Department of Medical Biochemistry and Biophysics, Division of Chemistry II,
Karolinska Institutet, 171 77 Stockholm, Sweden

Studies of leukotriene C_4 synthase isoenzymes and the cysteinyl leukotriene receptors in human endothelial- and mast cells

by

Mattias Sjöström

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Abstract

Leukotriene (LT) C_4, the parent compound of LTD_4 and LTE_4, is generated when LTA_4 is conjugated with GSH by either leukotriene C_4 synthase (LTC_4S) or microsomal GSH S-transferase (MGST) type 2 or 3. Together these lipid mediators cause contractions particularly in airway smooth muscle and the microcirculation, leading to bronchconstriction, plasma extravasation and edema formation as well as increased mucus secretion from epithelial cells and recruitment of inflammatory cells.

Human umbilical vein endothelial cells (HUVEC) were found to generate LTC_4 via MGST2 while the levels of LTC_4S protein and mRNA were below the detection limit in these cells. Human mast cells expressed two functional LTC_4 producing enzymes; LTC_4S and MGST2 but no MGST3. Activity assays showed that LTC_4S was responsible for around 80%, of the LTC_4 production in these cells. Moreover, MGST2 was shown to prefer the free acid of LTA_4, the naturally occurring form of substrate, over the non-natural methyl ester, which is opposite to the preferences of LTC_4S. The rat orthologs of these enzymes were cloned and found to be highly similar to their human counterparts with amino acid identities of 86.7%, 79.6%, and 86.2% for LTC_4S, MGST2, and MGST3, respectively. LTC_4 as well as the FLAP inhibitor MK-886 was shown to inhibit all enzyme activities tested in the three enzymes pointing to a common, or at least overlapping, active site(s) for the different enzyme activities. Rats injected intra-peritoneally with lipopolysaccharide (LPS), showed a transient up-regulation of LTC_4S mRNA within one hour in almost all tissues. LTC_4S protein expression in brain, heart, liver, and adrenal gland increased 4.9-, 4.0-, 2.9- and 2.3-fold, respectively. No effects were detected for MGST 2 or -3. Together, the data from human and rat indicate that LTC_4S and MGST2 both serve as LTC_4 producers. However, their individual roles are not yet elucidated but possibly MGST2 exists for housekeeping purposes while LTC_4S is more active in certain situations concerning allergic- and inflammatory responses.

The effects induced by the cysteinyl leukotrienes are mediated via at least two G-protein coupled seven transmembrane spanning surface receptors, the $CysLT_1$ and $CysLT_2$ receptors. $CysLT_1$ mRNA and protein expression was detected in HUVEC and mast cells. The localization of the $CysLT_1$ receptor was determined in cord blood mast cells (CBMC) and cultured HMC-1 mainly to the surface membrane but also to some extent to granules and the cytoplasm. Calcium signalling induced by LTD_4 and LTC_4 indicated LTD_4 as a more potent agonist and the response could be completely inhibited by 1 μM of the $CysLT_1$ specific antagonist Zafirlukast, both results consistent with a $CysLT_1$-dominated scenario. HUVEC, however, besides $CysLT_1$, also expressed $CysLT_2$. When examined by quantitative RT-PCR, the mRNA ratio of $CysLT_2$ to $CysLT_1$ was determined to > 4300:1. Further, calcium responses elicited by cys-LTs and BAYu9773, a selective partial agonist for $CysLT_2$, indicated signalling mainly from this receptor. In addition, BAYu9773 completely blocked calcium signalling induced by leukotrienes while MK-571, a $CysLT_1$ specific antagonist, gave poor, if any, inhibition. Together, these data suggest that $CysLT_2$ is the dominant receptor in HUVEC. The expression of $CysLT_2$ in HUVEC grown in the presence of LPS or cytokines was also studied. LPS induced a transient suppression of mRNA while the effects of IL-1β and TNFα were at least 20% suppression lasting 120 min or more.

In light of these data it seems likely that the cys-LTs produced, in endothelial cells (EC) and mast cells, as they are exported, will be present in the vicinity and may very well act on the $CysLT_{1/2}$ surface receptors of these cells, or cells of the same type nearby, thereby creating an autocrine or paracrine signalling loop.

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This thesis is based on the following papers and manuscripts henceforth referred to by their roman numerals.

I. Sjöström, M., Jakobsson, P.-J., Heimburger, M., Palmblad, J. and Haeggström, J.Z. (2001) Human umbilical vein endothelial cells generate leukotriene C_4 via microsomal glutathione S-transferase type 2 and express the $CysLT_1$ receptor. Eur. J. Biochem. **268**, 2578-2586.

II. Sjöström, M., Jakobsson, P.-J., Juremalm, M., Ahmed, A. Nilsson, G., Macchia, L. and Haeggström, J.Z. (2002) Human mast cells express two leukotriene C_4 synthase isoenzymes and the $CysLT_1$ receptor. Biochim Biophys Acta. **1583**, 53-62.

III. Schröder, O., Sjöström, M., Qiu, H., Jakobsson, P.-J. and Haeggström, J.Z. (2002) Molecular cloning of rat microsomal glutathione S-transferases: selective in *vivo* induction of leukotriene C_4 synthase by lipopolysaccharide. *Submitted*

IV. Sjöström, M., Johansson, A.-S., Schröder, O., Qiu, H., Palmblad, J. and Haeggström, J.Z. (2003) Dominant expression of the $CysLT_2$ receptor accounts for calcium signaling by cysteinyl-leukotrienes in human umbilical vein endothelial cells. Arterioscler Thromb Vasc Biol. **23**, E37-41.

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Abbreviations

AA	Arachidonic acid; 5,8,11,14 eicosatetraenoic acid
aa	Amino acid(s)
ATP	Adenosine trisphosphate
BLT_1	Leukotriene B_4 receptor 1
BLT_2	Leukotriene B_4 receptor 2
BMMC	Bone marrow derived mast cells
$[Ca^{2+}]$	Calcium ion concentration
CBMC	Cord blood derived mast cells
COX	Cyclooxygenase
cys-LT	Cysteinyl-leukotriene
$CysLT_1$	Cysteinyl-leukotriene receptor type 1
$CysLT_2$	Cysteinyl-leukotriene receptor type 2
EC	Endothelial cell
ECL	Enhanced chemiluminescence
GSH	(Reduced) glutathione
HETE	Hydroxyeicosatetraenoic acid
HMC-1	Human mast cell line
HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
LPS	Lipopolysaccharide
LT	Leukotriene
LTA_4	5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTB_4	5(S), 12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetranoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTA_4H	LTA_4 hydrolase
LTC_4S	LTC_4 synthase
MAPEG	Membrane Associated Proteins in Eicosanoid and Glutathione metabolism
MGST	Microsomal GSH S-transferase
PAF	Platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)
PLA_2	Phospholipase A_2
PCR	Polymerase chain reaction
PG	Prostaglandin
$rLTC_4S$	Rat LTC_4S
rMGST	Rat MGST
RBL-1	Rat basophilic leukaemia cell line
Sf9	Spodoptera frugiperda (fruit fly)
SMC	Smooth muscle cells
T-TBS	Tween-20 in Tris-buffered saline
5-LO	5-lipoxygenase
UDP	Uracil diphosphate
UTR	Untranslated region

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INTRODUCTION

Inflammation is a state of conflict or even war in the body. The purpose can either be to drive out and destroy invaders or just a reaction to tissue damage after which re-building of the afflicted area can take place. Defending forces are leukocytes among which neutrophils, monocytes and macrophages are counted. The battle stages are numerous, for example the joints in rheumatoid arthritis, the skin in psoriasis or the airways in asthma. In its classical form, the conflict is manifested by redness, swelling, heat and pain.

Among the weaponry are the eicosanoids, derived from arachidonic acid (AA). These can be divided into three major groups that are named after where they were first found: the prostaglandins (PGs) from prostate gland, the thromboxanes (TXs) from thrombocytes and the leukotrienes (LTs) from leukocytes. Like with every other weapon there are unpleasant side effects and over-eager usage is never good.

Leukotriene B_4 (LTB_4) and cysteinyl-leukotrienes (LTC_4, LTD_4, and LTE_4) are two classes of powerful lipid mediators that use at least two specific receptors each, BLT_1, BLT_2 and $CysLT_1$, $CysLT_2$, respectively, to mediate their effects. LTB_4 is a chemotactic agent that stimulates aggregation and adhesion of leukocytes to the endothelium, as well as subsequent diapedesis and infiltration into the injured tissue. Cys-LTs cause smooth muscle contraction particularly in the airways and microcirculation leading to bronchconstriction, plasma leakage, and possibly edema formation.

The enzymes catalyzing the committed step in the biosynthesis of cys-LTs, leukotriene C_4 synthase (LTC_4S), microsomal glutathione S-transferases 2 & 3 (MGST2 and MGST3) as well as the corresponding leukotriene receptors ($CysLT_1$ and $CysLT_2$) are important targets for drug development to help those afflicted by various inflammatory diseases such as allergy and asthma. In fact, several such drugs are already in use (montelukast / Singulair®, zafirlukast / Accolate ®). These enzymes and receptors are also the topic for this thesis.

BACKGROUND

The eicosanoids

The eicosanoids, after the Greek word *eikosa* meaning twenty, are a large group of biologically active lipids. They are all derived from unsaturated 20-carbon fatty acids such as eicosapentaenoic acid (20:5 ω3), arachidonic acid (20:4 ω6) and dihomo-γ-linolenic acid (20:3 ω6). Either of these three can be obtained directly from our diet or synthesized endogenously through desaturation and elongation of linoleic acid (18:2 ω6) (dihomo-γ-linolenic- and AA) or linolenic acid (18:3 ω3) (eicosapentaenoic acid) [295]. The linoleic- and linolenic acids can not be produced by mammals due to lack of enzymes introducing double bonds beyond carbon nine (C-9) and are hence classified as essential fatty acids. Thus, eicosanoid product formation is influenced by the balance of fatty acids in the diet. One such balancing factor may be the ω3 unsaturated fish oils, for example eicosapentaenoic acid, shifting product formation from the potent prothrombotic agent thromboxane $(TX)A_2$ and the inflammatory mediator LTB_4 towards the less potent TXA_3 and LTB_5 [18, 63, 142, 148].

Biosynthesis of eicosanoids

Arachidonic acid release

Under basal conditions the concentration of free AA in the cell is low due to incorporation in the cell membrane [118]. AA can be generated via phospholipase C using phosphatidylinositol-bisphosphate (PIP_2) as substrate to form inositol-trisphosphate (IP_3) and diacylglycerol (DAG). DAG is then further metabolized into AA by diglyceride lipase [15, 225].

Further, AA esterified in the *sn*-2 position can be released from the membranes by the action of phospholipase (PL) A_2. This occurs after different forms of stimulation leading to an increase of intracellular calcium. There are several candidate phospholipases for this step, including group IV high molecular weight (85 kDa) cytosolic PLA_2 ($cPLA_2$), several isoforms of low molecular weight, secreted forms of PLA_2 ($sPLA_2$), and calcium independant PLA_2 ($iPLA_2$) [55]. However, it now seems clear that the cytosolic PLA_2 ($cPLA_2$), the only PL with preference for AA esterified in the *sn*-2 position, translocates to the nuclear membrane, and can act in concert with $sPLA_2$, particularly group II and V, to generate AA for eicosanoid biosynthesis [55, 56, 86, 245]. $cPLA_2$ enzyme has been purified from U937 cells and the cDNA cloned, revealing a deduced 751 amino acid sequence with a 45 aa sequence showing homology to protein kinase (PK) C [43, 44]. This homologous sequence is located in the N-terminal part of the enzyme and considered to be a putative Ca^{2+}-dependent phospholipid binding (CalB) domain, necessary for translocation [83].

It has been shown that $cPLA_2$ knockout mice produce poor offspring and that peritoneal macrophages from these mice do not produce LTB_4 or LTC_4 [20, 285]. Further, $cPLA_2$ deficient mice showed less symptoms than wildtype littermates in a collagen-induced arthritis model [106]. In addition, disruption of the $cPLA_2$ gene was shown to reduce pulmonary edema, PMN sequestration, and gas exchange deterioration caused by lipopolysaccharide (LPS) and zymosan administration, as well as acute lung injury caused by acid aspiration in a murine model of acute respiratory distress syndrome (ARDS) [192]. (For $cPLA_2$ review see [17, 55, 189]).

Subsequent to release, AA can enter several metabolic pathways; two of them are the cyclooxygenase pathway, generating prostaglandins and thromboxane, and the 5-lipoxygenase pathway for generation of leukotrienes and lipoxins.

Cyclooxygenase pathway

The metabolism of AA along the cyclooxygenase pathway starts with the action of the cyclooxygenase (COX) enzymes COX-1 and COX-2 previously known collectively as prostaglandin H (PGH) synthase, the target for aspirin. Both of these enzymes appear to share the same catalytic functions, the formation of PGH_2, and cellular compartmentalization, the inner and outer membrane of the nuclear envelope and the endoplasmatic reticulum (ER). However, differences in expression between cell types and regulation separate the two including the suggestion of COX-1 possibly being constitutively expressed for housekeeping synthesis of PGs for normal homeostasis etc., and COX-2 being the enzyme induced as part of inflammatory response. For review, see [85, 262].

From PGH_2, prostaglandins and thromboxane are synthesized via a number of specific enzymes such as PGD-, PGE-, PGF-, PGI-, and TXA-synthase(s). The

formed products, PGs and TXs, then exert their various effects via specific G-protein coupled receptors [193].

5-Lipoxygenase pathway

A second pathway accessible to AA, the 5-lipoxygenase pathway (Fig. 1), takes place mainly at the nuclear membrane. After AA is made available by $cPLA_2$, 5-lipoxygenase (5-LO) proceeds with a dual catalytic action, oxygenation and dehydration, leading to double-bond rearrangement and epoxide formation to form 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and 5(S)-*trans*-5,6-*oxido*-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene A_4, LTA_4), respectively. The very unstable epoxide intermediate LTA_4 can then be further metabolized by either LTA_4 hydrolase (LTA_4H), yielding 5(S),12(R)-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (LTB_4), or conjugated with reduced glutathione by LTC_4 synthase (LTC_4S), microsomal glutathione S-transferase type 2 or 3 (MGST2 or MGST3) (or cytosolic GSTs outside the scope of this thesis) yielding 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-*trans*-11,14-*cis* eicosatetraenoic acid (LTC_4). LTC_4 is then exported out of the cell and by γ-glutamyl transpeptidase and dipeptidase activities further metabolized into 5(S),6(R)-S-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTD_4) and 5(S),6(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTE_4), respectively [241, 249].

5-Lipoxygenase *(EC 1.13.11.34)*

The enzyme metabolizing AA into 5-HPETE and LTA_4, 5-lipoxygenase (5-LO) [228, 256], has been purified from a number of sources including human and porcine leukocytes [228, 230, 283], rat basophilic leukaemia cells [87, 109], and murine mast cells [256]. It has been characterized as a soluble, monomeric enzyme with a molecular weight of around 72-80 kDa and a dependency on Ca^{2+} and ATP for optimal function [109, 228, 230, 232]. The enzyme also binds one atom of non-heme iron per molecule, which during lipoxygenase catalysis is oxidized from the resting ferrous state (Fe^{2+}) to the active ferric state (Fe^{3+}) by lipid hydroperoxides such as 5-HPETE [234].

No crystal structure is yet available for 5-LO but from the 3D-structure of the homologous rabbit 15-LO, some data regarding the substrate anchoring site could be extrapolated [84]. It was suggested that mammalian lipoxygenases share the feature of a hydrophobic substrate-binding pocket with the depth and width regulating the specific mechanism of the enzyme, i.e. a deep pocket for hydrogen abstraction at C-7 followed by oxygenation at C-5 and a shallow pocket for hydrogen abstraction at C-17 and specific C-15 oxygenation [84]. In addition, rabbit 15-LO was found to contain an N-terminal domain with a so called β-fold, similar in structure to those found at the C-terminus of various lipases [84]. From molecular modelling and site-directed mutagenesis, 5-LO was also found to contain an N-terminal β-barrel domain involved in Ca^{2+}-binding and translocation/membrane association of the enzyme [101].

Localization of 5-LO in resting cells has been determined to the cytosolic compartment for human neutrophils, nuclear compartment for mouse bone marrow-derived mast cells (BMMC) and both compartments for RBL-1 cells and rat alveolar macrophages (AM) [26, 39]. Upon cell activation, 5-LO translocates (Fig. 2) from these sites to the nuclear envelope with the exception of the cytosolic pool in rat AM [26]. It was suggested that the 5-LO binding of calcium, mentioned above, increases the hydrophobicity of the enzyme and thereby attracts it to the nuclear membrane

[101, 231]. Furthermore, it was shown that translocation of 5-LO to the nuclear membrane in neutrophils changed the substrate preference of the enzyme, making it more efficient in producing LTA_4 [108].

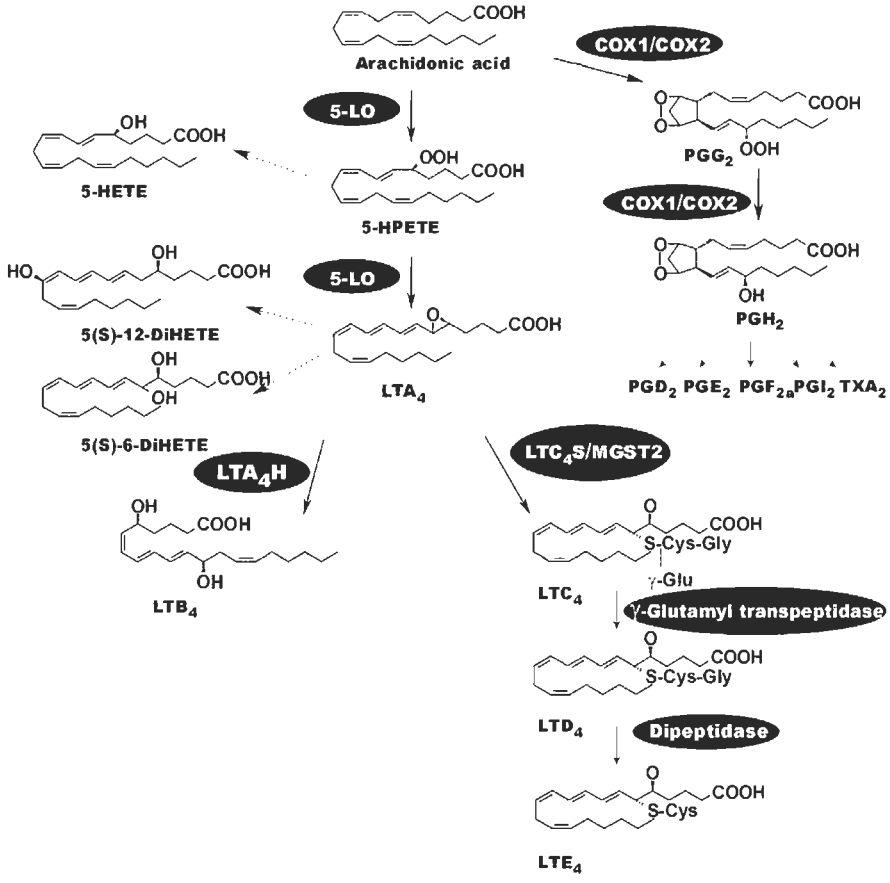

Fig. 1 *Leukotriene synthesis along the 5-lipoxygenase pathway.*

The translocation patterns of 5-LO and 15-LO in mouse macrophages were recently determined [40]. 5-LO was found in the nuclear envelope after translocation from the cytosol induced by increased Ca^{2+} levels. In contrast, 15-LO did not translocate, suggesting that this difference in compartmentalization has a possible regulatory effect on the levels of leukotrienes versus 15(S)-hydroxyeicosatetraenoic acid [40]. A second Ca^{2+}-independent mode of enzyme regulation was reported recently when 5-LO was shown to be phosphorylated by p38 mitogen-activated protein kinase-activated protein kinase (MAPKAP kinase 2) [291]. Stimulation of poly-

morphonuclear leukocytes (PMNL) with PAF and AA together with induction of MAPKAP kinase 2 by sodium arsenite led to a 4-fold increase in activity.

Other regulatory mechanisms can possibly be found in 5-LO interactions with other proteins. Studies have reported interaction with growth factor receptor-bound protein 2 and cytoskeletal proteins *in vitro* [151], as well as interaction with coactosin-like protein (CLP) and tumor growth factor type β receptor I-associated protein 1 (TRAP-1) [219]. Furthermore, 5-LO was shown to interact with the ribonuclease Dicer, a protein involved in gene silencing by RNA interference [218].

The cDNA has been cloned from several species including human [59, 170], rat [12] and mouse [37] with the deduced amino acid identity between them ranging from 93-96%. The sequence revealed six conserved histidines of which two, His372 and His550, by mutagenic analysis, were shown to be important for iron binding together with the C-terminal Ile673 [102, 119, 195, 234, 305, 306]. His367 and Asn554 were suggested to possibly be replaceable ligands to iron [100, 233].

The human 5-LO gene has been mapped to chromosome 10 which differentiates it from most other human LOs localized on chromosome 17p13 [269]. It has been isolated and characterized and shown to span over 14 exons and 13 introns in a total length of over 82 kilo base pairs (kbp) [80]. The promoter contains a set of 5 GC-boxes, for possible interaction with Sp1 and/or Egr-1 transcription factors, but no TATA or CCAT patterns [258], features typical for so called house-keeping genes. Furthermore, DNA-methylation of the core promoter was shown to regulate 5-LO expression and treatment of U937 and HL-60TB cells with the demethylating agent 5-aza-2'-deoxycytidine (AdC) upregulated expression of 5-LO primary transcripts and mature mRNA [284].

5-LO knockout mice have been shown to develop normally and are apparently healthy [38]. The response to endotoxin shock is the same as for wild type mice but they do not suffer lethal effects from PAF-induced shock and have less severe symptoms from ear inflammation induced by AA [38]. The AA response however, could be almost completely blocked by the COX-inhibitor indomethacin in 5-LO (-/-) mice but not in wild type mice suggesting a more complex interconnection between PGs and LTs in inflammatory responses [90]. Moreover, 5-LO (-/-) mice were shown to be prone to *Klebsiella pnemoniae* infection as a result of reduced LT biosynthesis leading to impaired antimicrobial host defense [11]. In addition, 5-LO null mice also exhibited a reduced airway responsiveness to metacholine and lower levels of serum immunoglobulins [117]. Further, cross-breeding of 5-LO (-/-) mice with low density lipoprotein receptor (LDLR) deficient mice, which is an animal model for severe atherosclerosis, did not lead to viable homozygous offspring but showed a more than 26-fold decrease in aortic lesion development for the 5-LO heterozygots [177]. This is also in line with a recent study concerning the role of the 5-LO pathway in atherosclerosis, suggesting possible treatment with antileukotrienes [266].

5-Lipoxygenase activating protein (FLAP)

FLAP was discovered during the development of LT biosynthesis inhibitors or more specifically the inhibitor MK-886 [227]. This inhibitor was reported to block biosynthesis of LTs in intact cells but had no effect on conversion of AA by purified 5-LO enzyme. However, it was reported that 5-LO translocation to the membrane was prevented and reverted by MK-886 and that an 18 kDa protein previously detected in human leukocytes might have a significant role in this effect [227, 229]. FLAP was

later isolated, cloned, and characterized as an 18 kDa protein with three membrane spanning domains that was required for cellular LT synthesis [58, 180]. Moreover, FLAP presence and absence was correlated with LT synthesis in a number of cells and the protein was also shown to be upregulated by dimethylsulfoxide in HL-60 cells, again correlating with their increasing LT synthesis ability [222]. Through transfection of Sf9 cells with 5-LO and FLAP the stimulatory effect of the latter protein on AA utilization was shown together with an increased efficacy in turnover of 5-HPETE to LTA_4 [2]. In the same study, FLAP was suggested to stimulate 5-LO by acting as an AA transfer protein which is close to the current belief that FLAP "presents" the substrate, AA, to 5-LO.

The FLAP gene is localized to chromosome 13, spans over 31 kb in length [78] and is divided into five exons separated by four introns, an organization shared with LTC_4S (see below) [78, 138]. The FLAP promoter contains a possible TATA box, an AP-2 binding site and a potential glucocorticoid response element (GRE).

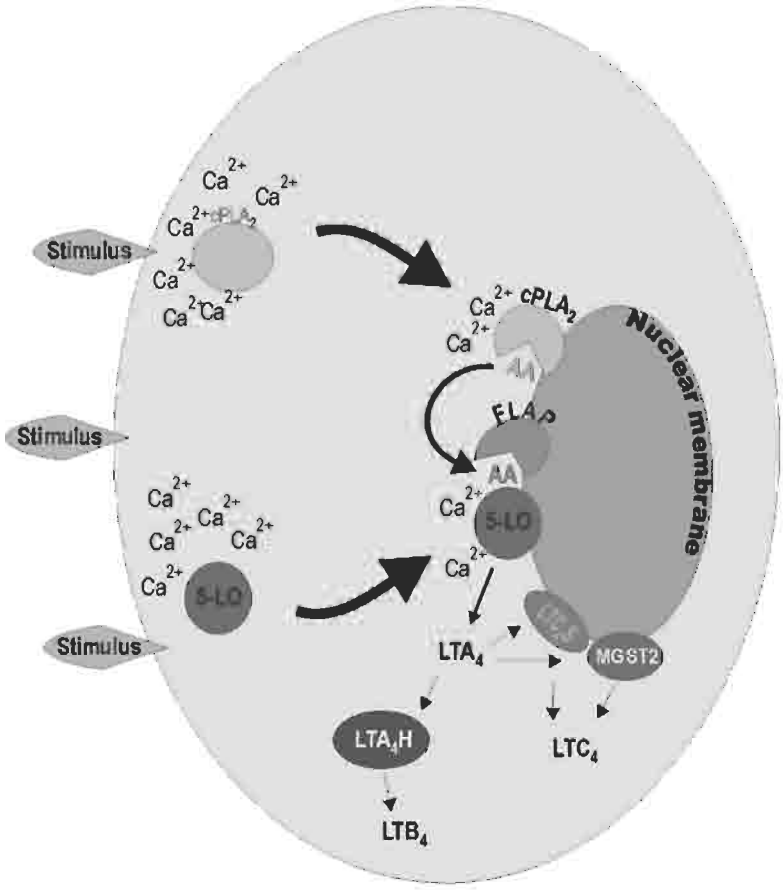

Fig. 2 *5-LO and $cPLA_2$ translocates to the perinuclear membrane where leukotriene biosynthesis commences.*

FLAP deficient mice develop normally but have a blunted inflammatory response to topical AA and have increased resistance to platelet-activating factor-induced shock, compared to wild-type mice. Also, edema associated with Zymosan A-induced peritonitis is markedly reduced in animals lacking FLAP [33]. Further, the severity of collagen-induced arthritis in FLAP-deficient mice is substantially reduced when compared with wild-type animals [93].

Leukotriene A_4 hydrolase *(EC 3.3.2.6)*

Leukotriene A_4 hydrolase (LTA_4H) transforms LTA_4 into the powerful chemotactic mediator LTB_4 [22, 76]. It was first purified from human leukocytes [235] but has since been detected in most tissues and cells even those without 5-LO activity, e.g., endothelial cells (EC), erythrocytes, fibroblasts and T-cell lines [42, 72, 79, 175, 292]. The enzyme is typically monomeric with an approximate mass of 69 kDa [98, 235] residing in the cytosol. However, membrane- and nuclear-associated LTA_4H have been described in studies of rat liver microsomes [96] and rat alveolar macrophages [25].

The cDNA has been cloned and expressed from several sources including rat, mouse, and human [81, 97, 183]. The deduced amino acid sequence is 610 amino acids long and over 90% conserved between the three species. From sequence comparisons with certain zinc proteases and exopeptidases, typically thermolysin and aminopeptidase M, a zinc binding motif was discovered and further work showed that the enzyme contains one zinc atom bound to His295, His299, and Glu318 as determined by site-directed mutagenesis combined with metal analysis [98, 176, 181]. The metal has been shown to be essential for the catalytic function of the enzyme [98]. Further, LTA_4H also possesses an aminopeptidase activity, which cleaves the N-terminal arginine moiety from tripeptides such as Arg-Gly-Asp, Arg-Gly-Gly and Arg-His-Phe with similar efficacy (k_{cat}/K_m) as for the epoxide hydrolase activity ($LTA_4 \rightarrow LTB_4$)[99, 182, 209, 293]. However, no natural substrate has yet been presented for the peptidase activity [91, 201].

The LTA_4H gene is located to chromosome 12, spans over 35 kbs in length and is divided into 19 exons [163]. A possible promoter region contains no TATA box but several putative cis-elements including a phorbol-ester-response element (AP-2) and two xenobiotic-response elements (XREs) [163].

The crystal structure of LTA_4H in complex with the inhibitor bestatin, was recently solved and revealed a protein folded into three domains, N-terminal, catalytic and C-terminal. The catalytic domain is very similar in structure to that of thermolysin, the prototype of a zinc metalloprotease [111, 277]. Together the three domains form a zinc-binding cleft from where a deep hydrophobic pocket leads into the enzyme. A computer model of enzyme-substrate interactions suggested that the C-7 to C-20 fatty acid backbone of the substrate LTA_4 fits into this hydrophobic pocket in an L-shaped binding conformation [277].

Both enzyme activities of LTA_4H are "suicide-inhibited" through covalent modification of the enzyme by its substrate LTA_4, or the isomers LTA_3 and LTA_5 [66, 206, 208]. Mutagenic studies identified Tyr-378 as the aa binding LTA_4 during inactivation and mutation of this residue to phenylalanine (Y378F) or glutamic acid (Y378Q) abolished the suicide-inactivation and increased k_{cat} 2.5-fold compared to wild type enzyme [187].

LTA_4H deficient mice develop normally and are healthy [34]. It was reported that LTA_4H is required for the production of LTB_4 in an *in vivo* inflammatory response and that LTB_4 is responsible for the characteristic influx of neutrophils. Moreover, mice lacking LTA_4H were shown to be resistant to PAF induced shock, proving LTB_4 to be a mediator in this experimental model [34].

Transcellular metabolism

While LTC_4 production has been reported from numerous tissues and LTA_4H is expressed in virtually all cells, 5-LO expression is essentially restricted to leukocytes. One possible solution to this uneven equation is transcellular biosynthesis (Fig. 3), meaning the transportation of one product or intermediate from a donor cell to another recipient cell for further metabolism. The phenomenon was first demonstrated in 1976 when it was shown that platelet-derived prostaglandin endoperoxides, through interaction with an enzyme from the arterial wall led to prostacyclin formation [31, 165, 184]. Erythrocytes, which lack 5-LO, were early shown to produce LTB_4 when incubated with exogenous LTA_4 due to their LTA_4H content [72].

Albumin and/or phopholipid bilayers may help to stabilize the otherwise highly unstable epoxide LTA_4 during transport from donor- to recipient cell [71, 73]. Later, cys-LT production has been reported from coincubations of neutrophils with endothelial cells, mast cells, or smooth muscle cells (SMC) [42, 50, 68, 158, 159]. Apparently, the potential for platelets to participate in transcellular metabolism is highly species-dependent. Thus, while platelets were concluded to play a role in cys-LT biosynthesis in man, partly due to their reactivity and number [64, 158, 160], they were shown to lack LTC_4S activity in rabbits [239].

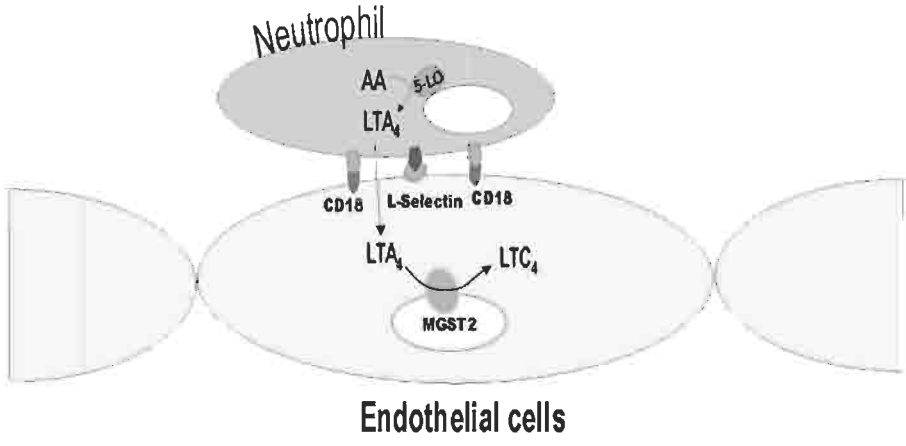

Fig. 3 *Cysteinyl leukotriene biosynthesis in endothelial cells relies on transcellular metabolism.*

The necessity of adhesion between donor- and acceptor cell has been shown by blocking the cell-cell interactions in several coincubations. Thus, antibodies against CD18 and L-selectin were shown to prevent cys-LT formation in a system of polymorphonuclear neutrophils (PMN) and EC from kidney glomeruli [23]. Similar results were obtained with PMNL-perfused isolated rabbit heart *in vitro* and PMNL-

perfused rabbit coronary artery *in vivo* [238]. Modulation of the NO pathway by L-arginine and N-monomethyl-L-arginine was also shown to affect these events in a similar setting [28]. Further, when bone marrow grafts of 5-LO and LTA_4H-deficient mice were used in a zymosan-induced peritonitis model, the LTB_4 levels measured in the lavage fluid indicated that the transcellular route of production plays a significant role and may be responsible for around 20% of the total amounts of LTB_4 generated *in vivo* [67].

Leukotriene C_4 synthase *(EC 2.5.1.37)*

Leukotriene C_4 synthase conjugates GSH with LTA_4 to form LTC_4, a powerful lipid mediator in itself and the parent compound of LTD_4 and LTE_4. Together, these three compounds are referred to as cys-LTs (previously slow reacting substance of anaphylaxis or SRS-A) and cause smooth muscle contraction, plasma leakage, edema formation, and increased mucus secretion from epithelial cells [51, 52, 61, 149, 152].

History

The first description and initial characterization of this enzyme came from experiments on crude separation of the particulate and soluble fraction of RBL-1 cells, where it was shown that the conjugation of LTA_4 with GSH was more specific than previously known enzymatic conjugations of GSH with 2,4-dinitrochlorobenzene and 2,3-dichloronitrobenzene [303]. Three years later, the first partial purification was reported from guinea pig lung [304]. Enzyme activity has since been described from several sources (table 1). Among these, the enzyme has been purified partially [304] or to apparent homogeneity from KG-1, U-937, and THP-1 cells [196, 199, 213, 214], along with bovine platelets [279], and human lung [215]. LTC_4 synthesizing ability was also reported in EC, vascular SMC [68, 69], synoviocytes [296], and brain tissue [281], which all lack 5-LO, and consequently have to rely on a transcellular mechanism for LTA_4 supply, for example from neutrophils [212, 237].

Functional features

Upon purification and characterization [199, 213], LTC_4S was reported to be an 18 kDa integral membrane protein, and suggested to be active as a homo-dimer *in vitro* [196]. The activity was stimulated by phosphatidylcholine and divalent cations especially Mg^{2+}, whereas the second substrate, GSH, was also needed for maintaining the enzyme stability. When purified (> 10 000-fold) from DMSO differentiated U-937 cells, the K_m values for LTA_4 and GSH were determined to 19.6 μM and 1.83 mM, respectively, with a V_{max} of 2-4 μmol/min/mg [199]. This is in agreement with data obtained from LTC_4S purified to homogeneity from THP-1 cells exhibiting K_m values for LTA_4 and GSH of 9.9 μM and 1.7 mM, respectively, and a V_{max} of 4.1 μmol/min/mg [196]. Furthermore, it is also in line with data on partially purified enzyme from guinea pig lung yielding K_m values for LTA_4 and GSH of 3 μM and 2.3 mM, respectively, but with a much lower V_{max} of 108 nmol/ 3 min/mg reflecting the contaminating proteins in the enzyme preparation [304]. Interestingly, it was also observed that for the methyl ester of LTA_4 (LTA_4-ME), the Michaelis constant was five times higher (K_m = 16 μM) but the turnover four times higher V_{max} = 420 nmol/ 3 min/mg protein suggesting that LTA_4-ME is almost as good substrate as the free acid. This property also made it possible to use LTA_4-ME for studying the inhibitory

effects of the natural product LTC_4. The enzyme was shown to be competitively inhibited with an IC_{50} of 2.1 μM [304].

The subcellular localization of LTC_4S has recently been determined. The enzyme is present in the outer, but not inner, nuclear membrane and peripheral endoplasmatic reticulum. In contrast, FLAP is localized to the inner rather than outer nuclear membrane. This would indicate that LTB_4 and LTC_4 are synthesized in different subcellular compartments and suggests that LTC_4 needs to be transported back to the cytoplasmic side of the membrane before export out of the cell [41]. The export is facilitated by the ATP dependent plasma membrane multidrug resistance associated protein-1 (MRP-1) [126, 144, 150]. Outside the cell, LTC_4 is further metabolized into LTD_4 and LTE_4 by γ-glutamyl transpeptidase and dipeptidase, respectively.

Table 1. *Tissues / cells expressing LTC_4 synthase activity.*

Occurrence in (tissue / Cell)	**Reference**
RBL-1 cells	[10, 125, 303]
Human lung mast cells	[157]
Mouse bone marrow mast cells	[220]
Human eosinophils	[289]
Human monocytes	[294]
Mouse mastocytoma cells	[272]
U-937 cells	[198]
Human platelets	[273]
Mouse mast cells	[188]
Rat kidney	[217]
Human mast cells	[260]
Purified from (tissue / cell)	**Reference**
Guinea pig lung	[304]
KG-1 cells	[213]
U-937	[198]
THP-1	[197]
Human lung	[215]
HL-60 cells	[250]
Bovine platelets	[279]

Cloning of human LTC_4S

The human LTC_4S cDNA and gene have been cloned and characterized [16, 145, 214, 290]. The cDNA showed an open reading frame of 450 bp flanked by a 54 bp non-coding 5'-region and a 187 bp untranslated 3'-region (UTR) including a 72 bp poly(A)$^+$ tail and a polyadenylation signal ATTAAA. The gene is 2.51 kb in total and contains five small exons (136, 100, 71, 82, and 257 bp, respectively) matching exactly the sizes of the exons of the homologous protein FLAP. The total gene size, however, is quite different since the FLAP gene spans > 31 kb.

The 5' UTR of the LTC_4S gene does not contain a TATA or CAAT box but does reveal DNA binding motifs for transcription factors such as SP-1, AP-1, and AP-2 as well as CREB/ATF. Also, a STAT binding motif is situated in the first intron. Characterization of the promoter implied a function for an SP-1 site and a putative initiator element (Inr) in non-cell-specific expression as well as a role for a Kruppel-like transcription factor and SP-1 in cell-specific regulation of the gene [307]. In THP-1 cells an SP-3 was found and added to the SP-1 for regulation of LTC_4S [253].

The cDNA sequence encodes a protein of 150 amino acid residues with a calculated mass of 16 567 Da and a pI of 11.05. The deduced aa sequence also contains two cysteine residues, two consensus PKC phosphorylation sites for apparent downregulation of the activity [5, 95], a potential N-linked glycosylation site as well as three membrane spanning domains.

Mutagenetic analysis showed that Arg51 is a catalytically important residue, which supposedly opens the epoxide ring of LTA_4 [147]. Moreover, mutations of Arg51 to His or Lys provided a fully active enzyme whereas an exchange for Thr or Ile abolished catalytic function, indicating that a positively charged residue in this position is important for catalysis. Further, mutation of Tyr93 to Phe not only increased K_m, which was the case for two other tyrosine mutants Y59F and Y97F, but also decreased catalytic efficiency to 1/260 compared to wild type enzyme. These data suggest that Tyr93 is the specific tyrosine, common in GSH transferases, acting as a base to generate the thiolate anion [127, 147, 286].

Regulatory effects of cytokines on the LTC_4S gene have been described with regard to eosinophilic cell lines and mast cells [188, 190, 248]. Thus, IL-3 was shown to upregulate LTC_4S together with the other enzymes in the 5-LO pathway in mouse bone marrow derived mast cells primed with IL-10 and c-kit ligand [188]. In contrast IL-4, downregulated the activity in the same system [190]. However, IL-4 together with SCF proved both an effective primer of anti-IgE dependant cys-LT and PGD_2 production in cord blood derived human mast cells (CBMC), which was further enhanced by co-priming with IL-3 and IL-5. In addition, IL-4 priming increased the expression of LTC_4S protein and functional activity in a dose- and time-dependent manner [113].

Mice deficient in LTC_4S develop normally and are fertile [134]. Bone marrow derived mast cells (BMMC) from LTC_4S (-/-) mice also exhibit similar exocytosis capacity and capability to produce PGD_2, LTB_4 and 5-HETE as BMMC from wild type animals. However, Zymosan A-induced peritoneal plasma extravasation as well as development of IgE-induced passive cutaneous anaphylaxis in the ear is significantly impaired in LTC_4S deficient mice.

Interestingly, two case studies in human new-born babies linked a deficiency in LTC_4 synthesis with a possible fatal neurological syndrome [171, 172].

The MAPEG family and LTC_4S isoenzymes

LTC_4S is a member of the MAPEG (membrane associated proteins in eicosanoid and glutathione metabolism) super family, a group of enzymes with widespread origin and highly diversified biological properties and functions [123]. As mentioned above, LTC_4S share almost identical exon sizes, has a similar gene organization but carries no functional similarities with FLAP. However, two other human enzymes in the MAPEG family also have LTC_4 synthesizing ability, i.e. MGST2 and MGST3.

Microsomal glutathione S-transferase type 2 (MGST2) (EC 2.5.1.18)

Like LTC_4S, MGST2 can conjugate LTA_4 with GSH to form LTC_4, which is the committed step in the biosynthesis of cys-LT. Human MGST2 contains 147 amino acids with a predicted molecular mass of 16.6 kDa and a calculated pI of 10.4 [121]. The primary structure is 44% identical to LTC_4S, with highly conserved regions like amino acids 48-62, where 13 of 15 residues match. The amino acid sequence is also 33% identical to FLAP but is only 11% identical with the previously characterized MGST1. The chromosomal localization of MGST2 was determined to 4q28-31, using fluorescent in situ hybridization. The apparent K_m values of MGST2 and LTC_4S for LTA_4 (at 5 mM GSH) were determined to 41 μM and 7 μM, respectively. Besides the LTC_4 synthase activity, MGST2 also conjugates 1-chloro-2,4-dinitrobenzene with GSH and has peroxidase activity shown by its ability to reduce 5-HPETE to 5-HETE with an apparent K_m of 7 μM [122]. High levels of human MGST2 mRNA is found as a ~ 0.6 kb transcript in liver, spleen, skeletal muscle, heart, adrenal glands, pancreas, prostate, and testis. Low levels can also be detected in lung, brain, placenta and bone marrow. Expression of the protein has also been found in liver, EC, and to a small extent in the lung using an MGST2 specific polyclonal antibody [124]. The fact that protein expression was not significantly detected in several of the tissues showing mRNA expression indicates posttranscriptional regulation of this enzyme.

Microsomal glutathione S-transferase type 3 (MGST3) (EC 2.5.1.18)

Another member of the MAPEG family is MGST3. The human cDNA encodes a polypeptide of 152 amino acids with a predicted molecular mass of 16.5 kDa and a calculated pI of 10.2 [122]. The amino acid sequence is 36% and 27% identical to MGST2 and LTC_4S, respectively, but has only 22% identity to MGST1 and 20% to FLAP. Amino acids 31-38 are highly conserved with respect to MGST2 (7/8 identical) and further comparison with LTC_4S shows that the second PKC phosphorylation motif, which is not present in MGST2, is also found in MGST3. However, a region encompassing residues 46-50, which is highly conserved among MGST2, LTC_4S and FLAP, is not present in MGST3 [122].

The human MGST3 gene has been mapped to chromosome 1q23 and mRNA is predominantly expressed in human heart, skeletal muscle, and adrenal cortex but also in brain, placenta, liver, kidney, testis, ovary, pancreas, and thyroid gland. Expression is barely, if at all, observed in lung, thymus and peripheral blood leukocytes. Unfortunately, no antiserum against MGST3 is available for studies of the corresponding protein expression.

Membrane preparations from Sf9 cells expressing human recombinant MGST3 was found to produce 998 ± 296 pmol of LTC_4/mg protein/15 min [122]. The peroxidase activity mounted to 5 nmol 5-HETE/mg protein/min and had an apparent K_m for

5-HPETE of 21 μM. In contrast to MGST2, MGST3 does not conjugate 1-chloro-2,4-dinitrobenzene with GSH with or without *N*-ethylmaleimid treatment [122].

It should also be noted that an additional member of the MAPEG family, previously known as MGST1-L1, has been identified as a GSH-dependent, membrane-bound, and inducible form of prostaglandin E synthase [123].

Leukotriene B_4 receptors

History

Leukotriene B_4 was the first leukotriene to be structurally characterized and was initially isolated from rabbit leukocytes [21]. It was established as a potent lipid mediator of allergic and inflammatory reactions [240, 251]. The leukocytes were shown to be targets for LTB_4 which induced several functional responses including chemotaxis, aggregation and enhanced adherence to EC [24, 77]. At higher concentrations LTB_4 also elicited degranulation and superoxide anion formation [252]. LTB_4 was also shown to be involved in some immunoregulatory responses (e.g induction of suppressor T-cells) [226] and alveolar macrophages were reported to recruit PMNL via this mediator [166]. Furthermore, mice with bacterial pneumonia had bronchiolar lavage fluid containing high amounts of this compound and exogenously added LTB_4 was reported to help clear the infection [54]. It is now clear that this small molecule plays a role in several pathological conditions like bronchial asthma, rheumatoid arthritis, psoriasis, and inflammatory bowel disease [88, 92, 120, 282].

To mediate these effects, LTB_4 uses at least two G-protein coupled surface receptors termed BLT_1 and BLT_2.

Leukotriene B_4 receptor 1 (BLT_1)

Cloning and expression

The cDNA of the principal, high-affinity, LTB_4 receptor, now termed BLT_1, was first cloned from a human B-lymphoblast cDNA library under the name chemoattractant receptor-like 1 (CMKRL1) [211] and has also been misidentified as a novel P2 purinoreceptor [4] but was later characterized as an LTB_4 receptor mediating chemotaxis of transfected Chinese hamster ovary (CHO) cells [298]. The receptor has now been cloned and characterized from human, rat, mouse, and guinea-pig as a seven transmembrane (7-TM) spanning, G-protein coupled, surface receptor [114, 167, 169, 278, 298, 302] (see Fig. 4). It is expressed almost exclusively in inflammatory cells like leukocytes and also in thymus and spleen [211, 299]. The open reading frame consists of 1056 bp translated into 352 amino acids with an approximate mass of 43 kDa. The primary structure exhibits homology to some chemokine receptors like those for fMLP, C5a, and LXA_4 [211, 298].

The gene encoding human BLT_1 was reported to be about 5.5 kb and is located on chromosome 14q11.2-q12 [200]. Neither TATA nor CAAT- boxes are found in the vicinity of the transcription initiation site but the region is reported to be GC-rich. However, a region located about 80 bp upstream of the initiator sequence was reported as essential for transcription. Furthermore, Sp1 was determined as a major transcription activator by electrophoretic mobility shift assay and site-directed mutagenesis studies. Moreover, methylation of CpG-sites in the promoter appears highly important for cell specific BLT_1 expression as they were found methylated to

a high degree in HeLa and HepG2 cells not expressing BLT_1 whereas the corresponding sites were not methylated in BLT_1-expressing cells, such as U937 and THP-1 [136].

BLT_1 deficient mice develop normally and show normal calcium mobilization and chemotactic response to stimuli such as PAF and C5a. However, these mice failed to show an influx of neutrophils in the peritoneal cavity in response to LTB_4 and had a blunted response in an ear model using AA as the inflammatory agent. Furthermore, female, but not male, BLT_1 knockout mice were resistant to PAF induced anaphylactic shock elucidating an unexpected sex-related connection [103].

BLT_1 has also been suggested as an possible co-receptor for HIV virus [210] but conflicting data have also been presented [167].

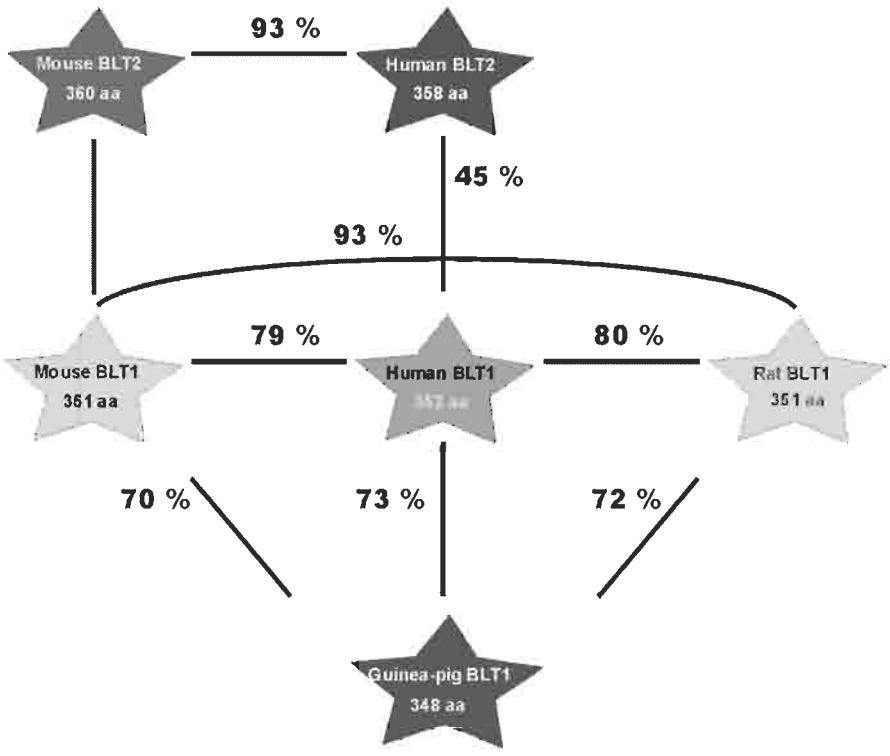

Fig. 4 *BLT receptor amino acid identities between species.*

Ligands and antagonists

When examined in COS-7 cells expressing BLT_1, LTB_4 was the most effective ligand in inhibiting binding of ^{3}[H]LTB_4 (K_i = 0.38 nM) followed by 20-OH-LTB_4 (K_i = 7.6 nM), 12-oxo-LTB_4 (K_i = 7.6 nM), and 20-COOH-LTB_4 (K_i = 190 nM), whereas compounds with a 6-*trans* configuration and a 12S hydroxyl group were much less effective. These data suggest that the *cis* configuration at C-6, the 12 (R)-hydroxyl moiety and the 20-carboxyl group are important structural elements for interaction with BLT_1 [298]. These data are also in agreement with earlier binding studies performed with PMNL [19] and eosinophils [194]. The binding constant (K_d)

for LTB_4 in COS-7 cells expressing BLT_1 was reported to be 0.154 nM, which is in line with later work on transfected HEK 293 cells (K_d = 1.2 nM) [299].

Functionality of the receptor in CHO cells stably expressing BLT_1 was confirmed when the cells were reported to migrate towards a 1 nM LTB_4 gradient in a Boyden-chamber assay [298].

Several antagonists have been developed for the BLT receptors and some are reported as BLT_1 specific like U-75302 (Ono pharm.) and CP105696 (Pfizer), while others are suggested to be effective also against BLT_2 (see below), such as ZK-158252 (Schering) and CP 195543 (Pfizer). U-75302 was also reported as a weak agonist of BLT_1 [300].

Inhibition of BLT_1 using the antagonist CP 105696 was reported to reduce lipid accumulation and monocyte infiltration in apoE(-/-) mice compared to control. However, CP 105696 had no significant effect on atherosclerotic lesion size in apoE(-/-) mice possessing the null alleles for monocyte chemoattractant protein-1 (MCP-1(-/-) x apoE(-/-)), suggesting that MCP-1 and LTB_4 may either interact or exert their effects by a common mechanism [3].

Leukotriene B_4 receptor type 2 (BLT_2)

Cloning and expression

Less is known about the second LTB_4 receptor partly because it was discovered later but also due to the fact that many early results and effects have probably automatically been attributed to BLT_1. It was cloned and characterized from human and mouse in the year 2000 by four independant groups, although named JULF2 instead of BLT_2 by some [133, 280, 287, 301]. Like BLT_1, BLT_2 has seven transmembrane spanning regions and is G-protein coupled.

The full-length open reading frame (ORF) of human BLT_2, containing two in-frame methionines, can encode two similar proteins with slight difference in length of 358 and 389 amino acids, respectively, whereas the corresponding mouse protein contains 360 aa [133, 274, 280, 287, 301]. Surprisingly, the ORF of BLT_2 was located within the promoter of the BLT_1 gene, the first report of such an organization ("ORF in promoter") in mammals, suggesting a complex regulatory system for gene transcription [301].

Human BLT_2 is homologous to BLT_1 (Fig. 4), with an amino acid identity of 45.2% (for 358 amino acids). Homology to the mouse BLT_2 is very high (92.7%) possibly indicating conservation of an important gene through evolutionary stages [301]. BLT_2 is expressed ubiquitously, in contrast to BLT_1, which is expressed predominantly in leukocytes.

Ligands and antagonists

Differences in affinity for ligands and antagonists between BLT_1 and BLT_2 are invaluable tools in elucidating receptor relationships and functionalities.

The BLT_2 has been described as a low affinity receptor and BLT_1 as the high affinity receptor for LTB_4. The membrane fraction of HEK 293 cells stably expressing the BLT_2 exhibited a binding constant (K_d) for LTB_4 of 22.7 nM compared to 1.1 nM for BLT_1 [301]. In contrast, radioligand binding assays using membranes prepared from COS-7 cells transfected with BLT_2 cDNA displayed high affinity (K_d = 0.17 nM) for ^{3}[H]LTB_4. However, the K_i value for inhibition of ^{3}[H]LTB_4-binding

by LTB_4 was more than three times higher for BLT_2 than BLT_1 [287]. Other ligands were also reported to have intermediate to high affinity like, 12-epi-LTB_4, 12-(R)HETE, 12-(S)HETE, 15-(S)-HETE, 15-(S)-HPETE and 20-COOH-LTB_4 [287, 300]. Stimulation of BLT_2-transfected CHO-cells and HeLa cells with LTB_4 resulted in increased calcium levels and caused a dose-dependant inhibition of forskolin-activated adenylyl cyclase in the CHO-cells [280, 301].

Known BLT_1 antagonists like U-75302 (Ono pharm.) and CP105696 (Pfizer) had little or no effect on LTB_4-binding to BLT_2 whereas ZK158252 (Schering), LY255283 (Lilly) and CP195543 (Pfizer) were efficient towards BLT_2 and are hence considered BLT_2 specific (LY255283) or dual-specific (ZK158252 and CP195543) [300].

Cysteinyl leukotriene receptors

History

The cys-LTs, described in 1940 as "slow-reacting substance of anaphylaxis" or SRS-A [137], are potent mediators of allergy and inflammation. From the initial biological discoveries, it took almost 40 years until the chemical structure of SRS-A had been determined [191]. The clinical importance of SRS-A for anaphylactic reactions and the development of symptoms characteristic of asthma was established in 1960 [27, 60, 62, 153]. Now it is well established that the cys-LTs induce smooth muscle contractions, particularly in airways and microcirculation, leading to bronchconstriction, plasma extravasation, and edema formation, as well as increased mucus secretion from epithelial cells and recruitment of inflammatory cells [51, 52, 61, 149, 152]. Together, these effects can account for all cardinal signs of asthma.

The biological responses elicited by cys-LTs are signalled via at least two receptors termed $CysLT_1$ and $CysLT_2$ [107, 155, 202, 242, 275]. A very early mentioning of cys-LT receptors came with the development of FPL-55712, which was originally termed an SRS-A inhibitor [8]. Subsequently, this compound was found to inhibit cys-LT induced contractions of guinea pig tracheal and parenchymal smooth muscle [128, 141]. In contrast, FPL-55712 was also reported to enhance the contractile response induced by histamine and carbachol [140], an effect suggested to originate from COX inhibition [140].

The presence of a receptor that is not antagonized by this compound was indicated using binding studies with LTD_4 and FPL-55712 in guinea pig ileum-, lung parenchyma-, and tracheal smooth muscle [74, 75]. Later it was shown that inhibition of γ–glutamyl transpeptidase with L-serine borate, thereby preventing the conversion of LTC_4 to LTD_4, abolished the antagonistic effect by FPL-55712 towards LTC_4-induced contractions, which further indicated the presence of a second receptor [263, 264]. Finally, pharmacological data were presented, which confirmed the presence of a second cys-LT receptor in human pulmonary vein that was resistant to known antagonists like ICI 198615, MK-571 and SKF 104353 but inhibited by the antagonist BAYu9773 [143].

Receptor mediated effects of cys-LTs have been described for a number of cells and tissues [128, 244, 264, 265, 288] but the studies have seldomly elucidated which receptor(s) is involved.

The classification of the receptors is initially based on the effects of known cys-LT antagonists towards one subtype, arbitrarily named $CysLT_1$, in contrast to a sub-

type which is not inhibited, and thus termed $CysLT_2$ [46]. This classification has recently been corroborated by molecular data from the cloning of two distinct receptors [107, 155, 202, 242, 275].

Cysteinyl leukotriene receptor 1($CysLT_1$)

Cloning and expression

The $CysLT_1$ receptor has been cloned and characterized from human [155, 242] and mouse [162, 168]. The human cDNA encodes a 337 amino acid protein with a calculated Mw = 38,549 while the mouse cDNA potentially encodes two receptors, a shorter of 339 residues matching the human $CysLT_1$ fairly well and a longer with a 13 amino acid extension at the N-terminus. The shorter mouse sequence and the human sequence are 87% identical at the amino acid level [162] (Fig. 5). Human $CysLT_1$ receptor mRNA was found in human spleen, peripheral blood leukocytes and lung SMC as well as lung macrophages and expression of mRNA as well as protein has later been shown in a number of tissues from different species (table 2) [155, 242]. The gene has been mapped by in situ hybridization to chromosome X (Xq13-Xq21) [155].

$CysLT_1$ receptor deficient mice are fertile and develop normally but macrophages from these mice do not mobilize Ca^{2+} in response to LTC_4 or LTD_4 [161]. Further, plasma extravasation, but not neutrophil infiltration, was diminished in these mice when subjected to zymosan A-induced peritoneal inflammation and IgE-mediated passive cutaneous anaphylaxis [161].

Ligands and antagonists

The relative potency of the agonists LTD_4, LTC_4, and LTE_4, as judged from calcium mobilization assays in HEK-293 cells transfected with human $CysLT_1$ ($hCysLT_1$), are in the order with the corresponding EC_{50} values 2.5 nM, 24 nM, and 240 nM, respectively [242]. This is mainly in line with similar studies of the human receptor [143, 155] but differ from mouse [162, 168], in which the reported rank order of potency is $LTD_4 > LTC_4 = LTE_4$. No effect was detected when LTB_4 was tested as agonist [162]. In previous studies carried out with human bronchial preparations, supposedly harbouring the $CysLT_1$ receptor, the rank order of agonists was reported to be $LTD_4 = LTC_4 > LTE_4$ [143, 186] or even $LTD_4 = LTC_4 = LTE_4$ [29, 30, 143, 186]. However, these data are difficult to interpret since the relative contribution from the $CysLT_1$ and $CysLT_2$ receptor was not assessed. In addition to cys-LTs, acetyl choline and UDP have been reported to act as ligands of the $CysLT_1$ receptor [173, 179] and in fact, the human $CysLT_1$ has some homology to purinoreceptor P2Y [179]. Many pharmaceutical companies have developed a number of different $CysLT_1$ antagonists of different potencies and specificities, such as MK-0476 (montelukast / Singulair®) [129], ICI 204,219 (zafirlukast / Accolate ®) [139], SKF 104,353 (pobilukast) [185], ONO 1078 (pranlukast / Onon®) [204], MK-571 [130], and BAYu9773 [49] (table 3), of which some are used clinically to treat asthma.

Cysteinyl leukotriene receptor 2 ($CysLT_2$)

Cloning and expression

The $CysLT_2$ receptor has been cloned and characterized from human [107, 202, 275] and mouse [116, 205]. Further, a rat orphan GPCR (RSBPT32) was tentatively identified as a $CysLT_2$ receptor [107] and a rat $CysLT_2$ cDNA was later cloned by PCR and compared to the mouse ortholog [116].

The human $CysLT_2$ cDNA ($hCysLT_2$) encodes a protein of 346 amino acids with 38% identity to the human $CysLT_1$ receptor [107]. The mouse and rat $CysLT_2$ receptor cDNAs ($mCysLT_2$ and $rCysLT_2$) are truncated at both ends compared to the human ortholog and encode 309 aa proteins with calculated molecular masses of 35.3 kDa [116]. The amino acid identity is 65% between $hCysLT_2$ and $mCysLT_2$, 73% between $hCysLT_2$ and $rCysLT_2$, and 84% between $rCysLT_2$ and $mCysLT_2$ [107, 115, 116] (Fig. 5).

$CysLT_2$ receptor mRNA seems to be almost ubiquitously expressed but the strongest signals have been reported in heart, brain, spleen, peripheral blood leukocytes (PBLs), placenta, lymph node, and adrenal gland [107, 202, 275]. In addition to direct detection of mRNA or protein expression, $CysLT_2$ receptor expression has been analyzed pharmacologically in human pulmonary vein SMC [143, 244], guinea-pig trachea [288], and ileum [35, 82].

The $hCysLT_2$ gene has been mapped to chromosome 13q14, a region linked to atopic asthma [107], whereas the corresponding mouse gene resides on the central region of chromosome 14 [116].

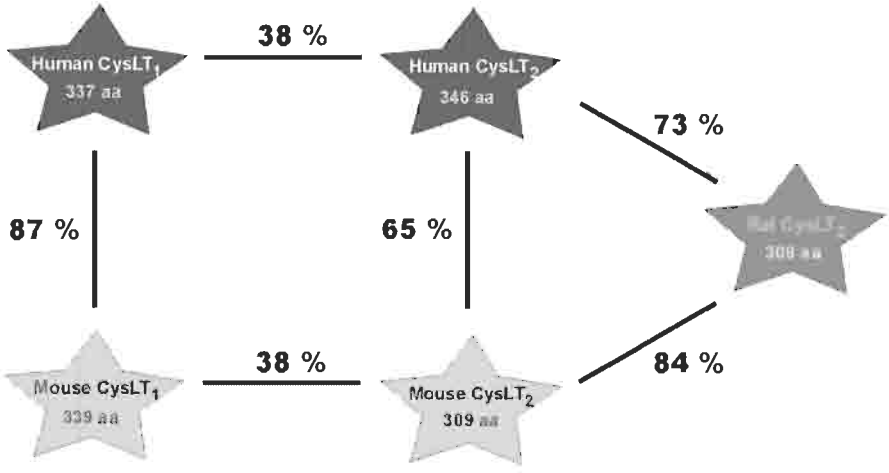

Fig. 5 *Cys-LT receptor amino acid identities between species.*

Ligands and antagonists

The $CysLT_2$ receptor responds to the same agonists as $CysLT_1$ but with a different rank order of potency, i.e., $LTC_4 \geq LTD_4 >> LTE_4$, as judged from assays of calcium mobilization and ligand binding studies [107, 202, 275]. The EC_{50} values reported from calcium mobilization studies in HEK 293T cells range between 2-10 nM for

LTC_4, 2-6 nM for LTD_4, and 50-330 nM for LTE_4 [202, 275]. The human $CysLT_2$ receptor is not antagonized by the common $CysLT_1$ antagonists such as, MK-0476 (montelukast / Singulair®), ICI 204,219 (zafirlukast / Accolate ®), ONO 1078 (pranlukast / Onon®), or MK-571 [107, 202, 275]. However, the $CysLT_{1/2}$ dual antagonist BAYu9773 was shown to be effective as inhibitor of [^{3}H]LTD_4-binding and calcium mobilization with an IC_{50} value of around 300-600 nM [107, 202]. In contrast to the human enzyme, mouse $CysLT_2$ is antagonized by the human $CysLT_1$ specific inhibitor Pranlukast [205]. At present, no specific $CysLT_2$ antagonist is available.

Table 2. *$CysLT_1$ and $CysLT_2$ receptor expression determined by either mRNA or protein detection in human and mouse.*

Species	Tissue	Receptor expression				Reference
		$CysLT_1$		$CysLT_2$		
		mRNA	protein	mRNA	protein	
Human	Spleen	+		+		[155, 242]. [107, 202, 275]
	PBL	+	+	+		[70, 155, 242].[107, 202, 275]
	Lung	+	+			[70, 155, 242]
	Placenta	+		+		[242, 275]
	Colon	+				[242]
	Heart			+		[107, 202, 275]
	Brain			+		[107, 202, 275]
	Lymph node			+		[107, 202]
	Adrenal gland			+		[202]
	EC	+	+	+		[259]
	Mast cells	+	+			[260]
	Eosinophils	+	+			[207]
	Nasal mucosa	+	+			[257]
Mouse	Lung	+		+		[161, 168, 205]
	Skin	+				[205]
	Macrophages	+	+	+		[161, 205]
	Trachea	+				[168]
	Spleen	+		+		[205]
	Small intestine			+		[205]
	Adrenal gland	+		+		[205]

Signalling of leukotriene receptors

Guanosine (G) protein-coupled receptors (GPCRs) represent the single largest family of cell surface receptors involved in signal transduction. It is estimated that several hundred distinct members of this receptor family in humans direct responses to a wide variety of chemical transmitters, including biogenic amines, amino acids, peptides, lipids, nucleosides, and large polypeptides. These transmembrane receptors are involved in such diverse physiological processes as neurotransmission, cellular me-

tabolism, secretion, cellular differentiation, and growth as well as inflammatory and immune responses. Many currently used therapeutics act by either activating (agonists) or blocking (antagonists) GPCRs [105].

G-proteins are made up of three different subunits α, β, and γ. Subsequent to receptor activation, the receptor-ligand complex stimulates the G-protein to release its α-subunit coupled to GTP (G_α-GTP), which then triggers a second specific event, for example, stimulation of adenylate cyclase [268].

There are at least 18 different subtypes of α-subunits identified which can be divided into four different subfamilies (1) the 'G_s' subfamily that stimulates adenylyl cyclase (G_s and G_{olf}); (2) the $G_{i/o}$ subfamily that inhibits adenylyl cyclase (G_{i1}, G_{i2}, G_{i3}, G_{o1}, G_{o2}, G_{o3}, G_z, G_{t1}, G_{t2}, and G_{gust}); (3) the '$G_{q/11}$' subfamily that activates phospholipase C β (G_q, G_{11}, G_{14}, and $G_{15/16}$), and (4) the '$G_{12/13}$' subfamily that activates the Na^+/H^+ exchanger pathway (G_{12} and G_{13}). Depending on the subtype(s) of the G protein α subunit that a given GPCR interacts with, a single or a combination of effectors can be activated [297]. Pertussis toxin (PTX) can block the inhibition of adenylate cyclase by catalyzing the covalent modification of G_i, leaving the enzyme in its activated state [268]

Signalling by BLT receptors

Early work by Goetzl and coworkers characterized the receptor(s) for LTB_4 as a specific surface receptor coupled to guanine nucleotide binding proteins, G_i and G_o. In line with these early data, CHO-cells expressing the BLT_1 receptor exhibited a chemotactic response towards low concentrations of LTB_4 in a pertussis-toxin sensitive manner [298], which was shown also for the same cell type expressing BLT_2 [301]. Calcium mobilization in CHO-cells expressing either BLT_1 or BLT_2 and pretreated with PTX, was only around 50% of the response in non-treated cells [298, 301]. LTB_4-induced inhibition of adenylyl cyclase in CHO-cells was affected differently by pertussis-toxin (PTX) depending on the type of receptor. Thus, PTX abolished 80% of the adenylyl cyclase inhibition in the cells expressing BLT_1, whereas BLT_2 expressing cells were unaffected, suggesting involvement of a PTX-insensitive G-protein, G_z [301]. These results suggest that LTB_4 signalling through BLT_1 and BLT_2 is connected to both PTX-sensitive and PTX-insensitive G-proteins.

In the first structural characterization of the BLT_1 it was suggested that the receptor as well as the ligand, LTB_4, undergo conformational changes upon binding to each other [13].

Signalling by CysLT receptors

It has been known for more than a decade that ligand induced signalling through the cys-LT receptors is a G protein-coupled event [48]. More recently, experiments with PTX treatment of HEK-293 cells stably expressing the $CysLT_1$ receptor or *Xenopus laevis* oocytes injected with $CysLT_1$ receptor cRNA, had no or limited blocking effect (~ 10%) on calcium mobilization or calcium activated chloride conductance, suggesting limited involvement of $G_{i/o}$-linked signalling routes and rather a $G_{q/11}$-linked pathway [155, 242]. This sensitivity, however, has earlier been suggested to vary with cell type and possibly with cellular differentiation [47, 48, 243]. The induced calcium mobilization in transfected HEK-293 cells was also suggested to originate from internal stores and was unaffected by removal of extracellular calcium [242], which is in contrast to previous studies [48] and indeed SKF 96365, an inhibi-

tor of receptor-operated calcium channels, has been shown to block LTD_4-induced contraction in human airways [89].

Dimerization/Oligomerization of receptors

GPCRs have recently joined the list of cell surface receptors that dimerize and several studies have attempted to clarify the role of oligomerization, particularly dimerization, for receptor activity [57, 105, 131, 224]. Dimerization has been shown to alter the ligand binding, signaling, and trafficking properties of these receptors. In some cases heterodimerization is required for efficient agonist binding and signalling, and in others, heterodimerization appears to lead to the generation of novel binding sites. Recent studies have shown that GPCRs heterodimerize with closely related receptors, resulting in the modulation of their function [57, 131]. Indeed, a very recent study on BLT_1 suggested that dimerization was crucial for binding to the appropriate G-protein trimer and thus also for signalling [14]. If this phenomenon occurs with CysLT receptors as well it could be a factor to help explain some of the differences in results observed in similar experimental settings. Indeed, recombinant $hCysLT_1$ was shown to form both dimers and oligomers when expressed in *E. coli* [70] a phenomenon also seen in HUVEC and HMC-1 (Sjöström, unpublished data).

Table 3. *CysLT receptor antagonists.*

Name	Chemical name	Target receptor	Reference
(Montelukast/ Singulair®) (MK-0476 / L 706,631)	(1-(((1(R)-(3-(2-(7-chloro-2-quinolinyl)-(E)-ethenyl)phenyl)(3-2-(1-hydroxy-1-methylethyl) phenyl)propyl)thio)methyl) cyclopropane) acetic acid sodium salt	$CysLT_1$	[129]
(Zafirlukast/ Accolate®) (ICI 204,219)	(4-(5-cyclopentyloxcarbonyl amino-1-methylindol-3-ylmethyl)-3-methoxy-N-o-tolylsulfonyl benzamide)	$CysLT_1$	[267]
Pobilukast (SKF 104,353)	(2(S)-hydroxyl-3(R)-carboxyethyl thio)-3-[2-(8-phenyloctyl)phenyl] propanoic acid)	$CysLT_1$	[185]
(Pranlukast / Onon®) (ONO 1078)	(4-oxo-8-[4-(4-phenylbutoxy)-benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran)	$CysLT_1$	[203]
MK-571 / L 660,711	(3-(3-(2-(7-chloro-2-quinolinyl) ethenyl)phenyl)((3-dimethyl amino-3-oxopropyl)thio)methyl) thio)propanoic acid	$CysLT_1$	[130]
BAYu9773	(6(R)-(4'-carboxyphenylthio)-5(S)-hydroxy-7(E),9(E),11(Z),14(Z)-eicosatetraenoic acid)	$CysLT_1$/ $CysLT_2$	[49]

METHODS

The methods indicated in this thesis and used in the original papers are well known and well established within the research field of biochemistry and molecular biology. They are listed below as a reference to the papers in which they appear, where a detailed description of the method also can be found.

Method	**Paper**
Cell culturing (HMC-1, HUVEC, RBL-1, Sf9)	I, II, III, IV
Isolation of mast cells from cord blood	II
RNA isolation	I, II, III, IV
Northern blot	I, II, IV
RT-PCR	I, II, III, IV
Amino acid sequencing	I, II, III, IV
Protein expression	I, II, III
Cloning	III
Membrane preparation	I, II, III
SDS/PAGE	I, II, III
Western blot	I, II, III
Enzyme activity assays	I, II, III
HPLC analysis	I, II, III
Calcium mobilization	II, IV
Immunostaining	II

AIMS OF THE PRESENT THESIS

The hemodynamic effects of cys-LTs have been known for long as well as their ability to constrict coronary vessels resulting in reduced cardiac output [261]. Specific LTC_4 synthesis has also been observed in endothelial cells [42, 68] and, until recently, LTC_4S was considered the only enzyme catalyzing the reaction. However, with the identification of MGST2 and MGST3 and the finding of MGST2 protein in EC membranes [121, 122, 249] there was a need to clarify which enzyme(s) are responsible for LTC_4 production in these cells. MGSTs are generally regarded as detoxifying enzymes that are ubiquitously expressed, particularly in the liver. On the other hand, LTC_4S is typically expressed in cells derived from the bone marrow. With the discovery of an intrinsic LTC_4 synthase activity of MGST2, it was of interest to investigate its potential participation in LTC_4 synthesis from myeloid cells. For this purpose, we chose mast cells, with their prominent role in allergy and asthma and well known production of cys-leukotrienes in response to different stimuli like A23187 or anti-IgE [104, 156, 216, 221, 246]. Thus, we set out to investigate expression, characteristics, and functionality of the LTC_4 producing enzymes in these cells using HUVEC, HMC-1 [32], and CBMC.

The above mentioned hemodynamic effects together with stimulatory effects on both the synthesis of PAF, induction of PMNL adherence, surface expression of P-selectin, and secretion of von Willebrand factor [53, 174] are mediated by cys-LTs linked to a receptor and signal transduction pathway. The recently cloned $CysLT_1$ [155, 242] and $CysLT_2$ [107, 202, 275] receptors were interesting candidates to investigate. Thus, we attempted to elucidate the expression, functional state, regulation and role of these receptors on endothelial and mast cells.

RESULTS

LTC_4 synthase and MGST2 in HUVEC and mast cells (paper I and II)

Expression of LTC_4S and MGST2 in HUVEC and mast cells (paper I and II)

Using Northern blot analysis of total RNA the levels of LTC_4S and MGST2 mRNA in HUVEC and HMC-1 were investigated. One major and one minor MGST2 transcript was detected in each cell type. The size of the major band (0.7 kbp) matched previously reported data very well and the shorter band may be interpreted as a less abundant splice variant of MGST2.

LTC_4S mRNA was, as expected, found in HMC-1 cells, whereas no such signal was detected in HUVEC. These findings were also verified using the much more sensitive method of RT-PCR yielding the same results. Since also MGST3 is a potential candidate for LTC_4 production, we used RT-PCR to possibly detect expression of this enzyme. However, no mRNA signal was observed from either cell type.

Polyclonal antisera directed against a specific region of MGST1, MGST2 or LTC_4S were employed in subsequent Western blot experiments. No pertinent bands were detected using the antisera against MGST1 on membrane fractions from either cell type. The antiserum against MGST2 detected a distinct immunoreactive band of the expected size in both HUVEC and HMC-1 membrane fractions. No cross-

reactivity was seen against recombinant MGST3. In contrast, the serum against LTC_4S detected a relevant protein band in HMC-1 cells, whereas no band was detected in HUVEC.

Relative LTC_4S and MGST2 enzyme activity in HMC-1 (paper II)

A modified batch of LTA_4 proved to be a useful tool for detection of MGST2-specific LTC_4 synthase activity. Thus, using this particular batch of substrate, MGST2 but not LTC_4S synthesized an additional product, a postulated LTC_4 isomer, which is slightly more polar than LTC_4 and therefore separable on RP-HPLC [249]. Since both HUVEC and HMC-1 cells expressed active MGST2 protein they were also able to produce the isomer. Further, by comparing the amounts of LTC_4/LTC_4 isomer produced by HMC-1 membrane fractions (containing both LTC_4S and MGST2) after incubation with different preparations of substrate, we could calculate a relative MGST2 activity to around 18%.

Based on results presented above, we concluded that MGST2 was responsible for the production of LTC_4 in HUVEC whereas mast cells rely on both enzymes.

Comparison of substrate preferences for recombinant MGST2 and LTC_4S (paper I)

Recombinant human MGST2 was expressed in Sf9 cells from which the 100 000 *x* g membrane fraction was isolated and used for enzyme characterization. It is known that LTC_4S turns over the unnatural methyl ester derivative of LTA_4 (LTA_4-ME) more efficiently than the naturally occurring free acid. We therefore decided to investigate the substrate specificity of MGST2, using activity assays and RP-HPLC, to see if the two enzymes share this catalytic feature. For MGST2, the free acid of LTA_4 was turned over into 28% more product (LTC_4) than was the methyl ester (LTC_4-ME). These results should be compared to previous reports on partially purified LTC_4S where LTA_4-ME was shown to be almost 4 times more efficient as substrate as compared to LTA_4 [304]. From Hanes plot we could determine values of V and K_m. Since no purified enzyme was available we could not determine a k_{cat}/K_m value but instead chose the expression V/K_m (pmol/min^{-1}x M^{-1}) which takes into account both turnover and substrate affinity. This value was almost four times higher for LTA_4 free acid than for the methyl ester.

Product inhibition of MGST2 (paper I)

We also examined the possibility of the enzyme being feedback-inhibited by LTC_4. Thus, LTC_4 formation was effectively inhibited by the product LTC_4 with an IC_{50} of around 1 μM, which is about half the IC_{50} obtained in similar experiments for LTC_4S [304]. LTD_4 and LTE_4 proved to be much weaker inhibitors with IC_{50} values of 16 and 17 μM, respectively.

Molecular cloning and characterization of rat LTC_4S, MGST2, and MGST3 (paper III)

The rat is a very useful species for studies of inflammatory symptoms and diseases. However, the probable differences in cDNA structure and biochemical characteristics of the enzymes of interest to us, LTC_4S, MGST2, and MGST3, compared to human,

were not known. Thus, to be able to fully benefit from this model we first needed to clone and characterize the rat orthologs of the human enzymes.

Amino acid sequence of rat LTC_4S, MGST2, and MGST3: comparison to human orthologs.

The cloned rat LTC_4S ($rLTC_4S$) cDNA, like the human, encodes a 150 amino acid sequence with almost identical calculated Mw and slightly lower pI. The overall amino acid identity to human and mouse was 86.7% and 94.7%, respectively, which was corroborated by another group [1]. Further, the two potential PKC phosphorylation sites present in the human and mouse enzymes [145, 146, 290] were present at Ser28-Ala29-Arg30 and Ser111-Ala112-Arg113 (numbering according to the $rLTC_4S$ sequence). Also, the putative FLAP inhibitor binding domain in the first hydrophilic loop of the enzyme was preserved but with Thr41 and Tyr50 exchanged for serine and phenylalanine, respectively, compared to the human protein.

Rat MGST2 (rMGST2) cDNA contained an open reading frame encoding a polypeptide of 147 amino acids with an identity of 79.6% to the human protein, and 42.7% to $rLTC_4S$. [121]. One of the points of differences was found in the so called "FERV-region" (using single letter amino acid code), a pattern (residues 46-49) conserved between the human enzymes LTC_4S, MGST2, and FLAP [121]. In rMGST2 that pattern was changed to "FERI".

Rat MGST3 (rMGST3) amino acid identity was 86.2% to the human ortholog, 36.5% to rMGST2, and 27% to $rLTC_4S$.

LTC_4 synthase activity

As might be expected from the structural similarities between the human and rat enzyme, $rLTC_4S$ also showed high degree of similarity to the human ortholog regarding catalytic features. Thus, K_m for the recombinant enzyme, using LTA_4 and LTA_4-ME as substrate was calculated to 18.8 μM and 19.8 μM, in good agreement with the human enzyme [199]. We used the expression V/K_m to compare the two substrates, which indicated that LTA_4-ME was the better substrate, in accordance with human LTC_4S [304]. In contrast, rMGST2, as its human counterpart (paper I), turned over the free acid of LTA_4 more efficiently than the methyl ester.

Rat MGST3 failed to show any significant LTC_4 synthase activity. Interestingly, the important residues Arg51 and Tyr93, previously reported to function as proton donor and base in human LTC_4S [147], were conserved, suggesting a different and yet unknown function for these residues in rMGST3.

Inhibition by MK-886 and cys-LTs

Similar to the human orthologs, the LTC_4 synthase activity of $rLTC_4S$ and rMGST2 could be inhibited by $LTC_4 > LTD_4 > LTE_4$ in decreasing order of potency. The IC_{50} values for inhibition of LTC_4S and MGST2 were determined as shown in table 4.

Further, the general GSH transferase activity and peroxidase activity of rMGST2 listed in table 4 as well as the peroxidase activity of MGST3 was equally sensitive to inhibition by LTC_4 (table 4). Similarly, the FLAP inhibitor MK-886 was tested and found to inhibit all enzyme activities of the three enzymes, i.e., LTC_4 synthase activity (LTC_4S and MGST2), GSH transferase activity (MGST2), and peroxidase activity (MGST2 and MGST3), which points to a common, or at least overlapping, active site(s) for the different enzyme activities (table 4).

Table 4. *Inhibition of LTC_4S, MGST2, and MGST3 by MK-886 and cys-LTs.*

Enzyme	Activity	Inhibitor	IC_{50} [μM]
LTC_4S	$LTA_4 \rightarrow LTC_4$	MK-886	4.5 ± 0.5
	$LTA_4 \rightarrow LTC_4$	LTC_4	3.0 ± 0.3
		LTD_4	44.2 ± 10.3
		LTE_4	84.5 ± 13.0
MGST2	$LTA_4 \rightarrow LTC_4$	MK-886	7.5 ± 0.6
	5-HPETE →5-HETE		9.0 ± 2.4
	Conjugation with CDNB		8.3 ± 1.8
	$LTA_4 \rightarrow LTC_4$	LTC_4	2.6 ± 0.3
	5-HPETE →5-HETE		2.3 ± 0.3
	Conjugation with CDNB		2.8 ± 0.4
MGST3	5-HPETE →5-HETE	MK-886	6.4 ± 1.2
	5-HPETE →5-HETE	LTC_4	2.9 ± 0.4

Effects of systemic inflammation on the expression levels of rat LTC_4S, MGST2, and MGST3

Studies of LTC_4S in isolated cells *in vitro* have demonstrated that cytokines such as IL-3, IL-4, and IL-5 [112] as well as TGF-β [223] are involved in the regulation of expression. However, no such studies had been done on MGST2 or MGST3 expression. We therefore investigated the effect of systemic inflammation, induced by LPS, on these three enzymes in the rat.

Expression of rat LTC_4S, MGST2, and MGST3.

In normal unstimulated rats LTC_4S mRNA was detected in high levels in ileum, lung, skeletal muscle, spleen, colon, lower levels in liver and kidney, and barely measurable levels in heart, brain, and adrenal gland, in agreement with previous results for the human enzyme [270]. At the protein level, LTC_4S was detectable in all tissues except spleen.

For rMGST2, the expression of mRNA also correlated well with previous results on the human enzyme [121] and was mainly found in liver, adrenal gland, ileum, and colon. However, using our polyclonal peptide antibody, the rMGST2 protein was

only detected in liver, ileum, colon, and brain, possibly due to differences in post-transcriptional regulation or an insufficient sensitivity of the Western blot assay.

The MGST3 mRNA was ubiquitously expressed in rat as in human, with strong expression in heart, liver, and adrenal cortex with the liver showing a point of difference compared to the weak expression in human [122]. Unfortunately, MGST3 antiserum was not available so protein expression could not be analyzed.

Specific induction of rat LTC_4S by intraperitoneal injections of LPS.
When rats were injected intraperitoneally with LPS (2 mg/kg bodyweight) and studied over a 6h period, LTC_4S mRNA was transiently upregulated within one hour in all tissues except ileum and spleen. The strongest elevation was observed in heart, brain, and adrenal gland. This upregulation was also seen at the protein level in brain, heart, liver, and adrenal gland where the increase was 4.9-, 4.0-, 2.9- and 2.3-fold, respectively. This tissue-specific upregulation of LTC_4S appeared with a time-lag between mRNA and protein expression, suggesting involvement of tissue-bound cells rather than blood borne cells adhering to the endothelium. Likely candidates would be mast cells, macrophages or even parenchymal cells as reported recently for rat hepatocytes [255] and mouse brain choroid plexus [271]. No similar effects of LPS could be detected for MGST2 or MGST3 mRNA or MGST2 protein, suggesting that these enzymes are not primarily involved in inflammatory reactions but perhaps in "house-keeping" functions.

Cysteinyl leukotriene receptors in HUVEC and mast cells (paper I, II & IV)

As mentioned previously, cys-LTs have documented hemodynamic effects in monkey [261] together with stimulatory effects on endothelial cells to synthesize PAF, induce surface expression of P-selectin and adherence of PMNL, as well as secretion of von Willebrand factor [53, 174]. These effects are probably mediated via one or more cell surface receptors for cys-LTs and the recently cloned $CysLT_1$ and $CysLT_2$ receptors [107, 155, 202, 242, 275] appeared to be interesting candidates to investigate.

HUVEC and mast cells express the $CysLT_1$ receptor (paper I and II)
Using total RNA isolated from HUVEC, we were able to pick up and amplify a 947 bp cDNA band through RT-PCR. The band was sequenced and matched the published sequence of $CysLT_1$ [155]. Further, the microsomal fraction isolated from HUVEC homogenates was analyzed by Western blot, using a polyclonal antiserum raised against recombinant $CysLT_1$. Two bands matching the positive control were detected but we could not determine whether both or just one of them were correct. However, the stronger band was greatly reduced and the weaker completely disappeared when the antiserum was pre-incubated with purified receptor protein indicating specific antibody targeting of the $CysLT_1$ protein.

The presence of $CysLT_1$ in HUVEC was corroborated by another group reporting upregulation of the receptor in HUVEC by long-term treatment with IL-1β [94].

During the search for $CysLT_1$ in HUVEC, we included samples from another cell type, which play an important role in leukotriene biosynthesis, allergy and asthma, namely the mast cell. By the same techniques used for HUVEC, plus North-

ern blot, we found that human mast cells, i.e., cultured HMC-1 or mast cells derived from cord blood (CBMC), expressed the $CysLT_1$ receptor.

Subcellular localization of $CysLT_1$ in mast cells (paper II)
By immunohistochemical staining, we could localize $CysLT_1$ to the surface membrane of mast cells, as expected, but also to the granules or cytoplasm, which was more surprising. The granular/cytoplasmic staining might be explained by the presence of the receptor on intracellular membranes or internal processing of the protein.

Mast cells express a functional $CysLT_1$ receptor (paper II)
The functionality of the receptor on mast cells was verified by calcium mobilization experiments showing concentration-dependent responses to LTC_4 and LTD_4 with the latter compound yielding the stronger signals.

The $CysLT_1$-specific receptor antagonist Zafirlukast (1 μM) completely inhibited the calcium response while MK-571 (1 μM) afforded a strong but incomplete inhibition. This difference in potency between the two antagonists can be explained by the fact that the inhibition constant (IC_{50}) of MK-571 is about five times higher than for Zafirlukast [155, 242].

Dominant expression of $CysLT_2$ in HUVEC (paper IV)
Using quantitative RT-PCR on total RNA isolated from unstimulated HUVEC, we compared the expression of $CysLT_2$ mRNA with that of $CysLT_1$ mRNA and found that the former receptor was expressed in huge excess, in a ratio of about 4300:1. Attempts were made to verify the expression at the protein level by Western blot but the commercially available antibody proved very unspecific, targeting several bands in the standard protein mix.

A functional $CysLT_2$ receptor accounts for cys-LT- induced calcium signalling in HUVEC (paper IV)
Calcium mobilization experiments in HUVEC showed responses to LTD_4 and LTC_4 (100 nM). However, unlike the situation in mast cells, the signal strength obtained from LTD_4 was only slightly greater than that of LTC_4, a relative potency far below that expected for $CysLT_1$ mediated signalling [155, 242]. In addition, the dual $CysLT_{1/2}$ antagonist BAYu9773 (100 nM), that is also a selective agonist for $CysLT_2$ [275], invoked a calcium response of similar magnitude as the leukotrienes. Challenge with LTD_4 (100 nM, 1 min) prior to treatment with BAYu9773 blocked the Ca^{2+}-response indicating that the receptor was occupied or desensitized by the natural agonist. Moreover, the $CysLT_1$ specific antagonist MK-571 (1 μM) afforded a weak, if any, inhibition of subsequent LTD_4- or LTC_4-induced (100 nM) calcium mobilization. In contrast, BAYu9773 completely blocked these Ca^{2+} signals. Together, these results strongly suggest that $CysLT_2$ is the dominant cys-LT receptor on HUVEC. During the process of writing this thesis, another group published similar results regarding expression of $CysLT_2$ on HUVEC [154]

Cytokines and LPS regulate the expression of $CysLT_2$ mRNA in HUVEC (paper IV)

$CysLT_2$ mRNA expression in HUVEC, cultivated for 0-120 min in the presence or absence of LPS, IL-1β or TNFα, was evaluated with (semi-)quantitative RT-PCR. LPS stimulation caused a transient down-regulation of mRNA to levels corresponding to 30% of control, which returned to normal levels after 120 min. IL-1β suppressed the level of $CysLT_2$ mRNA to approximately 50% of the levels of non-stimulated cells which only returned to about 80% after 120 min. TNFα had a similar effect on the level of $CysLT_2$ mRNA as IL-1β. Thus, TNFα decreased the $CysLT_2$ mRNA level to approximately 40% after 30 min, which returned to 60% after 60 min and stayed unchanged up to 120 min. Hence, our data suggest that these cytokines may suppress $CysLT_2$ expression.

DISCUSSION

This thesis has focused on the expression and regulation of leukotriene C_4 producing enzymes and the cysteinyl leukotriene receptors in human endothelial- and mast cells.

The potential role of MGST2 vs. LTC_4S

Glutathione S-transferases, soluble or membrane bound, are multi-functional enzymes. Their biological roles are thought to include detoxification of xenobiotics, the metabolism of drugs, and protection from oxidative stress caused by lipid peroxidation [7, 9, 164].

MGST2 conjugates 1-chloro-2,4-dinitrobenzene with glutathione and has peroxidase activity, as shown by its ability to reduce 5-HPETE to 5-HETE with an apparent K_m of 7 μM [122]. In addition, it possesses LTC_4 synthase activity with an apparent K_m of 28 μM for the lipid substrate LTA_4 (paper I). With the limited number of available studies and the lack of pure enzyme for biochemical characterization, it is difficult to speculate which enzyme activity, or activities, are the most physiologically relevant. Only comparing the apparent value of K_m for LTA_4 of MGST2 expressed in Sf9 cells with that of purified LTC_4 synthase (~10 μM) [197], suggests that LTC_4S is the primary enzyme for this reaction, which however, does not rule out a role for MGST2. The cys-LT production of endothelial cells via transcellular routes is well documented [42, 68, 158, 159], a reaction which seems to rely on MGST2 alone ([249], paper I). These cells do not express LTC_4S, as judged by Northern blot, RT-PCR, and Western blot, but instead MGST2. The biosynthesis of LTC_4 by MGST2 was shown to be feedback inhibited in the same fashion as LTC_4S with similar IC_{50} values for the two enzymes. Interesting is also the fact that the active site of MGST2 seems better suited for accommodation of the free acid of LTA_4, the naturally occurring substrate, than for the methylated substrate. This is a reversal of the situation for LTC_4S.

The notion of MGST2's LTC_4 synthase activity as a disposal mechanism for excess LTA_4 [110] without further consequence does not appear likely. To release such a potent mediator, known to affect the vascular wall, in the direct vicinity just to eliminate excess LTA_4 seems like a poor strategy. In addition, mast cells were found to express both LTC_4S and MGST2 and also employ both for LTC_4 synthesis in a 4:1 ratio, again, a scenario pointing to MGST2 as more than a "by chance metabolizer" of

LTA_4. Hence, it seems possible that MGST2 can contribute to mast cell LTC_4 biosynthesis *in vivo*, a function developed from metabolism of endogenous toxic agents. It should be noted that one group claimed that tissues from LTC_4S (-/-) mice showed almost no LTC_4 synthase activity except testis [135]. However, this study used LTA_4-ME as substrate which is expected to yield low activity due to the substrate preference of MGST2.

These and previously mentioned results, however, are pertaining to unstimulated cells. In *vivo* stimulation of rats (intraperitoneal injection) with LPS results in an increase of LTC_4S mRNA and protein in a number of tissues with 2-5 times upregulation of protein in adrenal gland, liver, heart, and brain, in that order. A similar increase in expression was reported from another group doing studies on retinoic acid stimulated RBL-1 cells [1]. The effect in rat appears to be tissue-specific rather than a result of blood borne cells regarding lag times of mRNA and protein synthesis. Mast cells, macrophages, and even parenchymal cells would then be likely candidates for this upregulation. This also implies that LTC_4S, through regulated cys-LT production, directly or indirectly is involved in the general symptoms of inflammation like fever, hypotension, tachycardia, and fatigue. Interestingly, LTC_4S mRNA was recently detected in mouse choroid plexus [271] and cys-LTs were found in 5-fold increased levels in cerebrospinal fluid following experimental brain injury [247]. However, it should be noted that LPS stimulation of the monocyte-like cell line THP-1, suggests that LPS has a downregulating effect on LTC_4S in those cells [254].

In contrast to LTC_4S, LPS treatment had no detectable effect on either MGST2 protein or MGST2 or -3 mRNA. This indicates that MGST2 (and -3) have a more "house-keeping" function while LTC_4S is induced to meet an increased need, as has been discussed for COX [262] and recently the down-stream PGE synthases, mPGES-1 and cPGES [276]. The failure to respond to LPS and the notion of a "house-keeping role" is to some extent strengthened by studies in mice [135] and seems likely also for humans when regarding similarities in tissue distribution and homology of the enzymes.

Possible common or overlapping active site(s) for MAPEG enzymes

The MAPEG family is composed of a number of enzymes with at least some similarity in their features [123]. FLAP and LTC_4S despite their difference in gene size have identical exon/intron organization and the exons 2 through 5 are identical in size while the flanking exons differ minimally in the 5'- and 3'-ends.

When the FLAP inhibitor MK-886 was used to inhibit the conversion of LTA_4 to LTC_4 by $rLTC_4S$ and rMGST2, the compound was found to be almost as potent as LTC_4 itself. Furthermore, LTC_4 and MK-886 were found to inhibit the peroxidase activity of rMGST2 and rMGST3 as well as the general GSH-transferase activity of MGST2 with similar potency. This indicates that the active sites are structurally related and have similar architectures in all three enzymes (Fig. 6). In addition, it was recently shown that another MAPEG family member, mPGES-1, was also inhibited by MK-886 [45], which further suggests a common active site for the enzymes of the MAPEG family. If that is the case, the future development of inhibitors for a specific enzyme in the group may become difficult, since it could have unforeseen and possibly unwanted effects on other family members. On the other hand, it may allow the

development of inhibitors against multiple targets, e.g., mPGES-1, LTC_4S, and MGST2, to achieve a greater anti-inflammatory effect.

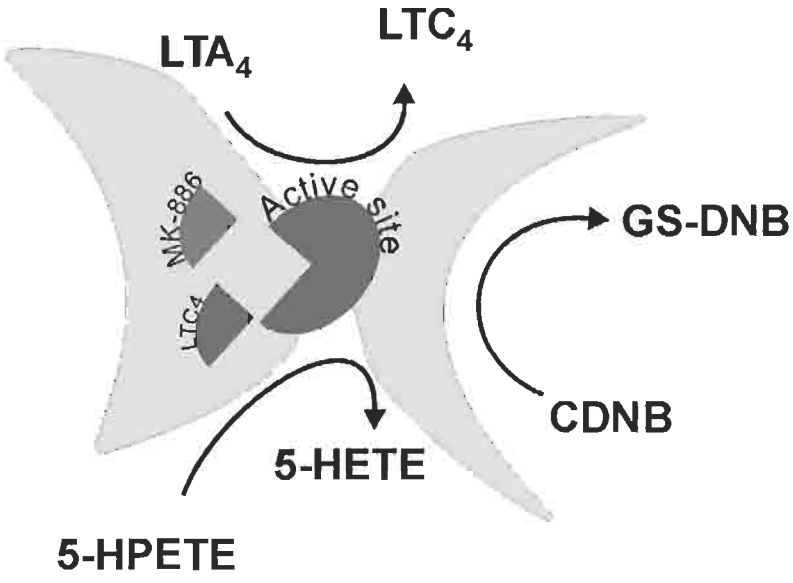

Fig. 6 *Schematic of the possible common or overlapping active site(s) where MK-886 and LTC_4 block the enzymatic activities of rat MGST2 (LTC_4 synthase activity, GSH-conjugation to CDNB and peroxidase activity), MGST3 (GSH-conjugation to CDNB and peroxidase activity), and LTC_4S (LTC_4 synthase activity).*

CysLT1 receptor expression in mast cells

The effects of cys-LTs are mediated via at least two G-protein coupled, seven trans-membrane spanning surface receptors termed $CysLT_1$ and $CysLT_2$. These receptors have been characterized and classified not only by sensitivity to antagonists but also by cloning and seem to have a different tissue distribution as well.

Human mast cells isolated from cord blood express at least one of these receptors namely $CysLT_1$ as described above (paper II). Immunohistochemistry indicated receptor expression, not only on the surface as expected, but possibly also on granules and/or in the cytoplasm. The significance of these results is difficult to interpret but one interesting speculation could be that the $CysLT_1$ receptor is located on granules for direct signalling by LTD_4 and LTC_4 to release histamine, PGD_2 etc (Fig. 7). However, another possibility is that the $CysLT_1$ protein is merely being tagged by the antiserum as it is being processed in the cell.

In any event, the $CysLT_1$ receptor was shown to be functional and responded to LTD_4 and LTC_4 with a concentration-dependant Ca^{2+}–signal (Fig. 7). Ca^{2+}-signalling through the $CysLT_1$ receptor has also been shown in mast cells by another group as a

result of UDP-challenge suggesting that $CysLT_1$ accepts a wider range of agonists than previously known [179]. Indeed, acetylcholine was shown to use the $CysLT_1$ receptor to induce contractions in rat aortic smooth muscle cells [173].

Mellor *et al.* also suggested that another cys-LT receptor, with higher sensitivity to LTC_4, was induced in mast cells by long-time stimulation with the cytokine IL-4. Cys-LTs together with UDP was reported to trigger the production of IL-5, TNF-α, and macrophage inflammatory protein (MIP)-1β in these cells [178]. Interestingly, this production occurred without concomitant release of histamine or generation of PGD_2.

A similar result regarding regulation of expression was obtained for $CysLT_1$ in earlier studies on airway myocytes, where IFN-γ was shown to upregulate the receptor mRNA expression and enhance the response to LTD_4 [6]. Further, very recent studies also showed that the $CysLT_1$ receptor in bronchial smooth muscle cells was upregulated by TGF-β, IL-13, and IFN-γ but not IL-4 [65].

Without excluding the presence of a $CysLT_2$ or even a $CysLT_3$ in mast cells, there seems little doubt that $CysLT_1$ is the dominant receptor in unstimulated cells and although preliminary data (not presented) suggest that $CysLT_2$ mRNA is also expressed in mast cells, the relevance of this finding is questionable. However, more studies are needed to clarify how the expression pattern of CysLT receptors is changed, by cytokines or other cell stimuli, to complete the picture.

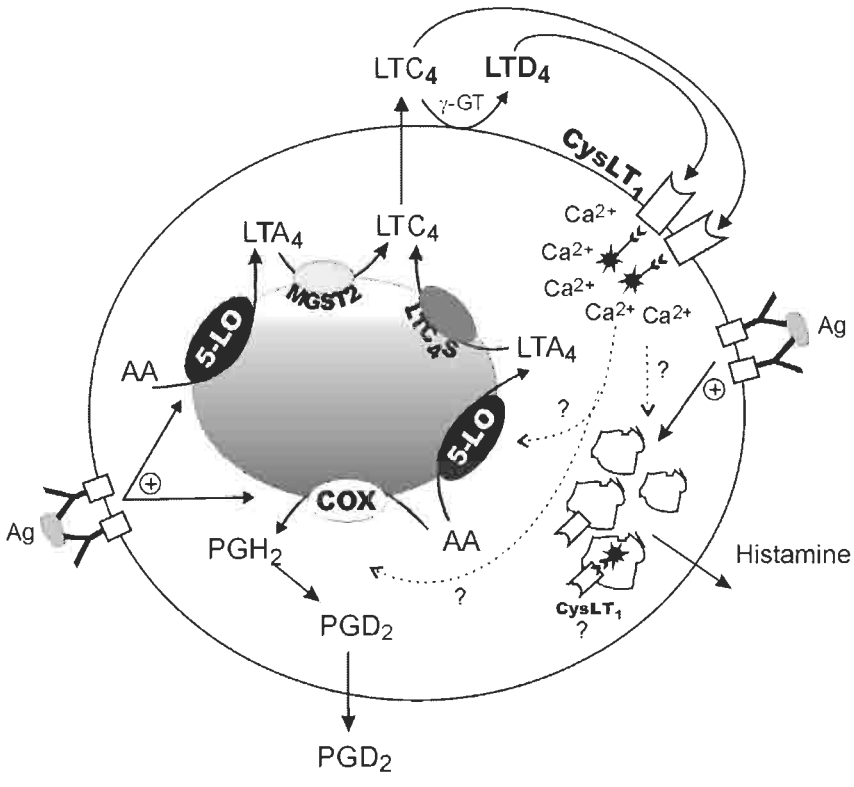

Fig. 7 *Schematic of possible cys-LT receptor placement in mast cells and effect of ligand binding in an autocrine/paracrine fashion.*

Cysteinyl leukotriene receptors in endothelial cells

Cys-LTs have been shown to constrict coronary vessels, reduce cardiac output [261] and was implicated in cardiac ischemia [36]. 5-LO products and LTs were also recently indicated by immunohistological, genetic and pharmacological data to play a role in atherosclerosis and ischemic heart disease [3, 177, 266]. Furthermore, transcellular cys-LT generation in EC, using LTA_4 donated from PMNL, was shown to induce coronary vasospasm and elicit inflammatory changes in the vasculature [236]. We have shown that HUVEC express both the $CysLT_1$ and the $CysLT_2$ receptors but to different extents (Fig. 8). $CysLT_1$ receptor mRNA and protein are expressed in HUVEC as described in (paper I) and seems to be upregulated, at least at the mRNA level, by long-term treatment with IL-1β [94]. Indeed, the $CysLT_1$ receptor has been implicated for the signal transduction in some contraction-relaxation responses in the vessel wall during inflammatory states [161] and has been shown to be expressed in peribroncheal SMC [155]. However, the dominant cys-LT receptor expressed in EC seems to be $CysLT_2$, as judged by the relative amounts of mRNA ($CysLT_2$/$CysLT_1$ >4300:1) and the agonist and antagonist response-profile. Through the cloning and characterization of the cys-LT receptors [107, 155, 202, 242, 275] it was implied that LTD_4 is a 10-100 times more effective ligand to $CysLT_1$ than LTC_4 and that they had similar potency towards $CysLT_2$. Calcium mobilization signals in HUVEC elicited by LTD_4 are of similar magnitude as those of LTC_4, which could be interpreted as a predominant $CysLT_2$ response without excluding a contribution by $CysLT_1$. Furthermore, the weak effect of the $CysLT_1$ antagonist MK-571 on cys-LT induced Ca^{2+}-signalling and its total abolishment by the dual $CysLT_{1/2}$ antagonist BAYu9773, together with the partial agonistic effect of BAYu9773 (only attributable to $CysLT_2$), clearly shows that $CysLT_2$ is the dominant receptor and that $CysLT_1$ plays a very minor, if any, role in the response. Interestingly, $CysLT_2$ mRNA was also found in certain mouse heart EC [116] as well as other human heart cells including purkinje cells, SMC, and heart muscle [107, 132, 202, 275].

It should be noted, however, that various cell stimuli may change the expression profile of $CysLT_1$ and $CysLT_2$ in EC (and other cells as well). Thus, stimulation of HUVEC by LPS, TNFα or IL-1β, for up to two hours, downregulated the $CysLT_2$ mRNA expression. This is in contrast to the report about upregulation of $CysLT_1$ mRNA by IL-1β after 24h [94]. In our hands no significant change could be established in $CysLT_1$ mRNA expression with either stimulus for up to two hours. However, one might speculate that there exists a scenario where longer time-periods of stimulation by cytokines reduce the level of $CysLT_2$ in favour of $CysLT_1$.

Both $CysLT_2$ and $CysLT_1$ have been implicated as possible receptors mediating vascular events such as contraction and relaxation [132, 161]. The presence of both receptors has also been shown in vascular and surrounding cell types as reviewed above. Recently, both cys-LT receptors were also suggested to have a role in atherogenesis [154]. However, in view of our data on ECs, it seems fair to state that signal transduction induced by cys-LT is mediated via the $CysLT_2$ receptor and that previously documented effects exerted by cys-LTs on these cells such as stimulated synthesis of PAF, induction of P-selectin and secretion of von Willebrand factor [53, 174] could be results of this signalling.

One interesting question is also what conclusion may be drawn from the fact that both endothelial- and mast cells synthesize cys-LTs as well as express the appro-

priate receptors. It is very tempting to speculate that these cys-LTs will then act as ligands for receptors on the same cell or similar cells nearby creating a autocrine/paracrine loop (Fig. 8), which has also recently been suggested by another group [154]. To what effect is not clear but LTD_4-induced signalling through cys-LT receptors have previously been reported to increase AA release [48] and recently a positive feedback for cys-LTs and UDP to induce cytokine production in hMCs was argued [178].

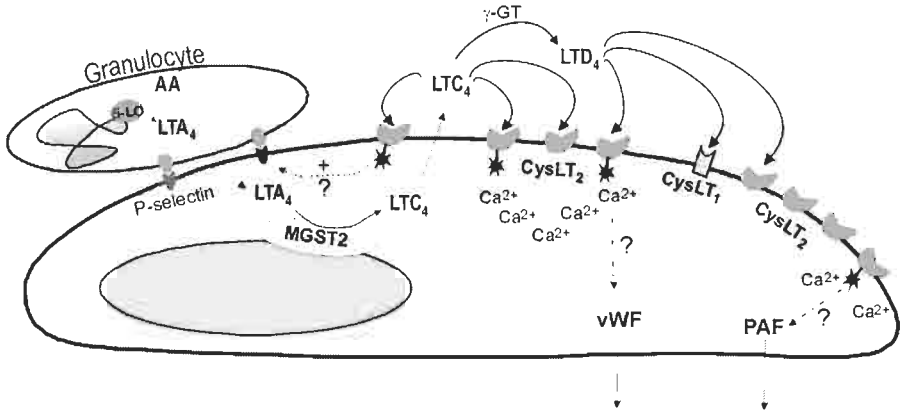

Fig. 8 *Summary of cys-LT biosynthesis and the effect of possible autocrine/paracrine regulation in endothelial cells.*

Concluding remarks

In the present thesis work, we have studied certain aspects of cys-LT biosynthesis and signalling. On several occasions, the results have been surprising and modified our notion regarding the molecular and cellular mechanisms for LT biosynthesis and its potential biological impact. The data also raise a number of questions and point to possible routes for further investigations. Thus, we have established that MGST2, rather than LTC_4S, is the major enzyme catalyzing the committed step in LTC_4 biosynthesis in HUVEC. In addition, MGST2 may contribute to LTC_4 synthesis in myeloid cells, as judged by its expression in mast cells. The relative roles and biological significance of these enzymes, however, is not clear but data like the selective LPS induction of LTC_4S in rats, without effect on MGST2 or MGST3, points to a more house-keeping role for the MGSTs. It will be interesting to investigate the effects of other proinflammatory agents together with animal models of enzyme deficiencies, to verify if this notion holds true.

The dominant expression of $CysLT_2$ in HUVEC and its implications for cys-LT-dependent vascular responses is intriguing. Together with other reports in the literature, our data suggest that cys-LTs may be involved in the pathogenesis of atherosclerosis and cardiovascular diseases. Further work is clearly needed to clarify

the exact role of $CysLT_2$ and will be greatly facilitated when $CysLT_2$ specific antagonists and $CysLT_2$ deficient mice become available.

The strong expression of $CysLT_1$ in human mast cells is also an interesting finding that has been corroborated by other groups. It implies that cys-LTs act on mast cells for a particular functional purpose that we have not yet recognized. Since mast cells themselves are equipped with all enzymes necessary for cys-LT biosynthesis, one can envisage autocrine and paracrine signalling loops involved in mast cell communication and functional regulation. Considering the role of mast cells in inflammatory and allergic reactions, it is tempting to speculate that cys-LTs may influence the secretory response and/or synthesis of lipid mediators in these cells. As these studies continue, new insights concerning the biological actions of cys-LTs will hopefully emerge, which in turn may elucidate the mechanisms of action for anti-leukotriene drugs as well as novel targets for pharmacological intervention.

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