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Lipoxin A₄ Attenuates Microvascular Fluid Leak During Inflammation

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Abstract

Background—The release of proinflammatory cytokines during inflammation disturbs the endothelial barrier and can initiate significant intravascular volume loss. Proinflammatory cytokines also induce the expression of anti-inflammatory mediators, such as lipoxin, which promote the resolution of inflammation. Our hypothesis is that lipoxin A₄ (LXA₄) reverses the increased microvascular fluid leak observed during inflammatory conditions.

Materials And Methods—Microvascular fluid leak (L_p) was measured in rat mesenteric venules using a micro-cannulation technique. L_p was measured under the following conditions: 1) LXA₄ (100nM) alone (n=5), 2) LXA₄ (100nM) administered after endothelial hyperpermeability induced by a continuous perfusion of 10nM platelet activating factor (PAF) (n=5), 3) LXA₄ (100nM) perfused after inflammation induced by a systemic bolus of 10mg/kg lipopolysaccharide (LPS) (n=5), and 4) LXA₄ (100nM) perfused after LPS-induced inflammation during inhibition of c-Jun N-terminal kinase (n=4).

Results—LXA₄ alone slightly increased L_p from baseline (L_p -baseline=1.05±0.03, L_p -LXA₄=1.55±0.04; p<0.0001). PAF increased L_p fourfold (L_p -baseline=1.20±0.10, L_p -PAF=4.49±0.95; p<0.0001). LXA₄ administration after PAF decreased L_p 66% versus PAF alone (from 4.49±0.95 to 1.54±0.13; p=0.0004). LPS-induced inflammation increased L_p over 2-fold (L_p -baseline=1.05±0.03, L_p -LPS=2.27±0.13; p<0.0001). LXA₄ administration after LPS decreased L_p 42% versus LPS alone (from 2.27±0.13 to 1.31±0.05; p<0.0001). The effect of c-Jun N-terminal kinase inhibition during LPS-induced inflammation attenuated the decrease in leak cause by LXA₄ by 51% (p=0.0002).

Conclusion—After either LPS or PAF, LXA₄ attenuated the intravascular volume loss caused by these inflammatory mediators. LXA₄'s activity may be partly mediated by the c-Jun N-terminal kinase signaling pathway. These data support an anti-inflammatory role for LXA₄ and suggests a potential pharmacologic role for LXA₄ during inflammation.

Keywords

Shock; Lipoxins; Inflammation; Sepsis; Lipoxin A₄

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INTRODUCTION

Lipoxins are trihydroxytetraene-containing eicosanoids that are generated within the vascular lumen through platelet-leukocyte interactions. The formation of lipoxins occurs during a variety of conditions, including inflammation and ischemia-reperfusion injury. In the original description by Serhan in 1985, lipoxins represented a unique but growing class of mediators with anti-inflammatory actions [1]. The spectrum of lipoxins' anti-inflammatory activity include inhibiting formyl-methionyl-leucyl-phenylalanine (fMLP) and leukotriene B₄-induced chemotaxis [2], preventing neutrophil adhesion and transmigration through the endothelium [3,4], and diminishing the production of chemokines in intestinal mucosa and fibroblasts [5,6]. Furthermore, lipoxins have been found to promote the resolution of inflammation by stimulating non-phlogistic phagocytosis of activated macrophages and monocytes [7] and inhibiting the production of superoxide ion [8].

During sepsis and shock, inflammatory cytokines initiate events that can lead to a significant loss of intravascular volume. The loss of microvascular integrity following sepsis is among the cascade of events that has been associated with systemic inflammatory response syndrome, multiple organ failure, and significant morbidity and mortality [9,10]. As the insult from sepsis or shock ensues anti-inflammatory mediators, such as lipoxins, are released and the presence of lipoxin in the inflammatory milieu heralds the resolution phase of inflammation [11]. Several studies have supported the actions of lipoxins as endogenous anti-inflammatory mediators that may act to limit the inflammatory process [1–8]. Since most inflammatory reactions are self-limiting, it seems likely that signals which limit inflammation also would limit leukocyte recruitment and tissue injury. In fact, there is compelling evidence that the white cell-platelet interactions which form during inflammation, also lead to the formation of lipoxins, which in turn, act as the stop signals which limit the inflammatory cascade [12,13].

The purpose of this study was to determine the effect of lipoxin on modulating microvascular fluid leak. Our hypothesis was that lipoxin A₄ reverses the increased microvascular fluid leak observed during inflammatory conditions and that the effect of lipoxin A₄ is dependent on white cell-platelet interaction. The ability of lipoxin A₄ to modulate microvascular fluid leak was examined in three ways. First, the effect of lipoxin A₄ on microvascular fluid leak was examined in the basal state. Second, the effect of lipoxin A₄ was explored during a hyperpermeable state induced by the acute inflammatory agent, platelet activating factor, in an environment that excluded the involvement of white cells and platelets. Next the effect of lipoxin A₄ was determined during systemic inflammation induced by systemic lipopolysaccharide that included the influence of white cells and platelets. Finally, the signalling pathway of lipoxin A₄ were examined by determining its effects during inflammation induced by LPS during inhibition of the c-Jun N-terminal kinase pathway.

MATERIALS AND METHODS

Institutional Animal Care and Use Committee approval for this study was obtained and appropriate protocols for vertebrate animals research were followed.

Solutions

Mammalian Ringer's solution was prepared with 135 mmol/L NaCl, 4.6 mmol/L KCl, 2.0 mmol/L CaCl₂, 2.46 mmol/L MgSO₄, 5.0 mmol/L NaHCO₃, 5.5 mmol/L dextrose, 11.04 mmol/L HEPES acid, and 9.03 mmol/L HEPES salt (Research Organics, Cleveland, OH). A 1% bovine serum albumin (BSA) solution was prepared before each experiment and added to all perfusion solutions (BSA crystallized, Sigma Chemical Co., St. Louis, MO).

The test perfusates consisted of rat red blood cell markers, 1% BSA solution, and test mediator(s). The test mediators included Lipoxin A₄ (Sigma), platelet activating factor (PAF, Sigma), the c-Jun N-terminal kinase antagonist, SP600125 (Biomol International Inc.), and *E. Coli* lipopolysaccharide (LPS; O55:B5, Sigma). The LPS was dissolved in 0.9% normal saline and administered as a systemic 10 mg/kg bolus through the femoral vein and then subsequently as a 0.5 mg/ml continuous perfusion into the study post-capillary mesenteric venule.

Animal Preparation

Adult female Sprague Dawley rats (250–310 g; Hilltop Lab Animals Inc., Scottsdale, PA) were anesthetized with sodium pentobarbital (60 mg/kg body weight). The small bowel mesentery was gently exposed and positioned over a quartz pillar for examination on an inverted microscope (Diaphot, Nikon; Melville, NY). The mesentery was continuously bathed in mammalian Ringer's solution.

Mesenteric post-capillary venules, 20–30 μm in diameter and at least 400 μm in length, were identified based on flow patterns. The study vessels were confined to 2–3 convergence points from true capillaries and were devoid of smooth muscle cells. Because leukocyte adherence can affect microvascular fluid leak, only venules without leukocyte adherence or side branches were chosen for use in the experiments.

Red blood cells used as flow markers were obtained by harvesting red blood cells from adult female Sprague Dawley rats one week prior to experimentation. The blood was centrifuged to remove the plasma and buffy coat, and then washed three times in 15 ml of mammalian Ringer's solution.

Measurements of Microvascular Fluid Leak

Microvascular fluid leak (L_p) was determined by the modified Landis micro-occlusion technique. The assumptions and limitations of this method have been described in detail [14]. Briefly, single post-capillary mesenteric venules were cannulated with a micropipette that contained mammalian Ringer's solution, marker red cells, and 1% BSA. A water manometer connected to the micropipette enabled manipulation of the hydrostatic pressure in the vessel. To measure transmural water flux (J_v), the vessel was occluded at a position distal to the cannulation site, permitting the intravascular pressure to equilibrate with the pressure in the micropipette as set with the water manometer. As fluid moved out of the vessel and into the interstitium, marker red cells advanced out of the micropipette and down the vessel toward the occluder.

Video recordings were taken of the marker red cells in the vessel after occlusion for several seconds. Initial cell velocity (dl/dt) was obtained by recording marker red cell position at three different time points during each of three separate vessel occlusions. Transmural water flux per unit area (J_v/S) was calculated by the following equation: $J_v/S = (dl/dt)(r/2l)$, where r is the capillary radius and l is the initial distance between the marker red cell and the occluded site.

Microvascular fluid leak (L_p) was determined on the basis of a modified version of Starling's equation for fluid filtration: $J_v = L_p S[(P_c - P_i) - \sigma \Delta\pi]$, where P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, σ is the osmotic reflection coefficient, and $\Delta\pi$ is the colloid osmotic pressure gradient. Assuming the interstitial hydrostatic pressure (P_i) was near zero and the osmotic reflection coefficient (σ) and colloid osmotic pressure gradient ($\Delta\pi$) remain constant, L_p was calculated from the slope of the regression of transmural water flux per unit surface area (J_v/S) on hydrostatic pressure (P_c) derived from several occlusions at three different hydrostatic pressures: $L_p = (J_v/S)/P_c$. A

minimum of three different pressures (range, 35–80 cm H₂O) was used for each estimation of L_p . Time studies have documented the stability of this model over time [15].

Experimental Design

In all of the studies, each individual venule was initially cannulated and perfused with 1% BSA/Ringer's solution for 10 minutes, and L_p measurements were obtained as baseline controls for all the studies.

Effect of Lipoxin A₄ on Microvascular Fluid Leak—To examine the effects of LXA₄ on L_p , study venules were cannulated and, baseline L_p was measured. Then, 100 nM LXA₄ was continuously perfused into the study venule and L_p measurements were obtained at 5-minute intervals for 30 minutes (n = 5).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During PAF-induced Hyperpermeability—To examine the direct effect of LXA₄ on modulating endothelial hyperpermeability during an inflammatory state, a continuous local perfusion of the inflammatory agent PAF was administered in order to stimulate inflammatory conditions in the absence of white cells and platelets. This was accomplished by an initial cannulation of a mesenteric post-capillary venule and measurement of baseline L_p , followed by a continuous perfusion of PAF (10 nM). L_p was measured at 5-minute intervals for 30 minutes (PAF control, n = 5). The effect of LXA₄ during PAF-induced endothelial hyperpermeability was examined in a new set of rats and baseline L_p measured. Venules were then perfused for 3 minutes with PAF after which L_p was measured. Venules were immediately recannulated with PAF (10 nM) plus LXA₄ (100 nM) and L_p measured at 5-minute intervals for 30 minutes (n = 5).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation—Systemic inflammation was induced by an intravenous bolus of LPS (10 mg/kg) into the femoral vein followed 15 minutes later with cannulation of a mesenteric post-capillary venule and continuous perfusion of the venule with LPS (0.5 mg/kg) (LPS control, n = 5). Because the mechanism of action of LXA₄ is thought to be dependent on the interaction between white cells and platelets, the effect of LXA₄ was then measured during an inflammatory state that included the presence of formed blood elements including platelets and white cells. Therefore in another set of animals, LPS (10 mg/kg) was administered as an intravenous bolus into the femoral vein followed 15 minutes later by cannulation of a mesenteric post-capillary venule and continuous perfusion of the venule with LPS (0.5 mg/kg) plus LXA₄ (100 nM). L_p measurements were obtained at 5-minute intervals for 30 minutes (n = 5).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation and JNK Signal Pathway Inhibition—To examine the effect of LXA₄ on hydraulic permeability during LPS-induced inflammation and JNK signaling pathway inhibition, baseline L_p was measured as described above. The venule was then continuously infused with the JNK specific inhibitor SP600125 (10^{-5} M) for 30 minutes, after which, the micropipette removed was the venule. A bolus of LPS (10 mg/kg) was then given intravenously and the venule under study was recannulated 15 minutes later. LPS (0.5 mg/kg) and LXA₄ (100nM) were infused continuously along with SP600125 (10^{-5} M). L_p measurements were obtained at 5-minute intervals for 30 minutes (n = 4).

Statistical Analysis

Differences between data point means within each study group were analyzed by Repeated Measures ANOVA with post hoc analysis. Differences between group means were analyzed

by ANOVA. L_p measurements for groups treated with JNK-pathway inhibition were compared by calculating the area under the curves (AUC) as described previously [16,17,18]. Comparisons of the AUC were evaluated for significance using ANOVA and Fisher's protected least significance. Statistical significance was set at an alpha error of 5%. All values for L_p are shown as mean $\pm$ SEM $\times 10^{-7}$ cm s⁻¹cmH₂O⁻¹. All statistical analyses were calculated using *Stat View* Version 5.0.1 (1992–1998, Cary, NC).

RESULTS

Effect of Lipoxin A₄ on Microvascular Fluid Leak

Continuous administration of LXA₄ slightly increased L_p compared to baseline values (L_p baseline = 1.05 ± 0.03 ; L_p LXA₄ = 1.55 ± 0.04 , $p < 0.0001$). The increase in microvascular fluid leak was maximal at 5 minutes. Changes in L_p were transient and returned to baseline values by twenty minutes (Figure 1).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During PAF-induced Hyperpermeability

PAF increased L_p fourfold by 10 minutes (from 1.20 ± 0.10 to 4.49 ± 0.95 ; $p < 0.0001$). Administration of LXA₄ after PAF-induced hyperpermeability decreased L_p 66% versus PAF alone (from 4.49 ± 0.95 to 1.54 ± 0.13 ; $p = 0.0004$) (Figure 2).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation

LPS-induced inflammation increased L_p over 2-fold from baseline (L_p baseline = 1.05 ± 0.03 , L_p -LPS = 2.27 ± 0.13) ($p < 0.0001$). Administration of LXA₄ after LPS-induced inflammation decreased L_p 42% versus LPS alone (from 2.27 ± 0.13 to 1.31 ± 0.05 ; $p < 0.0001$) (Figure 3).

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation and JNK Signal Pathway Inhibition

LXA₄ administration after LPS during JNK inhibition with SP600125 prevented the 45% decrease observed with LXA₄ during LPS. JNK inhibition attenuated the effect of LXA₄ after LPS by 51%. (AUC: LPS alone = 33.50 ± 1.40 ; AUC: LPS + LXA₄ = 18.32 ± 0.70 ; AUC: LXA₄ + LPS during JNK inhibition = 25.46 ± 1.11 ; $p = 0.0002$).

DISCUSSION

Lipoxins were described for the first time by Serhan and his colleagues in 1984 [1]. Since their discovery, lipoxins have been found to modulate the inflammatory cascade by modulating the activity of several cell types. Lipoxins and their analogues have been found to inhibit leukocyte chemotaxis [19], margination [20], and transmigration across endothelial cells [21], as well as preventing neutrophil IL-8 chemokine release [22,23] and superoxide formation [22,24]. In monocytes, lipoxins stimulate non-phlogistic phagocytosis of apoptotic leukocytes [25]. Within the endothelia, lipoxins inhibit P-selectin mobilization and attenuates CD11/CD18 expression preventing neutrophil accumulation within the microvasculature and thus neutrophil-dependent vascular injury [21]. The major pathway identified by which lipoxins limit neutrophil-dependent vascular injury occurs through receptor antagonism of leukotrienes. [19,26–28]. By reducing those signals that promote the prolongation of the inflammatory cascade, these lines of evidence suggest that lipoxins possess anti-inflammatory properties and may act as endogenous “braking signals” during inflammation.

Despite numerous studies pointing to the potent anti-inflammatory activities of lipoxins, there is a paucity of research that examines the direct effect of lipoxin on mesenteric

endothelia. The purpose of this study was to determine the effect of lipoxin A₄ on modulating mesenteric microvascular fluid leak. We hypothesized that lipoxin A₄ would reverse the increase in microvascular fluid leak caused by inflammation and that the effect of lipoxin A₄ is dependent on white cell-platelet interaction.

Our first purpose was to examine the direct effect of lipoxin A₄ on the venular endothelium under basal condition. We found that in the basal state, continuous administration of lipoxin A₄ lead to an increase in microvascular fluid leak. In human bronchial epithelial cells, exposure to lipoxin A₄ stimulated a cytosolic calcium increase through a mechanism involving the lipoxin A₄ receptor and protein kinase A activation [29]. Increases in endothelial intracellular calcium ion concentration have also been closely linked to increases in microvascular fluid leak [30,31]. It is thought that the intracellular increase in calcium ion activates protein kinase A, which phosphorylates endothelial myosin light-chain kinase, resulting in contraction of the cytoskeleton and opening of intracellular gap junctions and thereby increasing the amount of fluid leaking out of the vascular space and into the interstitium [9].

Our second specific aim was to investigate the direct effect of lipoxin A₄ on the endothelium during an inflamed state. Since previous work has shown that the anti-inflammatory effects of lipoxin A₄ are mediated through a mechanism involving white cells and platelets, we chose an inflammatory agent, platelet activating factor, that directly induces endothelial hyperpermeability [32,33]. Additionally the micro-occlusion technique we use can measure water movement across the endothelial barrier in the absence of any formed blood elements. These conditions allowed us to interrogate the effect of lipoxin A₄ directly on endothelial cells. We found that lipoxin A₄ administration during endothelial hyperpermeability induced by platelet activating factor attenuated the 4-fold increase in microvascular fluid leak due to platelet activating factor alone by 66%. This effect was surprising because previous studies have shown that formed blood elements are essential for mediating the effects of lipoxin A₄ [12,13]. The ability by lipoxin A₄ to attenuate endothelial hyperpermeability in the absence of platelets and PMN's suggests a novel interaction of lipoxin A₄ directly with endothelial cells.

Our third purpose was to investigate lipoxin A₄'s ability to modulate microvascular fluid leak in the presence of formed blood elements including white cells and platelets. We found that lipoxin A₄ administration during lipopolysaccharide-induced inflammation attenuated the 2-fold increase in microvascular fluid leak due to LPS alone by 42%. This data further support the importance of white cells and platelets in the activity of lipoxin. Lipoxin generation is dependent on white cells and platelets and a major pathway for the biosynthesis of lipoxin A₄ is via platelet-polymorphonuclear neutrophil interaction [12,13]. Located in cells of myeloid origin, 5-lipoxygenase generates leukotriene A₄ from arachidonic acid. Leukotriene A₄ is rapidly taken up by platelets and is then converted to lipoxin A₄ through the oxygenase activity of platelet 12-lipoxygenase. Our findings are supported by previous studies that demonstrated that lipoxin A₄ inhibits lipopolysaccharide induced proliferation and reactive oxygen species production in macrophages [34]. The underlying mechanisms by which lipoxins interact to disrupt the inflammatory cascade induced by lipopolysaccharide include inhibition of activation of NF-κB and AP-1, both of which are involved in the signaling mechanisms of the inflammatory cascade [22]. Furthermore, lipoxins are known to attenuate the expression of adhesion molecules on leukocytes and endothelial cells [3], diminish the production of chemokines in intestinal mucosa [5] and inhibit the production of superoxide ion [8].

Our final specific aim was to determine if the mechanism of action by lipoxin A₄ was mediated through the c-Jun N-terminal kinase pathway. The JNK family and the p38 family

of kinases belong to the stress-activated protein kinase (SAPK) superfamily. Members of the SAPK family are activated in response to a gamut of stimuli and stressors that include cytokines, osmotic shock, oxidative stress, and UV irradiation [35]. Although pretreatment of mice with the JNK pathway inhibitor SP600125 protects against experimentally-induced colitis [36], it is not clear whether JNK signaling is also a key regulator of fluid leak. Our data show that the JNK antagonist, SP600125, may influence fluid leak regulated by lipoxin A₄.

Little is known about the ability of lipoxin A₄ to inhibit microvascular fluid leak during inflammation. We have demonstrated that lipoxins are anti-inflammatory mediators that modulate microvascular fluid leak during inflammation and that this may occur via both white cell-platelet dependent and independent mechanisms. The ability to attenuate the increase in microvascular fluid leak during inflammatory conditions identifies lipoxins as a mediator that promotes the resolution of inflammation and thereby suggests a potential pharmacologic role for LXA₄ during sepsis and trauma.

Selected Abbreviations

LXA ₄	Lipoxin A ₄
LPS	Lipopolysaccharide
PAF	Platelet Activating Factor
L _p	Microvascular Fluid Leak
J _v	transmural water flux

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Figure 1.

Effect of Lipoxin A₄ on Microvascular Fluid Leak. LXA₄ slightly increased L_p compared to baseline values (L_p baseline = 1.05 ± 0.03 ; L_p LXA₄ = 1.55 ± 0.04 , $p < 0.0001$). Error bars indicate the standard error of the mean. Units for L_p are $\times 10^{-7} \text{ cm} \cdot \text{s}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$. The * denotes a significant change from baseline.

Figure 2.

Effect of Lipoxin A₄ on Microvascular Fluid Leak During PAF-induced Endothelial Hyperpermeability. PAF increased L_p fourfold from 1.20 ± 0.10 to 4.49 ± 0.95 ($p < 0.0001$). Administration of LXA₄ after PAF-induced inflammation decreased L_p 66% versus PAF alone (from 4.49 ± 0.95 to 1.54 ± 0.13 ; $p = 0.0004$). Error bars indicate the standard error of the mean. Units for L_p are $\times 10^{-7} \text{ cm} \cdot \text{s}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$. The * denotes a significant change from baseline; the † denotes a significant change from perfusion with PAF alone.

Figure 3.

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation. LPS-induced inflammation increased microvascular fluid leak over 2-fold from baseline (1.05 ± 0.03 to 2.27 ± 0.09 ; $p < 0.0001$). Administration of LXA₄ after LPS-induced inflammation decreased L_p 42% from LPS alone (from 2.27 ± 0.13 to 1.31 ± 0.05 ; $p < 0.0001$). Error bars indicate the standard error of the mean. Units for L_p are $\times 10^{-7} \text{ cm} \cdot \text{s}^{-1} \cdot \text{cm H}_2\text{O}^{-1}$. The * denotes a significant change from baseline; The † denotes a significant change from perfusion with LPS alone.

Figure 4.

Effect of Lipoxin A₄ on Microvascular Fluid Leak During LPS-induced Inflammation and JNK Pathway Inhibition. LXA₄ administration after LPS during JNK inhibition with SP600125 prevented the 45% decrease observed with LXA₄ during LPS. JNK inhibition attenuated the effect of LXA₄ after LPS by 51%. (AUC: LPS alone = 33.50 ± 1.40 ; AUC: LPS + LXA₄ = 18.32 ± 0.70 ; AUC: LXA₄ + LPS during JNK inhibition = 25.46 ± 1.11 ; $p = 0.0002$). Error bars indicate the standard error of the mean. Units for the AUC are ($\times 10^{-7}$ cm $\cdot$ s⁻¹ $\cdot$ cm H₂O⁻¹)(min⁻¹). The * denotes a significant decrease from LPS alone; The † denotes a significant increase compared to LPS + LXA₄.