ability to stimulate the resolution of inflammation.

Keywords

statin; lipoxin; soluble epoxide hydrolase; inflammation; lung

INTRODUCTION

Statins are potent cholesterol-lowering agents with pleiotropic anti-inflammatory properties. Inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase does

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not explain all of these drugs’ anti-inflammatory actions.\textsuperscript{1} Of interest, statins can regulate innate and adaptive immune responses,\textsuperscript{2, 3} as well as endothelial and epithelial cell function.\textsuperscript{4-6} Prospective human clinical trials have provided strong evidence for statin-mediated anti-inflammation, as individuals with normal plasma cholesterol that were randomized to receive statin therapy had marked decrements in both C-reactive protein, monitored as a marker of systemic inflammation, and cardiovascular events.\textsuperscript{7} In several animal models of injury or inflammation, statins also display protective actions, including sepsis\textsuperscript{8}, rheumatoid arthritis,\textsuperscript{9} asthma,\textsuperscript{10} emphysema\textsuperscript{11} and acute lung injury (ALI).\textsuperscript{12} Mechanisms remain to be identified.

Acute respiratory distress syndrome (ARDS) and ALI have an annual incidence in the USA of more than 200,000 adults and still carry a 1 year mortality of greater than 40\%.\textsuperscript{13} There is no specific medical therapy for these conditions. Resolution of ARDS and ALI is an active process characterized in part by clearance of polymorphonuclear leukocytes (PMNs) from the lung. The natural resolution of inflammation occurs via local biosynthesis of braking signals, such as lipoxins (LXs) and 15-epi-LXs (aka aspirin-triggered LXs) at sites of inflamed tissue.\textsuperscript{14} 15-epi-LXs belong to a new genus of lipid mediators that are agonists of resolution and regulate airway inflammation\textsuperscript{14, 15} and ALI.\textsuperscript{16, 17} 15-epi-LXs are locally produced via cell-cell interactions between leukocytes and resident cells during multicellular host responses to injury, inflammation, and microbial invasion (reviewed in reference \textsuperscript{14}). These lipid mediators display diverse counter-regulatory actions in pico to nanomolar amounts, including inhibition of PMN functional responses\textsuperscript{18, 19} and interactions with the endothelium,\textsuperscript{20} as well as stimulation of mucosal epithelial bacterial killing\textsuperscript{21} and macrophage clearance of apoptotic PMNs.\textsuperscript{22} Statins display many of the same anti-inflammatory properties as 15-epi-LXs. Both inhibit leukocyte adhesion, regulate cytokine and chemokine expression, induce endothelial and inducible nitric oxide synthase, lower matrix metalloproteinase expression and activation, increase tissue inhibitor of matrix protease and inhibit nuclear factor-κB activation (editorial in reference \textsuperscript{23} on reference \textsuperscript{24}) with the new property for 15-epi-LXA\textsubscript{4} of also promoting active resolution.\textsuperscript{25}

15-epi-LXs were first described as arachidonic acid derived products generated during interactions between cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LO).\textsuperscript{26} Aspirin-acetylated COX-2 converts arachidonic acid to 15-\textit{R}-hydroxyeicosatetraenoic acid (15-\textit{R}-HETE) that is transformed by 5-LO to 15-epi-LXs.\textsuperscript{26} In addition to aspirin-acetylated COX-2, cytochrome P450 enzymes (CYP450s) are also capable of catalyzing 15-\textit{R}-HETE production from arachidonic acid.\textsuperscript{27} Moreover, CYP450s can also convert arachidonic acid to epoxyeicosatrienoic acids (EETs)\textsuperscript{28} that have anti-inflammatory properties when present in micromolar quantities.\textsuperscript{29} EETs are further metabolized by soluble epoxide hydrolase (sEH) to their corresponding diols, dihydroxyeicosatrienoic acids (DHETs).\textsuperscript{30} Soluble epoxide hydrolase inhibitors increase LX generation and decrease plasma levels of pro-inflammatory cytokines to promote resolution of inflammatory responses.\textsuperscript{31} Here, we report that statin-initiated 15-epi-LXA\textsubscript{4} generation proceeds via transcellular biosynthesis during human PMN - epithelial cell interactions that in the lung promotes resolution of ALI.
RESULTS

Lovastatin increases 15-epi-LXA\(_4\) formation during interactions between human PMNs and airway epithelial cells

In the presence of lovastatin (1 \(\mu\)M hydroxy acid), human PMNs co-incubated with tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\))-primed airway epithelial cells (Calu-3) generated significant amounts of 15-epi-LXA\(_4\) (2.82 \(\pm\) 0.62 fold greater than Calu-3 alone (705 pg/ml), \(p < 0.05\) for \(n = 3\) independent experiments) (Figure 1A). In the absence of PMNs, lovastatin did not augment 15-epi-LXA\(_4\) production by the cytokine-primed Calu-3 cells (1.02 \(\pm\) 0.34 fold change). In addition, there was no significant increase in 15-epi-LXA\(_4\) by incubations of cytokine-primed Calu-3 cells with PMNs in the absence ofLovastatin (1.44 \(\pm\) 0.22 fold change). Statin-triggered 15-epi-LXA\(_4\) formation correlated with increasing PMN numbers relative to Calu-3 cells (Figure 1B). For purposes of comparison, 15-epi-LXA\(_4\) generated at a cell ratio of 0:1 (PMNs: airway epithelial cells) was assigned a value of 1 (mean, 541 pg 15-epi-LXA\(_4\)/ml) and changes in 15-epi-LXA\(_4\) amounts were expressed as a fold change for each cell ratio tested [1:1 (1.6 \(\pm\) 0.19 fold increase); 2:1 (2.8 \(\pm\) 0.85 fold increase) and 5:1 (3.8 \(\pm\) 0.8 fold increase); \(p < 0.05\) for \(n = 5\) independent experiments] (Figure 1B). In addition to Calu-3 cells, lovastatin-triggered 15-epi-LXA\(_4\) formation during human PMN interactions with primary normal human bronchial epithelial (NHBE) cells differentiated at an air-liquid interface (5:1 ratio, PMNs:NHBE, 927 pg 15-epi-LXA\(_4\)/ml, mean for \(n = 4\) and with the distal human airway epithelial cell line A549 (5:1 ratio, PMNs:A549, 2.14 ng 15-epi-LXA\(_4\)/ml, mean for \(n = 3\)). In further experiments, Calu-3 cells were used principally for ease of culture and cost considerations. These results indicate that a statin can increase 15-epi-LXA\(_4\) generation in human cells via transcellular biosynthesis and that interactions between cytokine primed-airway epithelial cells and PMNs can enhance this biosynthetic reaction.

Lovastatin is well characterized as an inhibitor of HMG CoA reductase, but mevalonate (100 \(\mu\)M) did not block the statin-triggered 15-epi-LXA\(_4\) formation (Figure 1C), indicating that lovastatin’s mechanism for 15-epi-LXA\(_4\) generation is distinct from its regulation of isoprenoid metabolism. Although independent from exogenous mevalonate, the actions of lovastatin were shared with simvastatin, another lipophilic statin (Figure 1C). Of interest, the broad acting LO inhibitor, nordihydroguaiaretic acid (NDGA), significantly inhibited lovastatin-triggered 15-epi-LXA\(_4\) formation by over 80% (Figure 1C), indicating that LO activity is critical to the compound’s biosynthesis.

To identify which biosynthetic enzymes for LXs and 15-epi-LXs are expressed in these cell types, we next determined gene expression using semi-quantitative RT-PCR for sEH, 15-LO-1, 15-LO-2 and 5-LO (Figure 1D), as well as the related genes 5-LO activator protein (FLAP), 12-LO and COX-2 in Calu-3 cells, Calu-3 cells exposed to TNF-\(\alpha\) (1 ng/ml, 24h), freshly isolated human PMNs and NHBE exposed to interleukin-13 (10 ng/ml, 96h). sEH mRNA was present in all three cell types. In contrast, 15-LO-1 and 15-LO-2 mRNA were only expressed in cytokine-primed NHBE, and 5-LO was only expressed in PMNs and Calu-3 cells (Figure 1D). FLAP was only present in PMNs, and low levels of 12-LO mRNA were present in freshly isolated PMNs from peripheral blood (Supplementary Figure 1S).
indicating likely minor platelet contamination. Increased COX-2 mRNA expression was observed only in cytokine-primed airway epithelial cells (Supplementary Figure 1S). Together, these findings indicate that neither cell type alone contains the genes required for 15-epi-LXA₄ biosynthesis; thus, providing a rationale for enhanced statin-triggered 15-epi-LXA₄ generation via transcellular biosynthesis during cell-cell interactions.

**Lovastatin increases 14,15-EET generation**

To identify potential biosynthetic intermediates in lovastatin-triggered 15-epi-LXA₄ formation, lipid extracts from PMN and airway epithelial cell cultures (5:1, PMNs:Calu-3) were analyzed by RP-HPLC. In addition to 15-epi-LXA₄ (Figure 2A), statin exposed cells also had a significant increase in materials with the retention time of 14,15-epoxyeicosatriene (14,15-EET) by charged aerosol detection (CAD) (Figure 2B). The statin mediated increase in both 14,15-EET and 15-epi-LXA₄ was markedly increased by the sEH inhibitor AUDA (Figure 2A and 2B). The actions of AUDA on statin-triggered 15-epi-LXA₄ formation were concentration-dependent between 0.01 – 1 μM (Figure 2C). AUDA (0.01-1 μM) consistently increased the amount of 14,15-EET ~10 fold higher than 15-epi-LXA₄ (Figure 2D).

**Exogenous addition of 14,15-EET increases 15-epi-LXA₄ biosynthesis**

To determine if the increased 14,15-EET reflected a role in statin-initiated transcellular 15-epi-LXA₄ biosynthesis, PMNs alone were activated in the presence of exogenous 14,15-EET. No epithelial cells or lovastatin were present in these incubations. When exposed to exogenous 14,15-EET and activated with the divalent cation ionophore A23187, PMNs generated substantial amounts of 15-epi-LXA₄ (Figure 3A). There was no significant production of 15-epi-LXA₄ by PMNs activated in the absence of 14,15-EET. The effects of 14,15-EET on 15-epi-LXA₄ generation in this system were regioselective and not shared by the related isomers 5,6-EET, 8,9-EET or 11,12-EET (Figure 3B). There was a bell-shaped dose response relationship between 14,15-EET and 15-epi-LXA₄ generation with a maximal response at 100 pmoles (Figure 3C). Amounts of 14,15-EET greater than 100 pmoles gave a submaximal response; however, this decreased response was not statistically significant. In contrast, significant changes in leukotriene B₄ (LTB₄) levels in these same incubations were only observed at doses of 14,15-EET greater than 100 pmoles (Figure 3D), suggesting that the increase in 15-epi-LXA₄ with 14,15-EET was not secondary to decreased 5-LO conversion of arachidonic acid to LTB₄. In addition, the omega oxidation products of LTB₄ and 15-epi-LXA₄ were not significantly altered by addition of the EET.

The selective 5-LO inhibitor AA861 and the cPLA₂ inhibitor methyl arachidonyl fluorophosphonate both inhibited EET stimulated 15-epi-LXA₄ and LTB₄ formation. In contrast, a CYP450 enzyme inhibitor 17-octadecynoic acid (17-ODYA) increased the amount of both 15-epi-LXA₄ and LTB₄ in incubations with PMNs and 14,15-EET, likely secondary to this compound’s additional actions as a suicide inhibitor of 20-hydroxylase metabolism of eicosanoids. Incubation of 14,15-EET with recombinant 5-LO confirmed that 14,15-EET is not directly converted to 15-epi-LXA₄ or other tetraene containing products. To determine if the relationship between 14,15-EET and 15-epi-LXA₄ was...
bidirectional, PMNs were activated in the presence of 15-epi-LXA₄ (1 μM), but there was no detectable 14,15-EET generated.

**Lovastatin promotes the resolution of acute lung injury by increasing 15-epi-LXA₄ formation in vivo**

To investigate the in vivo impact of the in vitro findings with airway epithelial cells and PMNs, lovastatin (0.2 or 2 mg/kg) or vehicle was administered (i.v.) 15 min prior to ALI, and lung leukocyte infiltration was determined 18h later during the early resolution phase of this model. Lovastatin was given in mg/kg doses based on its in vivo activity in other murine models of lung inflammation.bronchoalveolar lavage fluids (BALFs) after ALI contained increased numbers of total leukocytes, especially macrophages (Mϕ) and PMNs, compared to BALFs from control animals receiving sterile saline (0.9%) (Figure 4A and 4B). At this time point, lovastatin significantly decreased total BALF cells in a dose dependent manner. Low dose lovastatin (0.2 mg/kg) decreased BALF PMNs, but only the higher dose (2 mg/kg) significantly decreased both Mϕ and PMNs. Of interest, administration of lovastatin in the absence of acid injury also displayed anti-inflammatory properties in blocking PMNs (P < 0.05) and increasing Mϕ trafficking related to low level inflammation associated with the surgical procedure. The protective actions of lovastatin for BALF PMNs were also evident in the lung parenchyma with decreased numbers of cells positive for the PMN marker Ly-6G (Figure 4C). Concomitant with decreased lung leukocyte infiltration 18h after acid injury, lovastatin treated mice also had significantly higher 15-epi-LXA₄ levels in BALFs (266 ± 46 pg/ml) compared to vehicle (131 ± 29 pg/ml, P < 0.05) or uninjured, statin-exposed animals (76 ± 34 pg/ml, P <0.05) (Figure 4D). When tested in parallel, lovastatin’s actions on airway leukocytes, in particular on airway PMNs, were similar in magnitude to direct administration of 2 mg/kg of 15-epi-LXA₄ (Figure 4E and 4F). In addition, lovastatin and 15-epi-LXA₄ gave additive inhibition of airway inflammation (Figure 4E and 4F). 14,15-EET also decreased airway PMN trafficking after ALI.

**DISCUSSION**

Results presented here have identified biosynthetic pathways for statin triggered 15-epi-LXA₄ formation during cell-cell interactions between human PMNs and airway epithelial cells. Neither cell type alone was independently capable of substantial generation of 15-epi-LXA₄, yet together they collaborated to produce this anti-inflammatory and pro-resolving mediator. Lovastatin’s induction of 15-epi-LXA₄ was shared with simvastatin, but not blocked by the addition of mevalonate, indicating that these statin properties were not solely secondary to inhibition of HMG CoA reductase. In contrast, 15-epi-LXA₄ production was dependent on LO activity. Unexpectedly, lovastatin also increased 14,15-EET formation during PMN-airway epithelial cell interactions, suggestive of decreased sEH activity, and in separate experiments the potent sEH inhibitor AUDA markedly increased 15-epi-LXA₄ production. 14,15-EET displayed a regioselective induction in 15-epi-LXA₄ formation that was distinct from its effects on LTB₄ generation. 14,15-EET was not a direct substrate for 5-LO mediated conversion, so this CYP450 enzyme-derived product indirectly altered arachidonic acid metabolism to favor 15-epi-LXA₄. These cell-cell interactions in vitro were
also present in vivo in an experimental model of PMN-airway epithelial cell interactions. Lovastatin dampened acid-initiated ALI in a dose dependent manner, in particular decreasing PMN trafficking to the lung and increasing 15-epi-LXA₄ levels in BAL fluids. Exogenous addition of 15-epi-LXA₄ also displayed tissue protective actions that were additive with lovastatin’s effects. Together, these results are the first to (a) identify statin-triggered 15-epi-LXA₄ generation by human cells, (b) determine its transcellular biosynthetic routes, (c) assign roles for CYP450 intermediates in its biosynthesis and (d) uncover 15-epi-LXA₄ as an in vivo mechanism for statin’s anti-inflammatory actions.

Interactions between PMNs and tissue resident cells are critical to innate immunity. When damaged by injury or exposed to potential pathogens, mucosal epithelial cells rapidly signal for PMN accumulation and activation to maintain mucosal barrier integrity and host defense. Because PMN anti-microbial effector mechanisms can also have an unintended capacity to damage host tissues, the interplay between PMNs and epithelial cells is tightly regulated to control the intensity of acute mucosal inflammation and prevent extensive tissue damage (reviewed in reference 35). To this end, counter-regulatory and pro-resolving mediators can stop PMN activation, block the release of epithelial pro-inflammatory mediators, enhance macrophage clearance of apoptotic PMNs and increase expression of epithelial anti-microbial peptides (reviewed in reference 14). Transient exposure of human bronchial epithelium to HCl upregulates LX receptors and LXs enhance restitution of the injured epithelium and potently block acid-triggered cytokine release and neutrophil trans-epithelial migration. By inducing 15-epi-LX generation during PMN-epithelial cell interactions, statins initiated the formation of mediators with autocrine anti-inflammatory and pro-resolving actions for these cells. In the presence of statins, epithelial cells exposed to increased inflammation in the form of greater numbers of activated PMNs gave a dose-dependent increase in 15-epi-LXA₄ production. Together, these findings uncovered new roles for transcellular biosynthesis in statin-mediated promotion of pro-resolving mediator generation to counter acute PMN-rich inflammation at mucosal epithelial interfaces.

15-epi-LXs are generated in vivo during inflammation in human disease. While CYP450 enzymes can generate 15R-HETE as a biosynthetic intermediate for 15-epi-LXs, novel biosynthetic pathways were recently elucidated in rat myocytes in which atorvastatin and/or pioglitazone can trigger atypical interactions between post-translationally modified COX-2 and 5-LO. While these findings link statin-mediated generation of 15-epi-LXA₄ to tissue protection in rat myocardium, the results presented here build on those with rat myocytes to broaden the implication for statin triggered 15-epi-LXA₄ generation to mucosal anti-inflammation with human cells and murine tissues in vivo. Of interest, statins altered CYP450 metabolism of arachidonic acid to increase levels of 14,15-EET that in turn increased 15-epi-LXA₄ production by indirect means. Exogenous addition of 14,15-EET to activated human PMNs led to 15-epi-LXA₄ generation with a bell-shaped dose-response relationship that may have resulted from cytotoxicity at the higher doses of 14,15-EET as LTB₄ generation was also inhibited. Whereas 5,6-EET can serve as a direct substrate for platelet lipoxigenase, evidence presented here did not support direct LO conversion of 14,15-EET to epi-LXs. 14,15-EET is the most abundant EET in the lung, and here the influence of 14,15-EET was regioselective, indicative of specific lipid-protein interactions.
In addition, a sEH inhibitor markedly increased both 14,15-EET and 15-epi-LXA\(_4\) formation. Inhibition of sEH during acute inflammation can also increase LX formation \textit{in vivo}.\(^{31}\) The exact mechanism for the positive influence of 14,15-EET on LX and 15-epi-LX biosynthesis remains to be elucidated.

Both EETs and LXs display anti-inflammatory actions, but their concentration responses are distinct and target dependent. In nano to micromolar amounts, EETs can inhibit NF-κB, release of pro-inflammatory mediators and leukocyte adhesion.\(^{29,41}\) In sharp contrast, LXs and 15-epi-LXs display these protective actions yet with an IC\(_{50}\) in the pico to nanomolar range and activate specific G-protein coupled receptors.\(^{19,42}\) Results here with statins serendipitously uncovered the capacity for EETs to promote 15-epi-LX production. In the presence of lovastatin and the sEH inhibitor AUDA, the generation of EETs and 15-epi-LXA\(_4\) each increased in an approximate 10:1 ratio of 14,15-EET to 15-epi-LXA\(_4\). In addition, both 14,15-EET and 15-epi-LXA\(_4\) blocked PMN trafficking \textit{in vivo} in acid-initiated ALI. Thus, these results suggest that the anti-inflammatory actions of EETs may result from endogenous generation of 15-epi-LXs that activate pro-resolving circuits in inflammation.

Statins appear to have particularly beneficial actions in several forms of lung disease. Several observational studies have suggested a link between pre-existing statin use and improved outcomes in ALI and sepsis.\(^{43-46}\) Recent data analyses from the Normative Aging Study revealed that statin use attenuated the decline of lung function in the elderly, including among past and present smokers,\(^{47}\) and the Veterans Affairs Health Care System database revealed a protective effect for statins against the development of lung cancer.\(^{48}\) In addition, statin use is associated with improved allograft function after lung transplantation.\(^{49}\) In model systems, statins inhibit cigarette smoke induced emphysema, pulmonary hypertension and lung inflammation.\(^{5,6,12}\) While less information is available on 15-epi-LX generation in human illness, these mediators were identified in plasma\(^{50}\) and in the airways of human subjects with chronic obstructive pulmonary disease.\(^{37}\) Here, lovastatin decreased ALI initiated airway inflammation by triggering an increase in lung 15-epi-LXA\(_4\). When administered with lovastatin, 15-epi-LXA\(_4\) blocked lung inflammation in an additive manner. Together, these findings indicate 15-epi-LXA\(_4\) biosynthesis is an endogenous mechanism that underlies statins’ protective actions in the lung.

In conclusion, statins initiated the endogenous biosynthesis of 15-epi-LXs during PMN-epithelial cell interactions \textit{in vitro} and in mucosal inflammation \textit{in vivo}. Statins promoted 15-epi-LXA\(_4\) generation by decreasing sEH activity to increase 14,15-EET that influences arachidonate conversion to 15-epi-LXs. Thus, the present results have uncovered endogenous 15-epi-LX generation as a pivotal mechanism involved in statin’s known anti-inflammatory actions and suggest that via 15-epi-LXA\(_4\) biosynthesis statins can activate resolution circuits \textit{in vivo}. Given the observed clinical benefits for statin therapy, our findings raise the possibility that enhancing 15-epi-LX production may also enhance the anti-inflammatory actions of statins.
METHODS

PMN Isolation and Incubations

Fresh venous blood (~180 ml) was obtained from healthy volunteers who had not taken any medication for at least two weeks, and PMNs were isolated as in reference 32. The protocol was approved by the Partners Healthcare institutional review board and written informed consent was obtained from all subjects. Freshly isolated PMNs were suspended (10 x 10^6 PMNs/ml) in PBS containing 130 mg/l calcium (Ca^{2+}) and 100 mg/l magnesium (Mg^{2+}). In select experiments, PMNs were exposed (30 min, 37°C) to 5,6-EET, 8,9-EET, 11,12-EET or 14,15-EET (0 – 10 μM) or 15-epi-LXA_4 (1μM) followed by A23187 (5 μM, 15 min, 37°C). For incubations with inhibitors, PMNs were exposed (10 min, 37°C) to methyl arachidonoyl fluorophosphonate (10 μM), AA-861 (20 μM), ODYA (20 μM) or vehicle (0.1% ethanol) prior to cell activation. All incubations were stopped with 2 volumes of cold methanol (2:1, vol:vol) and samples stored at -20°C.

Airway Epithelial Cell Culture and Incubations

Human bronchial epithelial cells (Calu-3 cells) were cultured to confluence (~3 x 10^6 cells), then exposed (24h) to TNF-α (1ng/ml) prior to the addition of freshly isolated human PMNs (15 x 10^6 PMNs/ml) for 30 min (37°C) in Hanks’ balanced salt solution containing Ca^{2+} and Mg^{2+} (Invitrogen) in the presence or absence of lovastatin hydroxy acid (Cayman Chemical, 1μM) as in reference 34. In some experiments, Calu-3 cells were exposed (15 min, 37°C) to NDGA (5μM), mevalonate (100 μM), simvastatin (10 μM) or AUDA (0-1μM) prior to addition of PMNs. Incubations were stopped with cold methanol (2:1, vol:vol) then samples were stored at -20°C.

Primary NHBE cells (Clonetics-BioWhittaker, San Diego, CA) were maintained in culture for 21 days to obtain a differentiated cell population with a mucociliary phenotype as in reference 17. Differentiated NHBE were exposed to TNF-α (1 ng/ml, 24h) and then PMNs were added with lovastatin (1μM) or vehicle (0.1% ethanol) for 30 min (37°C). Incubations were stopped and stored as above.

Lipid Mediators

To identify eicosanoids, materials were first extracted with C_{18} Sep-Pak cartridges (Waters, Milford, MA). Prostaglandin B_2 (PGB_2) (100 ng) was added to each sample as an internal standard for extraction recoveries. Materials in the methyl formate eluate (i.e., 15-epi-LXs) were brought to dryness under a gentle stream of N_2, resuspended in 1 ml of methanol and kept at ~20°C. 30% of the methyl formate fraction was analyzed by RP-HPLC (Agilent 1100 series; Agilent Technologies, Palo Alto, CA) equipped with an Ultrasphere C18 (250 x 4.6 mm, 5μm; Phenomenex, Torrance, CA) column and coupled to a photodiode array detector (ultraviolet and visible range). In addition, a second HPLC system coupled to a Corona® CAD™ detector (Charged Aerosol Detector; ESA, Chelmsford, MA) enabled detection of select lipid mediators in the low picogram range. The mobile phase was methanol–distilled H_2O–glacial acetic acid (70:30:1, vol/vol/vol) as phase one (to-30 min) and a linear gradient with methanol (100%) as phase two (30-65 min) at an initial flow rate of 0.5 ml/min (to-30 min) followed by 1 ml/min (30 min-65min). The criteria used for...
identification included retention time, UV spectra and CAD. 15-epi-LXA₄ and LTB₄ were monitored by both RP-HPLC and ELISA (Neogen); PGB₂ by HPLC-DAD and EETs by HPLC-CAD since they do not possess specific chromophores.

**RT-PCR**

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) and residual DNA was removed by DNAse I (Invitrogen Life Technologies, Carlsbad, CA). After reverse transcription (Sensiscript, Qiagen, Valencia, CA), PCR was performed using 1μg cDNA/reaction with specific primers for human sEH (40 cycles) (Forward: TGTAATAGCCCAGAAGGCCCCAG, Reverse: ACATCTGAGGAAGGCAGAACAG); 15-LO-1 (45 cycles) (Forward: CCGACCTCGCTATCAAAGAC, Reverse: GGATGACCATGGGCAAGAG); 15-LO-2 (45 cycles) (Forward: ATCAGGACGTTCACGGCGAGG, Reverse: CCAGGAACAGCTCGTTTTCCTG); FLAP (40 cycles) (Forward: GCAGCAACGGCAGATGAGGAACAGG, Reverse: GCCAGCAACGGACATGGAACAGG); 5-LO (40 cycles) (Forward: GGTGAATCCGCTATCAAAGAC, Reverse: GGATGACCATGGGCAAGAG); 12-LO (40 cycles) (Forward: TGGACACTGAAGGCAGGGC, Reverse: GGCTGGGAGGCTGAATCTG); and COX-2 (40 cycles) (Forward: TTCAAATGAGATTGTGGGAAAATTGCT, Reverse: AGATCATCTCTTGACGTATCTT). Human β-actin was used as internal control.¹⁷

**Acid-Initiated Acute Lung Injury**

All animal protocols were approved by the Harvard Medical Area Animal Institutional Review Board. Hydrochloric acid (0.1 N HCl, pH 1.5, 50 μl, endotoxin free; Sigma-Aldrich) was selectively instilled into the left mainstem bronchus of anesthetized mice (FVB, male, 10–12 wk; Charles River Laboratories) via a 24-gauge angiocatheter inserted intratracheally as in reference ¹⁷. To select animals, lovastatin (0.2 or 2 mg/kg), 15-epi-LXA₄ (2 mg/kg), 14,15-EET (2 mg/kg), the combination of lovastatin and 15-epi-LXA₄ (2 mg/kg each) or vehicle (<1% ethanol) was administered (i.v., 100 μl) 15 min before HCl instillation. 18h after HCl, BAL was performed with 2 × 1 ml PBS with 0.6 mM EDTA, cell-free supernatants were obtained and 15-epi-LXA₄ levels measured by ELISA (Neogen). Cells in BALFs were resuspended in PBS and enumerated by hemocytometer. Cytospins were performed by cyt centrifuge (STATspin) (265 × g) and leukocyte differentials determined after Wright-Giemsa stain (Sigma), counting ≥200 cells per slide. Lungs were fixed in IHC zinc buffer and paraffin embedded for immunostaining with LY-6G (1:50 dilution).

**5-LO Incubations**

14,15-EET (100 μM) was incubated with 20 μg of potato 5-LO (Biomol) (30°C, 10 min) in 0.1 M K₂HPO₄ buffer (pH 6.3). Lipoxigenase activity was measured by spectrophotometer in the standard assay mixture at 0 and 10 min, monitoring at 301, 270 and 234 nm.
Statistical Analysis

Values for eicosanoid levels were analyzed by Student’s t-test and analysis of variance (ANOVA). Data are presented as the mean ± SEM; P value < 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Jain MK, Ridker PM. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. Nature Reviews Drug Discovery. 2005; 4(12):977–87. [PubMed: 16341063]
2. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. Nature Medicine. 2000; 6(12):1399–402.
3. Mira E, et al. Statins induce regulatory T cell recruitment via a CCL1 dependent pathway. J Immunol. 2008; 181(5):11. [PubMed: 18566364]
4. Murphy DM, et al. Simvastatin attenuates release of neutrophilic and remodeling factors from primary bronchial epithelial cells derived from stable lung transplant recipients. Am J Physiol Lung Cell Mol Physiol. 2008 Mar; 294(3):L592–9. [PubMed: 18203812]
5. Takahashi S, et al. Reversal of elastase-induced pulmonary emphysema and promotion of alveolar epithelial cell proliferation by simvastatin in mice. Am J Physiol Lung Cell Mol Physiol. 2008 May; 294(5):L882–90. [PubMed: 18310229]
6. Taraseviciene-Stewart L, et al. Simvastatin causes endothelial cell apoptosis and attenuates severe pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol. 2006 Oct; 291(4):L668–76. [PubMed: 16698853]
7. Ridker PM, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. N Engl J Med. 2008 Nov 20; 359(21):2195–207. [PubMed: 18997196]
8. Merx MW, et al. HMG-CoA reductase inhibitor simvastatin profoundly improves survival in a murine model of sepsis. Circulation. 2004; 109(21):2560–5. [PubMed: 15123521]
9. Leung BP, et al. A novel anti-inflammatory role for simvastatin in inflammatory arthritis. J Immunol. 2003; 170(3):1524–30. [PubMed: 12538717]
10. McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. J Immunol. 2004; 172(5):2903–8. [PubMed: 14978092]
11. Lee JH, et al. Simvastatin inhibits cigarette smoking-induced emphysema and pulmonary hypertension in rat lungs. American Journal of Respiratory & Critical Care Medicine. 2005; 172(8):987–93. [PubMed: 16002570]
12. Fessler MB, et al. A role for hydroxy-methylglutaryl coenzyme a reductase in pulmonary inflammation and host defense. American Journal of Respiratory & Critical Care Medicine. 2005; 171(6):606–15. [PubMed: 15591471]
13. Ware LB, Matthay MA. The acute respiratory distress syndrome. N Engl J Med. 2000 May 4; 342(18):1334–49. [PubMed: 10793167]
14. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nature Reviews Immunology. 2008; 8(5):349–61.
15. Haworth O, Levy BD. Endogenous lipid mediators in the resolution of airway inflammation. European Respiratory Journal. 2007; 30(5):980–92. [PubMed: 17978156]
16. El Kebir D, et al. 15-Epi-lipoxin A4 Inhibits Myeloperoxidase Signaling and Enhances Resolution of Acute Lung Injury. American Journal of Respiratory & Critical Care Medicine. 2009; 180:311–9. [PubMed: 19483113]
17. Fukunaga K, Kohli P, Bonnans C, Fredenburgh LE, Levy BD. Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury. J Immunol. 2005; 174(8):5033–9. [PubMed: 15814734]
18. Clish CB, O’Brien JA, Gronert K, Stahl GL, Petasis NA, Serhan CN. Local and systemic delivery of a stable aspirin-triggered lipoxin prevents neutrophil recruitment in vivo. Proceedings of the National Academy of Sciences of the United States of America. 1999 Jul 6; 96(14):8247–52. [PubMed: 10393980]
19. Jozsef L, Zouki C, Petasis NA, Serhan CN, Filep JG. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit peroxynitrite formation, NF-kappa B and AP-1 activation, and IL-8 gene expression in human leukocytes. Proceedings of the National Academy of Sciences of the United States of America. 2002 Oct 1; 99(20):13266–71. [PubMed: 12235371]
20. Paul-Clark MJ, Van Cao T, Moradi-Bidhendi N, Cooper D, Gilroy DW. 15-epi-lipoxin A4-mediated induction of nitric oxide explains how aspirin inhibits acute inflammation. Journal of Experimental Medicine. 2004 Jul 5; 200(1):69–78. [PubMed: 15238606]
21. Canny G, et al. Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. Proceedings of the National Academy of Sciences of the United States of America. 2002 Mar 19; 99(6):3902–7. [PubMed: 11891303]
22. Godson C, Mitchell S, Harvey K, Petasis NA, Hogg N, Brady HR. Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. Journal of Immunology. 2000 Feb 15; 164(4):1663–7.
23. Levy BD. Myocardial 15-epi-lipoxin A4 generation provides a new mechanism for the immunomodulatory effects of statins and thiazolidinediones. Circulation. 2006; 114(9):873–5. [PubMed: 16940203]
24. Birnbaum Y, et al. Augmentation of myocardial production of 15-epi-lipoxin-a4 by pioglitazone and atorvastatin in the rat. Circulation. 2006; 114(9):929–35. [PubMed: 16908763]
25. Schwab JM, Chiang N, Arita M, Serhan CN. Resolvin E1 and protectin D1 activate inflammation-resolution programmes. Nature. 2007 Jun 14; 447(7146):869–74. [PubMed: 17568749]
26. Clara J, Serhan CN. Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proceedings of the National Academy of Sciences of the United States of America. 1995; 92(21):9475–9. [PubMed: 7568157]
27. Clara J, Lee MH, Serhan CN. Aspirin-triggered lipoxins (15-epi-LX) are generated by the human lung adenocarcinoma cell line (A549)-neutrophil interactions and are potent inhibitors of cell proliferation. Mol Med. 1996 Sep; 2(5):583–96. [PubMed: 8898374]
28. Karara A, Dishman E, Blair I, Falck JR, Capdevila JH. Endogenous epoxyeicosatrienoic acids. Cytochrome P-450 controlled stereoselectivity of the hepatic arachidonic acid epoxygenase. J Biol Chem. 1989 Nov 25; 264(33):19822–7. [PubMed: 2584196]
29. Capdevila JH, Harris RC, Falck Jr. Microsomal cytochrome P450 and eicosanoid metabolism. Cell Mol Life Sci. 2002 May; 59(5):780–9. [PubMed: 12088278]
30. Moghaddam MF, Grant DF, Cheek JM, Greene JF, Williamson KC, Hammock BD. Bioactivation of leukotoxins to their toxic diols by epoxide hydrolase. Nature Medicine. 1997; 3(5):562–6.
31. Schmelzer KR, Kubala L, Newman JW, Kim IH, Eisrich JP, Hammock BD. Soluble epoxide hydrolase is a therapeutic target for acute inflammation. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102(28):9772–7. [PubMed: 15994227]
32. Romano M, Chen XS, Takahashi Y, Yamamoto S, Funk CD, Serhan CN. Lipoxin synthase activity of human platelet 12-lipoxigenase. Biochem J. 1993 Nov 15; 296(Pt 1):127–33. [PubMed: 8250832]
33. Shak S, Reich NO, Goldstein IM, Ortiz de Montellano PR. Leukotriene B4 omega-hydroxylase in human polymorphonuclear leukocytes. Suicidal inactivation by acetylenic fatty acids. Journal of Biological Chemistry. 1985; 260(24):13023–8. [PubMed: 2997155]
34. Morimoto K, et al. Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. J Immunol. 2006 Jun 15; 176(12):7657–65. [PubMed: 16751413]

35. Leitch AE, Duffin R, Haslett C, Rossi AG. Relevance of granulocyte apoptosis to resolution of inflammation at the respiratory mucosa. Mucosal Immunol. 2008 Sep; 1(5):350–63. [PubMed: 19079199]

36. Bonnans C, Fukunaga K, Levy MA, Levy BD. Lipoxin A4 regulates bronchial epithelial cell responses to acid injury. Am J Pathol. 2006 Apr; 168(4):1064–72. [PubMed: 16565483]

37. Vachier I, et al. Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. J Allergy Clin Immunol. 2005 Jan; 115(1):55–60. [PubMed: 15637547]

38. Ye Y, et al. Phosphorylation of 5-lipoxygenase at ser523 by protein kinase a determines whether pioglitazone and atorvastatin induce proinflammatory leukotriene B4 or anti-inflammatory 15-epi-lipoxin a4 production. J Immunol. 2008; 181(5):9.

39. Balazy M. Metabolism of 5,6-epoxyeicosatrienoic acid by the human platelet. Formation of novel thromboxane analogs. Journal of Biological Chemistry. 1991; 266(35):23561–7. [PubMed: 1748634]

40. Zeldin DC, et al. The rabbit pulmonary cytochrome P450 arachidon acid metabolic pathway: characterization and significance. J Clin Invest. 1995 May; 95(5):2150–60. [PubMed: 7738183]

41. Morin C, Sirois M, Echave V, Gomes MM, Rousseau E. EET displays anti-inflammatory effects in TNF-alpha stimulated human bronchi: putative role of CPI-17. Am J Respir Cell Mol Biol. 2008 Feb; 38(2):192–201. [PubMed: 17872494]

42. Fierro IM, et al. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit human neutrophil migration: comparisons between synthetic 15 epimers in chemotaxis and transmigration with microvessels endothelial cells and epithelial cells. J Immunol. 2003 Mar 1; 170(5):2688–94. [PubMed: 12594298]

43. Almog Y, et al. Prior statin therapy is associated with a decreased rate of severe sepsis. Circulation. 2004 Aug 17; 110(7):880–5. [PubMed: 15289367]

44. Irish Critical Care Trials G. Acute lung injury and the acute respiratory distress syndrome in Ireland: a prospective audit of epidemiology and management. Crit Care. 2008; 12(1):R30. [PubMed: 18312626]

45. Kruger P, Fitzsimmons K, Cook D, Jones M, Nimmo G. Statin therapy is associated with fewer deaths in patients with bacteremia. Intensive Care Med. 2006 Jan; 32(1):75–9. [PubMed: 16283159]

46. Liappis AP, Kan VL, Rochester CG, Simon GL. The effect of statins on mortality in patients with bacteremia. Clin Infect Dis. 2001 Oct 15; 33(8):1352–7. [PubMed: 11565076]

47. Alexeeff SE, Litonjua AA, Sparrow D, Vokonas PS, Schwartz J. Statin use reduces decline in lung function: VA Normative Aging Study. American Journal of Respiratory & Critical Care Medicine. 2007 Oct 15; 176(8):742–7. [PubMed: 17673694]

48. Khurana V, Bejjanki HR, Caldito G, Owens MW. Statins reduce the risk of lung cancer in humans: a large case-control study of US veterans. Chest. 2007 May; 131(5):1282–8. [PubMed: 17494779]

49. Johnson BA, Iacono AT, Zevei A, McCurry KR, Duncan SR. Statin use is associated with improved function and survival of lung allografts. American Journal of Respiratory & Critical Care Medicine. 2003 May 1; 167(9):1271–8. [PubMed: 12615629]

50. Chiang N, Bermudez EA, Ridker PM, Hurwitz S, Serhan CN. Aspirin triggers antiinflammatory 15-epi-lipoxin A4 and inhibits thromboxane in a randomized human trial. Proceedings of the National Academy of Sciences of the United States of America. 2004 Oct 19; 101(42):15178–83. [PubMed: 15471991]
Figure 1. Lovastatin triggers 15-epi-LXA$_4$ biosynthesis by activated human airway epithelial cells and PMNs

(A) 15-epi-LXA$_4$ levels were determined after Calu-3 cells were exposed to TNF-α (1 ng/ml, 24h, 37°C) and then to human PMNs in the presence or absence of lovastatin (1 μM, 30 min, 37°C) (see Methods). (B) Dose dependent relationship between PMNs relative to Calu-3 cells was determined for 15-epi-LXA$_4$ generation by lovastatin exposed cells. (C) Select incubations with PMNs and Calu-3 cells (5:1 ratio) were carried out in the presence of lovastatin (1 μM), mevalonate (100 μM), simvastatin (10 μM), NDGA (LO inhibitor, 5 μM) or vehicle (0.1% ethanol). (D) Representative RT-PCR for pivotal lipoxin biosynthetic
genes in Calu-3 cells (without and with exposure to TNF-α), freshly isolated human PMNs, and NHBE exposed to IL-13 (10 ng/ml, 96h, 37°C). Results are expressed as the mean ± SEM for n ≥ 3 independent experiments. *P < 0.05 by ANOVA, n.s. = not significant.
Figure 2. Lovastatin and the sEH inhibitor AUDA increase 14,15-EET and 15-epi-LXA4 formation
Neutrophils (PMNs) were incubated with TNF-α-exposed Calu-3 cells (5:1, PMNs:Calu-3) in the presence of lovastatin (1 μM) and in some cases AUDA (1 μM) for 30 min (37°C). Lipids were extracted and analyzed by RP-HPLC using (A) absorbance at 301 nm (Inset, UV spectrum for (i.) non-specific materials at 13.3 min and (ii.) for 15-epi-LXA4 at 13.7 min) or (B) charged aerosol detector (CAD) (see Methods). Authentic materials are shown in the upper panels. (C,D) Concentration response to AUDA (0.01 – 1 μM) on statin-triggered 15-epi-LXA4 formation relative to (C) vehicle and (D) 14,15-EET by TNF-α-exposed Calu-3.
cells and PMNs. 15-epi-LXA$_4$ levels were determined by ELISA. Results are expressed as mean ± SEM (n = 3 independent experiments). *P < 0.05 relative to no AUDA and **P < 0.05 compared to 14,15-EET by ANOVA.
Figure 3. Regiospecific influence of 14,15-EET on 15-epi-LXA₄ biosynthesis by activated human PMNs
Freshly isolated human PMNs were activated with A23187 in the presence of EETs (1 μM) or vehicle (0.1% ethanol). After extraction, 15-epi-LXA₄ levels were measured by (A) RP-HPLC (Authentic 15-epi-LXA₄ is shown in the upper panel) or (B) ELISA (see Methods). Concentration response for 14,15-EET (10 – 10,000 pmoles) on (C) 15-epi-LXA₄ and (D) LTB₄ formation. Values represent the mean ± SEM for n ≥ 3. *P < 0.05 by Student’s t-test.
Figure 4. Lovastatin promotes 15-epi-LXA4 formation in vivo and ALI resolution
Lovastatin (0.2 or 2 mg/kg Statin) or vehicle (0.9% saline) were given i.v. 15 min before HCl-initiated ALI and BALFs were obtained 18h later. (A) Total BALF cells were enumerated, and (B) the number of BALF macrophages (Mϕ) and neutrophils (PMN) were determined (see Methods). (C) Immunostaining for Ly-6G (1:50 dilution) in murine lung tissue obtained 18h after acid-induced ALI in the absence (upper panels) or presence of lovastatin (lower panels). PMNs are highlighted by arrows and original magnifications are indicated. (D) 15-epi-LXA4 levels were determined by ELISA in BALFs. For purposes of
direct comparison, resolution of lung leukocyte infiltration at 18h was determined by monitoring (E) total BALF leukocytes and (F) BALF PMNs in mice administered (2 mg/kg, i.v., 100 μl) lovastatin, 15-epi-LXA₄, 14,15-EET, the combination of 15-epi-LXA₄ and lovastatin (2 mg/kg each) or vehicle (1% ethanol) 15 min prior to intratracheal acid. Values represent the mean ± SEM (n ≥4 from at least 3 independent experiments). *P < 0.05 compared to no acid or statin, **P < 0.05 compared to vehicle and #P < 0.05 compared to 15-epi-LXA₄ alone.