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Leukotriene Signaling in Atherosclerosis and Ischemia

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Abstract
Introduction The inflammatory process of atherosclerosis is associated with several pathophysiological reactions within the vascular wall. The arachidonic acid released by phospholipase A_2 serves as substrate for the production of a group of lipid mediators known as the leukotrienes, which induce pro-inflammatory signaling through activation of specific BLT and CysLT receptors.
Discussion Leukotriene signaling has been implicated in early lipid retention and foam cell accumulation, as well as in the development of intimal hyperplasia and advanced atherosclerotic lesions. Furthermore, the association of leukotrienes with degradation of extracellular matrix has suggested a role in atherosclerotic plaque rupture. Finally, studies of either myocardial or cerebral ischemia and reperfusion indicate that leukotriene signaling in addition may be involved in the development of ischemic injury.
Conclusion Both leukotriene synthesis inhibitors and leukotriene receptor antagonists have been suggested to induce beneficial effects at different stages of the atherosclerosis process.

Key words Inflammation · Intimal hyperplasia · Leukotriene receptors · Lipoxygenase · Myocardial infarction

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Leukotriene biosynthesis from phospholipids

The proinflammatory lipid mediators known as the leukotrienes (LTs) were initially described by their bioactivity referred to as Slow Reacting Substance of Anaphylaxis (SRS-A; [1]), which was discovered after challenge of animal lungs with snake venom [2]. This observation has subsequently been explained by LT synthesis induced by phospholipase A_2 (PLA_2), which is the major enzymatic component of snake venom. PLA_2 consists of several groups of both secretory and cytosolic forms [3]. The cytosolic PLA_2 ($cPLA_2$) displays a calcium-dependent activation, and has a specificity for phospholipids with arachidonic acid (AA) bound at the sn-2 position. It is the resulting liberation of AA, which provides the substrate for the formation of LTs (Fig. 1) [4]. The subsequent enzymatic step, lipoxygenation of AA by 5-lipoxygenase (5-LO) and its activating protein FLAP (Fig. 1), yields the unstable product LTA_4 [4]. Subsequently, the LT synthesis follows two distinct pathways. Hydrolysis of LTA_4 leads to formation of LTB_4, a potent chemoattractant and leukocyte activator [5]. On the other arm of the pathway, LTA_4 is conjugated with glutathione by the action of LTC_4 synthase (Fig. 1). The latter enzymatic step leads to the formation of LTC_4, D_4 and E_4 (collectively referred to as the cysteinyl-LTs) (Fig. 1), which have been associated with broncho- and vasoconstriction [5].

Although the major interest for PLA_2 in the context of atherosclerosis has been focused on their effects on lipoproteins [6], the participation of PLA_2 in leukotriene formation may result in additional proatherogenic signaling from this enzymatic pathway. While the effects of different PLA_2 inhibitors on leukotriene biosynthesis today remains largely unknown, specific interventions within the LT

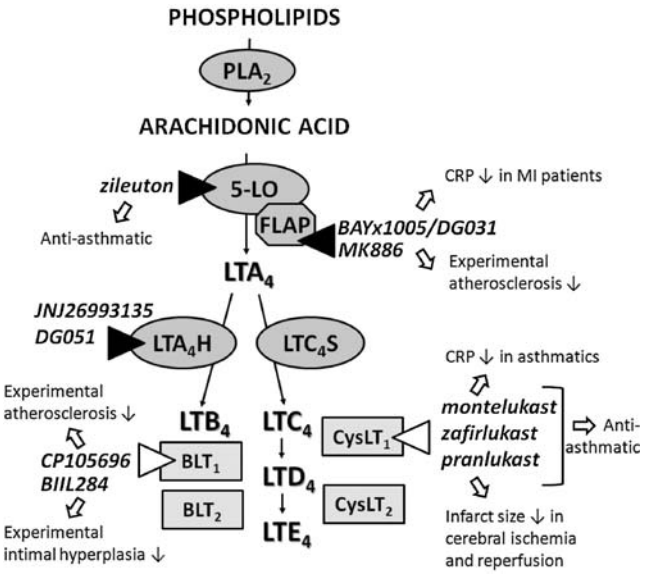


Fig. 1 The formation of leukotrienes (LTs) through the 5-lipoxygenase pathway of arachidonic acid metabolism, and the effects of LT inhibitors in different contexts. *Gray ovals* indicate the enzymes involved in LT biosynthesis; Phospholipase A_2 (*PLA_2*), 5-lipoxygenase (*5-LO*) with its activating protein (*FLAP*), LTA_4 hydrolase (*LTA_4H*), and *LTC_4* synthase (*LTC_4S*). *Rectangles* indicate the LT receptors. Examples of LT synthesis inhibitors are indicated by *black triangles*, whereas examples of LT receptor antagonists are indicated by *open triangles*

signaling have indicated beneficial effects on atherosclerosis development.

Leukotriene receptors

The biological actions of the LTs are transduced through 7-transmembrane G-protein-coupled receptors (GPCR). The two arms of the LT pathway have distinct receptors referred to as BLT receptors (activated by LTB_4) and CysLT receptors (activated by the cysteinyl-LTs), respectively (Fig. 1) [5]. While the BLT receptors are denoted BLT_1 and BLT_2, for the high and low affinity receptor subtypes, respectively, receptors activated by the cysteinyl-LTs are characterized by their sensitivity to available antagonists and are referred to as $CysLT_1$ and $CysLT_2$ [7, 8] The latter nomenclature probably only in part describes the cysteinyl-LT signaling, since limitation of the currently available CysLT receptor antagonists [8], as well as cross-reactivities between CysLT and purinoreceptors [9] indicate more complex receptor ligation properties for cysteinyl-LTs.

Proinflammatory leukotriene signaling in atherosclerosis

Although the use of statins may decrease both systemic and local inflammation in atherosclerosis, a potential further benefit could be anticipated by specific anti-inflammatory agents targeting key immune reactions in the atherosclerosis process. Ever since the concept of inflammation and atherosclerosis was raised, a number of inflammatory mediators has been explored as potential therapeutic targets in this disease [10]. The inflammatory response induced through LT signaling may be of particular interest in this context since several drugs targeting this pathway are either available or under development, as indicated in Fig. 1 [11]. Although there is a long tradition of treating asthma with anti-LTs [12], very little is known about the long term effects of the anti-asthmatic LT inhibitors on cardiovascular outcome. Some interesting indications can however be obtained from retrospective studies. For example, a randomized controlled trial of placebo versus either the $CysLT_1$ receptor antagonist montelukast (Fig. 1) or theophylline, reported significantly lower levels of CRP in montelukast-treated patients with severe asthma [13]. Although no follow-up of those patients was performed in terms of cardiovascular disease, a systemic anti-inflammatory effect of montelukast could indicate potential beneficial effects.

Although asthma may not be a classical co-morbidity of atherosclerosis, LTs have been implicated as potential mediators of cardiovascular risk in other inflammatory diseases. For example, in studies of patients with chronic obstructive pulmonary disease (COPD), the prevalence of ischemic heart disease is approximately twofold higher compared with the general population [14]. The beneficial effects of anti-LTs in reducing air-flow obstruction in asthma has been reproduced in COPD patients [14]. Furthermore, short time treatment with the LT synthesis inhibitor BAYx1005/DG031 (Fig. 1) has been evaluated in both COPD and in patients with a history of myocardial infarction [15, 16]. Although the treatment protocols used in both the latter two studies resulted in only modest inhibition of LTB_4 concentrations in either sputum or stimulated whole blood, the results suggested a tendency for decrease of inflammatory markers [15, 16]. Despite the limitations (small patient numbers, short time treatments, limited LT inhibition etc), these studies provide an initial suggestion as to a potential beneficial effect of an anti-LT treatment in atherosclerosis.

In addition to the lungs, the oral cavity may be another source of low grade chronic inflammation as a substrate for atherosclerosis development. Periodontal disease is for example associated with the development of early atherosclerotic lesions in the carotid artery [17], and an increased risk of stroke [18] and myocardial infarction [19]. The notion of LTs as common mediators of atherosclerosis and periodontal disease was recently suggested in a study associating high levels of cysteinyl-LTs in gingival crevicular fluid with an increased carotid artery wall thickness

[20]. Chronic inflammation in the oral cavity can also be measured in the saliva, in which high LT concentrations have been demonstrated [21, 22], and reported to be inhibited by zileuton [23], an anti-asthmatic LT synthesis inhibitor (Fig. 1). Although hitherto not evaluated in the context of cardiovascular disease, those studies not only suggest the oral cavity as a site of chronic inflammation, but also indicate that the saliva may be a possible source for measures of LTs as biomarkers.

As a final example of co-morbidities in which LTs could act as a potential link to atherosclerosis, subjects with obstructive sleep apnea (OSA) exhibit significantly higher LTB_4 synthesis in *ex vivo* stimulated neutrophils compared with control subjects [24]. Interestingly, the LTB_4 production in subjects with OSA was significantly correlated to the carotid artery diameter in the latter study [24], further supporting the notion of LTs as potential mediators of the increased cardiovascular risk associated with different inflammatory conditions.

In addition to the studies implicating leukotrienes in co-morbidities of atherosclerosis, studies of genetic polymorphisms have established significant associations for the LT pathway with early signs of atherosclerosis [25, 26], as well as the development of stroke and myocardial infarction [27, 28]. Furthermore, mechanistic studies have implicated the LT pathway at several different stages of the atherosclerosis process (Fig. 2) [29]. For example, LT signaling has been suggested to be involved in the initiation of atherosclerosis, through both lipid retention and thickening of the vascular wall, as well as in the changes of the endothelial homeostasis that characterize early atherosclerosis (Fig. 2). Subsequently, the potent LT-induced immunostimulatory actions have indicated a key role in atheroma development. Furthermore, some studies have indicated a role for the LT pathway in plaque rupture, causing thrombosis and vessel occlusion (Fig. 2). The plaque rupture induces ischemia in distal organs, such as the brain and the myocardium, and the contribution of LT signaling in stroke and myocardial infarction has been studied in models of ischemia and reperfusion. Taken together, atherosclerotic processes deteriorating healthy vessels and causing organ damage may at each stage represent a putative target for anti-LT therapy. The next sections of this review will focus on how the LT pathway potentially may interact at those different stages of atherosclerosis development, and eventually its ischemic complication, such as myocardial infarction and stroke (Fig. 2).

Lipid retention and modification

The early sign of lipid retention within the vascular wall is referred to as fatty streaks, which appear early in life and may either disappear or develop into more advanced atherosclerotic lesions (Fig. 2) [10]. Fatty streaks consist mainly of lipid laden macrophages, or foam cells [10]. As lipids are taken up into the vascular wall, they undergo oxidative modifications [10]. Several studies support a lipoxygenase-mediated oxidation of low density lipoproteins (LDL) [30, 31], mainly mediated trough the 15-lipoxygenase (15-LO) pathway [32]. However, recent findings have indicated an absence of 15-LO expression within atherosclerotic lesions [33], hence questioning its role as a driver of LDL oxidation *in vivo*. According to the latter study, 5-LO is the main lipoxygenase expressed within atherosclerotic lesions [33]. Since the role of 5-LO in LDL oxidations appears less convincing compared with the actions of 15-LO [34, 35], the atherogenic role of 5-LO may rather be dependent on its participation in the biosynthesis of LTs, and the pro-inflammatory signaling transduced through LT receptor activation [29]. The latter notion is supported by findings in atherosclerotic apolipo-

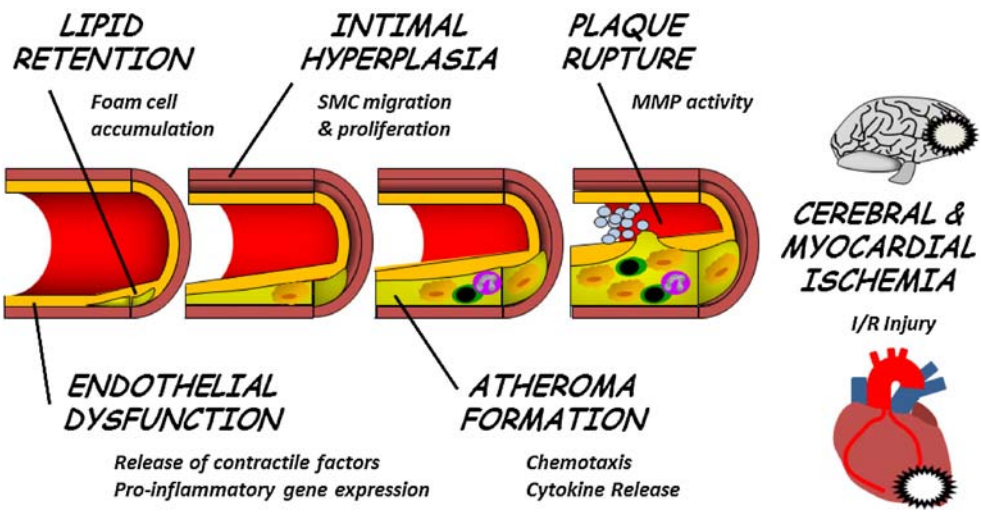


Fig. 2 The potential involvement of leukotrienes in the different stages of the atherosclerosis process eventually leading to cerebral and myocardial ischemia. Abbreviations: *SMC* smooth muscle cells, *MMP* matrix metalloproteinase, *I/R* ischemia and reperfusion

protein E (ApoE) knockout mice in which either genetic or pharmacological targeting of the BLT_1 receptor reduces lipid accumulation and foam cell infiltration [36–38].

Intimal hyperplasia

Another sign of early atherosclerosis is the thickening of the inner muscular layer of the vascular wall, which is referred to as intimal hyperplasia (Fig. 2) [10]. Cysteinyl-LT signaling through CysLT receptors mediates proliferation and migration of vascular smooth muscle cells (SMCs) [5]. In addition, the class of $CysLT_1$ receptor antagonists used in the treatment of asthma (Fig. 1) inhibits intimal hyperplasia after vascular injury in different animal models [5, 39]. Recently, it was discovered that SMCs within human atherosclerotic lesions also express receptors for LTB_4 [38, 40], mediating proliferation and migration of coronary artery SMCs [40]. The important signaling through these receptors has been supported by findings in $ApoE^{-/-}$ mice with a targeted BLT_1 receptor, which have fewer SMCs in their lesions [38]. Furthermore, BLT receptor antagonism reduces intimal hyperplasia after vascular injury in rats [5, 40]. Taken together, the LT-induced stimulation of SMCs suggests their involvement in the intimal hyperplasia associated both with early atherosclerosis and in-stent restenosis after coronary interventions [5].

Endothelial dysfunction

While in healthy individuals, the endothelium contributes to vascular homeostasis, atherosclerosis is characterized by a dysfunctional endothelium (Fig. 2). Cysteinyl-LT activation of endothelial cells have been reported to be transduced through constitutively expressed $CysLT_2$ receptors [41, 42] coupled to a release of relaxant factors [5]. However, while a proinflammatory environment down-regulates $CysLT_2$ receptor expression in human umbilical vein endothelial cells (HUVECs) [42], $CysLT_1$ receptor expression is induced in HUVECs under such conditions [43]. Furthermore, studies of isolated arteries have demonstrated endothelium-dependent contractions through $CysLT_1$ receptor signaling [44, 45]. Taken together, those observations suggest that a balance of CysLT receptor subtype expression may determine the LT-induced endothelium-dependent vasomotor responses. The latter notion has in addition received support from animal models. Directed endothelial expression of the human $CysLT_2$ receptor in mice induces an enhanced NO production in response to LTC_4, but does not change baseline systemic blood pressure compared with wild type mice [46]. Furthermore, although the LT synthesis inhibitor MK-886 (Fig. 1) does not alter mean arterial blood pressure in spontaneously hypertensive rats [47], this treatment prevents the rise in blood pressure observed in rats chronically treated by an NO synthesis inhibitor [48]. In summary, data from several studies using different *in vivo* and *in vitro* models suggest that cysteinyl-LT signaling may provide a key balance between a release of endothelium-dependent relaxant and constricting factors.

In contrast to this relatively extensive exploration of CysLT receptor signaling on endothelial cells, less is known about the signaling of the other arm of the LT pathway, through BLT receptors, in this context. Although healthy human arteries may not express receptors for LTB_4, an endothelial BLT_1 receptor expression is induced in atherosclerotic lesions [40]. Findings in animal models have in addition suggested that LTB_4-signaling through the BLT_1 receptor may also be associated with an endothelium-dependent release of vasoactive factors [49, 50].

In addition to direct action on the vascular tone, LT-induced activation of endothelial cells may also lead to changes in the transcriptional activity. In HUVECs, LTD_4 induces endothelial P-selectin expression through $CysLT_2$ receptor activation [51]. Furthermore, the CXC chemokines CXCL-2 [52] and IL-8 [53] are found among the most up-regulated genes after stimulation of HUVECs with LTD_4. The latter findings suggest that LT-induced activation of the endothelium in addition may participate in the starting point of the immune activation associated with the atheroma formation.

Atherosclerotic plaque formation

Foam cell accumulation and altered endothelial homeostasis will induce progressive recruitment of different populations of immune cells to the vascular wall, and eventually lead to the formation of the atherosclerorotic plaque, or atheroma (Fig. 2) [10]. This process is characterized not only by a reduction of the arterial lumen size, but also by the formation of a necrotic lipid core surrounded by a fibrous cap of extracellular matrix and SMCs (Fig. 2) [10]. The chemoattractant activity induced by LTB_4 through BLT_1 and BLT_2 receptors expressed on monocytes may play an important role in the continued accumulation of macrophages at the site of the initial foam cell infiltration [36]. Since macrophages represent a major source of 5-LO in the cardiovascular system, LTB_4-induced monocyte recruitment could potentially further exacerbate the inflammatory activity at sites of atherosclerotic lesions. Furthermore, LTB_4 also induces chemotaxis of other immune cells, such as T-lymphocytes, which accumulate in the vicinity of 5-LO-positive macrophages [52]. In a mouse model combining hyperinflammation and hyperlipidemia, through selective

abrogation of T-cell TGFβ signaling in ApoE$^{-/-}$ mice, a stimulatory effect of activated T-lymphocytes on LT synthesis has been demonstrated [54]. Activated T-lymphocytes up-regulate the expression of FLAP in macrophages, leading to an increased LTB_4 formation [54]. The increased LTB_4 formation that results from both monocyte recruitment and T-lymphocyte-mediated FLAP up-regulation, may lead to a vicious circle involving progressive recruitment of inflammatory cells and a maintained immune activation within the atherosclerotic lesion [29, 54]. Selective targeting of LTB_4 signaling could be key in blocking this vicious circle, as suggested by the capacity of different anti-leukotriene treatment strategies to reduce atheroma macrophage and T-lymphocyte content in hyperlipidemic mice (Fig. 1) [29].

Plaque rupture

Rupture of the fibrous cap will expose the content of the atheroma to the circulation, leading to platelet activation and thrombotic occlusion of the arterial lumen [10]. In a coronary artery, this process will lead to myocardial ischemia whereas in the cerbrovasculature, cerebral ischemia will occur (Fig. 2). The role of leukotrienes in plaque rupture has been suggested through transcriptional profiling of carotid endarterectomies, in which the constituents of the leukotriene pathway are expressed at higher levels in specimens obtained from patients with recent clinical signs of cerebral ischemia compared with asymptomatic patients [55]. One of the key components in plaque instability and rupture is the degradation of extracellular matrix by specific matrix metalloproteinases (MMPs) [56]. The production, secretion and activation of MMPs may be dependent on the generation of endogenous LTs, as suggested by the co-localization of 5-LO with MMPs in human carotid atherosclerotic lesions [55]. The latter observation was recently extended to endarterectomies derived from diabetic patients, in which 5-LO expression and LTB_4 production was associated with increased levels of MMP protein [57]. Furthermore, smokers exhibit a significant correlation between LTB_4 concentrations and MMP-9 activity in saliva [21]. Taken together, those studies suggest that both LTs and MMPs are simultaneously increased during an inflammatory response.

Those observations provide an initial suggestion of inhibited protease activities after pharmacological inhibition of LT signaling, with potential therapeutic implications for the prevention of plaque rupture.

Myocardial ischemia

In support of a link between leukotrienes and coronary plaque rupture, increased levels of urinary LTs have been reported in patients with acute coronary syndromes [58]. However, whether urinary LTs are markers of plaque rupture, or rather reflects the inflammatory response associated with myocardial ischemia remains to be established. Experimental myocardial ischemia indeed induces increased LT production [59, 60]. Several studies of transient ischemia in isolated perfused hearts derived from either rats or rabbits have in addition indicated an improved myocardial recovery after LT inhibition [59–62]. However, the results of *in vivo* ischemia and reperfusion have provided contradictory results. Either $CysLT_1$ receptor antagonism or LT synthesis inhibition induces a protective effect after myocardial ischemia followed by reperfusion in rabbits and cats, respectively [60, 63]. In contrast, different LT receptor antagonists have been reported not to alter the infarct size after myocardial ischemia and reperfusion in rats [61, 64], and both positive and neutral effects have been reported for LT synthesis inhibitors in dogs [65, 66]. In mice subjected to coronary ligation and reperfusion, the infarct size is not affected by either 5-LO knock out or CysLT receptor antagonism [67, 68]. In the latter model, 5-LO deficient mice have been reported to exhibit an even increased neutrophil infiltration and proinflammatory gene expression within the infarction area compared with wild type mice [67]. Although the these results raise a doubt as to the role of LTs in myocardial ischemia, a recent study of transgenic mice expressing a human endothelial $CysLT_2$ receptor, have reported an increased infarct size and leukocyte infiltration resulting from signaling trough this receptor [67]. However, the absence of atherosclerosis in the latter studies may limit the extrapolation of the findings to human coronary syndromes. As discussed above, the endothelial $CysLT_2$ receptor is rapidly down-regulated by pro-inflammatory stimuli *in vitro* [42]. Furthermore, microcirculatory changes induced during atherosclerosis have in addition been suggested by the exaggerated leukocyte recruitment and plasma leakage after ischemia and reperfusion in hypercholesterolemic LDLR$^{-/-}$ mice [69]. Although a role for LTs in this enhanced response was not established in the latter study [69], the LTB_4-induced leukocyte adhesion and migration, as determined by intravital microscopy in postcapillary venules, are significantly enhanced in LDLR$^{-/-}$ compared with wild type mice [70].

Cerebral ischemia

The notion of LT production in the ischemic brain was firstly raised by studies in gerbils [71], in which inhibitors of LT synthesis limit the damage after transient cerebral ischemia [72]. Subsequent studies in rats have associated beneficial effects with the cysteinyl-LT arm of the AA metabolism. For example, anti-asthmatic $CysLT_1$ receptor antagonists, such as pranlukast and montelukast, reduce the effects of both focal

and global ischemia provoked through either transient or permanent cerebral artery occlusion in rats [9, 73, 74] (Fig. 1). Interestingly, a purino-like GPRC named GPR17 has been shown to in addition to uracil nucleotides, also be activated by LTD_4 and LTC_4 [9]. This potentially dual CysLT and purinoreceptor is up-regulated in the ischemic brain, and silenced receptor expression *in vivo* mimics the neuroprotective effects of montelukast after 48 h of permanent focal ischemia.

Conclusion

The proinflammatory signaling induced by LT receptor ligation has been implicated at several stages of the atherosclerosis process, and may represent a putative target in the design of novel anti-inflammatory strategies to prevent myocardial infarction and stroke. Based on findings in different animal models of atherosclerosis [29], beneficial effects could be anticipated by anti-LT treatments. Although some findings in humans based on either genetic associations [25–28] or LT measurements in different patient populations [20, 21, 24, 58], support this notion, it may currently be premature to conclude on what exact mechanisms to target in human disease. Some interesting information can however be obtained through studies of other pathologies in which anti-LT treatments already have been evaluated [12, 14]. In the latter context, the effects of LT inhibition is of particular interest in the context of diseases associated with an increased cardiovascular risk, such as COPD and periodontitis [14, 20]. The challenge is now to extrapolate experimental findings to a clinical setting in order to identify patients groups that would benefit from an inhibited LT signaling.

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