5-Lipoxygenase Pathway in Experimental Abdominal Aortic Aneurysms


Objective—The impact of leukotriene production by the 5-lipoxygenase (5-LO) pathway in the pathophysiology of abdominal aortic aneurysms (AAAs) has been debated. Moreover, a clear mechanism through which 5-LO influences AAA remains unclear.

Approach and Results—Aneurysm formation was attenuated in 5-LO−/− mice, and in lethally irradiated wild-type mice reconstituted with 5-LO−/− bone marrow in an elastase perfusion model. Pharmacological inhibition of 5-LO—attenuated aneurysm formation in both aortic elastase perfused wild-type and angiotensin II–treated LDLr−/− mice, with resultant preservation of elastin and fewer 5-LO and MMP9-producing cells. Separately, analysis of wild-type mice 7 days after elastase perfusion showed that 5-LO inhibition was associated with reduced polymorphonuclear leukocyte infiltration to the aortic wall. Importantly, 5-LO inhibition initiated 3 days after elastase perfusion in wild-type mice arrested progression of small AAA. Human AAA and control aorta corroborated these elastin and 5-LO expression patterns.

Conclusions—Inhibition of 5-LO by pharmacological or genetic approaches attenuates aneurysm formation and prevents fragmentation of the medial layer in 2 unique AAA models. Administration of 5-LO inhibitor in small AAA slows progression of AAA. Targeted interruption of the 5-LO pathway is a potential treatment strategy in AAA. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: aneurysm • aorta • immune system • inflammation • leukotriene A

Abdominal aortic aneurysms (AAAs) remain an important cause of cardiovascular mortality and is the 15th leading cause of death from any cause in men aged ≥55 years.12 AAA is a multifactorial disease associated with aging, smoking, and hypertension, where chronic inflammation in the aortic wall and protease-mediated degradation of structural matrix proteins contribute to aortic dilatation and rupture.13 Surgery is the only established treatment as there are currently no medical therapies to delay the onset or prevent AAA. Pharmacological approaches that slow aneurysm growth by 50% could delay surgery by >5 years and will also reduce risk of potentially fatal rupture.3 Thus, understanding the pathogenesis of AAA is critical to developing novel therapies.

Leukotrienes mediate inflammatory responses in various cardiovascular diseases such as atherosclerosis and aortic valve disease.10 5-Lipoxygenase (5-LO) is the key enzyme in leukotrienes biosynthesis,11 catalyzing the initial steps in the conversion of arachidonic acid to the unstable leukotriene precursor, LTA4.12 LTA4 is a highly unstable compound and cannot be stored by cells for long periods.12 The efficient use of endogenous arachidonic acid by 5-LO requires a helper protein, 5-LO activating protein (FLAP).

To date, studies investigating the role of 5-LO in AAA have been equivocal. Zhao et al14 initially reported a functional role of the 5-LO pathway in aneurysm formation and found that 5-LO/apolipoprotein E–deficient (ApoE−/−) mice fed a cholate-rich diet had a reduced incidence of aortic aneurysms. Consistent with this, BLT1/ApoE−/− mice that lack the high-affinity LTB4 receptor to 5-LO exhibited attenuated aneurysm formation in an angiotensin II (Ang II) infusion model.15 A similar effect was reproduced using a BLT antagonist in the same model and was associated with attenuation in aortic wall infiltration by macrophages.16 Expressions of ALOX5 and ALOX5AP, the genes encoding 5-LO and FLAP, respectively, have been reported to be higher in human AAA compared with control aorta.18 Furthermore, LTB1 production by polymorphonuclear leukocytes (PMNL) seems to be elevated in patients undergoing aneurysmectomy.16,17,18 However in epidemiological studies, the 7 known single nucleotide polymorphisms of ALOX5AP were not associated with human AAA,19 suggesting the lack of a strong genetic association between the 5-LO pathway and AAA. Moreover, aneurysm formation...
and aortic wall inflammation were not attenuated in Ang II–infused hyperlipidemic 5-LO/ApoE−/− mice or in ApoE−/− mice treated with a FLAP inhibitor.20 Collectively, these data suggest an unclear role of this pathway in the pathogenesis of aortic aneurysm formation.

As a result of the critical role of 5-LO in inflammation, several compounds targeting the 5-LO pathway have been developed.21 To resolve the debate of the role of 5-LO in AAA, we used genetic and pharmacological approaches in 2 distinct mouse models of AAA and assessed the impact of 5-LO inhibition in AAA initiation as well as in treatment of small, developing AAA. We tested the hypothesis that 5-LO is critical to aneurysm formation, and that interfering with 5-LO pathway would reduce aneurysm formation. To this end, we aimed to comprehensively investigate the involvement of the 5-LO pathway in experimental AAA progression and to explore the relevance of the 5-LO pathway to human disease.

Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Genetic Loss or Pharmacological Inhibition of 5-LO–Attenuated Aneurysm Formation in an Elastase Perfusion Model

To evaluate whether 5-LO is involved in experimental aneurysm progression, we first compared aneurysm formation in wild-type and 5-LO−/− mice using the aortic elastase perfusion model. Genetic deletion of 5-LO resulted in a 71% reduction in aortic dilatation compared with wild-type controls at day 14 (wild-type: 113±14% [n=9] versus 5-LO−/−: 42±8% (N=9); P<0.05; Figure 1A). To further assess the role of the 5-LO pathway in AAA, subsequent studies were performed using oral and highly selective 5-LO inhibitor (AZD4407; Table I in the online-only Data Supplement; Figure 1B). Aneurysm formation was attenuated in the highest dose group (30 mg/kg per day) indicating that a high level of 5-LO inhibition is required for a phenotypic effect (Figure 1C). A dose-dependent increase in plasma compound exposure and inhibition of LTB4 production in blood was observed when mice were administered oral AZD4407 at 3, 10, and 30 mg/kg per day (Figure 1D and 1E) and was associated with a dose-dependent reduction in ALOX5 gene expression and MMP9 enzymatic activity in aortic tissue at day 14 (Figure 1F and 1G). Consistent with findings in Figure 1C, histological examination of aortic tissue sections indicated that there was less destruction of the elastic lamellae in mice treated with 30 mg/kg per day AZD4407 compared with controls or mice treated with lower doses (Figure 1H). Immune cell infiltration was also lower at day 14 in this high-dose group (Figure 1H; Figure IA in the online-only Data Supplement). Expression of the SMC marker smooth muscle α-actin was dose-dependently preserved with greater 5-LO inhibition and was associated with concomitant reductions in cleaved caspase-3 (Figure 1H). Expression of the leukotrienes receptors BLT1, and CysLT1, were also dose-dependently reduced in response to 5-LO inhibition (Figure 1I and 1J). Taken together, these data suggest that 5-LO activity is involved in aneurysm formation, and in part influences elastin morphology and MMP9 protease activity through leukotrienes production.

5-LO Inhibition Attenuated Aneurysm Formation in Ang II–Infused Hypercholesterolemic LDLr−/− Mice

We sought to confirm our results in a second model, and therefore we investigated the effect of 5-LO inhibition using LDLr−/− mice infused with Ang II for 28 days (Figure 2A).22–24 LDLr−/− mice infused with Ang II and fed control chow had an aortic diameter of 1.51±0.16 mm, those fed 10 mg/kg per day AZD4407 diet had a diameter of 1.10±0.13 mm, whereas mice fed 30 mg/kg per day AZD4407 diet had a diameter of 1.03±0.26 mm at day 28 (Figure 2B). Administration of 10 and 30 mg/kg per day of AZD4407 in the chow for 28 days inhibited 5-LO activity in the circulation in part leading to a 54% reduction in aneurysm formation (Figure 2C and 2D). The incidence of aneurysms was 100%, 0%, and 25% in control, 10 mg/kg per day AZD4407, and 30 mg/kg per day AZD4407 groups, respectively (P<0.05, defined by >50% increase in aortic size from baseline).22 Mortality at day 28 was 5.4%, 2.6%, and 6.1% in the control, 10 mg/kg per day AZD4407, and 30 mg/kg per day AZD4407 groups. The incidence of mortality occurred principally between days 5 and 7 after Ang II infusion as has been demonstrated in other studies, and necropsy demonstrated suprarenal AAA rupture. Treatment with AZD4407 prevented aortic rupture (Figure 2E) and preserved elastin morphology in the media compared with controls (Figure 2F). In addition, 5-LO inhibition decreased PMNL in the media and adventitia of the suprarenal aorta compared with control aneurysms (Figure 2F). However, there was no difference in Mac2 or 5-LO–positive cells at day 28 (Figure 2F). Plasma cytokine levels at day 28 were also similar as were aortic wall BLT1 and CysLT1 receptor expression levels (Figure IIE and IIF in the online-only Data Supplement). The reduction in aneurysm size was independent of an effect on systemic blood pressure or plasma levels of cholesterol, triglycerides, and high-density lipoprotein (Figure IIA–IID in the online-only Data Supplement). Collectively these data suggest that the 5-LO pathway is critical to experimental AAA formation, and that the 5-LO axis is in part regulated via aortic wall PMNLs.

Bone Marrow–Derived 5-LO Contributes to Aneurysm Formation

Based on the predominant expression of 5-LO in leukocytes, we postulated that 5-LO activity from bone marrow–derived cells plays an important role in aneurysm formation. To address this hypothesis, we performed elastase perfusion in chimeric mice after bone marrow transplantation from 5-LO−/− or wild-type donors to both 5-LO−/− and wild-type recipients.
Transplantation of 5-LO−/− bone marrow cells attenuated aneurysm formation in both 5-LO−/− and wild-type recipients, whereas aneurysm formation was unaffected in recipients that received bone marrow cells from wild-type donors (Figure 3B and 3C). Breakdown of elastin in the media was significantly attenuated in mice that received 5-LO−/− bone marrow cells (Figure 3D). Despite similar plasma cytokine levels at day 14 among groups (Figure IIIA in the online-only Data Supplement), immunohistochemistry indicated that PMNL infiltration to the aorta, and aortic wall BLT1 and CysLT1 receptor expression was suppressed in mice receiving 5-LO−/− cells (Figure 3E; Figure IIIB in the online-only Data Supplement). Thus, these data suggest that 5-LO activity in myeloid cells plays a predominant role in aneurysm formation in experimental AAA.

5-LO Inhibition Attenuates PMNL Infiltration in the Aortic Wall Early in Aneurysm Formation

To study the contribution of 5-LO activity early in aneurysm formation, we analyzed aortas at days 3 and 7 after elastase perfusion from mice pretreated with 30 mg/kg per day AZD4407 (Figure 4A). Mice treated with AZD4407 had a 20% reduction (AZD4407 treated: 23.16% above baseline versus control: 43.18% above baseline) in aortic size at day 7 after elastase
Figure 2. Effects of oral (dietary) inhibition of 5-lipoxygenase (LO) in the angiotensin II (Ang II) infusion model. A, Experimental design. B, In situ video micrometry of suprarenal aortic dilatation in mice compared with their baseline aortic diameter (n=8 per group). *P<0.05 pairwise comparison vs control. C, Plasma 5-LO inhibitor levels measured at day 28 by LC-MS/MS. *P<0.05 pairwise comparison vs control. D, Ex vivo whole blood–stimulated LTB4 production determined by EIA at day 28. *P<0.05 pairwise comparison vs control. E, Proportion of mice with ruptured aortic aneurysms. *P<0.05 pairwise comparison vs control. F, Representative histology sections exhibiting elastin fragmentation, IHC stains and quantification exhibiting polymorphonuclear leukocytes and macrophage infiltration, along with 5-LO–positive cells in the aorta of Ang II–infused hypercholesterolemic mice.
5-Lipoxygenase in AAA

PMNL infiltration in the aortic media was similar at day 3 between controls and treated mice, but day 7 was lower in 5-LO inhibitor-treated mice (Figure 4E). Mac2-positive cells increased from days 3 to 7 but did not differ between groups (Figure 4E). SMC marker protein smooth muscle α-actin was significantly higher in treated mice on day 7 indicating preservation of SMC phenotype (Figure 4E). Levels of cleaved caspase-3 in the media were higher in controls on day 3, suggesting more apoptosis in control mice (Figure 4E). 5-LO expression was similar in both controls and treated mice at days 3 and 7 and colocalized primarily with PMNLs (Figure 4F) but not with Mac2-positive cells (data not shown), whereas expression of BLT1 colocalized with the SMC-rich medial elastic lamellae (Figure 4G). Expression of CysLT1 also colocalized with the SMC medial layer (data not shown). Collectively, these data indicate that 5-LO activity could have a prominent effect on PMNL infiltration and SMC phenotype in the early stages of aneurysm formation.

5-LO Inhibition in Small Aneurysms Reduced Progression

To determine whether 5-LO inhibition could be used as a treatment strategy to attenuate aneurysm growth, we compared the effect of 30 mg/kg per day AZD4407 administered 5 days before elastase (prevention) to mice treated 3 days after elastase (treatment) (Figure 5A). AZD4407 attenuated aneurysm development in both the prevention and the treatment groups by day 14 (Figure 5B) and was associated with preservation of the elastin layers in the aortic media and attenuation of PMNL infiltration (Figure 5E). Plasma cytokine interleukin-1β and tumor necrosis factor α were lower in mice that received AZD4407 (Figure IVA in the online-only Data Supplement). In addition, 5-LO-positive cells and aortic wall BLT1 receptor expression were lower in mice that received AZD4407 (Figure 5E; Figure IVB in the online-only Data Supplement). Medial layer preservation was also observed among both groups of mice that received AZD4407 (Figure 5E). Although macrophage infiltration was equivalent at day 14 after elastase perfusion, cleaved caspase-3 expression was reduced in mice when 5-LO was inhibited (Figure IVB in the online-only Data Supplement). These data suggest that 5-LO could be targeted in small AAA and is associated with reduced PMNL infiltration and preserved SMCs.

ALOX5 Expression Is Reduced in Advance Stage Human Aneurysms

Movat staining from the intima, medial, and adventitial layers of the aortic wall in patients with AAA (Figure 6A) exhibited significant elastin loss compared with control aorta. Smooth muscle α-actin expression was also markedly lower in patients with AAA (Figure 6B). We observed few PMNLs and Mac2-positive cells in the aortic wall of patients with AAA (data not

Figure 3. Effects of adoptive transfer bone marrow transplantation of 5-lipoxygenase (LO). A. Experimental design. B. In situ video micrometry of infrarenal aortic dilatation in mice compared with their baseline aortic diameter (n=12 per group). *P<0.05 pairwise comparison vs control. C. Ex vivo whole blood-stimulated LTB4 production determined by EIA 4 weeks after adoptive transfer of bone marrow cells. *P<0.05 pairwise comparison vs control. D. Representative histology sections exhibiting elastin fragmentation in the aorta of elastase-perfused mice. E. Representative IHC stains exhibiting polymorphonuclear leukocytes infiltration in the aorta of elastase-perfused mice.
Figure 4. Time course evaluation of effects of oral (dietary) inhibition of 5-lipoxygenase (LO) in the elastase perfusion model. 

A, Experimental design (controls, n=4 per group and comparison, n=10 per group). B, In situ video micrometry of infrarenal aortic dilatation in mice compared with their baseline aortic diameter after perfusion. The day 14 data from Figure 1 (orange ellipse) is used to contextualize the continued aortic dilatation seen in mice in the elastase perfusion model. C, Plasma 5-LO inhibitor levels measured at days 3 and 7 by LC-MS/MS. *P<0.05 pairwise comparison vs control. D, Ex vivo whole blood–stimulated LTB4 production determined by EIA at days 3 and 7. *P<0.05 pairwise comparison vs control. E, Representative IHC stains and quantification exhibiting immune cell infiltration, smooth muscle cell marker expression, apoptosis, and 5-LO protein expression at days 3 and 7 of harvest in the aorta of elastase-perfused mice. *P<0.05 pairwise comparison vs control. F, Representative confocal IHC stains exhibit luminal polymorphonuclear leukocytes infiltration into the aorta of control mice at day 7. G, Representative confocal IHC stains exhibit BLT1r and smooth muscle cell colocalization in the aorta of control mice at day 7.
shown). Interestingly, we observed significantly higher ALOX5 expression in healthy control aorta compared with late stage AAA (Figure 6C).

### Discussion

Previous studies investigating the significance of 5-LO in aortic aneurysm formation have been equivocal. The current study is the first to comprehensively examine the 5-LO pathway using both genetic and pharmacological approaches to disrupt the 5-LO pathway in 2 complementary murine models. Our studies demonstrate this pathway is critical to experimental AAA formation and that disruption of 5-LO is associated with preservation of the elastic lamina, enhanced SMC marker smooth muscle α-actin expression and reductions in neutrophil count and MMP9 levels in the aortic media. Bone marrow chimeric studies indicate that 5-LO in myeloid cells determine aneurysm progression, whereas confocal studies suggest that neutrophils are an important source for 5-LO. Collectively, these data provide strong evidence that 5-LO inhibition is a potential treatment strategy for AAA disease.

Previous studies demonstrated that AAA is attenuated in ALOX5 LDLr−/−, and ALOX5 ApoE−/− fed an atherogenic diet containing cholate, an effect that was associated with a reduction in MIP-1α and MIP-2 inflammatory signaling. Subsequent observations reported that both BLT1 ApoE−/− mice and BLT receptor antagonist had attenuated AAA
formation after Ang II infusion for 28 days. In contrast, separate studies reported that AAA formation was not affected in either ApoE ALOX5 –/– mice or ApoE –/– mice treated with FLAP inhibitor (MK-0591) after Ang II infusion.20

Our findings differ from previous investigations potentially because of differences in the experimental strategies. We used LDLr–/– mice fed a hyperlipidemic diet and infused Ang II at 1000 mg/kg per minute, whereas Cao et al20 used ApoE<sup>+</sup> mice fed a normal chow diet and infused Ang II at 500 mg/kg per minute. It is possible that different genetic backgrounds could have an impact on the inflammatory response associated with aneurysm formation and the expression of the 5-LO pathway in the aorta. One consequence of using a lower dose of Ang II in the Cao study was that the incidence of aneurysm formation was significantly lower than in our study (30% versus 100% in controls). Potentially, this low incidence of AAA in their study could have hidden any effect that 5-LO deletion or FLAP inhibition had on aneurysm phenotype. With respect to the different pharmacological approaches used, we observed >90% inhibition of LTB<sub>4</sub> production in the blood of mice treated with AZD4407, whereas Cao et al reported 50% inhibition of LTB<sub>4</sub> production using MK-0591. Our studies indicate that a high level of 5-LO inhibition is required to achieve a protective effect on aneurysm phenotype and it is possible that the level of inhibition achieved by others was inadequate. The high level of 5-LO inhibition in blood needed to reduce aneurysm progression may relate to adequate exposure of compound within the aorta to inhibit 5-LO activity. This could be particularly important because it has been observed that the potency of some nonredox 5-LO inhibitors can be impaired in tissues.27,28 Supportive of this potential explanation is the fact that SMC marker smooth muscle α-actin expression, MMP9 levels, and 5-LO pathway gene expression in the aorta were dose-dependently affected by 5-LO inhibition.

Myeloid cells are known to be one of the sources of the 5-LO pathway. Our bone marrow chimera experiments demonstrated that 5-LO activity in myeloid cells is critical to aneurysm formation. Moreover, we demonstrated that 5-LO expression is predominantly associated with PMNLs in the aortic wall early in AAA development. However, in contrast to earlier reports,14,20,29 we did not demonstrate colocalization with macrophages. Prior studies have demonstrated that neutrophil counts are elevated in human AAA17,30,31 and have suggested a pathological role for neutrophils in AAA.32–37 Because LTB<sub>4</sub> is a potent chemokine for neutrophils and can also promote neutrophil survival by inhibiting apoptosis,38 the reduction in PMNL after 5-LO inhibition could be explained by reduced infiltration and decreased survival of neutrophils in the aortic wall. This reduction in neutrophil number between days 3 and 7 could be pivotal to the mechanism by which 5-LO inhibition attenuates aneurysm progression. SMC marker α-actin expression was reduced on day 3 in both control mice and those treated with the 5-LO inhibitor. However, by day 7 it seemed that SMC marker α-actin was again re-expressed in mice that had received the 5-LO inhibitor. This intriguing and novel finding suggests that SMC phenotype is reversible and that 5-LO inhibition can revert SMCs back to healthy state. Whether this effect could be explained by a direct action of leukotrienes on vascular SMCs or through an alternative mechanism linked to fewer neutrophils in the aneurysm cannot be deduced from our data but is worthy of further investigation. We also demonstrated a dose-dependent reduction in MMP9 expression with 5-LO inhibition, suggesting yet another mechanism by which 5-LO inhibition could attenuate aneurysm progression.

A clinically relevant finding in this study is that 5-LO inhibition could attenuate aneurysm progression of small or

Figure 6. Late stage human aneurysms and expression of genes encoding 5-lipoxygenase (LO) pathway proteins. A, Representative Movat staining in healthy control aorta (n=9 per group). Panels 1 and 2 are ×40 magnifications of select areas from the ×10 paticomicrograph. B, Representative smooth muscle cell marker expression in human samples with clinically diagnosed abdominal aortic aneurysms (AAA). Panels 1 and 2 are ×40 magnifications of select areas from the ×10 paticomicrograph. C, Relative fold change of mRNA transcripts, normalized to 18S, of 5-LO pathway protein encoding genes in non-AAA and AAA human samples. *P<0.05 vs patients without clinically diagnosed AAA.
developing AAA. The effect of 5-LO inhibition on aneurysm phenotype was similar whether 5-LO inhibition was initiated before elastase perfusion or after elastase in small AAA, indicating that 5-LO plays a prominent physiological role in aneurysm progression. We initially chose day 3 to start the treatment arm based on time course studies that suggested that inflammatory changes occur prominently during this postsurgical period in the elastase model. However, significant aortic dilation does not proceed until around day 7. To improve possible translation to the clinic, it will be important to assess whether 5-LO inhibition can have therapeutic effects on established aneurysms, by evaluating treatment initiated after 14 or 28 days and analyzing the aneurysm phenotype at later time points. The rapid formation of AAA in both the elastase perfusion and Ang II models have always been a limitation in the translation to human AAA, yet have provided important insights to our understanding of the disease. Because there is evidence that the inflammatory response is attenuated late in experimental and human aneurysm disease, it is not certain that 5-LO will play an active role in aneurysm progression in advanced AAA. In addition to producing proinflammatory leukotrienes, the 5-LO pathway is involved in the transcellular formation of lipoxins that can mediate inflammatory resolution. By targeting 5-LO activity early in experimental models, our current studies have primarily addressed the impact of the proinflammatory effects of this pathway on aneurysm formation. Future studies should also address whether treatment with a 5-LO inhibitor could have an impact on inflammatory resolution in experimental aneurysms and evaluate the effect that this could have on aneurysm phenotype.

The primary long-term objective of our studies is to determine whether 5-LO inhibition could be a therapeutic strategy to treat AAA progression in humans. There is currently no approved medication for AAA, and the small number of clinical trials that have been performed have failed to identify significant effects of existing medications on aneurysm progression. We have demonstrated that the 5-LO pathway is highly relevant early in experimental AAA using 2 different mouse models. Moreover, myeloid-derived 5-LO is critical in neutrophil recruitment in the aortic wall of early AAA. Given that the 5-LO pathway and inflammation are attenuated in late stage human and experimental AAA, our findings suggest the 5-LO pathway could be targeted early in AAA to prevent aneurysm progression.

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Disclosures
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References
Abdominal aortic aneurysm (AAA) is a top-20 cause of mortality overall and is the 15th leading cause of death in men aged >55 years. Surgery is the only established treatment as there are currently no medical therapies to delay the onset or prevent AAA. To this end, the current study is the most comprehensive and first to examine the 5-lipoxygenase pathway using both genetic and pharmacological approaches to disrupt this pathway in 2 complementary murine models in the study of AAA. These data demonstrate that this pathway is critical to experimental AAA formation and that disruption of 5-lipoxygenase is associated with preservation of the elastic lamina, enhanced SMC marker smooth muscle α-actin expression and reductions in neutrophil count and MMP9 levels in the aortic media. Collectively, these data provide strong evidence that 5-lipoxygenase inhibition could be used as a potential medical therapy for AAA in humans.
5-Lipoxigenase Pathway In Experimental Abdominal Aortic Aneurysms

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METHODS

Elastase perfusion model

The elastase perfusion model of abdominal aortic aneurysm formation in mice was utilized as previously described. Congenic age, weight and gender matched male C57BL/6J (Stock #000664, Jackson Laboratories, Bar Harbor, Maine) (WT) or 5-LO−/− mice (Stock #004155, Jackson Laboratories, Bar Harbor, Maine) were used for experiments (N=16/group). 5-LO−/− mice have no externally visible abnormal phenotype. Mice underwent 75/1mg/kg intraperitoneal ketamine/medetomidine anesthesia. Aorta were perfused with either saline or elastase (Lot #078K7018, Sigma Aldrich Inc., St. Louis, Missouri) for 5 and a-half minutes. Immediate mechanically induced aortic wall dilatation of 15-35% after perfusion with saline or elastase (0.47U/mL porcine pancreatic elastase) is followed by either no aneurysmal (perfused with saline) or aneurysmal (perfused with elastase) dilatation over 14 days. Mice were housed individually throughout experiments, and maintained at 70°F, 50% humidity, in 12-hour light-dark cycles per institutional animal protocols. All mice were fed ad libitum water and placed on diet with no restrictions on movement. All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. Video micrometry measurements of the maximum diameter of the aortic wall distal to the renal arteries were performed in situ before perfusion, following perfusion, and at the time of harvest using a Q-Color3 Optical Camera (Olympus Corp., Center Valley, Pennsylvania) attached to an operating microscope (Leica Microsystems, Bannockburn, Illinois) using QCapture Pro Software version 6.0 (QImaging Inc., Surrey, Canada). Infrarenal aortic diameter that exceeded the baseline Infrarenal aortic measurement by 50% were considered aneurysmal across all experiments. Change in aortic dilatation from baseline (%) was calculated by subtracting the infrarenal control diameter from the infrarenal maximal diameter divided by the infrarenal control diameter as determined by video micrometry. At the time of harvest following antegrade perfusion, the entire infrarenal aorta was explanted. The aortas (or aneurysms, when present) were either: 1) snap frozen in liquid nitrogen for analyses by polymerase chain reactions or protein extraction, or 2) processed for histology or immunohistochemistry.

Angiotensin II infusion model

Osmotic pumps (Alzet®, 2004, Durect Corp., Cupertino, California) containing either Ang II (1000 ng/kg/min, Sigma Aldrich Inc., St. Louis, Missouri) or saline were introduced in 10-week-old LDLr−/− male mice (Stock #002207, Jackson Laboratories, Bar Harbor, Maine) as previously described (N=8/group). Mice were housed individually throughout experiments, and maintained at 70°F, 50% humidity, in 12-hour light-dark cycles per institutional animal protocols. All mice were fed ad libitum water and placed on high fat diet (TD 88137, Harlan Teklad Inc., Indianapolis, Indiana) with no restrictions on movement. All mice had unlimited daily access to the chow (control or 5-
Bhamidipati et al.

LO adminixed) ad libitum in their cage, and had no restrictions on movement. Aneurysmal segments of the aortas (proximal to the renal arteries) were harvested after 28 days just like in the elastase perfusion model and processed for histology. Cardiovascular profile (heart rate, mean arterial pressure, systolic and diastolic blood pressure) was determined over 28 days. Conscious mice underwent measurements using Coda 8 (Kent Scientific Corporation, Torrington, Connecticut) tail-cuff system as described previously. Acclimatization cycles (N=10) were discarded, and average of the ensuing cycles (N=20) are reported. Measurements were obtained for 7 days prior to exposure to the 5-LO inhibitor, and then subsequently on day 0, 1, 3, 5, 7, 10, 14, 21 and 28 of Ang II infusion. At day 28, video micrometry measurements of the aortic wall diameter (proximal to the renal arteries) were performed in situ using a Q-Color3 Optical Camera (Olympus Corp., Center Valley, Pennsylvania) using QCapture Pro Software version 6.0 (QImaging Inc., Surrey, Canada). LDLr−/− mice (N=6) were harvested prior to experiments to establish baseline aortic diameters and based on these findings the 0.5mm baseline aortic diameter size was determined. Video micrometry measurements were compared to a baseline aortic diameter of 0.5mm, which was established as the arbitrary baseline aortic diameter from age, gender and weight matched LDLr−/− mice that were harvested separately.

Pre-injury Pharmacological studies

All mice were single housed throughout experiments and exposed to 5-LO inhibitor admixed into chow for 5-7 days prior to injury (i.e.: elastase perfusion or Ang II infusion). AZD4407 (AstraZeneca PLC, London, UK), a unique potent orally available non-redox inhibitor of 5-LO that has greater than 800-fold selectivity over other lipoxygenases and cyclooxygenases was utilized for experiments. Control chow and 5-LO inhibitor admixture chow at 3 mg/kg, 10 mg/kg and 30 mg/kg for experiments were formulated either at AstraZeneca R&D (Mölndal, Sweden) or through Research Diets Inc. (New Brunswick, New Jersey) based on a baseline 25g mouse weight. All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. Pre-exposure time points were selected based on in vivo 5-LO inhibitor PK levels, and LTB4 production determined in preliminary experiments (data not shown). Mice were weighed routinely throughout elastase (Day -5, 0, 7, and 14) and Ang II experiments (Day -7, 0, 1, 3, 5, 7, 10, 14, 21 and 28).

Post-injury Pharmacological studies

All mice were single housed throughout experiments and exposed to 5-LO inhibitor admixed into chow for 10 days after injury (i.e.: elastase perfusion). All mice had unlimited daily access to the chow (control or 5-LO admixed) ad libitum in their cage, and had no restrictions on movement. The post-injury time point was selected based on prior unpublished observations from our lab, which confirmed the presence of a small aneurysm at day 7 following elastase perfusion. Based on the pre-exposure
studies, we anticipate *in vivo* drug steady state to be reached by day 7 in mice exposed to the inhibitor at day 3 after elastase perfusion. Control chow and 5-LO inhibitor admixture chow at 3 mg/kg, 10 mg/kg and 30 mg/kg for experiments were formulated either at AstraZeneca R&D (Mölndal, Sweden) or through Research Diets Inc. (New Brunswick, New Jersey) based on a baseline 25g mouse weight.

**LTB₄ assay**

Whole blood obtained by cardiac puncture from mice (500 μl) using heparinized syringes and collection tubes containing 1 mg EDTA diluted in RPMI was incubated at 37°C for 30 minutes. Samples were subsequently incubated at 37°C for 20 minutes with DMSO (control) or 25μM calcium ionophore (A23187, Sigma Aldrich Inc., St. Louis, Missouri). The reaction was stopped using 10 μM AZD4407 in PBS. Microtiter plates were centrifuged at 2000 rpm, 4°C for 10 minutes. Supernatants were stored at -80°C until further processing. LTB₄ enzyme immunoassay (EIA), plate reading by iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories Inc., Hercules, California), and calculations to quantify absorbance were completed per EIA manufacturer recommendations (Cayman Chemical Co., Ann Arbor, Michigan).

**Plasma 5-LO levels**

Whole blood obtained by cardiac puncture from mice using heparinized syringes and collection tubes containing 1 mg EDTA was centrifuged at 2500 rpm for 10 minutes, 4°C. Plasma samples were stored at -20°C for further processing, and when ready were transferred to a pre-labeled plate for analysis by liquid chromatography mass spectrometry/ mass spectrometry (LC-MS/ MS) according to manufacturer's protocol (AstraZeneca R&D, Mölndal, Sweden).

**Plasma blood and urine metabolite profile**

Whole blood obtained by cardiac puncture from mice using heparinized syringes and collection tubes containing 1 mg EDTA was either immediately assayed for blood chemistry profiles or centrifuged at 2500 rpm for 10 minutes, 4°C. Complete blood count with differential in whole blood, and plasma cholesterol, high-density lipoprotein, and triglyceride levels were determined though the University of Virginia medical laboratories. Urine samples collected at day 14 following elastase perfusion studies were examined for urine LTE₄ by EIA per manufacturer’s protocol (Cayman Chemical Co., Ann Arbor, Michigan).

**Adoptive transfer bone marrow transplantation**

Adoptive transfer bone marrow transplantation experiments were performed as previously described. Briefly 6-week-old recipient mice were lethally irradiated and rescued through retro-orbital injection of 6 million bone marrow cells, isolated from
femurs of 6-week-old donor mice. Mice were placed on antibiotic prophylaxis for 3 weeks in their drinking water (2 weeks trimethoprim, 1 week fluoroquinolone) following adoptive transfer, and then subsequently switched to regular drinking water. Non-rescued control mice died between days 7 and 10 following irradiation. Chimeric mice underwent blood DNA analysis, obtained from saphenous vein aspiration 4 weeks following adoptive transfer. LT4 production in blood samples was quantified at 10-weeks following adoptive transfer for additional verification of successful bone marrow transplantation. Chimeric mice then underwent elastase perfusion as described following a two-week recovery period (N=12/group).

Gelatin zymography

Snap-frozen murine AAA samples were analyzed by gelatin zymography. Protein was extracted after harvest using Lysing Matrix D (MP Biomedicals, Solon, Ohio) and 1M Tris (hydroxymethyl) aminomethane buffer at pH 7.5, 5M Sodium Chloride, 1M Calcium Chloride and Triton X-100 homogenized at 4°C, while concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, Illinois). Electrophoresis was completed using 0.1% gelatin in a 10% sodium dodecyl sulfate polyacrylamide gel using equivalent volume of each fraction. Enzymatic activity was visualized as negative staining with Coomassie Brilliant Blue R-250 (Thermo Scientific, Rockford, Illinois). Relative densitometry analysis of lytic bands, adjusted for background, indicative of MMP activity was performed using Gel Doc™ XR+ System and Image Lab™ software (Bio-Rad Laboratories Inc., Hercules, California).

Histology, immunohistochemistry and confocal microscopy

Murine aortas were harvested at sacrifice for histological analysis after undergoing left ventricular puncture and 4% paraformaldehyde (PFA) followed by phosphate buffered saline (PBS) antegrade perfusion at physiologic pressure. Further fixation was achieved by overnight incubation in 4% PFA at 4°C followed by paraffin embedding and sectioning at 5μm. Human and murine paraffin embedded samples underwent microwave antigen retrieval, antibodies were bound and detected using VectaStain Elite Kit (Vector Laboratories Inc., Burlingame, California). Murine samples were embedded in the same orientation based on the identifying ligature from the aortotomy sight. The specimens were sectioned at 5μm through the entire length of the aorta and mounted (approximately 120 slides per aorta harvested). Screening stains with Hematoxylin and Eosin (H&E) and Modified Russell-Movat Pentachrome (Movat) for elastin were completed. These slides were used to determined the area of interest for further staining. Then Hematoxylin and Eosin (H&E), along with Modified Russell-Movat Pentachrome (Movat) for elastin layers, luna and toluidine blue staining for mast cells, Verhoeff-Van Gieson staining for elastin layers, and immunohistochemical (IHC) staining was completed. Antibodies for IHC staining were anti-rat Mac2 for macrophages (Cedarlane Laboratories, Burlington, Canada), anti-mouse anti-Neutrophil (Ly 6B.2) for PMNLs (AbD Serotec, Oxford, United Kingdom), anti-mouse CD3ε (M-20)
for T-lymphocytes (Santa Cruz Biotechnology Inc., Santa Cruz, California), anti-mouse
cleaved caspase-3 (Asp175) for apoptosis (Cell Signaling Technology, Inc., Danvers,
Massachusetts), anti-mouse SMαA (14A) (Santa Cruz Biotechnology Inc., Santa Cruz,
California), anti-rat 5-LOX (Cayman Chemical Co., Ann Arbor, Michigan), anti-human
BLTr1 (Cayman Chemical Co., Ann Arbor, Michigan), and anti-human CysLT1 (Cayman
Chemical Co., Ann Arbor, Michigan). Visualization color development was completed
using diaminobenzidine (Dako Corporation, Carpinteria, California). Positive controls
were included as per antibody manufacturer recommendations where paraffin
embedded mouse organ sections specific to augment the antibody were stained (e.g.
lymph node or spleen). A negative control included the same conditions, without the
antibody being used. Conditions for both sets of controls (i.e. positive and negative)
were first calibrated and then re-run with the slides of interest from our experiments.
This process was repeated for each lot/batch # of antibody used per stain during every
staining session. Images were acquired using AxioCam Software version 4.6 via 4X,
10X, and 40X objectives and an AxioCam MRc camera (Carl Zeiss Inc., Thornwood,
New York).

Paraffin sections were examined on a Zeiss LSM 510 confocal microscope
equipped with purple diode laser for 405 nm excitation (blue); an argon laser with 458,
477, 514, and 488 nm lines (blue-green); a 543 nm HeNe laser (red); and a 633 nm
HeNe laser (far-red) (Carl Zeiss Inc., Thornwood, New York). IHC antibodies were
optimized with appropriate fluorescently labeled detection antibodies (Alexa
fluorophores, Life Technologies Inc., Grand Island, New York) and excited by lasers for
acquisition through a 40X objective. ZEN lite 2011 (Carl Zeiss Inc., Thornwood, New
York) software was used to analyze, merge images, and optically zoom to 300X.

Real-Time PCR

RNA from snap frozen mouse aortic samples was isolated with TRIzol®
(Invitrogen Corp., Carlsbad, California) after homogenization according to the
manufacturer’s instructions. Total RNA quantification was determined using a
NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, Delaware).
Subsequently 0.5μg complimentary deoxyribonucleic acid (cDNA) was synthesized from
RNA using RNase H+ iScript reverse transcriptase (Bio-Rad Laboratories, Hercules,
California). Then cDNA was amplified by qPCR on a MyIq™ Color Real-Time PCR
Detection System (Bio-Rad Laboratories, Hercules, California). The 5-LO mutant (S: 5’-
ATC GCC TTC TTG ACG AGT TC-3’), common (S: 5’-GCA GGA AGT GGC TAC TGT
GGA-‘3’) and wildtype (S: 5’-TGC AAC CCA GTA CTC ATC AAG-’3) sequences were
obtained from Jackson Laboratories, Bar Harbor, Maine. 5-LO pathway Taqman®
primer and probe sets (Applied Biosystems Inc., Foster City, California) were used to
study amplicons in the aneurysm tissue samples, and compared to the amplification of
standards. Relative quantification with normalization to 18S using iQ5 optical system
software, version 2.1 (Bio-Rad Laboratories, Hercules, California) was completed.
Amplification by CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad
Laboratories, Hercules, California) was completed. Polymerase chain reactions amplified DNA transcripts of 5-LO and WT sequences in whole blood isolated from chimeric mice per manufacturer’s recommendations (Qiagen Inc., Valencia, California). Amplicons were resolved on a 1-2% agarose gel and quantified by Gel Doc™ XR+ System and Image Lab™ software (Bio-Rad Laboratories Inc., Hercules, California). We achieved near complete abrogation (99%) of the WT band in WT recipients of 5-LO–/– donor bone marrow cells. All fold changes were calculated by the method of ΔΔCt and are expressed as mean±sem compared with controls (as indicated).

Immunohistochemistry quantification

A circumferential area of interest (AOI) from IHC images was drawn to include the media and adventitial layers of the aorta. Threshold gated positive signal was detected within the AOI and quantified using Image-Pro Plus version 7.0 (Media Cybernetics Inc., Bethesda, Maryland).

Cytokine measurements

Plasma samples collected and stored at -80°C underwent quantification by mouse cytokine multiplex immunoassay (Bio-Rad Laboratories, Hercules, California) according to manufacturer’s protocol.

Human Samples

Male patients between the age of 55 and 75 years who underwent open elective aneurysmectomy for infrarenal AAA were identified, and tissue was taken from the anterior or lateral wall (N=9/group). De-identified (per IRB protocol) control abdominal aorta samples were obtained from organ donors between the age of 15 and 35 without clinical or macroscopic signs of aortic atherosclerosis at The University of Virginia, Charlottesville, Virginia. There are inherent limitations to interpreting results from comparisons between these two groups.

Study approval

Animal care and use were in accordance with the Guide for the Care and Use of Laboratory Mice 11. The animal protocol was approved by the University of Virginia Institutional Animal Care and Use Committee (#3634) in compliance with the Office of Laboratory Animal Welfare 12. Approvals for human sample investigations, including patient consent waiver, was obtained by the Human Investigation Committee at The University of Virginia, Charlottesville, Virginia (HSR #13178).

Statistics
Statistical analysis was performed using GraphPad Prism 5.0f for Mac OSX software (GraphPad Software, La Jolla, CA). Aortic dilation between groups was compared by single factor analysis of variance. When multiple groups were compared post hoc Tukey corrections were made to reduce false discovery rate. Histology and serology groups were compared using nonparametric 2-tailed Mann-Whitney U tests or by $\chi^2$ as appropriate (as no parametric distribution of the data was assumed). Survival was computed using Kaplan Meier plots, and compared by log rank tests. Comparisons were made against controls and considered statistically significant when $P$ value was equal to or less than 0.05. Numbers of mice per group used in experiments are shown in each figure, or within the methods section. Data are presented as mean±sem.
REFERENCES


Supplementary Figure I

A

Control 3 mg/kg 30 mg/kg

Macrophages

Mast cells

CD3ε T-cells
SUPPLEMENTARY FIGURES

Supplementary Figure I

Oral (dietary) inhibition of 5-LO in the elastase perfusion model reduces immune cell infiltration. (A) Representative IHC stains exhibiting macrophage, mast cell and CD3ε T-lymphocyte infiltration in the aorta of elastase perfused mice.
Supplementary Figure II

A

B

C

D

E

F

Saline  Angiotensin II  10 mg/kg  30 mg/kg

BLT1

CysLT1
Oral (dietary) inhibition of 5-LO in the Ang II infusion model is blood pressure, plasma lipid and circulating cytokine level independent. (A) Mean arterial blood pressure represented in mmHg measured over time in Ang II infused mice. (B) Plasma cholesterol levels in mg/dL measured in mice at day 28 following continuous Ang II infusion. (C) Plasma triglyceride levels in mg/dL measured in mice at day 28 following continuous Ang II infusion. (D) Plasma high-density lipoprotein levels in mg/dL measured in mice at day 28 following continuous Ang II infusion. (E) Plasma cytokine IL-1β, TNFα, CCL2, IL-2, IFN-γ, KC, CCL5, and IL-10 levels in pg/mL measured in mice at day 28 following continuous Ang II infusion. (F) Representative IHC stains exhibiting BLT1 and CysLT1 receptor expression in mice at day 28 following continuous Ang II infusion.
Adoptive transfer of bone marrow derived 5-LO mitigates aneurysm formation. (A) Plasma cytokine TNFα, CCL2, KC, and CCL5 levels in pg/mL measured at day 14 after elastase perfusion. (B) Representative IHC stains exhibiting BLT1 and CysLT1 receptor expression in the aorta of elastase perfused mice.
SUPPLEMENTARY FIGURES

Supplementary Figure IV

Prophylactic and therapeutic oral (dietary) inhibition of 5-LO mitigates aneurysm formation in the elastase perfusion model. (A) Plasma cytokine IL-1β, TNFα, CCL2, IL-2 levels in pg/mL measured at day 14 after elastase perfusion. *P < 0.05 pair-wise comparison versus control. (B) Representative IHC stains and quantification exhibiting macrophage infiltration in the aorta of elastase perfused mice, and representative IHC stains of cleaved caspase-3, BLT1 and CysLT1 receptor expression in the aorta of elastase perfused mice.
<table>
<thead>
<tr>
<th>Assay</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
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<tr>
<td>5-LO, human enzyme</td>
<td>525 nM (n=2)</td>
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<tr>
<td>LTB4 production, PMNL</td>
<td>9.9 ± 1.2 nM (n=30)</td>
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<tr>
<td>LTB4 production, human blood</td>
<td>39 ± 2.8 nM (n=30)</td>
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<tr>
<td>cysLT production, human blood</td>
<td>51 nM (n=2)</td>
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<tr>
<td>LTB4 production, mouse blood</td>
<td>240 nM</td>
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<tr>
<td>15-LO, human enzyme</td>
<td>&gt;10,000 nM</td>
</tr>
<tr>
<td>COX-1, human enzyme</td>
<td>&gt;10,000 nM</td>
</tr>
<tr>
<td>COX-2, human enzyme</td>
<td>&gt;10,000 nM</td>
</tr>
<tr>
<td>PGE2 production, human blood</td>
<td>&gt;10,000 nM</td>
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<tr>
<td>TXB2 production, human blood</td>
<td>&gt;10,000 nM</td>
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SUPPLEMENTARY TABLES

Supplementary Table I

Specificity of AZD4407 (AstraZeneca PLC, London, UK) in human and mice whole blood, and PMNL samples illustrates significant inhibition of LTB₄ production, and low non-specific activity against other lipoxygenases, and cyclooxygenases from the eicosanoid pathway.