The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease

Daniel Poeckel† and Colin D. Funk *

Departments of Physiology and Biochemistry, Queen’s University, 433 Botejail Hall, Kingston, ON, Canada K7L 3N6

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Leukotrienes (LTs) derived from 5-lipoxygenase (5-LO) activity are most widely known for their actions during acute inflammation and asthma. 5-LO/LT pathway involvement in cardiovascular disease (CVD) pathogenesis has come to the forefront based on provocative human genetic/population and animal studies leading to the hypothesis that this pathway promotes atherosclerosis, abdominal aortic aneurysm, and myocardial infarction/reperfusion injury via increased leucocyte chemotaxis, vascular inflammation and enhanced permeability, and subsequent tissue/matrix degeneration. A series of pre-clinical studies have tested this hypothesis by means of genetic or pharmacological inhibition of either the LT biosynthesis axis (5-LO, 5-LO-activating protein, LTA4 hydrolase, LTC4 synthase) or the cognate LT receptors. Here, we summarize, compare, and analyse these animal studies and relate their findings to human disease pathogenesis. We draw a complex picture of 5-LO/LT participation in cardiovascular disorders, which is further complicated by marked differences between species. Moreover, we discuss how the cytokine footprint of the respective pathological conditions determines the expression level and contribution of components of the pathway to the overall disease state. Current knowledge implies a role for 5-LO and LTs during the early/acute phase of CVD, but our understanding of a putative 5-LO/LT involvement in more advanced stages of CVD is limited, thereby preventing simple extrapolation of findings from animal studies to humans.

Keywords Leukotriene  •  5-Lipoxygenase  •  Atherosclerosis  •  Abdominal aortic aneurysm  •  Myocardial ischaemia/reperfusion  •  Inflammation

1. Introduction

Leukotrienes (LTs) are potent lipid mediators primarily known for their roles in pathophysiological settings. Derived from the polyunsaturated fatty acid arachidonic acid (AA), LTs constitute a class of local-acting messengers in addition to the cyclooxygenase (COX)-derived prostaglandins and thromboxanes. LTs are generated primarily in leucocytes at the nuclear envelope, where activated cytosolic phospholipase A2 liberates AA from membrane phospholipids. 5-Lipoxygenase (5-LO) interacts with a ‘scaffold-like’ protein designated 5-LO-activating protein (FLAP) that facilitates transfer of AA to 5-LO, for incorporation at the nuclear envelope, where activated cytosolic phospholipase A2 liberates AA from membrane phospholipids. 5-Lipoxygenase (5-LO) interacts with a ‘scaffold-like’ protein designated 5-LO-activating protein (FLAP) that facilitates transfer of AA to 5-LO, for incorporation of molecular oxygen into the backbone of AA in a two-step reaction. The first reaction yields 5-hydroperoxyeicosatetraenoic acid (5-HPETE), followed by a dehydration step that results in the unstable epoxide LTA4. As considerable amounts of 5-HPETE may escape the conjugated system, resulting in two hydroxyl groups at C-5 and C-12 and a triene structure coined LTB4. This derivative represents the most powerful pro-inflammatory product of 5-LO (Table 1). However, 5-HETE is a precursor of 5-oxo-ETE, an eicosanoid with potent biological functions (e.g. eosinophil chemotaxis, Table 1).1 LTA4 has a half-life of less than 3 s at physiological pH.2 Despite the evanescent nature of LTA4, it can undergo transformation by several mechanisms. First, it may be transferred to soluble LTA4 hydrolase, presumably aided by a fatty acid carrier protein. LTA4 hydrolase opens the epoxide ring by water attack in the conjugated system, resulting in two hydroxyl groups at C-5 and C-12 and a triene structure coined LTB4. This derivative represents the most powerful pro-inflammatory product of 5-LO (Table 1). LTB4 is exported from the cell or can potentially act in the nucleus as a modulator of transcription.3 Second, 5-LO and FLAP may associate with membrane-integral LTC4 synthase,4 which conjugates LTA4 with glutathione in a thioether bond at C-6. This derivative known as LTC4 is exported by transporters such as the multidrug resistance-associated protein to the extracellular milieu, where it undergoes cleavage of the glutathione moiety to LTD4 and LTE4 by extracellular peptidases (Figure 1).5 Notably, LTC4, LTD4, and LTE4 (referred to as cysteinyll-LT,

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1 Present address: Celzome AG, Meyerhofstrasse 1, 69117 Heidelberg, Germany.
† Corresponding author. Tel: +1 613 533 3242; fax: +1 613 533 6880, Email: funk@queensu.ca
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cysLTs) have potent but distinct biological activities, first discovered in relation to allergy and asthma (Table 1).5,6 The third fate of LTA4 is the transfer (by directed export or diffusion) to adjacent cells, such as from neutrophils to platelets, allowing further metabolism to either LTs or another class of lipid mediators known as lipoxins, which possess unique biological effects (Table 1)7 that will not be discussed here.

Besides 5-LO, there are other LOs that carry out stereospecific oxygenation at either C-12 or C-15 and their products are implicated in chemotaxis and tumorigenesis,8,9 but may also be subject to further metabolism, yielding products with functions in homeostasis and resolution of inflammation.7,9 The 12/15-LO enzyme apparently possesses both pro-inflammatory and anti-inflammatory functions and is present in some tissue macrophages but not in circulating monocytes.9 Of importance, the expression of enzymes of the LT pathway is strictly regulated and depends on the cell type, as well as the activation state of the cell.

The actions of LTs are mediated by a series of G protein-coupled receptors (Figure 1). These cell-surface receptors display a tissue-specific expression pattern and are classified into receptors for LTB4 (BLT1, BLT2), as well as those for cysLTs, CysLT1, CysLT2, plus a newly discovered LTE4-specific receptor known as CysLTE that was identified in dual CysLT1/CysLT2 knockout mice (Table 2).10 GPR17, which binds uracil nucleotides, may be another receptor for LTD4/LTC4,11 although this has been questioned recently.12 BLT1 transduces the pro-inflammatory effects of LTB4 mainly in inflammatory cells and BLT2 functions as a low-affinity LTBl/LTBl/LTBl/LTBl hydroxy-heptadecatrienic acid receptor on inflammatory cells and other tissues. CysLT receptors are found on airway smooth muscle cells (SBCs), B-lymphocytes, eosinophils and monocytes (CysLT1), mast cells and macrophages (CysLT1 and CysLT2), and endothelial cells (mainly CysLT2) (see Back and Hansson13 and references therein). They transduce a variety of biological responses (e.g. smooth muscle CysLT1 evokes bronchoconstriction; Tables 1 and 2). Furthermore, LTB4 and other unsaturated fatty acids have been reported to bind the nuclear receptor PPARa, possibly regulating transcription of genes related to inflammation—resolution and lipid degradation.3 In general, LTs typically act as ‘autacoids’ near their origin, via capillaries or the interstitial space. As the end product of peptidase activity on cysLTs, LTE4 is the only detectable LT in the urine.14 LTB4 can be detected in saliva.15

Table 1 Biological effects of 5-LO pathway-derived lipid mediators

| 5-oxo-ETE | Chemoattractant (granulocytes)119 |
| 5-HETE  | Weak neutrophil/eosinophil activator, pulmonary vasoconstriction120–122 |
| LTB4    | Neutrophil chemotaxis and adhesion5,123 |
| CysLTs  | Enhance post-capillary venule permeability, bronchoconstriction, mucus secretion (asthma), vascular smooth muscle constriction, eosinophil and monocyte chemotaxis and activation5,17 |
| Lipoxins| Reduce neutrophil chemotaxis and extravasation, stimulate non-phlogistic monocyte attraction/macrophage phagocytosis, reduce oedema, reduce pain signals7,18 |

Figure 1 Biosynthesis and signalling in the 5-lipoxygenase/leukotriene pathway. An inflammatory cell synthesizes leukotriene (LT) lipid mediators that exit the cell via specific transporters [(8), (9)], for activation of the LTB4 receptors BLT1/BLT2 or the cysLT receptors CysLT1/CysLT2/CysLTE. Alternatively, LTA4 may undergo transcellular metabolism (small blue cell). See text for details. AA, arachidonic acid; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; ETE, eicosatetraenoic acid; FLAP, 5-LO-activating protein; LXA4, lipoxin A4.
LTs are best known for their pro-inflammatory activities and most investigators have considered only their detrimental effects in pathological conditions including asthma and cardiovascular disease (CVD). LT modifier drugs are in use for human asthma treatment and are in logical conditions including asthma and cardiovascular disease (CVD). However, LTs also perform important functions in several important cardiovascular pathological settings.

2. 5-LO/LT pathway and atherosclerosis

2.1 Background
Atherosclerosis, a decades long process in humans starting with lipid deposition in the arterial wall and inflammatory cell recruitment into the intimal layer, is a prime contributor to CVD. The contributions of inflammation and immune mechanisms to this process are now well appreciated and the risk of plaque rupture, resulting in thrombosis and subsequently myocardial infarction or stroke, is directly associated with the inflammatory cell content of the plaque. The potential role of LTs in atherosclerosis has been studied in various facets of the pathology using animal models, primarily in genetically engineered mice.

2.2 LO and LT receptor expression in atherosclerosis

Of circulating cells, neutrophils, eosinophils, mast cells and monocytes express 5-LO in varying levels, depending on the activation state. In human atherosclerotic lesions, 5-LO is present in macrophages, foam cells, mast cells and dendritic cells (DCs). During the maturation of monocytes to tissue macrophages, 5-LO expression is upregulated. Nonetheless, under resting (non-phlogistic) conditions, the enzyme is normally inactive. Several factors, mainly associated with an inflammatory state of 5-LO-expressing cells and their local environment, drive 5-LO activation. External signals including cytokines (IL-8) and pathogenic factors (IML, zymosan) but also oxidative or genotoxic stress promote intracellular Ca\(^{2+}\) mobilization and/or induction of the MAP kinase cascade, culminating in 5-LO phosphorylation and increased Ca\(^{2+}\)-dependent binding to the nuclear membrane. At the 5-LO catalytic centre, an enhanced oxidative state favours oxidation of its non-heme iron to the ferric form, allowing for the execution of the catalytic reaction and eventually, LT formation (Figure 1).

12/15-LO in monocytes/macrophages, on the other hand, may be inversely regulated. During the onset of local tissue inflammation and promoted by Th1 type cytokines, the levels of 12/15-LO-products drop quickly, presumably because 12/15-LO expressing macrophages are sequestered or emigrate, e.g. through lymphatic vessels. Subsequently, activated 12/15-LO-negative macrophages enter the tissue. This effect reverts only after days, when the resolution phase of inflammation begins, pointing toward a role for 12/15-LO products in general tissue homeostasis and during late phase inflammation.

2.2.1 Early atherogenesis
In early atherogenesis, intimal inflammation is set off by retention of lipoproteins (VLDL, LDL) in the arterial wall. Here, roles for both 5-LO and 12/15-LO products have been postulated. The 5-LO product LTB\(_4\) enhances this inflammatory response by several mechanisms, one of which is the induction of pro-inflammatory cytokine release from BLT-expressing leucocytes. These include IL-6, monocyte chemoattractant protein-1 (MCP-1) and TNF\(_\alpha\), all of which have been linked to pro-atherosclerotic functions. These deleterious effects are aggravated by increased lipid hydroperoxide levels (giving rise to several reactive oxygen species). Thus, 12/15-LO is able to oxidize cholesterol-linoleic acid, a main component of LDL. This ‘oxidized’ LDL is retained in the vascular wall and activates the adjacent endothelium, inducing the expression of adhesion molecules (VCAM-1) that facilitate leucocyte binding and diapedesis. Additionally, tissue macrophages bind and ingest oxLDL via scavenger receptors (class B), resulting in M1 type activation and the subsequent release of Th1 cytokines (IL-1\(\beta\), TNF\(_\alpha\)) to attract further inflammatory cells. TNF\(_\alpha\) is an important early pro-atherogenic cytokine, and IL-1\(\beta\) is a promoter of leucocyte adhesion to endothelial cells (ECs). By preventing maturation and regression of local DCs in atherosclerotic arteries, oxLDL promotes a rise in DC numbers with the capacity to release more of the pro-inflammatory Th1 cytokine IL-12. Clearly, oxLDL is perceived as a ‘danger signal’, which induces clearance of oxLDL by macrophages, associated with a local inflammatory reaction.

CysLTs produced under these conditions by macrophages or DCs activate ECs via CysLT\(_1\) receptors. Notably, in a non-phlogistic state, ECs express predominantly CysLT\(_2\) receptors, which is linked to enhanced NO release and vasorelaxation. In contrast, some pro-inflammatory conditions are connected to CysLT\(_2\) downregulation and CysLT\(_1\) induction, resulting in vessel contraction and blood pressure elevation. This effect might occur during early to advanced atherosclerosis.

SMC migration plays an important role in the initiating stages of atherogenesis. Importantly, LTB\(_4\) activates these cells via BLT\(_1\) receptors. During progression of atherosclerosis, arterial tissue becomes responsive to cysLTs, indicating a vasoconstrictive function for CysLTs on SMCs as well.

2.2.2 Plaque remodelling/inflammation and acute complications
As atherosclerosis progresses in humans with persisting characteristics of inflammation, 5-LO would be activated with LTB\(_4\) acting as a chemotaxin to recruit more inflammatory cells. In macrophages,
this recruitment may be mediated by BLT1 and BLT2. T lymphocytes are also recruited via BLT1, allowing secretion of metalloproteinases upon LTB4 stimulation and thus, promoting plaque destabilization. Of note, these cells are more common in the adventitial layer than in the intimal region of an inflamed artery. A putative link between inflammation and adaptive immunity is further corroborated by the fact that dendritic cells are also responsive to LT stimulation. In contrast, ECs and inflammatory signalling (LPS, IFNγ) down-regulates BLT1 expression presumably in a negative feedback mechanism. In contrast, ECs and SMC respond with BLT1 upregulation, thereby enhancing the pro-atherogenic effect of LT signalling. The action of LTs on these cells results in intimal hyperplasia. Eventually, cumulative necrosis of foam cells re-inforces the local release of pro-inflammatory 5-LO products, amplifying a cycle that presumably leads to plaque destabilization and rupture.

### 2.3 Animal models of atherosclerosis targeting the 5-LO/LT pathway

In 2001, genetic studies using atherosclerosis-susceptible and -resistant mouse strains identified the 5-LO gene (Alox5) as a potential candidate for atherosclerosis. Subsequent studies with 4- to 6-month-old LDL receptor-deficient (LDL-R−/−) mice expressing only one Alox5 allele fed an atherogenic diet for 8 weeks indicated a >90% reduction in atherosclerotic lesions. These results, based on a very small study cohort (n = 4), could not be corroborated in a subsequent study using larger numbers of homozygous 5-LO knockout mice. In this study, Zhao et al. used 5-LO−/− apolipoprotein (apoE)−/−, as well as 5-LO−/− LDLR−/− mice on normal chow or atherogenic/western diet and examined lesions between 5 and 12 months of age, without observing any differences to the respective control mice. Only the youngest group of 5-LO−/− apoE−/− mice, fed an atherogenic diet for 2 months, showed a modest reduction (−24%) in lesion area. Additional studies by Cao et al. did not detect any dependence of lesion size on the presence or absence of Alox5 in apoE−/− mice. Male and female mice were analysed separately after 2 months of atherogenic diet, and female mice were studied after 6 months on western diet. Recently, we reported that 5-LO−/− mice on an apoE−/− background fed normal chow diet for 6 months had a similar atherosclerotic burden as apoE−/− control mice, but a slight synergistic effect toward reduced lesions was apparent with concomitant 12/15-LO deficiency. However, these differences were only observed in female but not in male mice, indicating gender-specific roles for LOs in atherosclerosis. Studies targeting 5-LO are summarized in Table 3. On the basis of recent findings about gender-dependent regulation of 5-LO activity and the differential effect of sex hormones on 5-LO pre-activation, future studies will have to address the roles of these enzymes in atherosclerosis with particular focus on gender-specific effects.

Pharmacological studies with 5-LO inhibitors have yielded mixed results (Table 4). L739010 supplied in an atherogenic diet for 8 weeks was without effect in apoE-deficient male and female mice. However, in LDLR−/− male mice treated with ZD4407 for 15 weeks, a reduction of 40% in the intima-media thickness was detected compared with untreated controls. A dual COX/5-LO inhibitor, licofelone, had similar effects and reduced inflammation in atherosclerotic rabbits. More convincing evidence for a role of the 5-LO pathway in atherosclerosis came from studies targeting FLAP. With progressing atherosclerosis, increased expression of FLAP and CysLT1 was determined in aorta extracts. In agreement with these results and the fact that FLAP expression is highly upregulated in a murine model of atherosclerosis, FLAP inhibition with MK-886 conferred reduced lesion size and less macrophage infiltration in 6-month-old female apoE−/−LDLR−/− mice fed western diet for 4 months and reduced lesions after only 4 weeks treatment in another model of aggravated atherosclerosis. Interestingly, in this model, presumably 5-LO-expressing macrophage numbers were unchanged, but 5-LO negative T cells were reduced by 50%. However, it should be mentioned that FLAP inhibitors such as MK-886 possess off-target effects, so it cannot be ruled out that some of the observed effects are due to the inhibition of secondary targets.

Genetic deletion or pharmacological inhibition of BLT1 in apoE knockout mice caused less leucocyte recruitment into atherosclerotic lesions and protection against cholesterol deposition and the subsequent formation of foam cells. A significant reduction of T-cell accumulation, which normally occurs in the vicinity of lesional macrophages, was observed in BLT1-deficient mice underlining the putative role of BLT1 in atherosclerosis.

### Table 3 Atherosclerosis studies and the 5-LO/LT pathway: studies in mice with 5-LO deficiency (5-LO+/− or 5-LO−/−)

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Diet, time point</th>
<th>n (Control group in parentheses)</th>
<th>Gender</th>
<th>Outcome (related to control group)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLR−/− 5-LO+/−</td>
<td>Chow 2–4 months + Ath 2 months</td>
<td>4 (4)</td>
<td>M + F</td>
<td>Lesions ↓ (−96%)</td>
<td>Mehrabian et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO+/−</td>
<td>Chow, 6–12 months</td>
<td>54 (33)</td>
<td>M + F</td>
<td>No difference</td>
<td>Zhao et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO+/−</td>
<td>Chow 2.75 months + Ath 2 months</td>
<td>13 (16)</td>
<td>M + F</td>
<td>Lesions ↓ (−24%)</td>
<td>Zhao et al.</td>
</tr>
<tr>
<td>LDLR−/− 5-LO+/−</td>
<td>Chow 2.75 months + western 3 months</td>
<td>6 (21)</td>
<td>M + F</td>
<td>No difference</td>
<td>Zhao et al.</td>
</tr>
<tr>
<td>apoE−/− 5-LO+/−</td>
<td>Chow 2 months + Ath 2 months</td>
<td>14 (16)</td>
<td>M</td>
<td>No difference</td>
<td>Cao et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO+/−</td>
<td>Chow 2 months + Ath 2 months</td>
<td>19 (13)</td>
<td>F</td>
<td>No difference</td>
<td>Cao et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO+/−</td>
<td>Chow 2 months + western 6 months</td>
<td>18 (15)</td>
<td>F</td>
<td>No difference</td>
<td>Cao et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO−/−</td>
<td>Chow 6 months</td>
<td>7 (10)</td>
<td>M</td>
<td>No difference</td>
<td>Poeckel et al.</td>
</tr>
<tr>
<td>ApoE−/− 5-LO−/−</td>
<td>Chow 6 months</td>
<td>7 (9)</td>
<td>F</td>
<td>No difference</td>
<td>Poeckel et al.</td>
</tr>
</tbody>
</table>

Ath, atherogenic ('Paigen') diet.
the importance of LTBA₂–BLT₁ signalling in T cells. Of interest, BLT₁ deletion significantly reduced lesion formation only in early (4–8 weeks) stages of atherosclerosis, but these differences had disappeared after 19 weeks on western diet. Likewise, pharmacological targeting of BLT₁ with CP-105696 in apoE⁻/⁻ or LDLR⁻/⁻ mice promoted a reduction of lesion size after 5 weeks treatment. CysLT₁ inhibition by montelukast appears to mimic these effects in apoE/LDLR double deficient mice or rabbits fed atherogenic diet. An overview of preclinical studies in mice targeting other components of the 5-LO/LT pathway is given in Table 4.

Interestingly, studies addressing the role of 12/15-LO have yielded similar variable results. Murine studies have most often reported beneficial effects of 12/15-LO deletion on atherosclerotic plaque formation, and enhanced atherosclerosis when 12/15-LO or human beneficial effects of 12/15-LO deletion on atherosclerotic plaque for- similar variable results. Murine studies have most often reported ben-

Table 4 Atherosclerosis studies and the 5-LO/LT pathway: Other mouse studies targeting the 5-LO/LT pathway

<table>
<thead>
<tr>
<th>Pharmacological/genetic target</th>
<th>Diet, Time point, Genotype</th>
<th>Gender</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-LO (L739,010)</td>
<td>Ath, 8 weeks (apoE⁻/⁻)</td>
<td>M + F</td>
<td>No difference</td>
<td>47</td>
</tr>
<tr>
<td>5-LO (ZD4407)</td>
<td>Western, 15 weeks (LDLR⁻/⁻)</td>
<td>M</td>
<td>Intima media thickness ↓ (−40%)</td>
<td>49</td>
</tr>
<tr>
<td>FLAP (MK-886)</td>
<td>Western, 4 months (apoE⁻/⁻ LDLR⁻/⁻)</td>
<td>F</td>
<td>Lesion size ↓</td>
<td>52</td>
</tr>
<tr>
<td>FLAP (MK-886)</td>
<td>Chow, 4 weeks (apoE⁻/⁻/dnTGFβRII)</td>
<td>M + F</td>
<td>Lesion size ↓</td>
<td>51</td>
</tr>
<tr>
<td>BLT₁ (CP-105,696)</td>
<td>Western, 5 weeks (apoE⁻/⁻ or LDLR⁻/⁻)</td>
<td>M + F</td>
<td>Lesion size ↓</td>
<td>54</td>
</tr>
<tr>
<td>BLT₁⁻/⁻</td>
<td>Western, 6 or 12 weeks (apoE⁻/⁻)</td>
<td>M + F</td>
<td>Lesion size ↓</td>
<td>54</td>
</tr>
<tr>
<td>BLT₁⁻/⁻</td>
<td>Western, 4, 8 or 19 weeks (apoE⁻/⁻)</td>
<td>M + F</td>
<td>Lesion size ↓</td>
<td>43</td>
</tr>
<tr>
<td>CysLT₁ (montelukast)</td>
<td>Western, 16 weeks, apoE⁻/⁻ LDLR⁻/⁻</td>
<td>M + F</td>
<td>No difference (8 and 19 weeks)</td>
<td>56</td>
</tr>
</tbody>
</table>

*dnTGFβRII, dominant negative TGFβ type II receptor on CD4⁺/CD8⁺ T cells.

some short-term studies, whereas the majority of longer studies did not detect any effect of 5-LO inhibition. The presently available data suggest a role for the LT pathway during early atherosclerosis and implies that targeting the LT pathway in the early stages of disease progression might be advantageous. In the next section, we offer a rationale for this hypothesis based on cytokine regulation of LT-pathway proteins.

2.4 The 5-LO/LT pathway in atherosclerosis: a regulatory role for cytokines?

Murine models of atherosclerosis represent accelerated disease development with an early, acute inflammatory phase that progresses to an advanced state with characteristics of chronic inflammation. In this rapid atherogenic process, oxLDL-activated macrophages participate in Th1 cytokine expression. Notably, the most commonly used mouse models are on a C57BL/6 background, which is characterized by a strong Th1 and less prominent Th2 immune response. The majority of CD4⁺ T cells identified in early atherosclerotic lesions are of the Th1 phenotype, which may primarily reflect their interaction with oxLDL-presenting macrophages or DCs via T-cell receptor-MHC II complex recognition, described for humans. Also, monocytes migrating into atherosclerotic lesions predominantly display the M1 phenotype. The 5-LO/LT pathway could be active during the early phase.

Severe hypercholesterolaemia promotes an apparent switch from Th1 to Th2 effector type in apoE⁻/⁻ mice, connected to reduced T cell and macrophage activation states. Accordingly, in advanced lesions of mice, most intimal foam cells display a DC-like phenotype (CD11c⁺) with no detectable 5-LO expression. Cultured murine DCs exposed to the Th2 cytokine IL-10 down-regulated FLAP expression and suppressed LTBA₄ biosynthesis. Conversely, 5-LO deletion or blockade of LTBA₄–BLT₁ signalling increased IL-10, an anti-atherogenic cytokine, in murine splenic cells. Therefore, available data support the concept that a role for 5-LO in murine atherosclerosis may be restricted to the early phase, where LTs promote inflammation. An 'eicosanoid switch', known to occur in some mixed cell populations under inflammatory conditions, whereby 5-LO-containing macrophages switch from synthesizing pro-inflammatory mediators in favour of pro-resolving agents like resolvins, protectins, and lipoxins
would not be expected to occur in advanced atherosclerotic lipid-laden lesions with entrapped macrophages/foam cells, accelerated leucocyte apoptosis and consequent chronic inflammation. Defects in the signalling pathways or the biosynthesis of pro-resolving agents or impaired receptor function, promoted by aberrant cytokine signalling in advanced lesions, might be the underlying cause for chronic inflammatory mechanisms to prevail over pro-resolving processes.\(^{18,69}\)

### 2.5 Limitations of animal models

Animal models potentially bear the risk of compensatory mechanisms due to genetic modification of the target gene that render the results difficult to interpret. Another caveat is species differences between mice and humans. For instance, 5-LO expression in intimal atherosclerotic lesions varies between mice and humans; also, 5-LO and 12/15-LO appear to be differentially regulated in inflammatory cells of mice and humans with the murine 12/15-LO producing mainly 12-HPETE, while its human counterpart primarily synthesizes 15-HPETE. Notably, both products may have opposing effects in inflammation.\(^{8}\) Moreover, atherogenesis in mice differs in several facets from the human pathology. Thus, T cells, whose presence in all stages of atherosclerotic lesions is acknowledged, are underrepresented in murine models of atherosclerosis.\(^{70,71}\) Despite these shortcomings, animal models afford an invaluable means to study the effects of directed genetic overexpression, deletion or pharmacological inhibition of key enzymes of the LT cascade in a physiological setting that cannot be achieved in humans.

### 2.6 5-LO/LT pathway disparities between murine and human atherosclerosis

Advanced human plaques show differences in 5-LO expression compared with mouse lesions. In human lesions, 5-LO\(^+\) cells were identified in macrophages, DCs, mast cells, and neutrophils,\(^{55}\) and notably, these 5-LO\(^+\) cells are present in the neointimal region, whereas in mice, they are restricted to the adventitial layer.\(^{46}\) With increasing age, these adventitial macrophages form clusters with T cells, independent of the severity of atherosclerosis. Intimal inflammatory reactions are connected to distinct adventitial inflammation responses, whereby B lymphocytes, plasma cells, and T cells conglomerate with macrophages.\(^{38,72}\) 5-LO\(^+\) cells accumulate around new blood vessels, a common feature between mice and humans.\(^{46}\) In human atherosclerotic plaque specimens, the quantity of 5-LO\(^+\) cells even increased during progression from early to late phase coronary heart disease.\(^{55}\) Moreover, the elevated 5-LO activity was found to be associated with BLT\(_1\)-mediated matrix metalloproteinase (MMP) release from T cells, promoting plaque instability.\(^{37,73}\) Human lesions demonstrate detectable expression levels for all major components of the LT cascade, i.e. FLAP, LTA\(_4\) hydrolase, and LTC\(_4\) synthase, as well as BLT\(_1\)/BLT\(_2\) and CysLT\(_1\)/CysLT\(_2\) receptors.\(^{55}\) Taken together, in advanced human atherosclerosis, a role for 5-LO is likely, which is distinct from its role in early atherogenesis (Figure 2). This presence of the 5-LO/LT pathway in advanced lesions is not found in mouse models, which might be due to: (i) rapid progression of atheroma growth in mice vs. slower, often interrupted progression in humans (i.e. initial fatty streaks might remain dormant for many years in humans, until certain factors promote the progression of some lesions into an advanced state);\(^{74,75}\) (ii) advanced human plaques display a higher degree of instability and risk to rupture than murine plaques; (iii) temporal dissociation in the Th1/Th2 ‘balance’ at distinct lesion stages between mice and humans.

### 3. 5-LO/LT pathway and abdominal aortic aneurysm pathogenesis

Aneurysm pathogenesis includes local inflammation of the aortic wall, immune cell infiltration in the adventitial layer, and medial degeneration by macrophage-derived proteases. Human aneurysm tissue

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**Figure 2** Participation of the 5-LO pathway in the development and progression of atherosclerosis in mice and humans. ‘Engineered’ murine atherosclerosis is accelerated and develops within months, whereas human atherosclerosis progresses over decades. Some studies indicate roles for 5-LO (expression indicated by triangles) in the early/acute stages of atherosclerosis in mice and humans, but only in the advanced stage of the human pathology. Other components of the 5-LO pathway (green triangle) are increasingly expressed in advanced human atherosclerosis. See text for details. LTA\(_4\)H, LTA\(_4\) hydrolase; LTC\(_4\)S, LTC\(_4\) synthase; EC, endothelial cell; SMC, smooth muscle cell.
reveals the presence of 5-LO, LTA4 hydrolase, and BLT1/BLT2 receptors predominantly associated with the abdominal aortic aneurysm (AAA) intraluminal thrombus and in macrophage-rich adventitial areas. In a hyperlipidemic atherogenic diet-induced AAA model, 5-LO expression is found in adventitial granulomas, often clustered with 5-LO negative T cells next to an extensive vasa vasorum. A distinct inflammatory/immune reaction takes place at these loci. In this model, atherogenic diet induces the development of AAA in the subadipocytic region of the abdominal aorta of apoE−/− mice. In this regard, apoE−/− 5-LO−/− mice assessed after 8 weeks on atherogenic diet were largely protected against aneurysms (incidence 2 of 17 vs. 16 of 34 in control apoE−/− 5-LO+/− mice). 5-LO may contribute to aneurysm formation through pro-inflammatory LTs via enhanced secretion or activity of MMP-2 and of the inflammatory chemokine macrophage inflammatory protein (MIP)-1α from macrophages. This effect appears to be mediated by LTD4 binding to CysLT1 receptors on macrophages of hyperlipidemic 5-LO expressing mice, but does not occur in 5-LO-deficient counterparts.

In the angiotensin II (AngII) AAA model, a role for LTBA was suggested based on a study using apoE−/−BLT1−/− mice. In this model, AngII is infused by osmotic minipumps at the concentration of 1 µg/min/kg body weight over the course of 4 weeks. AAA develops quite rapidly, often preceded by aortic dissection and formation of a thrombus in the aortic wall, followed by a fibrotic response and enlargement of the vessel. ApoE−/−BLT1−/− mice showed a diminished AAA incidence compared with apoE−/− controls. Matrix-destabilizing MMP-2 and MMP-9, as well as MCP-1 levels, along with leucocyte numbers, were found to be reduced in aortic tissues of apoE−/−BLT1−/− mice, consistent with an abrogation of LTBA signalling, which is known to induce MCP-1 release from mononuclear leukocytes in an autocrine manner. Interestingly, apoE−/−BLT1−/− mice, like apoE−/−5-LO−/− mice, had lower levels of plasma MIP-1α, implying a central role for this chemokine in 5-LO-mediated aneurysm formation. Studies in other cell types indicate that LTDA might be responsible for MIP induction. Also, pharmacological inhibition of BLT1 with CP-105 696 reduced AAA incidence in this model. Although these studies indicate a pro-active role for the 5-LO pathway in aneurysm pathogenesis, there is also evidence to the contrary. We analysed both genetic and pharmacological inhibition of the 5-LO pathway in murine AAA models, like apoE−/− mice, consistent with an abrogation of 5-LO activity of MMP-2 and of the inflammatory chemokine macrophage inflammatory protein (MIP)-1α from macrophages. In this model, AngII is infused by osmotic minipumps at the concentration of 1 µg/min/kg body weight over the course of 4 weeks. AAA develops quite rapidly, often preceded by aortic dissection and formation of a thrombus in the aortic wall, followed by a fibrotic response and enlargement of the vessel. ApoE−/−BLT1−/− mice showed a diminished AAA incidence compared with apoE−/− controls. Matrix-destabilizing MMP-2 and MMP-9, as well as MCP-1 levels, along with leucocyte numbers, were found to be reduced in aortic tissues of apoE−/−BLT1−/− mice, consistent with an abrogation of LTBA signalling, which is known to induce MCP-1 release from mononuclear leukocytes in an autocrine manner.

In summary, the role of the 5-LO/LT pathway in murine AAA pathogenesis is somewhat ambiguous at this stage, because there is distinct variability between murine models. Permitting some speculation, 5-LO-derived LTs may contribute to the recruitment of macrophages and T cells in early AAA pathogenesis in some pre-clinical models. Enhanced Th2 signalling could abort 5-LO-mediated pro-inflammatory events and with increasing structural deterioration of the medial wall matrix tissue, inflammatory reactions expand that may or may not be dependent on 5-LO. On the basis of human tissue studies, intraluminal thrombus form at AAA and upregulate expression of LT pathway enzymes, associated with LTBA-mediated neutrophil recruitment into the thrombus; so targeting the 5-LO pathway could potentially reduce aneurysm or associated pathology. Murine AAA models, much like the atherosclerosis models mentioned above, differ from the human situation in the temporal pattern of AAA development. For instance, in AngII-induced AAA, aneurysm evolution even precedes atherosclerotic lesion development and is evident within days, which is in clear contrast to the decades long AAA development and expansion and atherosclerotic lesion progression in humans. Targeting the 5-LO pathway for treatment of AAA pathogenesis awaits further pre-clinical studies perhaps in other larger animal models.

4. 5-LO/LT pathway and myocardial ischaemia reperfusion injury

Activated polymorphonuclear leucocytes (PMNL), an abundant source of LTs, are key players in the early pathogenesis of ischaemia–reperfusion injury. These cells adhere to ECs and initiate an inflammatory response, resulting in PMNL extravasation, secretion of pro-inflammatory and platelet-activating mediators, release of aggressive reactive oxygen species, and subsequent increase in vascular permeability and tissue damage. Given that the vasoactive role of cysLTs and their receptors has been known for many years, it is not surprising that 5-LO-derived cysLTs are involved in ischaemic injury. Besides enhancing microvascular constriction and vascular permeability, CysLTs also reduce myocardial contractility and cardiac output. CysLTs are produced by PMNL and EC in a transcellular fashion, in which ECs convert PMNL-derived LTA4 into LTC4 via glutathione-S-transferase (Figure 1). LTC4, which promotes leucocyte transmigration into tissues, is increased during acute myocardial infarction. Following the initial phase of neutrophil infiltration and concomitant biosynthesis of inflammatory mediators, CysLT2 expression in the heart is upregulated. As this upregulation was only apparent 48 h, but not 3 h, after reperfusion, the role for this...
receptor in the post-infarct response is probably restricted to a later stage of disease aetiology.99

Further supporting a role of 5-LO in MI, LTB4 was shown to mediate neutrophil chemotaxis to ischaemic tissue.99,100 Genetic studies a few years ago provided exciting evidence that certain haplotype types of the FLAP and LTA4 hydrolase genes (Alox5sap and LT44H, respectively) were associated with an increased risk of myocardial infarction/stroke. However, this excitement has been tempered more recently by a large number of genetic studies showing either no association or only modest evidence for involvement.101–104

In animal models, experimental ischaemia causes elevated LT production in the damaged tissue and evidence suggests that 5-LO products play a detrimental role to tissue recovery. One such feature is cystLT-mediated perivascular oedema, which directly affects cardiac function.99 Inhibiting LTC4 formation and action by infusion of anaesthetized rabbits with the FLAP inhibitor BAY-x1005 caused a drastic improvement in the consequences of coronary artery ligation 72 h post-surgery compared with vehicle-infused animals (ECG recordings and mortality).94,105 Other 5-LO-, FLAP-, or dual 5-LO/COX-inhibitors applied in models of myocardial infarction have shown similar beneficial effects (measuring infarct size).106–109 Mice overexpressing the human BLT1 receptor displayed strongly increased leucocyte infiltration in a lung ischaemia/reperfusion model, along with increased pro-inflammatory circuits.100 In dogs, a 5-LO inhibitor reduced infarct size and ischaemia,10,111 whereas an LT antagonist failed to do so in other studies.112 No effect of LT blockade was apparent in a rat model of myocardial ischaemia.113 Furthermore, a study using 5-LO-deficient mice, which underwent coronary artery ligation (30 min) followed by reperfusion (24 h), did not reveal any difference in infarct area between knockouts and control mice.114 These findings show that neither a clear target-inhibition profile exists for components of the 5-LO pathway nor is there consistency over different species and models. Further complication is added by apparently organ-specific effects of 5-LO inhibition. Thus, whereas Alox5 gene disruption caused no effect on cardiac ischaemia,114 it reduced the severity of kidney ischaemia.115 In cerebral ischaemia, the situation is not entirely clear. 5-LO−/− mice did not differ from control mice in infarct size after 60 min transient or permanent oxygen deprivation.116 On the other hand, post-ischaemic inflammation in the brain is characterized by ample recruitment of neutrophils and microglia with concomitantly elevated LT levels (reviewed elsewhere91). Following reperfusion, LTC4 levels rise particularly in the hippocampal area and forebrain.117 In rats, cystLT levels peaked 3–24 h and 7 days post-reperfusion, indicating roles for LTs during the subacute and late/chronic phase of cerebral ischaemia pathology,118 one of which is an increase in permeability of the blood–brain barrier in the early response.93 CysLT2 and CysLT1 receptors are expressed in the epithelium of the barrier, as well as in the brain, as determined by mRNA analysis.91 Of importance, rats and mice treated with CysLT1 antagonists (montelukast, ONO-1078) showed significantly reduced ischaemic brain injury under acute and chronic conditions.91 In summary, there is some evidence to support a potential role of the 5-LO pathway in ischaemic stroke, but the reason for the failure to detect differences in cerebral infarct size of 5-LO deficient mice vs. the control group remains elusive. Organ-specific deletion or overexpression of an LT pathway component represents an advantageous methodology to resolve many inconsistent results. In this respect, we recently reported that endothelium-specific overexpression of the CysLT2 receptor aggravates myocardial injury/reperfusion after 48 h and conversely, its inhibition via the CysLT2 antagonist BAY-u7773 abolished this effect. CysLT2 overexpression was shown to enhance vascular permeability, as well as the expression of pro-inflammatory transcription factors and adhesion molecules (Egr-1, VCAM-1, ICAM), which facilitate leucocyte transmigration.95

Taken together, animal studies have not led to a unified, unequivocal model for the LT pathway in myocardial ischaemia—reperfusion injury. The variable findings in animal models may firstly be related to the specific target of inhibition or deletion (5-LO, FLAP, LTC4 synthase, or the LT receptors). Whereas most studies targeting 5-LO or FLAP support their role in myocardial infarction pathogenesis, studies targeting LT receptors are mixed.94,95 This might indicate that additional receptors than those blocked are involved in transducing the LT effects. Alternatively, pharmacological blockade might suffer from incorrect dosing or off-target actions. Future studies should involve a systematic dissection of each component within the 5-LO/LT pathway.

5. Conclusion and future directions

During the last few years there has been a resurgent focus on the 5-LO/LT pathway as a potential target in CVD. The complexity of the 5-LO/LT pathway participation in mechanisms contributing to CVD is evident based on the many studies alluded to in this review. Limitations of these studies often result from the ‘snapshot’ punctual nature of analysing a single time point in CVD pathogenesis that makes it difficult to gain systematic insight into 5-LO-driven or -independent processes. Murine and human CVD aetiology differ with respect to the 5-LO/LT pathway, and even within murine studies, the nature of the applied model (for atherosclerosis, AAA, or ischaemia/reperfusion injury) influences the conclusions. Whereas a role for 5-LO-derived LTs in early stages of murine and human atherosclerosis, AAA, and reperfusion injury is cogent based on their effects in chemotaxis and induction of pro-inflammatory responses, the 5-LO pathway appears to play a distinct role in advanced human atherosclerosis but not in advanced murine disease. Targeting specific LT G protein-coupled receptors rather than upstream targets involved in LT synthesis may be a superior strategy for future CVD therapeutic interventions based on extensive past experience with other pathways (e.g. via angiotensin II and adrenergic receptors), although this remains to be determined. Conditional knockouts and comprehensive translational studies should serve better than the traditional, simplistic ‘one model’ approach to understand the complex effects exerted by 5-LO products. Understanding the cytokine milieu during distinct stages of CVD progression will be crucial to elucidate how the expression of members of the 5-LO/LT pathway is regulated. There is little doubt that 5-LO plays important roles in many facets of CVD, but the challenge for future studies will be to clearly dissect these activities in a temporal and cell- and tissue-specific context in order to provide a solid basis for potential therapeutic interventions.

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