

A new treatment for severe pulmonary arterial hypertension based on an old idea: inhibition of 5-lipoxygenase

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Abstract

It has been generally accepted that severe forms of pulmonary arterial hypertension are associated with inflammation. Plasma levels in patients with severe pulmonary arterial hypertension show elevated levels of interleukins and mediators of inflammation and histologically the diseased small pulmonary arterioles show infiltrates of inflammatory and immune cells. Here, we review the literature that connects pulmonary hypertension with the arachidonic acid/5-lipoxygenase-derived leukotrienes. This mostly pre-clinical background data together with the availability of 5-lipoxygenase inhibitors and leukotriene receptor blockers provide the rationale for testing the hypothesis that 5-lipoxygenase products contribute to the pathobiology of severe pulmonary arterial hypertension in a subgroup of patients.

Keywords

leukotrienes, inflammation, cell phenotype shift, gene transcription

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Introduction

Severe pulmonary arterial hypertension (PAH) occurs as an idiopathic and sometimes hereditary process as well as one associated with congenital heart disorders, HIV infection, collagen vascular diseases, chronic liver disease, and schistosomiasis.¹ Although PAH has not classically been regarded as an inflammatory process, the presence of inflammatory cells in vascular lesions was, in fact, recognized many years ago. Donald Heath initially brought attention to mast cells in and around pulmonary vascular lesions² and lymphocytes were later documented using immunohistochemistry.³ Elevated blood levels of the proinflammatory cytokines IL-1 and IL-6 were reported in 1995⁴ and it is now believed that their degree of elevation is related to clinical outcomes.⁵

Several recent reviews have sought to consider the importance of inflammation in PAH within an overall pathobiological context.^{6–9} This question of “How important is inflammation?” will ultimately be answered by clinical trial data.

A series of early experimental studies linked the lung circulation and pulmonary hypertension (PH) to 5-lipoxygenase (5-LO) metabolites of arachidonic acid, particularly leukotrienes (LTs) (Table 1). Tian et al. recently reviewed the roles of LTs in PH.¹⁰ In this perspective, we review the history of our evolving knowledge about LTs and pulmonary vascular responses. We also consider features of the 5-LO enzyme that, though less well appreciated, suggest the possibility for novel roles in transcriptional regulation apart from its enzymatic role in generating bioactive LTs. Ultimately, we endeavor to examine the strength of the evidence which links pulmonary vascular diseases and 5-LO and to build a case for treating patients with severe forms of PAH with 5-LO inhibitors.

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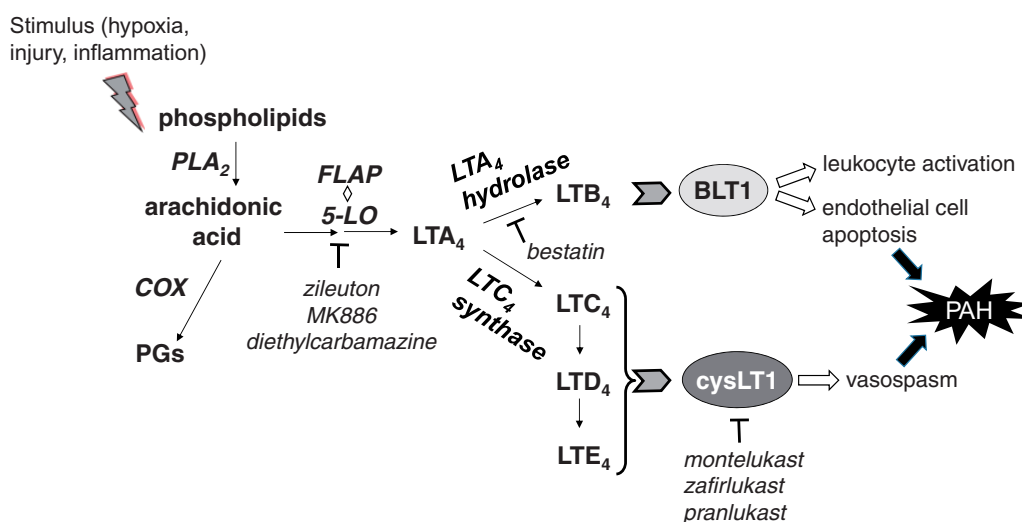
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Table 1. Leukotrienes and the lung circulation.

Experimental conditions	Results	References
Newborns with persistent pulmonary hypertension	Tracheal aspirate LTC ₄ , LTE ₄	Stenmark et al. ⁵⁰
Isolated perfused rat lung	LTE ₄ causes vasoconstriction	Feddersen et al. ⁵²
Isolated perfused rat lung	Leukotriene synthesis and receptor blockers inhibit hypoxic vasoconstriction	Morganroth et al. ⁵⁵
Isolated perfused rat lung	Vasoactive responses to 5-LO metabolites	Voelkel et al. ⁵³
Isolated perfused rat lung	LTC ₄ production during hypoxic vasoconstriction	Morganroth et al. ⁴⁴
Isolated perfused rat lungs	Mmetabolism of LTB ₄ ,LTC ₄	Harper et al. ⁴⁵
Rats	Diethylcarbamazine inhibits hypoxic pulmonary hypertension	Morganroth et al. ⁵⁸
Rats, monocrotaline	Diethylcarbamazine inhibits pulmonary hypertension and inflammation	Stenmark et al. ⁵⁸
Isolated perfused rat lung	Hydrogen peroxide- induced vasoconstriction blocked by 5-LO inhibitor	Burghuber et al. ⁵⁴
Rat, ferret, guinea pig, rabbit lung	Calcium ionophore- stimulated leukotriene production	Westcott et al. ⁵¹
Isolated perfused rabbit lung, anti-IGE	Leukotriene production and vasoconstriction	Walmrath et al. ⁵⁹
Rat, Sugen/hypoxia model of severe PAH	Diethylcarbamazine reduced established PAH and vessel obliteration	Al Hussein et al. ⁶⁶

**Fig. 1.** The 5-lipoxygenase biosynthesis-response pathway in PAH and pharmacologic strategies for its interruption.

Various stimuli are capable of activating the phospholipase A₂ (PLA₂)-mediated hydrolysis of arachidonic acid (AA) from membrane phospholipids. Free AA can be metabolized by cyclooxygenase (COX) enzymes to prostaglandins, or by 5-lipoxygenase (5-LO) in concert with 5-LO activating protein (FLAP) to leukotriene A₄ (LTA₄). LTA₄ is either hydrolyzed by LTA₄ hydrolase to form LTB₄, or conjugated with glutathione to form LTC₄. The glutathione moiety of LTC₄ subsequently undergoes serial amino acid hydrolysis to yield LTD₄ and then LTE₄. LTC₄, LTD₄, and LTE₄ are collectively termed cysteinyl LTs, as they all contain the amino acid cysteine. LTB₄ and the cysteinyl LTs exert their biological actions by binding to G protein-coupled receptors, namely, BLT1 and cysLT1, respectively. Pharmacological agents interrupting global LT biosynthesis include inhibitors of 5-LO (zileuton, diethylcarbamazine) or FLAP (MK886), while bestatin selectively inhibits LTA₄ hydrolase; selective inhibitors of LTC₄ synthase are in development. Selective antagonists to cysLT1 include montelukast, zafirlukast, and pranlukast, while selective BLT1 antagonists are in development. The actions of LTB₄-BLT1 and of LTC₄/LTD₄/LTE₄-cysLT1 mediate various aspects of the pathophysiology pertinent to PAH.

Lipoxygenases, leukotrienes, and leukotriene receptors

Lipoxygenases (or LOs) are a group of fatty acid dioxygenase enzymes that oxidize polyunsaturated fatty acids to hydroperoxy derivatives which, in turn, are further metabolized to bioactive lipid mediators. They are also key players in the regulation of cellular redox homeostasis, itself an important modulator of gene expression. The human

genome contains six LO genes, each of which encodes a distinct enzyme with specific biochemical activity. Here, we focus on 5-LO (Fig. 1), which oxygenates arachidonic acid at its C-5 carbon to first generate 5-HPETE and then LTA₄. The 5-LO gene has been mapped to human chromosome 10,¹¹ and it is worth noting that the expression level of 5-LO changes during lung development.¹² A major advance was the recognition that 5-LO must work in concert with

5-LO activating protein (FLAP), which binds and presents arachidonic acid to the oxygenase enzyme.^{13,14} Under the influence of leukotriene C₄ synthase, LTA₄ is glutathionylated to LTC₄, which can be further converted to LTD₄ and then LTE₄; collectively, LTC₄, D₄, and E₄ are termed peptido-LTs or cysteinyl-LTs (cysLTs). Mast cells and eosinophils are recognized as the major cellular sources for cysLTs. In contrast, the enzyme leukotriene A₄ hydrolase (LTA₄H) hydrolyzes LTA₄ to the potent chemotactic mediator LTB₄; neutrophils and macrophages are considered the major cellular sources for LTB₄. Initially, cysLTs, in particular LTC₄, were a focus of research in pulmonary arterial responses because they were known to cause contraction of bronchi,¹⁵ accounting for the bioactivity long known as slow-reacting substance of anaphylaxis and implicated in allergic diseases such as rhinitis and asthma.

Both cysLTs and LTB₄ exert their biological actions via multiple G protein-coupled receptors, which differ in their ligand specificity, affinity, cellular distribution, and signal transduction. Comprehensive reviews of LT biology and receptors exist¹⁶; for our purposes, it is sufficient to know that the major receptors mediating the classical actions of cysLTs and LTB₄, respectively, are CysLT1 and BLT1. Polymorphisms of the genes encoding both LT synthetic enzymes^{17,18} as well as receptors have been described, and some of these have been investigated in cohorts of patients with atopic disorders.^{19,20}

Over the last four decades, the recognized biological actions of LTs have expanded well beyond smooth muscle contraction and chemotaxis, and a sampling of these can be found in references.^{21–37} For example, 5-HETE and cysLTs are now recognized to stimulate cell proliferation and have been implicated in the development of colon and prostate cancer;^{36,37} LTC₄ appears to be a major trigger of stress-induced oxidative damage.²¹

The expression of LT biosynthetic enzymes as well as LT receptors are under the control of transcriptional and epigenetic mechanisms, in particular DNA methylation, and are themselves modulated by a variety of cytokines, growth factors like TGF beta, hormones, and inflammatory mediators.^{38–41} Interestingly, there is a gender difference in the formation of leukotrienes by stimulated neutrophils: those obtained from females produce several-fold higher amounts than those from males.³⁹

From pulmonary vasoconstriction to pulmonary vascular remodeling

Early research in PH was dominated by investigations of the mechanism of hypoxic vasoconstriction.⁴² Initially described as a pulmonary vascular reflex,⁴³ hypoxic vasoconstriction could be studied in isolated perfused lungs.^{44,45} Because mast cells were widely recognized to synthesize and release cysLTs,^{46,47} Heath's description of mast cell hyperplasia in human lungs from patients with severe PAH² provided a rationale supporting the investigation of a role of LTs in

PH,^{44,48} and systemic hypoxia was considered a possible stimulus for mast cell degranulation.⁴⁹ A collaboration between investigators at the Cardiovascular Pulmonary Research lab at the University of Colorado and Robert Murphy, who—while on sabbatical in the lab of Bengt Samuelsson at the Karolinska Institut in Stockholm—had elucidated the structure of LTs and named them,⁴⁶ was highly productive and generated much of the foundational information on these mediators in PH. Among the key observations were that cysLTs were produced and released in lungs^{44,45,50,51} during vasoconstriction,^{52–54} including acute hypoxic vasoconstriction.⁵⁵ However, while Naeije et al.⁵⁶ reported that high doses of the 5-LO inhibitor diethylcarbamazine failed to inhibit acute hypoxic pulmonary hypertension in anesthetized dogs, LT synthesis inhibitors had salutary actions in rat models of chronic pulmonary hypertension.^{57,58} Subsequently, other stimuli, including intravascularly presented anti-IgE antibodies, were shown to elicit release of LTs, and neutrophils and lung vessels were demonstrated to cooperate in LT generation.^{59,60} A number of years later, the FLAP inhibitor MK866 was likewise demonstrated to inhibit chronic hypoxia-induced PH in rats.⁶¹ Finally, Wright et al.⁶² documented gene and protein expression of both 5-LO and FLAP in the lung endothelial cells of patients with idiopathic pulmonary arterial hypertension (IPAH). The latter finding was important because prior to this discovery, the dogma had been that endothelial cells (and indeed, other nonmyeloid cells) do not express 5-LO. While the molecular mechanisms explaining aberrant expression of LT forming enzymes in vascular cells in PAH remain to be elucidated, it is intriguing to speculate that mutual interactions between 5-LO, p53, and beta-catenin, which appear to be part of the “cancer paradigm” of severe PAH are involved.

Work conducted in the lab of Joseph Loscalzo in Boston confirmed that normal pulmonary arterial endothelial cells in culture did not express 5-LO and did not generate LTs; overexpression using adenoviral 5-LO in these cells was required to enable the production of LTB₄ and cysLTs in response to stimulation with the calcium ionophore A23187.⁶³ Overexpression of 5-LO worsened, while the 5-LO inhibitor zileuton and the FLAP inhibitor MK866 inhibited, the development of PH in the monocrotaline rat model of PAH.⁶⁴ Because the BMPR2 gene is the most frequently mutated gene in hereditary forms of PAH, Loscalzo's lab explored the connection between impaired BMPR2 signaling and pulmonary expression of 5-LO in the pathogenesis of PAH; BMPR2 heterozygous mice did not spontaneously develop PH, but they did so after intratracheal instillation of an adenoviral 5-LO construct.⁶⁵

Al Hussein et al.⁶⁶ studied the VEGF receptor antagonist (Sugen 5416)/chronic hypoxia rat model of severe angio-obliterative PAH and found elevated levels of LTC₄ in the hypertensive lung tissues and also that the 5-LO inhibitor diethylcarbamazine prevented and reversed the angio-obstruction in this model. The drug also partially reversed

the right ventricular hypertrophy. While there is a paucity of data on myocardial leukotriene levels, myocardial mast cell numbers increase in heart failure⁶⁷ and one study shows increased expression of 5-LO, LTC₄-synthase and the CystLT1 in biopsy tissues obtained from human ischemic myocard.⁶⁸ In aggregate, this body of research provided a strong link between PAH and 5-LO/LTs.

Leukotriene B₄

While the focus of attention in regards to PH and the 5-LO pathway had for many years been on cysLTs as potential mediators, a possible mechanistic role for LTB₄ had never been seriously considered. This was reasonable, given the prevailing views that cysLTs were contractile actors whereas the major job description ascribed to LTB₄ had been neutrophil chemotaxis.

These long-held assumptions were ultimately turned on their head by elegant studies performed in the laboratory of Mark Nicolls at Stanford University. They identified LTB₄ as a major player in lung vascular endothelial cell apoptosis and angio-obliteration using a model in which athymic rats (lacking regulatory T-lymphocytes) are treated with Sugen 5416. This model may be particularly representative of pathogenic events in forms of severe PAH with an underlying immune disorder, such as those associated with systemic sclerosis and lupus erythematosus. In these rats, lung LTB₄ levels were high and bestatin – an inhibitor of LTA₄ hydrolase – prevented and reversed severe PAH. Perivascular lung macrophages were identified as the major source of the overproduced LTB₄. Further studies revealed – quite unexpectedly – that macrophage-derived LTB₄ itself was the major driver of endothelial cell apoptosis.⁶⁹ These novel preclinical findings were of obvious clinical interest and led to the randomized LIBERTY trial of treatment of patients with severe PAH with the LTA₄ hydrolase inhibitor bestatin. Disappointingly, when the data were analyzed by the sponsoring drug company across the entire cohort, the bestatin treatment arm was not different from the placebo arm. Unfortunately, the study results have not yet been published.

In hindsight, two possible shortcomings afflicted the design of the LIBERTY trial. First, many of the patients enrolled were on background treatment with prostacyclin analogs, and continuous infusion of prostacyclin has itself been reported to result in a reduction in the high lung tissue levels of LTB₄ found in PAH patients not treated with prostacyclin.⁷⁰ Second, it was not determined whether those patients enrolled in the trial actually exhibited elevated plasma LTB₄ levels, as is frequently the case in patients with scleroderma-associated PAH,⁶⁹ or if those that did preferentially demonstrated a reduction with bestatin treatment. For these reasons, the LIBERTY trial failed to definitively answer the question of whether LTB₄ plays an important role in some patients with severe PAH. It also remains unclear whether there are immunocompetent

patients with LTC₄ (as opposed to LTB₄)-centered forms of severe PAH.

Does 5-LO participate in the control of gene transcription?

Since the actions of LTs require them to be secreted by source cells into the extracellular space where they can bind to receptors on the surface of target cells, it had long been assumed that the enzymes involved in their biosynthesis would be localized at the plasma membrane. It was, therefore, quite surprising to learn that FLAP is constitutively localized at the nuclear envelope, while 5-LO translocates to this same site from a soluble resting compartment upon cell activation (reviewed in 71 and 72). LTC₄ synthase is also constitutively localized to the nuclear envelope, whereas LTA₄H is predominantly cytosolic. Such a pattern of localization – which has been confirmed by numerous investigators – makes it obvious that LTs are generated at or very near to the nucleus (Fig. 2). Even more surprising is the further observation that in many cell types, including alveolar macrophages, mast cells, and recruited neutrophils, the soluble compartment in which 5-LO resides in resting cells is not the cytosol, but the nucleoplasm.^{71–74} In resting alveolar macrophages, immunoelectron microscopic visualization localized most nuclear 5-LO to the euchromatin region of the nucleus – the zone containing chromosomes

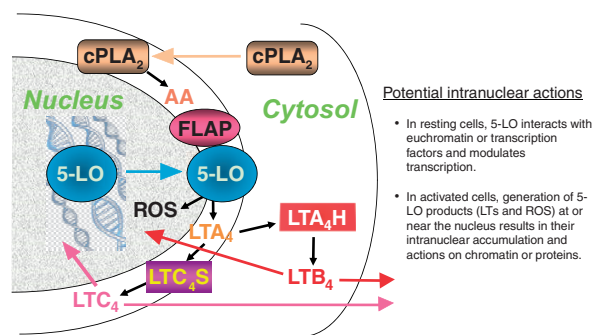


Fig. 2. The nucleus as a site of LT biosynthesis and potential actions. FLAP, 5-LO, and LTC₄ synthase (LTC₄S) are integral nuclear membrane proteins, whereas LTA₄ hydrolase (LTA₄H) is cytosolic; PLA₂ (cPLA₂) and 5-LO reside in the cytosol and nucleoplasm, respectively, but translocate to the nuclear membrane upon stimulation. Even in the absence of stimulation, 5-LO is predominantly found in the euchromatin region of the nucleus, where actively transcribing genes are localized, placing it in position to potentially participate in transcriptional control. Upon stimulation, translocation of cPLA₂ to the nuclear membrane leads to AA hydrolysis from phospholipids. The AA binds to FLAP, which presents it to 5-LO, which itself has translocated to the inner nuclear membrane; 5-LO-mediated catalysis leads to generation of both LTs and reactive oxygen species (ROS), both of which may accumulate and interact within the nucleus with either DNA itself or with transcription factors or co-activators, such as NFκB. Such interactions may influence transcriptional programs involved in inflammation and vascular remodeling pertinent to PAH.)

uncoiled to permit active gene transcription – rather than in the heterochromatin region containing coiled sections of chromosomes.¹⁴ Upon cell activation resulting in LT biosynthesis, 5-LO redistributed from the euchromatin region to the inner membrane of the nuclear envelope. This observation fueled the speculation that 5-LO could be participating in the regulation of transcriptional phenomena in a noncanonical manner independent of its enzymatic actions (see the recent review by Haefner et al.⁷⁵ and Fig. 2). Using an immunoprecipitation strategy, Frank Fitzpatrick and Robert Lepley did identify 5-LO bound to the transcription factor protein NFκB,⁷⁶ which is known to be of critical importance for the transcription of a number of genes encoding cytokines and other mediators of inflammation. Moreover, a role for NFκB has been described in models of PAH.^{77–80} Further investigative efforts are necessary to clarify the intriguing question of whether there is an enzyme activity-independent role for 5-LO as a transcriptional regulator.

5-Lipoxygenase and the pulmonary hypertension-cancer overlap

Apoptosis-resistant cell growth, phenotypic switching of cells, inflammation, angiogenesis, and participation of stem cells are hallmarks that are shared between cancer and angio-obliterative forms of severe PAH. One view is that in severe angioproliferative PAH wound healing has gone awry. Harold Dvorak has called cancer “the wound that never heals.” In this context, it is of interest that 5-LO, together with heme oxygenase 1, also plays a critical role in wound healing.⁸¹ Recent publications confirm expression of 5-LO in cancer tissues,^{82–84} associate cysLT receptor signaling with tumor angiogenesis and metastasis,⁸⁵ and demonstrate that inhibition of 5-LO inhibits cell growth in chronic myeloid leukemia⁸⁶ and in solid tumors.^{87,88} One recent study has described p53-dependent expression of 5-LO⁸⁹ and induction of apoptosis of prostate cancer cells by 5-LO inhibition.⁹⁰ Clearly, however, the nature of the relationship between 5-LO or LTs and apoptosis is complex and likely cell-specific, as we have noted previously that LTB₄ promotes pulmonary endothelial cell apoptosis.⁶⁹ In contrast, apoptotic cancer cells suppress 5-LO in tumor-associated macrophages.⁹¹ Clinical trials that evaluate the efficacy of 5-LO inhibitors in cancer patients are largely lacking.

Summary and conclusion

The preclinical data obtained in PH are clear and consistent: LTs are generated in the human lung⁹² and by the “sick lung circulation,”⁹³ and inhibitors of 5-LO abrogate the development of PAH in animal models. Without doubt, chronic inflammation is a salient contributing factor to the remodeling seen in hypertensive pulmonary vessels. Additionally, the key enzymatic components required for

LT synthesis – 5-LO, FLAP, and LTA₄ hydrolase – are expressed in the lung vessels from patients with severe PAH.^{62,69}

A small study conducted with patients diagnosed with COPD and cor pulmonale found that oral administration of the cysLT1 receptor blocker zafirlukast acutely lowered the pulmonary arterial pressure on average by 23%.⁹⁴ There is a convincing rationale for designing and conducting additional clinical trials testing drugs acting on the 5-LO pathway in patients with severe forms of PAH. These may include drugs inhibiting the 5-LO itself, which has the advantage of blocking production of both LTB₄ and cysLTs. Alternatively, antagonists of cysLT and/or BLT receptors could be considered. Some drugs exhibiting pertinent actions are already available and could be repurposed; e.g., zileuton, diethylcarbamazine, montelukast, and zafirlukast.⁹⁵ Other drugs have been developed by pharmaceutical companies for other indications but were never marketed. The risk/benefit ratio using these agents is likely to be acceptable, and at least in the case of 5-LO inhibitors, dose titration of these drugs in such trials can be guided by measuring plasma and urinary levels of LT metabolites. However, while the expression of 5-LO and of LTA₄ hydrolase in the vascular lesions of patients with IPAH can be seen as a reflection of the cells’ phenotypic shift and suggestive that 5-LO metabolic products are directly or indirectly involved in the pathobiology, it remains to be validated that plasma or urinary levels of LT metabolites accurately reflect LT synthesis by and release from the sick lung circulation.

The community of PH investigators and clinicians has begun to realize the need to transition from treatment of the disease with vasodilator drugs to testing new drugs designed to achieve disease modification. The repurposing of inhibitors acting on LT synthesis and actions and designing clinical trials to test whether there exists a 5-LO endotype of severe PAH appear to be justified, as these drugs have the potential to modify the pathobiology of severe PAH. Our patients deserve such trials to be pursued. For a first proof of concept trial, it would be preferred to enroll incident PAH patients that have been selected because they demonstrate significantly elevated plasma or urine LTC₄ or LTB₄ levels. A placebo control arm may not be necessary as long as the investigators monitor the leukotriene levels. Nonresponders would be identified as patients where 5-LO inhibition, i.e., clear reduction of leukotriene levels had been accomplished, did not result in clinical improvement.

Authors’ contribution

Both authors contributed equally to the writing.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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