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Studies on molecular properties and functional regulation of terminal leukotriene C_4 synthases and cysteinyl-leukotriene receptor signalling in human endothelium

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ABSTRACT

The transformation of the unstable intermediate leukotriene (LT) A_4 into the glutathione conjugate LTC_4, the parent compound of LTD_4 and LTE_4, is catalysed by leukotriene C_4 synthase (LTC4S) as well as microsomal glutathione *S*-transferase type 2 (MGST2) and type 3 (MGST3). Together, these eicosanoids also known as cysteinyl leukotrienes (cys-LT) are key mediators of immediate hypersensitivity reactions. However, recent evidence also supports their pivotal role in adaptive immune response as well as in the progression of inflammatory disease.

The rat orthologs of these LTC4S isoenzymes were successfully cloned and functionally expressed in Spodoptera frugiperda insect cells. The rat enzymes were found to be highly similar to their human counterparts with amino acid identities of 87%, 80%, and 86% for LTC4S, MGST2, and MGST3, respectively. As might be expected from the structural similarities between the human and rat enzyme, rat LTC4S also showed a high degree of analogy to the human ortholog regarding catalytic features. Thus, K_m for the recombinant enzyme, using the free acid and the methyl ester of LTA_4 as substrate, were calculated to 18.8 μM and 19.8 μM, respectively. In contrast, rat MGST2 converted the free acid of LTA_4 more efficiently than the methyl ester which is in accordance with the human counterpart. Whereas the LTC4S capacity was preserved in rat MGST2, rat MGST3 failed to show any significant LTC4S activity. Both, LTC_4 and the 5-lipoxygenase activating protein inhibitor MK-886 inhibited all respective enzymatic activities of the terminal LTC4S isoenzymes, i.e. LTC4S activity (LTC4S and MGST2), GSH transferase activity (MGST2), and peroxidase activity (MGST2 and MGST3), suggesting that the catalytic centres originate from structurally related overlapping active site(s).

Intraperitoneal injection of lipopolysaccharide (LPS) in rats lead to a transient increase of LTC4S mRNA in several tissues, particularly heart, brain, adrenal glands, and liver, within one hour followed by a 4.9-, 4.0-, 2.9, and 2.3-fold induction of LTC4S protein expression at six hours in brain, heart, liver, and adrenal gland, respectively, indicating that up-regulation of LTC4S might be triggered by systemic inflammatory signals and prime certain tissues for increased cys-LT biosynthesis. In contrast, no effects were detected for MGST2 and MGST3 suggesting that these enzymes do not contribute to LTC_4 formation during host-defence reactions, but may be involved in cys-LT biosynthesis for other, basal ”house-keeping” purposes.

Using *in situ* hybridization histochemistry and reverse transcription polymerase chain reaction (RT-PCR) the expression of MGST3 in the rat central nervous system (CNS) was investigated both, under normal conditions and after intraperitoneal injection of LPS. The broad distribution in the CNS was characterized by a strong signal in the hippocampal formation, the nuclei of the cranial nerves as well as the motor neurons in the spinal cord and sensory neurons in the dorsal root ganglia. A moderate signal was found in the cortex, thalamus, amygdala, and substantia nigra and a weak signal in the hypothalamus. However, no changes in the level of MGST3 mRNA expression in the CNS were found one, three, or six hours after LPS administration which do not support a role for MGST3 in the biosynthesis of pro-inflammatory cys-LT but rather suggest other functions, e.g. metabolic detoxication and neuroprotection.

Human umbilical vein endothelial cells (HUVEC) were found to abundantly express $CysLT_2$ mRNA in vast excess (>4000-fold) of $CysLT_1$ mRNA when examined by quantitative RT-PCR. Pro-inflammatory stimuli (LPS, Tumor necrosis factor α, and Interleukin-1β) caused a rapid (within 30 minutes) and partially reversible suppression of $CysLT_2$ mRNA levels. Challenge of HUVEC with BAY u9773, a partial $CysLT_2$ agonist, triggered diagnostic Ca^{2+} transients. LTC_4 and LTD_4 were demonstrated to be equipotent agonists, and their actions could be blocked by BAY u9773, which is also a dual $CysLT_1$ and $CysLT_2$ receptor antagonist, but not by the $CysLT_1$-selective antagonist MK571. Together, these data indicate that signalling events involving $CysLT_2$ might trigger functional responses involved in critical components of cys-LT dependent vascular reactions, which in turn have implications for ischemic heart disease and myocardial infarction.

LIST OF PUBLICATIONS

The present thesis is based on the following original articles which are reproduced with permission from the publishers:

I. Fetissov SO, Schröder O*, Jakobsson PJ, Samuelsson B, Haeggström JZ, Hökfelt T. Expression of microsomal glutathione *S*-transferase type 3 mRNA in the rat nervous system. *Neuroscience.* 2002;115:891-897.

II. Schröder O, Sjöström M, Qiu H, Stein J, Jakobsson PJ, Haeggström JZ. Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. *Biochem Biophys Res Commun.* 2003;312:271-276.

III. Sjöström M, Johansson AS, Schröder O, Hong Q, Palmblad J, Haeggström JZ. Dominant expression of $CysLT_2$ receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol.* 2003;23:37-41.

IV. Schröder O, Sjöström M, Qiu H, Jakobsson PJ, Haeggström JZ. Microsomal glutathione *S*-transferases: selective up-regulation of leukotriene C4 synthase during lipopolysaccharide-induced pyresis. *Cell Mol Life Sci.* 2005;62:87-94.

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ABBREVIATIONS

5-HETE	5(*S*)-hydroxy-8,11,14-*cis*-6-trans-eicosatretraenoic acid
5-HPETE	5(*S*)-hydroperoxy-8,11,14-*cis*-6-trans-eicosatretraenoic acid
5-LO	5-lipoxygenase
6-keto-$PGF_{1\alpha}$	6-keto-Prostaglandin $F_{1\alpha}$; 6-oxo-9α,11α,15(*S*)-trihydroxy-prost-13-en-1-oic acid
γ-GL	γ-glutamyl leukotrienase
γ-GT	γ-glutamyl transpeptidase
AA	Arachidonic acid; 5,8,11,14-*cis*-eicosatetraenoic acid
aa	Amino acid(s)
AP	Activating protein
BAL	Bronchoalveolar lavage
bp	Base pairs
CDNB	1-chloro-2,4-dinitrobenzene
CEBP	CCAAT/enhancer-binding protein
CHO	Chinese hamster ovary
COX-1	Cyclooxygenase type 1
COX-2	Cyclooxygenase type 2
CPBP	Core promoter-binding protein
cys-LT	Cysteinyl-leukotriene
$CysLT_1$	Cysteinyl-leukotriene receptor type 1
$CysLT_2$	Cysteinyl-leukotriene receptor type 2
DC	Dentritic cells
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
ECL	Enhanced chemiluminescence
ERK	Extracellular signal-regulated kinase
FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
GPCR	G protein-coupled receptor
GS-DNB	1-*S*-glutathionyl-2,4-dinitrobenzene
GSH	(Reduced) glutathione
GST	Glutathione *S*-transferase
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
IC_{50}	Half maximal inhibitory concentration
IgE	Immunoglobulin E
IL	Interleukin
kb	Kilo bases
K_m	Michaelis-Menten constant
LPS	Lipopolysaccharide
LT	Leukotriene
LTA_4	Leukotriene A_4; 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTA_4-FA	Free acid of LTA_4
LTA_4-ME	Methyl ester of LTA_4

LTB_4	Leukotriene B_4; 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid
LTC_4	Leukotriene C_4; 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTD_4	Leukotriene D_4; 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTE_4	Leukotriene E_4; 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTA4H	LTA_4 hydrolase
LTC4S	LTC_4 synthase
MAPEG	Membrane Associated Proteins in Eicosanoid and Glutathione Metabolism
MAPK	Mitogen-activated protein kinase
MGST1	Microsomal GSH *S*-transferase type 1
MGST2	Microsomal GSH *S*-transferase type 2
MGST3	Microsomal GSH *S*-transferase type 3
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
MIP-2	Macrophage inflammatory protein 2
mPGES-1	Microsomal prostaglandin E Synthase type 1
MRP1	Multidrug resistance protein 1
NEM	N-ethyl maleimide
PAF	Platelet activating factor
pI	Isoelectrical point
PL	Phospholipids
PLA_2	Phospholipase A_2
PCR	Polymerase chain reaction
PEA-3	polyomavirus enhancer activator 3
PG	Prostaglandin
PGD_2	Prostaglandin D_2; 9α,15(*S*)-dihydroxy-11-ketoprosta-5-*cis*-13-*trans*-dienoic acid
PGE_2	Prostaglandin E_2 ; 11α,15(*S*)-dihydroxy-9-ketoprosta-5-*cis*-13-*trans*-dienoic acid
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
RBL-1	Rat basophilic leukemia cell line
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
Sf-9	Spodoptera frugiperda
SNP	Single nucleotide polymorphism
SP	Stimulating protein
SRS-A	Slow-reacting substance of anaphylaxis
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
T-TBS	Tween-20 Tris-buffered saline
UDP	Uridine diphosphate
V_{max}	Maximal rate under saturating concentrations of substrate
Å	Ångström

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INTRODUCTION

Eicosanoids [Greek: eikosi = twenty] comprise a large group of biologically active lipids formed via enzymatic conversion of unsaturated 20-carbon fatty acids. The fatty acids acting as precursor in eicosanoid biosynthesis are eicosapentaenoic acid (20:5, ω3), aracidonic acid (20:4, ω6) and dihomo-γ-linolenic acid (20:3, ω6). Either of these three precursors can be obtained directly from our diet or synthesized endogenously through desaturation and elongation of linoleic acid (18:2, ω6) or linolenic acid (18:3, ω3) [209]. In mammalian cells, arachidonic acid (AA) is the most abundant 20-carbon polyunsaturated fatty acid and also the main precursor for eicosanoid biosynthesis [174]. AA is usually found in esterified form at the sn-2 position of membrane phospholipids. Consequently, phospholipase-dependent release of AA is a prerequisite for further transformation of the fatty acid and the initial step in eicosanoid generation [44].

AA is metabolized via three major enzymatic pathways: cyclooxygenase, lipoxygenase, and cytochrome P450. The principal eicosanoids formed from AA are prostaglandins (PG) and thromboxanes, leukotrienes and lipoxins as well as cytochrome P450 epoxygenase and ω-hydroxylase products. The transformation of the unstable intermediate leukotriene (LT) A_4 into the glutathione conjugate LTC_4, the parent compound of the cysteinyl leukotriene (cys-LT) family, is catalysed by the microsomal enzyme LTC_4 synthase (LTC4S) [92]. In addition, two other microsomal proteins, designated microsomal glutathione *S*-transferase type 2 (MGST2) and type 3 (MGST3), have recently been demonstrated to possess LTC4S activity [73,74,165]. The cys-LT (LTC_4, LTD_4, and LTE_4) are key mediators of immediate hypersensitivity reactions, but recent evidence also supports their pivotal role in adaptive immune response as well as in the progression of inflammatory disease [79]. The biological responses elicited by cys-LT are signalled via at least three G-protein coupled receptors.

Biosynthesis of cysteinyl leukotrienes

Cys-LT are a family of bioactive fatty acid mediators that were originally described in 1940 as the slow-reacting substance of anaphylaxis (SRS-A) [83]. Although substantial

evidence demonstrated the impact of SRS-A in mediating anaphylaxis and airway disease, it took almost four decades to define the chemical structure. Finally in 1979 the structure of SRS-A was elucidated and the parent molecule, LTC_4, was described [126], followed by its two biologically active metabolites, LTD_4 and LTE_4 [96,97,136,141].

Biosynthesis of cys-LT in cells of myeloid origin such as mast cells, basophils, monocyte/macrophages, and eosinophils is initiated by an increase in intracellular Ca^{2+} and activation of phospholipase A_2 (PLA_2) in response to agonist stimulation. Although multiple isoenzymes of phospholipase exist, recent evidence underlines the critical role of the high molecular weight, cytosolic isoenzyme in LT biosynthesis [20,195]. Enzymatic activity of cytosolic PLA_2 has previously been demonstrated to be dependent on translocation to the perinuclear membrane. In the same way, the perinuclear region has been identified as the site where all proteins involved in cys-LT formation are translocated or integrally situated [144,150,210]. On its release from membrane phospholipids [34], AA binds to 5-lipoxygenase activating protein (FLAP), an integral perinuclear membrane protein necessary in intact cells to stimulate the use of arachidonic acid by 5-lipoxygenase (5-LO). 5-LO translocates from either cytoplasm or nucleoplasm [24] to the perinuclear membrane depending on the cell source. The two catalytical reactions of this enzyme form 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently LTA_4 [159]. LTA_4 represents the common intermediate precursor of the two major bioactive classes of LT. The first is formed by the action of cytosolic LTA_4 hydrolase (LTA4H) and is comprised of the dihydroxy LT, LTB_4, and its metabolites. The other major class, cys-LT, requires the conjugation of LTA_4 with reduced glutathione (GSH) to form LTC_4 [215], a reaction carried out by LTC4S. In addition, several cytosolic and microsomal GSH *S*-transferases (GST), including MGST2 and MGST3, may catalyze this reaction. On its formation, LTC_4 is exported via a carrier-mediated mechanism [88], and further processed via sequential cleavage of glutamic acid and glycine residues from the GSH moiety of LTC_4 by γ-glutamyl transpeptidase (γ-GT) or γ-glutamyl leukotrienase (γ-GL) and dipeptidase yielding LTD_4 and LTE_4, respectively [137].

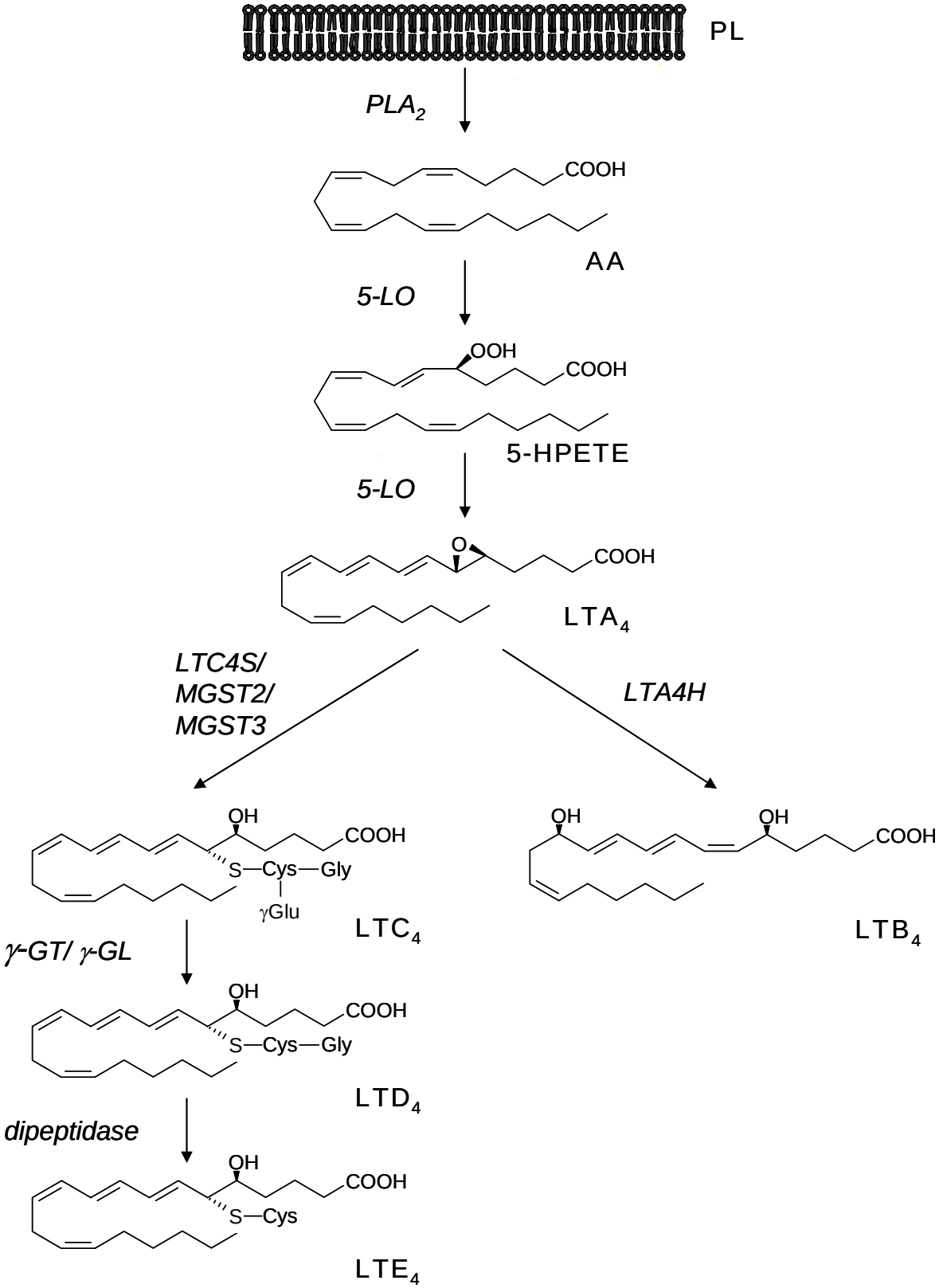

Figure 1: Biosynthesis of leukotrienes from membrane phospholipids (PL).

LTC_4 synthase (LTC4S)

Purification and biochemical characterization of LTC4S

LTC4S differs from common GST by its selectivity for LTA_4 and its analogs [215], microsomal localization, and inability to conjugate GSH with xenobiotics. This enzyme also exhibits differential susceptibility to inhibitors [8] and lacks immunoreactivity to antibodies for other known GST [127].

The first report demonstrating a unique LTC4S bioactivity originates from a crude separation of particulate and soluble cellular fractions from the rat basophilic leukemia cell line RBL-1 [7]. Specific antibodies were allowed for distinction of LTC4S from other microsomal and cystosolic GST in various cells [127,128]. LTC4S was long believed to be exclusively expressed in bone-marrow-derived cells such as basophils, eosinophils, mast cells, monocyte/macrophages, platelets [7,172,176,203,208] and in leukemic cell lines such as KG-1 [143] and THP-1 [128]. However, recent observations have indicated that LTC4S can be upregulated in parenchymal cells such as rat hepatocytes [169] and the choroid plexus of the cerebral ventricles of the mouse brain [178]. Parenchymal cells are rarely, if ever, equipped with all enzymes required for LT biosynthesis, implying that the substrate LTA_4 must be delivered via transcellular routes, e.g. via neighbouring activated leukocytes. Such cell-cell interactions have been demonstrated for transcellular biosynthesis of cys-LT along a platelet-neutrophil or an endothelial-neutrophil axis [33,46,49,101] and were recently shown to occur *in vivo* [48].

Following the recognition that LTC4S is a distinct microsomal protein with restricted distribution, the purification of this enzyme proved to be extremely difficult. Indeed, initial attempts of purification achieved only 10 to 21-fold enrichment [7,72]. Subsequently, LTC4S was purified up to 10.000-fold from human monocytic U-937 cells [215] based on the use of various bioactivity-stabilizing factors such as cations (Ca^{2+} and Mg^{2+}) and phospholipids, both of which were found to increase enzymatic activity [127]. Finally two independent groups succeeded in purifying the enzyme to apparent homogeneity from KG-1 and THP-1 cells [128,143]. The 18 kDa protein was proposed to function as a homo-dimer based on the size of the active fraction by gel filtration column chromatography [128]. Bioluminescence resonance energy transfer studies in living cells demonstrated that LTC4S forms a homo-oligomer [184], and fluorescence resonance energy transfer and cross linking studies suggested that LTC4S can form a hetero-dimer or a hetero-trimer with FLAP [107]. Finally,

calculation of a projection map of recombinant human LTC4S at a resolution of 4.5 Å by electron crystallography revealed that this enzyme functions as a trimer [164].

Initial kinetic data from partially purified enzyme derived from RBL-1 cells [7], platelets [176], and guinea pig lung [72,215] have been quite consistent, with K_m values of 7-36 μM for LTA_4 and 1.2-6.0 mM for GSH. Substantially pure LTC4S from dimethyl sulfoxide (DMSO)-differentiated U-937 cells has provided K_m values for LTA_4 and GSH of 19.6 μM and 1.83 mM respectively, with a V_{max} of 2-4 μmol × min^{-1} × mg^{-1} [127]. LTC4S purified to homogeneity from THP-1 cells exhibited K_m values for LTA_4 and GSH of 9.9 μM and 1.7 mM, respectively with a V_{max} of 4.1 μmol × min^{-1} × mg^{-1} [128]. In addition, the kinetic data obtained for the purified recombinant mouse protein were found to be similar [90].

Interestingly, a 4 to 5-fold higher K_m for the methyl ester of LTA_4 (LTA_4-ME) was observed for both human [107] and mouse [90] LTC4S, whereas catalytic conversion of the methyl ester was found to be 4-fold increased at the same time suggesting that LTA_4-ME represents an almost as good substrate as the free acid of LTA_4 (LTA_4-FA). Due to these properties the inhibitory effects of the natural product LTC_4 could be determined which revealed a competitive inhibition of the enzyme with an IC_{50} of 2.1 μM [107].

Structure/function relationships of LTC4S

In 1994, two groups independently succeeded in the molecular cloning of the cDNA of LTC4S [89,204]. The deduced 150 amino acid (aa) polypeptide sequence with a calculated molecular weight of 16.6 kDa and an isoelectrical point (pI) of 11.1 contains one putative N-glycosylation site and two potential protein kinase C (PKC) phosphorylation sites. The deduced aa sequence reveals 31% overall aa identity with FLAP. The identity at the N-terminal two-thirds of these proteins is 44% and includes the putative FLAP inhibitor binding site. Furthermore, the secondary structure analysis of the deduced polypeptide sequence of LTC4S indicates an almost identical hydropathy profile and similar topology compared with FLAP. These features of LTC4S are consistent with reports of down-regulation of the enzyme by PKC-activation and the ability of the FLAP inhibitor MK-886 to inhibit LTC4S activity. [1,58,80,170,192].

Mouse LTC4S also encodes for a 150 aa polypeptide and the deduced aa sequence is 88% identical to the human counterpart. Half of the 18 different aa residues in mouse LTC4S

reside in the C-terminus. However, the putative FLAP inhibitor binding domain, the potential N-glycosylation site, and the two PKC consensus sites are completely conserved in mouse LTC4S. Kinetic studies revealed that the differences in the aa residues do not significantly affect mouse LTC4S enzymatic function [89,90,204].

To elucidate the involvement of certain aa residues in substrate binding and catalysis, site-directed mutagenesis was carried out [91]. These studies revealed that Arg-51 and Tyr-93 act to provide acid and base catalysis for opening of the epoxide ring of LTA_4 (Arg-51) and stabilization of the thiolate anion of GSH (Tyr-93) to allow for conjugation (Figure 2). In support of the interspecies data indicating that the C-terminus is not important for enzymatic function it was furthermore demonstrated that deletion mutation of the 14 terminal C-terminal residues had no effect on kinetic parameters, proving that this region both lacks catalytic residues and does not play a role in structural integrity of the bioactive protein [91].

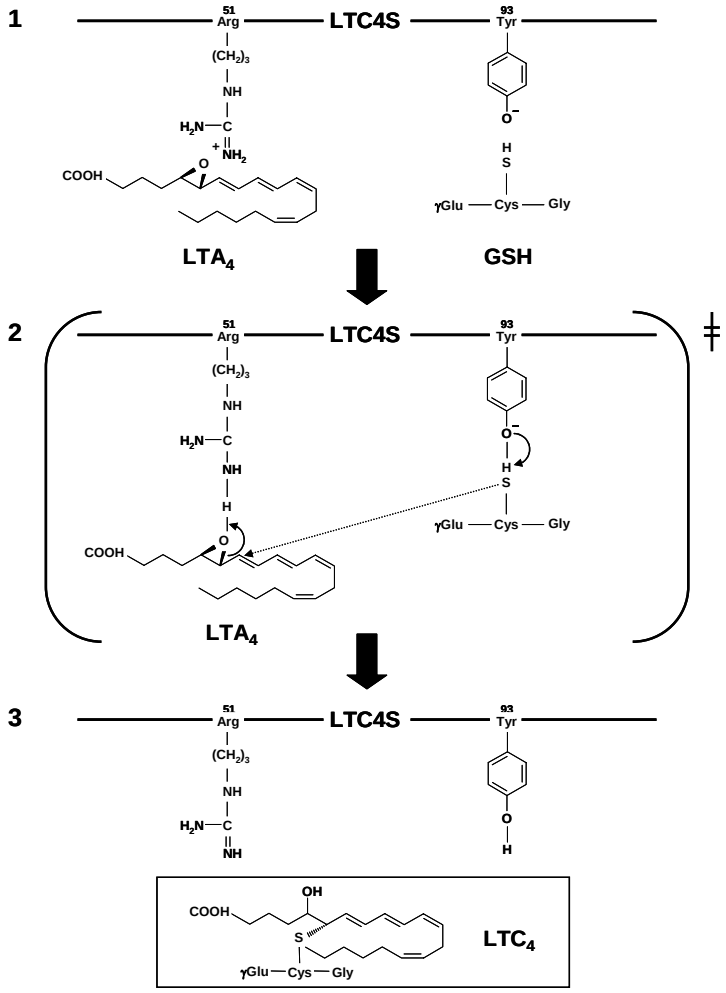

Figure 2: Proposed transition state for the LTC_4 conjugation reaction, with Arg51 as an acid catalyst for the epoxide ring opening and Tyr93 as a base catalyst for thiolate formation on GSH. Modified after [91].

The LTC4S gene

It took until 1996 before the human LTC4S gene was cloned and its complete nucleotide sequence was determined [18,145]. The gene spans 2.51 kilo bases (kb) and contains five small exons - 136, 100, 71, 82, 257 base pairs (bp) - interspersed with four introns. 5´ extension analysis of KG-1 cell mRNA revealed three putative transcription initiation sites in the human LTC_4 synthase gene, located 66, 69, and 96 nucleotides upstream of the ATG translation start site. The 5´ flanking region of human LTC4S gene contains typical features of genes that have been identified with multiple transcription initiation sites - a high G/C content and at least one consensus sequence for stimulating protein 1 (Sp1) binding 18 nucleotides upstream of the first transcription start site.

The 2.0 kb gene for mouse LTC4S exhibis an intron/exon organization identical to the human gene. The 5´ flanking sequence of the mouse LTC4S gene, however, contains only one transcription initiation site 64 bp upstream of the ATG start site and consensus transcription factor binding motifs for AP (activating protein)-2, CEBP (CCAAT/enhancer-binding protein), and PEA (polyomavirus enhancer A-binding protein)-3 [146].

Fluorescent *in situ* hybridization (FISH) with the P1 plasmid containing the gene for LTC4S localized this gene to the long arm of human chromosome 5, an area that corresponds to band 5q35 [18,145]. The LTC4S gene is located distal to the gene cluster for cytokines central to the Th2 cell phenotype and to the genes implicated in allergic inflammation [19,197]. This chromosomal organization is mimicked in mouse by an orthologous location of the LTC4S gene on mouse chromosome 11 [146].

Transcriptional regulation of LTC4S

Promoter analysis of the 2.1 kb 5´ flanking sequence of the human LTC4S gene revealed the ability of the proximal 226 bp genomic fragment (nucleotides 228 to 3 upstream of the ATG translation start site) to induce the expression of a reporter gene product in LTC4S expressing THP-1 cells [216] but not in K-562 cells, which do not express the gene. Deletional and mutational analysis of the 226 bp fragment showed the presence of a non-specific basal promoter region between nucleotides 122 and 56 upstream of the ATG translation start site and a cell-specific region further upstream. The non-specific region contains a Sp1 binding motif (GC box, -120 to -115) and an initiator element (CAGAC, -66 to

-62). This Sp-1 site binds Sp-1 and Sp-3 and is critical for transcription of the reporter gene. Mutation of a tandem CACCC motif (nucleotides -149 to -145 and -139 to -135) of the cell specific region reduced the expression of the reporter gene. Mutation of the CACCC motif also reduced the ability of both Sp1 and Kruppel-like factor Zf9/CPBP (core promoter-binding protein) to transactivate the reporter gene implying that these transcription factors are involved in cell-specific regulation of the LTC4S gene [216]. A single nucleotide polymorphism (SNP) at nucleotide -444(A-C) was reported to be higher in Polish patients with aspirin-intolerant asthma [160] as well as in African Americans with asthma and/or atopy [120], which is supported by functional data demonstrating an additional transcription factor binding site that increases the transcription rate of the gene [161]. In contrast, no such association was detected in American, Australian, Spanish, or Japanese populations [70,81,82,199]. In addition, this SNP did not determine the responsiveness to leukotriene antagonists in asthmatics. Recently, another SNP at nucleotide –1702 (G-A) was identified [163], however, no significant role for this polymorphism in asthma susceptibility in a Caucasian population as well as in Korean patients with aspirin-intolerant asthma was found [31,163]. In addition, a SNP at nucleotide 4 (G-A) in the first exon of the LTC4S gene was detected in 5 out of 141 allergic patients but in none of 110 non-allergic controls, suggesting that this polymorphism may be related to allergic diseases [213].

Although not analyzed by reporter gene assay, consensus PKC-responsive elements for AP-1 and AP-2 at nucleotides -807 and -877 upstream of the first ATG transcription initiation site may also be involved in the transcription regulation of the LTC4S gene as the induction of its enzyme activity has been reported in human erythroleukemia cells after treatment with phorbol myristate acetate (PMA) [177]. In addition, up-regulation of LTC4S has been reported in THP-1 cells treated with transforming growth factor (TGF)-β [154] and in *in vitro* derived human eosinophils developed with IL-3 and IL-5 [21].

Human mast cells derived in culture from cord blood mononuclear cells with stem cell factor (SCF), interleukin (IL)-6 and IL-10, washed and sustained with cytoprotective SCF reveal a remarkable induction of LTC4S by IL-4. Increased expression is apparent for the transcript, the protein, and for subcellular function [66]. The integrated response to cross linking of the membrane receptor for immunoglobulin (Ig)E, FcεR1, also increased more than 20-fold compared with cells maintained in SCF alone. In addition, a signal transducer activator of transcription (STAT)-6 binding motif has been identified 1.9 kb upstream of the ATG start site.

Mice deficient in LTC4S develop normally and are fertile [78]. Disruption of the murine LTC4S gene abrogated the ability of the strain-derived mast cells, obtained by bone marrow culture with IL-3, to express LTC4S transcript, protein, and function. Disruption of the gene vastly reduced or eliminated the capacity of various tissues to conjugate LTA_4-ME with GSH to form LTC_4-ME establishing LTC4S as the dominant enzyme in this pro-inflammatory pathway. Only the testis revealed normal LTA_4-ME conjugating activity, likely reflecting the activities of other GST. Bone marrow-derived mast cells from $LTC4S^{-/-}$ animals did not synthesize LTC_4 in response to IgE-dependent activation but generated normal amounts of PGD_2 and LTB_4 and also exhibited normal degranulation responses. There was an increase in 6-*trans*-LTB_4 diastereoisomers but not 5-hydroxyeicosatetraenoic acid (5-HETE) indicating decay of unused LTA_4 rather than shunting to the LTA4H pathway. In addition, a 50% reduction in IgE mediated passive cutaneous anaphylaxis of the ear in $LTC4S^{-/-}$ animals was observed revealing a prominent role of the cys-LT in augmenting mast cell dependent permeability [78]. Furthermore, substantial suppression of the increment in intraperitoneal permeability elicited by the injection of zymosan through its interaction with the monocyte/macrophage β-glucan receptor was found. These observations indicate that LTC4S is the major LTC_4-producing enzyme in tissues whose function includes mediation of the increase in vascular permeability in innate or adaptive immune responses.

Interestingly, two case studies in human new-born infants linked a deficiency in LTC_4 synthesis also with a fatal neurometabolic disorder [110,111].

LTC4S isoenzymes within the MAPEG protein family

Homology analysis of the nucleotide and the deduced aa sequence of LTC4S did not reveal a relationship with any member of the families of the cytosolic, mitochondrial or microsomal GST. However, a significant homology in the protein sequence was found for FLAP as outlined earlier in this thesis. Based on this homology four other related proteins were identified and termed MGST1 [43,121], MGST2 [73,165], MGST3 [74], and microsomal prostaglandin E Synthase (mPGES-1) [75,147]. Due to the functional properties of its members this protein superfamily was designated MAPEG (**M**embrane **A**ssociated **P**roteins in **E**icosanoid and **G**lutathione metabolism) [76]. The MAPEG members are all 16-18 kDa membrane-bound proteins with similar hydropathy profiles indicating 3-4 membrane-

spanning regions with similar topology. Multiple sequence alignment of the human representatives demonstrates six strictly conserved aa residues [22,76]. In addition to the six human proteins, several nonvertebrate members have also been identified in plant (i.e. Arabidopsis thaliana, Oryza sativa, and Ricinus communis), fungi (Aspergillus nidulans) and bacteria (Synechosystis, Escherichia coli, and Vibrio cholerae) [22,76].

MGST1 is involved in the cellular defence against harmful xenobiotics as well as metabolites produced as a consequence of oxidative stress. Substrates for its GST activity are lipophilic and electrophilic compounds with potential carcinogenic properties [6,122,124]. In contrast, LTA_4 and other epoxides are poor substrates for MGST1 [123,175]. Besides, MGST1 also possesses GSH-dependent peroxidase activity. Interestingly, MGST1 can physically interact with LTC4S and tightly bind LTC_4 [13], indicating an important role of MGST1 in the intracellular management of LTC_4.

mPGES-1 is a terminal isoenzyme involved in PGE_2 biosynthesis. In addition to its GSH-dependent PGES activity, mPGES-1 was found to have GSH-dependent peroxidase and GST activity, albeit at a low level [191]. Expression of the enzyme is up-regulated by pro-inflammatory stimuli and down-regulated by glucocorticosteroids, often in parallel with cyclooxygenase type 2 (COX-2) [60,93,106,125,181,190]. mPGES-1 is functionally coupled to COX-2 in marked preference to COX-1 [60,93,181,190,191] even though COX-1/mPGES-1 coupling may occur in the event of an increased supply of AA [125]. Expression of mPGES-1 has been reported to be associated with a large variety of physiologic and pathophysiologic conditions such as reproduction, fever, pain, inflammation, and various malignancies [35,36,183,193,198,211,214].

Microsomal glutathione S-transferase type 2 (MGST2)

Human MGST2 cDNA encodes a polypeptide of 147 aa residues with a predicted molecular mass of 16.6 kDa and a calculated pI of 10.4 [73]. The primary structure is 44% identical with LTC4S and 33% identical to FLAP [73]. A strictly preserved region, the so called “FERV” motif at position 46-49, constitutes MGST2, LTC4S, and FLAP as a subfamily within MAPEG. The chromosomal localization of MGST2 was determined to 4q28-31 by using FISH. High levels of human MGST2 mRNA as a ~ 0.6 kb transcript have been traced by Northern blot in liver, spleen, skeletal muscle, heart, adrenal gland, pancreas, prostate, and testis. Low levels were detected in lung, brain, placenta, and bone marrow. By

using a specific polyclonal antibody MGST2 protein has been found in liver, endothelial cells, and, albeit to a lesser extent, in lung. The limited tissue expression of the protein suggests posttranscriptional regulation of the enzyme. MGST2 displays diverse enzymatic functions. Like LTC4S, MGST has been found to catalyze the conjugation of LTA_4 with GSH. The apparent K_m value for recombinant human MGST2 and LTC4S expressed in Spodoptera frugiperda (Sf-9) insect cells were determined to be 41 μM and 7 μM, respectively [73]. In contrast to LTC4S, conversion of the free acid of LTA_4 is favoured over LTA_4-ME yielding up to 28% more product formation [171]. The apparent value of $V \times K_m^{-1}$ – the specificity constant $k_{cat} \times K_m^{-1}$ could not be performed as the amounts of enzyme were not known – for LTA_4-FA was almost four times as high as the value for LTA_4-ME. LTC_4 formation by recombinant human MGST2 was effectively inhibited by the product LTC_4 with an IC_{50} of ~ 1 μM [171]. Meanwhile, the two other cys-LT, LTD_4 and LTE_4, proved to be much weaker inhibitors with apparent IC_{50} values of 16 μM and 17 μM, respectively [171]. Finally, comparative studies in human umbilical vein endothelial cells (HUVEC) have shown that the main, if not only, LTC_4 producing enzyme in these cells is MGST2, indicating that this enzyme plays a critical role in transcellular biosynthesis of LTC_4 in the vascular wall [165,171]. In addition to its LTC4S activity, MGST2 also conjugates the xenobiotic substrate 1-chloro-2,4-dinitrobenzene (CDNB) with GSH [73]. Moreover, the enzyme also displays GSH-dependent peroxidase activity as shown by its ability to reduce 5-HPETE to 5-HETE with an apparent K_m of 7 μM [74].

Microsomal glutathione S-transferase type 3

MGST3 represents another human member of the MAPEG superfamily. Human MGST3 contains 152 aa residues with a predicted molecular mass of 16.5 kDa and a calculated pI of 10.2 [74]. The aa identity amounts to 27% and 36% to LTC4S and MGST2, respectively. The aa 31-38 are highly conserved with respect to MGST2 and further comparison with LTC4S identifies a common second PKC phosphorylation motif at position 110-112, which is not present in MGST2 [76]. However, at the site of the "FERV" motif in the MGST2/LTC4S/FLAP subfamily MGST3 and its subfamily features the pattern "FNCx1QRx2H", where x1 stands for a hydrophobic residue and x2 for a small aa. The human MGST3 gene has been mapped on the 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 [74]. Expression of MGST3 mRNA is barely, if at all, detectable in lung, thymus and peripheral leukocytes. Unfortunately, no suitable antiserum against MGST3 is available so far thus excluding studies of the corresponding protein expression. Microsomal preparations from Sf-9 insect cells expressing human recombinant MGST3 were found to produce 998 ± 296 pmol of LTC_4 × 15 min^{-1} × mg^{-1} [74]. In addition, this enzyme also displayed significant peroxidase activity mounting to 5 nmol × min^{-1} × mg^{-1} with an apparent K_m for 5-HPETE of 21 μM [74]. In contrast to MGST2, MGST3 was not found to conjugate CDNB with GSH with or without N-ethylmalemide (NEM), thus excluding GST activity of this protein [74].

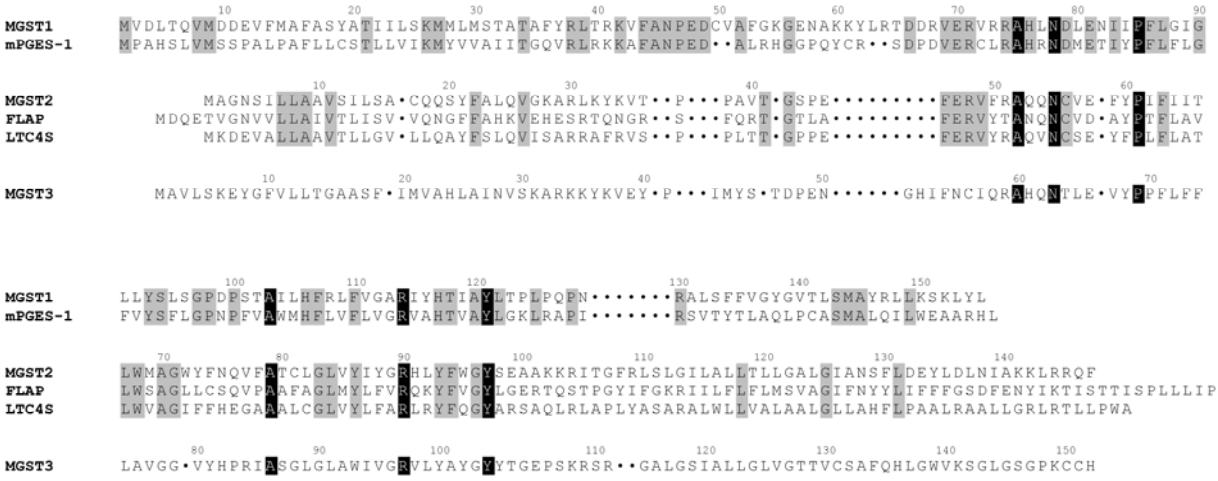

Figure 3: Multiple sequence alignment of the human MAPEG members. Strictly conserved residues are marked against a black background, conserved residues in one of the subfamilies against a grey background.

Cysteinyl leukotriene receptors

The biological responses elicited by cys-LT are signalled via specific stereoselective interaction with membrane-bound G protein-coupled receptors (GPCR) of the rhodopsin subfamily, termed as cysteinyl leukotriene receptors (CysLT) [37]. These receptors contain seven putative transmembrane-spanning domains with an extracellular amino terminus and an intracellular carboxy terminus. High-affinity ligand binding results in membrane-bound receptor conformational changes followed by activation of G protein GDP–GTP exchange, hydrolysis of GTP and intracellular signalling events [94].

Prior to the molecular identification of $CysLT_1$ and $CysLT_2$, receptor mediated effects have been described for various mammalian tissues, including man [37,118]. Many of those early studies examined the binding of radiolabelled LTD_4 to guinea pig lung membranes due to the high expression of LTD_4 binding sites on this tissue [138,148]. By applying a photoaffinity-labelled LTD_4 analog the guinea pig lung membrane CysLT was identified as a 45 kDa glycosylated protein and a 35 kDa non-glycosylated CysLT [117]. Specific cys-LT binding sites have also been reported on guinea pig ileum, uterus, and myocardium as well as on human lung, sheep lung, rat lung, and rat brain [37]. Initial binding and functional activation of human CysLT were defined in the U-937 cell line and this binding compared with that in human lung and guinea pig lung membranes [54]. The order of cys-LT potencies for competition of radiolabelled LTD_4 or agonist activation on the human $CysLT_1$ is LTD_4 > LTC_4 > LTE_4. Several structural classes of high-affinity human $CysLT_1$ antagonists were developed including three that have been shown to be clinically efficacious in chronic asthma, namely montelukast, zafirlukast, and pranlukast [57,151,182].

Functional characterization of cys-LT-induced contractile responses were also determined in tissues from a number of species including guinea pig trachea and lung, rat lung, ferret spleen, sheep bronchus and various vascular smooth muscle preparations [37]. The signal transduction pathway of CysLT activation has been primarily studied in cell lines and tissues. There, CysLT activation has been demonstrated to be predominantly coupled with mobilization of intracellular and extracellular Ca^{2+} [118]. For example, CysLT activation by LTD_4 in the human cell line THP-1 has been shown to result in an intracellular elevation of Ca^{2+} [28,38].

A second CysLT has been demonstrated on guinea pig trachea and ileum, ferret trachea and spleen, sheep bronchus, and on human pulmonary and saphenous vein preparations [37,55,85,86]. In guinea pig parenchymal preparation LTD_4 was observed to be a more potent contractile agent than LTC_4, whereas these two cys-LT possessed equal contractile potency on guinea pig trachea [29,85]. These findings indicated a predominance of the $CysLT_1$ subtype in guinea pig parenchyma and of $CysLT_2$ in the tracheal preparations. The rank order of agonist potency on the $CysLT_2$ was reported to be $LTC_4 = LTD_4 >> LTE_4$. With the exception of BAY u9773 which has been shown to antagonize both $CysLT_1$ and $CysLT_2$ functional responses, all other CysLT antagonists developed to date, selectively antagonize the $CysLT_1$ [194]. Contractions induced by LTC_4 in porcine pulmonary arteries are resistant to MK-571, a selective $CysLT_1$ antagonist, as well as to BAY u9773, suggesting

either species differences in response to these antagonists or the existence of a third CysLT [9].

There is also a large body of evidence for the existence of dualistic receptors for both cys-LT and nucleotides. Both types of mediators accumulate at the sites of inflammation, and inflammatory cells often co-express both P2Y and CysLT receptors. In rat microglia, the immune cells of the brain involved in the response to cerebral hypoxia and trauma, activation of $P2Y_1$ and CysLT receptors mediate co-release of nucleotides and cys-LT [10], which might, in turn, contribute to neuroinflammation and neurodegeneration. In human monocyte/macrophage-like cells, $CysLT_1$ receptor function is regulated by extracellular nucleotides via heterologous desensitization [27], and, in the same cells, montelukast and pranlukast, two selective $CysLT_1$ receptor antagonists [23], functionally interact with P2Y receptor signalling pathways [105]. Moreover, there are close structural and phylogenetic relationships between P2Y and CysLT receptors, which cluster together into the 'purine receptor cluster' of the rhodopsin family of GPCR, which also includes a large number of orphan GPCR [53]. Mellor *et al.* proposed that, in human mast cells, both the $CysLT_1$ receptor and a yet unidentified elusive receptor up-regulated by treatment with the pro-inflammatory cytokine IL-4 were responsive to both cys-LT and UDP [113]. Indeed, GPR17, a G_i protein-coupled orphan receptor at intermediate phylogenetic position between P2Y and CysLT receptors, was only recently shown to be specifically activated by both families of endogenous ligands, leading to both adenyl cyclase inhibition and intracellular calcium increase.

Cysteinyl leukotriene receptor 1 ($CysLT_1$)

The $CysLT_1$ has been cloned and characterized from human and mouse [99,162]. The human cDNA encodes a 337 aa protein with a calculated molecular weight of 38.5 kDa. The gene has been mapped by *in situ* hybridization on chromosome X (Xq13-21). Two isoforms of mouse LTC4S have been reported, a short splice variant consisting of 339 aa residues and a long variant encoding a protein extending 13 aa at the N-terminus. The shorter mouse variant is 87% identical to the human enzyme at the aa level.

The cRNA for the human $CysLT_1$ was expressed in *Xenopus* oocytes and dose-dependent LTD_4 activation of a Ca^{2+}-activated chloride channel was blocked by the selective $CysLT_1$ antagonist MK-571 [99]. In both the *Xenopus* melanophore signalling assay, and in a

human embryonic kidney (HEK-293T) cell calcium flux assay, the $CysLT_1$ did not couple via $G_{\alpha i}$ [99,162]. In monkey kidney Cos-7 cells expressing the $CysLT_1$, LTD_4 and LTC_4 activated calcium flux that was blocked by MK-571 [99]. In HEK-293T cells expressing $CysLT_1$, the agonist potency for calcium flux activation was $LTD_4 > LTC_4 > LTE_4$ with EC_{50} values of 2.5 nM, 24 nM, and 240 nM, respectively, whereas LTE_4 was also shown to be a partial agonist [162]. In addition, in HEK-293T cells expressing the CysLT1 receptor, LTD_4-induced Ca^{2+} mobilization was inhibited by structurally distinct $CysLT_1$ receptor antagonists in the order of potency pranlukast = zafirlukast > montelukast > pobilukast [162].

Human $CysLT_1$ mRNA was found to be expressed in spleen, peripheral blood leukocytes and to a lesser extent in lung, small intestine, pancreas and placenta [102,109]. Expression of the human $CysLT_1$ mRNA and protein was also shown in HUVEC [171] as well as in normal peripheral blood eosinophils [50,135], subsets of monocytes [172], macrophages, basophils, and in pre-granulocytic $CD34^+$ cells [50]. In cell lines, $CysLT_1$ mRNA could be detected in human monomyelocytic U-937 and HL-60 cells with an increase observed in DMSO-differentiated HL-60 cells [102].

Cysteinyl leukotriene receptor 2 ($CysLT_2$)

The open reading frame of the human $CysLT_2$ encodes a 347 aa protein, while both rat $CysLT_2$ and mouse $CysLT_2$ deduced polypeptides contain 309 aa, truncated by 16 and 21 residues at the N- and C-termini, respectively. The homology between human and rat, and human and mouse $CysLT_2$ is 73% and 65%, respectively. In contrast, the identity of the human subtypes $CysLT_1$ and $CysLT_2$ amounts to only 38%. The human $CysLT_2$ arises from a single exon gene on human chromosome 13q14, a region that has been identified by several groups as a polygenic asthma linkage [41,84]. The human $CysLT_2$ mRNA is expressed in peripheral blood leukocytes, lymph nodes, spleen, heart, and several regions of the central nervous system (CNS) [62,131,185], a tissue distribution similar to that reported for the mouse $CysLT_2$ [68]. *In situ* hybridization localized $CysLT_2$ mRNA also to the adrenal medulla, as well as the cardiac Purkinje cells. Particularly strong hybridization was noted in interstitial macrophages in the lung [62]. Coronary smooth muscle cells [77] express high levels of $CysLT_2$ mRNA and protein, suggesting a prominent function for this receptor in vascular responses. A SNP in human $CysLT_2$ was reported characterized by the substitution of methionine for valine at aa residue 202. The resultant polymorphic receptor, when cloned

and expressed heterologously, binds to LTD_4 and the receptor-selective partial agonist, BAY u9773, at 4-fold lower affinity than the wild-type receptor [189]. The presence of this polymorphic $CysLT_2$ variant conferred a substantially increased risk of atopy in inhabitants of Tristan da Cunha, a population characterized by both, a founder effect and a high prevalence of atopy. The incompletely overlapping distributions and distinct ligand-binding properties of $CysLT_1$ and $CysLT_2$ strongly suggest that they serve different functions *in vivo*.

GPR17

GPR17 has been cloned and characterized from human, rat, and mouse [32,200]. The human cDNA encodes a 339 aa protein with a calculated molecular weight of 37.9 kDa and a calculated pI of 9.4. The human receptor displays an 89.7% and 90.3% aa identity with rat and mouse GPR17, respectively. Both human and rat GPR17 were found to be highly expressed in the brain as well as in other organs typically undergoing ischemic damage such as kidney and heart with only little expression in the liver and lung. In addition, human GPR17 was also found in HUVEC and breast adenocarcinoma MCF7 cells, whereas no signal was found in human peripheral blood mononuclear cells, bronchial smooth muscle cells, myeloid U-937, hepatocellular carcinoma Hep-G2, cervix carcinoma (HeLa) and neuroblastoma SK-N-BE cells. In 1321N1 cells transfected with human GPR17 this receptor induced the appearance of concentration-dependent responses to LTC_4 and LTD_4 as well as to UDP, UDP-glucose and UDP-galactose. The agonist potency for calcium flux activation was LTC_4 >> LTD_4 with EC_{50} values of 0.3 nM and 7.2 nM, respectively. The respective EC_{50} values for adenyl cyclase inhibition by UDP, UDP-glucose and UDP-galactose were 1.1 nM, 12.0 nM and 1.1 nM, respectively. However, at the rat receptor UDP-glucose was found to be more potent than UDP, while UDP-galactose had no effect. In addition, the relative potencies of the cys-LT LTC_4 and LTD_4 were inverted in 1321N1 cells expressing rat GPR17. The $CysLT_1$ antagonists montelukast and pranlukast concentration-dependently inhibited the activation of both, the human and rat receptor by 100 nM LTD_4 with IC_{50} values in the double digit nanomolar range and similar potencies. Conversely, IC_{50} values for cangrelor, a $P2Y_{12}/P2Y_{13}$ antagonist, and the $P2Y_1$ receptor antagonist MRS2179 on human GPR17 were determined to be in the nanomolar range. These antagonists exhibited higher affinity on the rat receptor with IC_{50} values in the picomolar range. Moreover, MRS2179 was more potent than cangrelor. *In vivo* inhibition of GPR17 dramatically reduced ischemic damage in a rat

focal ischemia model, suggesting GPR17 as the common molecular target mediating brain damage by uracil nucleotides and cys-LT. However, further studies are mandatory to determine the precise role of this novel class of dual uracil nucleotides/CysLT receptors.

Cysteinyl leukotrienes and their receptors in pathophysiology

Allergy and asthma

Cys-LT have long been recognized for their powerful bronchoconstricting effects and their role in asthma. Dahlén and his coworkers were the first to report that administration of LTC_4 and LTD_4 constricted isolated bronchial tissues obtained from apparently normal lung tissue surrounding surgical specimens removed because of lung cancer [39,40,173]. They demonstrated that LTC_4 and LTD_4 at concentrations of 1 nM elicited an overall contractile effect similar to that observed after exposure to 1000 nM histamine, thereby establishing a potency ratio in this preparation of 1:1.000. In accordance, inhalation of an aerosol of LTD_4 was found to be about 1200-fold more potent than histamine as a bronchoconstrictor agonist [14]. Cys-LT are present in bronchoalveolar lavage (BAL) fluid collected from atopic human subjects after endobronchial challenge with allergen [205]. Increased urinary excretion of LTE_4 reflecting elevated cys-LT release are found both, in acute asthmatic exacerbations and aspirin-intolerant asthma [45,65,186]. Both, the 5-LO inhibitor zileuton [71] as well as $CysLT_1$-selective antagonists [5,59] have been demonstrated to be clinically efficacious in the treatment of asthma. The $CysLT_1$ antagonist pranlukast substantially attenuated both the early- and late-phase decrements in airflow in allergic asthmatic individuals challenged with allergen [59]. Oral $CysLT_1$ antagonists also attenuate bronchoconstrictive responses to challenges with exercise [116], cold air [152], and inhalation of adenosine [157]. Intravenous administration of montelukast, another $CysLT_1$-selective antagonist, substantially increased measures of airflow compared with placebo in a group of patients with acute asthmatic exacerbation who also received standard treatment with bronchodilators and glucocorticosteroids [26]. Taken together, these observations confirm the involvement of cys-LT in the development of airflow obstruction in both, experimentally induced and naturally occurring asthma in humans as well as the prominent role for $CysLT_1$ in acute exacerbations.

Inflammatory processes

Recruitment of effector leukocytes

In addition to bronchoconstriction, endobronchial instillation of LTE_4 into human subjects resulted in the subsequent influx of eosinophils and to a lesser extent, neutrophils, into the BAL fluid [87], suggesting the capacity for cys-LT to either directly or indirectly attract leukocytes to initiate inflammatory responses *in vivo*. Furthermore, cys-LT induced transendothelial migration of human $CD34^+$ peripheral blood-derived progenitor cells which are only equipped with $CysLT_1$ [15]. This suggests a prominent role for $CysLT_1$ in the regulation of hemopoietic progenitor cell trafficking. Interestingly, while cys-LT-mediated Ca^{2+} fluxes were completely blocked by pretreatment of the $CD34^+$ progenitor cells with montelukast, this $CysLT_1$ antagonist failed to block calcium fluxes in mature peripheral blood leukocytes, indicating that $CysLT_2$ or another, yet undefined receptor is acquired by leukocytes during hemopoietic development *in vivo*. Priming of human peripheral blood monocytes or monocyte-derived macrophages with recombinant human IL-4 or IL-13 increased their levels of $CysLT_1$ mRNA and protein expression as well as their chemotactic responses to LTD_4 *in vitro* [188]. Similarly, expression of $CysLT_1$ as well as chemotaxis to LTD_4 in an eosinophilic subline of HL-60 cells were enhanced by priming with recombinant IL-5 [187]. Both, $CysLT_1$ and $CysLT_2$ were localized to eosinophils, mononuclear cells, and resident mast cells in nasal biopsy tissue from humans with seasonal allergic rhinitis [51]. In another study, expression of $CysLT_1$ was up-regulated on $CD45^+$ leukocytes in nasal biopsy specimens obtained from subjects with aspirin-sensitive chronic rhinitis and nasal polyposis, compared with the same leukocyte subsets in biopsy specimens from non-aspirin-sensitive subjects with rhinitis and nasal polyposis [179]. In conclusion, these observations support the hypothesis that cys-LT may serve as chemotactic mediators and/or activating ligands for human effector leukocytes, and that the CysLT expression profiles of certain leukocytes are modified by cytokines and other mediators of inflammation produced in the microenvironment. *In vitro* studies support the capacity for cys-LT to serve as eosinophil chemoattractants via a $CysLT_1$-dependent mechanism [52].

Induction of cytokine generation by eosinophils and mast cells

Human eosinophils derived *in vitro* from umbilical cord blood mononuclear cells secrete IL-4 in response to cys-LT in a $CysLT_1$-dependent manner [11]. This response was also observed in permeabilized eosinophils, albeit in a profile not compatible with the function of either known CysLT receptor [12]. Moreover, the pretreatment of peripheral blood eosinophils with MK-886, an inhibitor of both FLAP and LTC4S, or with the 5-LO inhibitor AA-861, inhibited their release of IL-4 in response to other transmembrane stimuli, such as IL-16, RANTES, or eotaxin indicating an intracrine role for the cys-LT acting through an intracellular receptor in eosinophils. Indeed, a nuclear $CysLT_1$ receptor has recently been described in colorectal adenocarcinoma cells where it triggers a proliferative ERK 1/2 signal [130].

LTC_4 and LTD_4-mediated, PTX-resistant, calcium fluxes in human cord blood-derived mast cells expressing both CysLT [113,115] were completely blocked by pretreatment with MK-571, indicating the absolute requirement for $CysLT_1$ in this response. IL-4 primed mast cells markedly shifted their dose-response curve to LTC_4 compared with LTD_4, but did not alter $CysLT_1$ mRNA or cell surface protein expression [113], and only modestly up-regulated cell surface $CysLT_2$ protein [179]. Unexpectedly, IL-4 also markedly enhanced calcium fluxes in response to UDP, a nucleotide ligand previously thought to be specific for a purinergic receptor termed $P2Y_6$ [104]. The calcium flux in response to UDP was MK-571-sensitive and was cross-desensitized with LTC_4, but only incompletely with LTD_4. Chinese hamster ovary (CHO) cells stably expressing the long isoform of the mouse $CysLT_1$ receptor also respond to UDP with an MK-571-sensitive calcium flux [113]. IL-4-primed mast cells, but not unprimed replicates, secrete IL-5, TNF-α, and MIP-1β when stimulated with either LTC_4, LTD_4, or UDP [114,115]. Inhibition of $CysLT_1$ by MK-571 or of endogenous cys-LT production by MK-886 significantly attenuated the generation of IL-5 and TNF-α by mast cells activated by FcεRI cross-linkage [114]. These studies indicate that $CysLT_1$ and $CysLT_2$ have distinct yet complementary functions for mast cells, permitting cytokine generation through both autocrine and paracrine mechanisms. IL-4 could act by inducing the expression of an MK-571-sensitive receptor for both LTC_4 and UDP, or could alter the sensitivity and ligand specificity of constitutively expressed $CysLT_1$ by inducing posttranslational modification or heterodimerization with another receptor. Moreover, the induction of IL-5 generation by mast cells is an additional mechanism by which cys-LT could promote eosinophilia.

Dendritic cell maturation, phenotype, and activation

Initial recognition that cys-LT are involved in the maturation of dentritic cells (DC) emerged from studies of DC migration in mice lacking multi-drug receptor protein 1 (MRP1), which is required for export of LTC_4 to the extracellular space [95,206]. In a model of contact hypersensitivity induced by topical application of fluorescein isothiocyanate (FITC), DC migration was substantially attenuated in MRP1-deficient mice as compared with that observed in wild-type controls [156]. The migration defect could be reversed by the injection of LTC_4 or LTD_4 after FITC application. Migration in wild-type controls was blocked by local injection of MK-571, which acts as an antagonist of MRP1 at 25-fold higher doses than those required to block $CysLT_1$ [156]. In addition, DC from MRP1-deficient mice showed deficient *ex vivo* chemotactic responses to CCL19, a chemokine ligand specific for CCR7, which again was corrected by LTC_4 and LTD_4. In a subsequent study, myeloid DC cultured *ex vivo* from mouse bone marrow were found to express $CysLT_1$ receptor mRNA, as well as mRNA encoding 5-LO, FLAP, and LTC4S [100]. These DC generated both IL-10 and IL-12 when challenged *ex vivo* with the dust mite allergen Der f. Treatment of these DC with several $CysLT_1$-selective antagonists significantly blunted their Der f-mediated production of IL-10, but further enhanced formation of the Th1 cytokine IL-12. Moreover, whereas exogenous LTC_4, LTD_4, and LTE_4 failed to directly elicit IL-10 or IL-12 generation by otherwise unstimulated DC, each cys-LT amplified the Der f-mediated production of IL-10 and inhibited the generation of IL-12. Mice that received intranasal adoptive transfer of Der f-pulsed DC which had been costimulated with LTD_4 *ex vivo* developed enhanced eosinophilia and increased BAL fluid levels of IL-5 after an inhalation challenge with Der f compared with eosinophil counts and IL-5 levels in mice which received transfer of DC that had been stimulated with Der f alone. Finally, mice that received Der f-pulsed DC treated with pranlukast *ex vivo* and then challenged with Der f developed strikingly diminished BAL fluid levels of eosinophilia and IL-5 compared with the other groups. In addition to this report demonstrating that $CysLT_1$-selective antagonists can modify the cytokine profile of DC *in vitro,* a recent study by Okunishi and coworkers examined the role of cys-LT on DC function with the focus on its regulation in modulating immune response [135]. In a mouse model of asthma cys-LT were shown to boost both antigen-presenting capacity and cytokine production of DC. The $CysLT_1$-selective antagonists pranlukast and montelukast strongly suppressed these DC functions and subsequently led to an inhibition of Th1 and Th2 responses in the early stage of immune response. Moreover, treatment with pranlukast and montelukast

potently suppressed the development of antigen inhalation-induced eosinophilic airway inflammation, mucus production, and airway hyperreactivity *in vivo* during the early stage of immune response. This indicates that treatment with $CysLT_1$-selective antagonists in the early stage of sensitization may prevent development of various characteristic features of asthma via potently inhibiting DC function. These effects might be exhibited by up-regulation of PGE_2 since treatment with $CysLT_1$-selective antagonists increased biosynthesis of PGE_2 in the lung whereas pre-treatment with the COX-inhibitor indomethacin not only significantly decreased PGE_2 production but also reversed the allergic responses suppressed by pranlukast and montelukast including $CD4^+$ T cell and DC activation.

Allergen-induced pulmonary inflammation

Increased levels of LTC_4 and LTB_4 of up to 5 times were observed in BALB/c mice sensitized with an intraperitoneal injection of ovalbumin precipitated with aluminium and subsequently challenged with intranasal administration of ovalbumin for 24 h [63]. These increases were associated with widespread mucus occlusion of the airways, influx of predominantly eosinophils in airway tissues and BAL fluid, and airway hyperresponsiveness to intravenous methacholine. Intraperitoneal administration of the cys-LT inhibitors zileuton and MK-886 30 min before nasal allergen challenge reduced eosinophil infiltration in tissues and BAL fluid by ~ 85% and significantly blocked airway mucus release and plugging. In a model of chronic asthma, treatment with montelukast significantly reduced the airway eosinophil infiltration, mucus plugging, collagen deposition, and smooth muscle hyperplasia [64]. Levels of IL-4, IL-5, IL-10, and IL-13 mRNA in the lungs of challenged montelukast-treated mice were dramatically reduced compared with unchallenged controls. Collectively, these studies indicate that cys-LT, acting through the $CysLT_1$, initiate features of chronic inflammation and matrix remodelling in allergen-induced pulmonary disease, possibly by stimulating cytokine generation by resident inflammatory cells. The smooth muscle hyperplasia in the chronic model could also reflect direct proliferative effects, because LTD_4 is a mitogen for human bronchial smooth muscle cells when provided *in vitro* in combination with TGF-β, IL-13 [47], or epidermal growth factor [140]. However, it is noteworthy that airway hyperresponsiveness to methacholine was not affected by inhibition of cys-LT synthesis or the $CysLT_1$ receptor in either model. Indeed, mouse bronchial smooth muscle does not constrict in response to cys-LT [108,153], and the airways of transgenic mice

overexpressing the human $CysLT_1$ specifically in smooth muscle were hyperresponsive to LTD_4, but not to methacholine [212].

Microvascular permeability

Plasma protein leakage after intraperitoneal challenge with zymosan or IgE-dependent passive cutaneous anaphylaxis was reduced by ~ 50% in $LTC4S^{-/-}$ mice compared with the wild-type controls. Comparable decrements in plasma leakage were also observed in $CysLT_1$-deficient mice. These studies indicate the prominent role of cys-LT, acting through $CysLT_1$, in mediating increased vascular permeability in models of both innate and adaptive immunity.

Because LTC_4 is rapidly converted to LTD_4 in extracellular fluid by either γ-GT or the more functionally specific γ-GL, mouse strains lacking either or both enzymes were studied in the zymosan-induced model of peritonitis. Shi *et al.* showed that intraperitoneal injection of zymosan into γ-GL-deficient mice and γ-GT/γ-GL double-deficient mice led to the accumulation of LTC_4 in the peritoneal cavity, indicating that γ-GL is the enzyme responsible for the conversion of LTC_4 to LTD_4 in this model [167]. Plasma protein extravasation was not affected 1–24 h after zymosan injection, but neutrophil infiltration was significantly reduced 2–4 h after the injection in γ-GL-deficient mice indicating that extracellular conversion of LTC_4 into LTD_4 is not required for the increment of vascular permeability through $CysLT_1$; nevertheless LTD_4 may play a pivotal role in neutrophil recruitment. Surprisingly, however, neutrophil recruitment was not affected in either LTC4S- or $CysLT_1$-deficient mice subjected to the zymosan-induced peritonitis model [78,103].

Pulmonary fibrosis

Intratracheal or systemic administration of the chemotherapeutic agent bleomycin induces chronic pulmonary inflammation and fibrosis in mice. Several features of bleomycin-induced injury, including pulmonary macrophage and neutrophil recruitment, alveolar septal thickening, fibroblast accumulation, and collagen deposition were found to be significantly reduced in LTC4S-deficient mice compared with their wild-type littermates [16]. Unexpectedly, $CysLT_1$-deficient mice were not protected from this injury; instead, these mice

showed exaggerated alveolar septal thickening with reticular fibre deposition when compared with wild-type or LTC4S-deficient mice. Additionally, cys-LT levels in the BAL fluid of $CysLT_1$-deficient mice were ~ 2-fold greater than those recovered in the wild-type mice, with no change in the level of LTB_4 or PGE_2. In contrast, bleomycin-induced lung fibrosis was attenuated in $CysLT_2$-deficient mice generated by targeted gene disruption [17]. These results suggest that cys-LT are crucial to this macrophage-mediated chronic inflammatory and fibrotic insult, likely working through the $CysLT_2$.

Atherosclerosis

Human coronary arteries have been shown to be unresponsive to LTC_4 and LTD_4 [3,4]. However, cys-LT were potent constrictor agents in coronary arteries derived from patients with atherosclerosis. This vasoconstrictor effect was associated with an increased prevalence of autoradiographic [^{3}H]-LTC_4 binding sites but not those of [^{3}H]-LTD_4. This observation suggested that the induction of CysLT may be associated with this cardiovascular inflammatory disease. Expression of CysLT mRNA has also been determined in human atherosclerotic lesions which indicated that both receptor transcripts were present [180]. The relevance of cys-LT and their receptors are further discussed in the Discussion section.

Colon cancer

Cys-LT have also been shown to be released by tissues derived from patients with colon cancer [155]. In addition, exposing intestinal epithelial cells in culture to LTD_4 for extended period leads to an up-regulation of several proteins including COX-2 that have been associated with colon carcinogenesis [132,133,207]. Furthermore, $CysLT_1$ was recently reported to be overexpressed in colon tissues derived from cancer patients [129,133]. Interestingly, most studies have shown that LTC_4 did not induce the up-regulation of these proteins, and the $CysLT_1$ antagonist, ZM198615, blocked the LTD_4-induced responses. These data demonstrated that not all native ligands worked and that only a high concentration of the $CysLT_1$ antagonist blocked the induction of these proteins. While the data indirectly suggest that $CysLT_1$ was responsible for these responses, there appears to be some differences between this receptor and the classical $CysLT_1$ associated with human airway smooth muscle

contraction. The latter was activated by all native ligands and the $CysLT_1$ antagonists block the response at a 100-fold lower concentration. These variations in ligand potency suggest aa changes on the extracellular face of the receptor protein which may modulate binding of cys-LT. While $CysLT_2$ variants have recently been reported in atopic asthmatic subjects [189], there is no information as to whether CysLT variants are present in cancer cells. Further work is necessary to characterize the CysLT implicated in cellular transformation and to establish the potential role for cys-LT in colon cancer.

METHODOLOGY

The methods performed in the original articles comprise well established techniques within the research field of biochemistry and molecular biology. They are listed below as a reference to the articles in which they appear. Detailed descriptions of the applied protocols can be found in the respective method section.

Method	**Article**
Amino acid sequencing	II
Calcium mobilization	III
Cell culture	II,III
Cloning	II
Enzyme activity assays	II,IV
High-pressure liquid chromatography analysis	II,IV
In situ hybridization	I
Membrane preparation	II,IV
Northern Blot	IV
RNA-Isolation	I,II,III,IV
RT-PCR	I,II,III,IV
SDS/PAGE	II,IV
Western Blot	II,IV

AIMS OF THE PRESENT THESIS

The principle aims of the project were:

- To clone LTC4S isoenzymes from the rat.
- To characterize the structural and biochemical features of rat LTC4S isoenzymes.
- To study the tissue expression of rat LTC4S isoenzymes in the physiological state as well as in response to a systemic inflammatory situation.
- To elucidate expression, functional state and regulation of CysLT on endothelial cells.

RESULTS

Studies on molecular and catalytic properties as well as functional regulation of rat LTC4S isoenzymes

Rat LTC4S, MGST2, and MGST3 were cloned and functionally expressed in Sf-9 insect cells. The successful cloning of these known LTC4S isoenzymes not only allowed for side-by-side comparative investigations of their molecular and catalytic properties but also permitted the study of the functional regulation of these enzymes in response to a systemic stimulus *in vivo*.

Molecular and catalytic properties of rat LTC4S isoenzymes

The cloned rat LTC4S cDNA encodes a 150 aa sequence with a calculated molecular mass of 16.8 kDa and a pI of 9.69. The primary structure of the protein is 86.7% and 94.7% identical with human and mouse LTC4S, respectively. Two potential PKC phosphorylation sites are present at Ser28-Ala29-Arg30 and Ser111-Ala112-Arg113 and the putative FLAP inhibitor-binding domain is conserved between rat and mouse [89,90,204]. Here, the human enzyme differs and contains Thr41 and Tyr50 instead of Ser41 and Phe50.

Rat MGST2 contains an open reading frame encoding a polypeptide of 147 aa with a calculated molecular mass of 16.7 kDa and a pI of 9.58. The overall aa identity is 79.6% to the human protein, and 39.3% to rat LTC4S. Rat MGST3 contains 152 aa residues with a predicted molecular mass of 16.7 kDa and a pI of 9.54. Here, the primary structure is 86.2% identical to human MGST3, 29.9% to rat MGST2, and 23.4% to rat LTC4S. The LTC4S/MGST2/FLAP subgroup of the MAPEG family is characterized by the ”FERV” pattern at position 46-49, which is ”FERI” in rat MGST2. In this region MGST3 has a strikingly different structure with a ”FNCIQRAH” pattern, preceded by a four aa (NGHM) insertion.

As might be expected from the structural analogy between the human, mouse and rat LTC4S, all enzymes also show a high degree of similarity regarding their catalytic features. Apparent values of K_m and V_{max} for the recombinant rat enzyme, using LTA_4-FA and LTA_4-

ME as a substrate, were calculated to be 18.8 ± 2.9 μM and 56.2 ± 5.6 nmol × min^{-1} × mg^{-1} for LTA_4-FA, and 19.8 ± 2.5 μM and 81.3 ± 9.1 nmol × min^{-1} × mg^{-1} for LTA_4-ME, respectively. Employing the expression $V \times K_m^{-1}$ to compare the two substrates, LTA_4-ME was found to be the better substrate, which is in accordance with human LTC4S [215]. The K_m for GSH was determined to be 2.7 ± 0.3 mM and at concentrations of GSH >5 mM substrate inhibition was observed.

In contrast, rat MGST2 as its human counterpart converted LTA_4-FA more efficiently than LTA_4-ME. From incubations of rat MGST2 with LTA_4-FA K_m and V_{max} values were calculated to be 113.8 ± 33.7 μM and 2.5 ± 0.3 nmol × min^{-1} × mg^{-1}, respectively. Whereas the LTC4S capacity appeared to be preserved in rat MGST2, rat MGST3 failed to show any significant LTC4S activity. Interestingly, the important residues Arg51 and Tyr93, previously reported to function as a proton donor and base in human LTC4S [91], were conserved, suggesting a different and yet unknown function of these residues in rat MGST3.

As demonstrated for the human enzyme, rat MGST2 also displayed significant GST and peroxidase activity. Kinetic parameters for the peroxidase activity were determined from incubations of MGST2 with 5-HPETE and subsequent analysis of 5-HETE formation. Thus, values of K_m and V_{max} were calculated to be 16.8 ± 3.0 μM and 29.5 ± 1.9 nmol × min^{-1} × mg^{-1}, respectively. The ability of rat MGST2 to conjugate CDNB and GSH was calculated to 99 ± 2 nmol × min^{-1} × mg^{-1}. As for the human counterpart, no effect on GST activity could be observed by pretreatment of recombinant rat MGST2 with 1 mM NEM.

The only detectable enzyme activity for rat MGST3 was a GSH-dependent peroxidase activity versus 5-HPETE, and values for K_m and V_{max} were determined to be 31.0 ± 7.0 μM and 32.5 ± 4.0 nmol × min^{-1} × mg^{-1}, respectively.

Similar to the human orthologs, the LTC4S activity of rat LTC4S and rat MGST2 could be inhibited by LTC_4 > LTD_4 > LTE_4 in decreasing order of potency. In addition, the general GST and peroxidase activity of rat MGST2 as well as the peroxidase activity of rat MGST3 were equally sensitive to inhibition by LTC_4. Similarly, the FLAP inhibitor MK-886 was found to inhibit all enzymatic activities of the three rat proteins, i.e. LTC4S activity (LTC4S and MGST2), GSH transferase activity (MGST2), and peroxidase activity (MGST2 and MGST3). The respective IC_{50} values were calculated to be in the range of 2.3 to 3.0 μM for LTC_4 and 4.5 to 9.0 μM for MK-886 depending on the respective enzymatic activity of each specific protein.

Functional regulation of rat LTC4S, rat MGST2, and rat MGST3 in response to acute systemic inflammation

As already pointed out there is only limited data available regarding the regulation of LTC4S, mostly obtained from *in vitro* studies [66,154,216]. In addition, no such studies had been conducted on MGST2 and MGST3. The cloning and functional expression of rat LTC4S, rat MGST2, and rat MGST3 allowed for the first time side-by-side comparative investigations of all known enzymes with LTC4S activity *in vivo.* In order to mimic an acute systemic inflammation rats were injected with a single intraperitoneal dose of 2 mg × kg^{-1} lipopolysaccharide (LPS). Organ-specific expression of either of the three rat LTC4S isoenzymes on the mRNA and protein level at 1 and 6 h after injection was then compared with vehicle-treated control rats.

In unstimulated rats LTC4S mRNA was detected at high levels in ileum, lung, skeletal muscle, spleen, and colon. Low levels were found in liver and kidney, whereas LTC4S mRNA was barely measurable in heart, brain, and adrenal gland. This organ-specific expression profile is in good agreement with data obtained for the human enzyme. At the protein level, LTC4S was detectable in all tissues examined except spleen.

For rat MGST2, the mRNA expression pattern also correlated well with previous results on the human counterpart and was mainly found in liver, adrenal gland, ileum, and colon and to a minor extent also in lung and spleen. However, using a polyclonal peptide antibody, rat MGST2 protein could only be detected in liver, ileum, colon, brain, and adrenal gland but not in spleen and lung membranes, possibly due to differences in post-transcriptional regulation or an insufficient sensitivity of the immunoblot assay. Rat MGST3 was as ubiquitously expressed as in man, with strong expression in heart, liver, and the adrenal gland with the liver showing a point of difference compared with the weak expression in man. Unfortunately, MGST3 antiserum was not available so protein expression could not be analyzed.

When rats were injected intraperitoneally with LPS and studied over a 6 h period, LTC4S mRNA was transiently up-regulated within 1 h in all tissues except ileum and spleen. The strongest elevation in mRNA levels was observed in heart, brain, adrenal gland, and liver. This up-regulation was paralleled by an increase in the protein level in brain, heart, adrenal gland, and liver by 4.9-, 4.0-, 2.9-, and 2.3-fold, respectively.

In addition, a potential increase in LTC4S activity as a result of increased rat LTC4S protein was representatively screened in whole brain tissue. Indeed, an increased synthesis of

LTC4S in membrane preparations from LPS-treated rats as compared with vehicle-treated control rats was observed which, however, did not reach statistical significance. The difference between the overall increase in rat LTC4S mRNA and protein on the one hand, and the enzyme activity on the other, may be due to the fact that LTC4S activity is unstable and known to be affected by a number of factors. Therefore, activity measurements may be unreliable to use as an index of low levels of enzyme expression in complex tissues. In addition, rat MGST2 may also have accounted for some of the activity.

In contrast to rat LTC4S, expression of rat MGST2 mRNA and protein as well as rat MGST3 mRNA levels remained unchanged upon LPS stimulation over a 6 h period, suggesting that these enzymes are not primarily involved in inflammatory reactions but rather in "house-keeping" functions.

Furthermore, the cellular localization and regional distribution of rat MGST3 mRNA in the rat CNS (brain, spinal cord, and dorsal root ganglia) was investigated in detail using the *in situ* hybridization technique. Rat MGST3 mRNA seemed to be confined to the neurons. The broad distribution in the brain was characterized by a strong signal in the hippocampal formation and in the nuclei of the cranial nerves. A moderate signal was found in the cortex, thalamus, amygdala, and substantia nigra and a weak signal in the hypothalamus. When looking at the lower brainstem, spinal cord, and dorsal root ganglia, an intense MGST3 signal was found in the hindbrain reticular formation and in the motor nuclei such as the pontine gray, the nucleus of trapezoid body, and the facial nucleus. No signal could be detected in the cerebellum. In the spinal cord, the motor neurons were positive for MGST3 mRNA. In dorsal root ganglia an intense signal was found in the majority of the large neurons, while the small neurons had lower levels of MGST3 mRNA, some also being negative. No significant changes in the level of rat MGST3 mRNA in the brain were found at 1, 3, and 6 h after intraperitoneal LPS administration, corroborating our findings obtained with a different technical approach. In addition, these data further suggest that MGST3 in the rat CNS is not directly involved in the inflammatory response.

Studies on the expression of CysLT in human endothelial cells

Since cys-LT have been implicated in the pathogenesis of vascular inflammation, studies on the receptors involved in eliciting the effects of these lipid mediators are of

fundamental interest. In order to further elucidate the receptor profile in the vessel wall, studies on the expression of Cys-LT as well as their regulation and functionality were carried out in HUVEC.

Expression of CysLT in HUVEC

Using quantitative RT-PCR, HUVEC were found to abundantly express $CysLT_2$ in vast excess of $CysLT_1$. This finding was corroborated by another group reporting similar results [98]. Using different techniques the ratio of $CysLT_2$ mRNA to $CysLT_1$ mRNA was calculated as 500:1 to 4300:1. However, cautious interpretation of the absolute numbers is warranted since the value for $CysLT_2$ transcripts was obtained by extrapolation outside the linear range of the standard curve. Attempts were also made to verify the expression at the protein level, however, the commercially available antibody proved to be very nonspecific, targeting several bands in the standard protein mix.

Regulation of $CysLT_2$ mRNA in HUVEC by pro-inflammatory stimuli and functional response elicited by natural ligands

The expression of $CysLT_2$ mRNA in HUVEC was evaluated by semi-quantitative RT-PCR after cultivation with the cytokines TNF-α and IL-1β as well as LPS for up to 2 h. Stimulation of cells with LPS resulted in a transient down-regulation of mRNA levels of up to 30% compared with unstimulated cells, which returned to baseline levels after 120 min. TNFα down-regulated $CysLT_2$ mRNA expression to ~ 40% and ~ 60% of control at 30 min and 60 min, respectively. In contrast to LPS, suppression of $CysLT_2$ mRNA levels persisted after 120 min of incubation. IL-1β exerted a similar effect on $CysLT_2$ mRNA expression with a decrease of up to 50% compared with control which only returned to approximately 80% of unstimulated cells after 120 min. Hence, these data suggest that expression of the $CysLT_2$ may be suppressed by these pro-inflammatory stimuli.

In contrast to $CysLT_2$ mRNA, stimulation of HUVEC with TNF-α, IL-1β, and LPS did not result in alterations in the expression of $CysLT_1$ mRNA, indicating that regulation of $CysLT_1$ in HUVEC is not controlled by these cytokines and LPS.

Calcium mobilization experiments in HUVEC demonstrated significant effects in response to stimulation of cells with LTC_4 and LTD_4 in a concentration of 100 nM each. However, in contrast to mast cells the agonist activity of LTD_4 was only slightly greater than that of LTC_4, a relative potency far below that expected for $CysLT_1$ mediated signalling [99,162]. In addition, the dual $CysLT_{1/2}$ antagonist BAY u9773 (100 nM), which also has been demonstrated to be a selective agonist for the $CysLT_2$ [185], evoked a calcium response comparable to that induced by cys-LT. Challenge of HUVEC with 100 nM LTD_4 for 1 min prior to treatment with BAY u9773 inhibited the mobilization of Ca^{2+} indicating that the receptor had been occupied or desensitized by the natural ligand. Moreover, the $CysLT_1$ specific antagonist MK-571 in a concentration of 1 µM did not prevent subsequent Ca^{2+} mobilization induced by either LTC_4 or LTD_4, whereas BAY u9773 completely blocked the Ca^{2+} signal. Together, these results provide evidence that $CysLT_2$ represents the predominant CysLT on HUVEC and that this receptor accounts for intracellular Ca^{2+} mobilization induced by cys-LT in these cells [98].

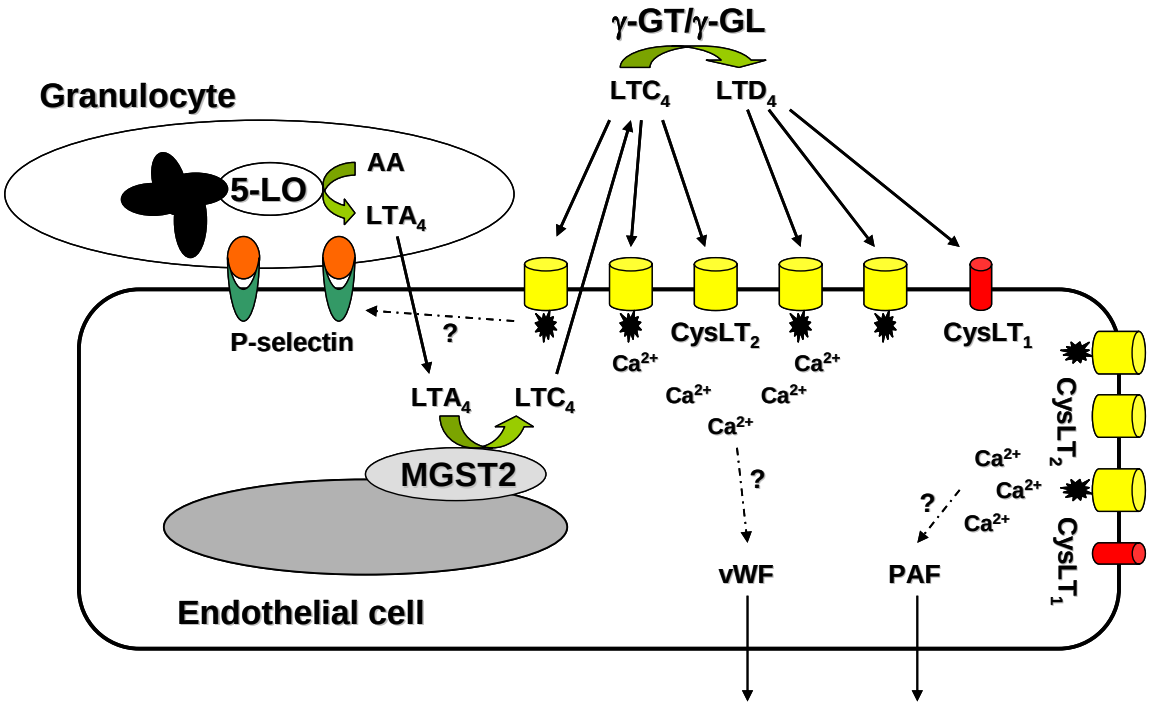

Figure 4: Proposed model of transcellular cys-LT biosynthesis and autocrine CysLT signalling in endothelial cells.

DISCUSSION

The results presented in this thesis expand our knowledge of both structural properties as well as biological functions of LTC_4 synthesizing enzymes. Moreover, the data yield further information with respect to the physiological role of CysLT in human endothelial cells.

Molecular and catalytic properties of LTC4S isoenzymes

Comparative inhibitor studies of all three LTC4S homologs with two active site-directed molecules, i.e. the enzyme product LTC_4 and the FLAP inhibitor MK-886, indicate that the catalytic centres of LTC4S, MGST2, and MGST3 originate from structurally related and overlapping active sites (Figure 5). In addition, MK-886 and LTC_4 were recently found to inhibit mPGES-1 as well, potentially linking a fourth enzyme activity to the same catalytic centre [106]. Hence, it seems feasible to design enzyme inhibitors, which will simultaneously target several members of the MAPEG family, e.g. LTC4S and mPGES-1, in order to yield improved anti-inflammatory action. On the other hand, future development of inhibitors for a specific enzyme of this protein family may be limited to possible unwanted or unforeseen side effects on other family members.

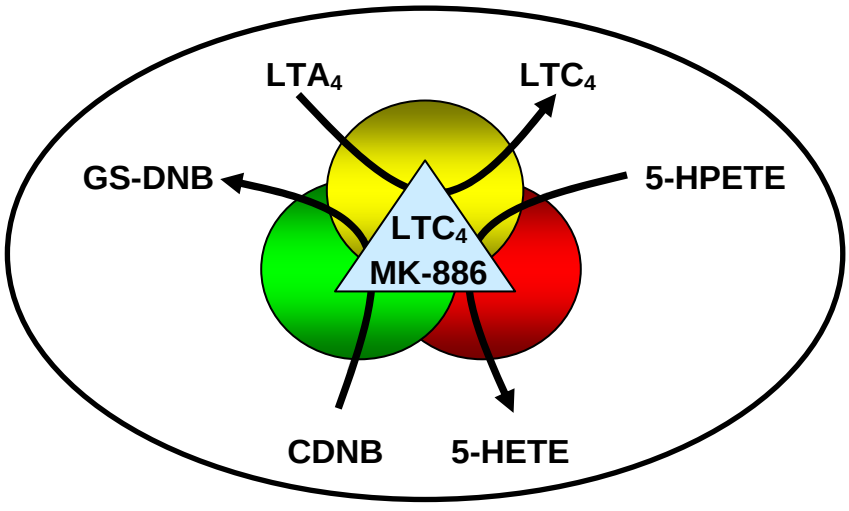

Figure 5: Model for the structural relationships between the active centres of LTC4S, MGST2, and MGST3. Note that MGST2 possesses all three catalytic activities, whereas LTC4S and MGST3 only exhibit LTC_4 synthase and peroxidase activity, respectively.

Previous work by Lam et al. has identified Arg51 and Tyr93 in human LTC4S as the proton donor and base in the conversion of LTA_4 to LTC_4 [91]. In addition, it has been proposed that the absence of this critical arginine in FLAP could explain the absence of LTC4S activity in this protein [76]. In as much as both the catalytic Arg and Tyr residues are conserved in rat MGST3, the lack of LTC4S activity challenges this structure-function hypothesis and suggests that evolution has preserved the corresponding residues (Arg59 and Tyr100) to fulfil other functions in MGST3, perhaps related to an as yet unidentified catalytic activity. In light of the present data, it may be of value to reexamine the catalytic properties of human MGST3, in particular the LTC4S activity.

Comparisons of the members of the MAPEG family have demonstrated identical putative membrane topography for all proteins, although the overall sequence identity is less than 20% at the aa level. The area of limited sequence homology corresponds to the C-terminal end of the first hydrophilic region, partly coinciding with a suggested AA binding site for FLAP, possibly involved in eicosanoid binding in other MAPEG members. Thus, the motif ”ER*XXX*A*XX*N*XX*D/E” was thought to represent a consensus sequence for interaction with AA and/or several of its oxygenation products. However, for MGST3 there is a significant difference in structure regarding the putative FLAP inhibitor binding domain (”FNCIQRAH” pattern and ”NGHM” insertion) and the ”ER*XXX*A*XX*N*XX*D/E” motif. The similar inhibitory constants for LTC_4 and MK-886 on peroxidase activity of both MGST2 and MGST3 imply that these structural dissimilarities do not prevent or diminish the effects of MK-886, indicating that additional structural elements are important for binding of this type of inhibitor, AA and/or several of its oxygenation products, at least in MGST3. Further mutagenic analysis is warranted to define the elements involved in this interesting segment of the MAPEG family members.

Role of LTC_4 synthesizing enzymes in the scope of a systemic inflammation

The successful cloning of rat LTC4S, MGST2, and MGST3 did not only yield novel information on the structure-function relationships of these enzymes but also allowed side-by-side comparative investigations of their expression *in vivo* for the first time. LTC4S has long been believed to be the sole enzyme capable of converting LTA_4 into LTC_4. After discovery of MGST2 and MGST3, two other proteins equipped with the enzymatic machinery to

conjugate LTA_4 with GSH, the physiology and pathophysiology of LTC_4 formation has become more complex. In fact, MGST2 was found to be the only protein in HUVEC to generate LTC_4 *in vitro* [171]. Induction of LTC4S by cytokines has been studied in several isolated mammalian cell types and cell lines however, none of these reports has tried to assess the relevance for the *in vivo* situation. Our data indicate that up-regulation of LTC4S may also be triggered by systemic inflammatory signals and prime certain tissues for increased cys-LT production (Figure 6), which in turn may elicit local inflammatory circuits provoking vasospasms and edema formation, particularly in heart, brain, liver, and adrenal gland. Moreover, LTC4S and cys-LT formation may directly or via secondary signals contribute to the general signs of systemic inflammation, e.g. fever, tachycardia, hypotension, and fatigue. In this context, LTC_4, but not LTD_4 and LTE_4, was recently shown to increase the biosynthesis of PGE_2 and 6-keto-$PGF_{1\alpha}$ via up-regulation of COX-2 in an *in vitro* model of inflammation [158]. The mitogen-activated protein kinase (MAPK) pathway was found to be the underlying signal transduction mechanism involved in this up-regulation. Furthermore, activation of MAPK appeared to be correlated with the phosphorylation of ERK-1/2 mediated via $CysLT_1$. In contrast to LTC4S, our *in vivo* model suggests that both MGST2 and MGST3 do not appear to be involved in increased LTC_4 biosynthesis during a systemic inflammatory response. Moreover, a recent report has demonstrated that $LTC4S^{-/-}$ mice exhibits undetectable levels of GST activity specific for LTA_4-ME in some tissues including the brain [78] indicating that other enzymes do not take over LTC_4 biosynthesis. Thus, it seems likely that MGST3, although widely distributed in the rat brain according to our immunohistochemical studies, is not primarily involved in the synthesis of LTC_4 production in the rat CNS but rather suggest other functions, e.g. in metabolic detoxication and neuroprotection.

Regarding the cellular origin of LTC_4, it seems likely that LPS challenge leads to an up-regulation of LTC4S in resident cells of the tissues rather than blood-borne cells that have adhered to the vascular wall. These cells may be resident mast cells, macrophages, or even parenchymal cells, as indicated by recent observations demonstrating up-regulation of LTC4S in rat hepatocytes and in mouse choroid plexus [169,178]. Additional immunohistochemical studies of the mouse brain demonstrated that LTC4S is selectively localized in the vasopressinergic magnocellular neurons of the hypothalamic paraventricular, supraoptic, and suprachiasmatic nuclei and in the retrochiasmatic area as well as in axons that emanated from these neurons to the pars nervosa of the pituitary gland thereby indicating that LTC_4 is involved in the regulation of arginine-vasopressin related physiological activities such as

water retention [168]. In contrast, immunoreactivity for LTC4S in the extrahypothalamic system was minor (bed nucleus of the stria terminalis, lateral habenular nucleus, midbrain central gray, medial amygdaloid nucleus, and ventral septal area) or even absent (cerebral cortex, neostriatum, cerebellum, and brain stem).

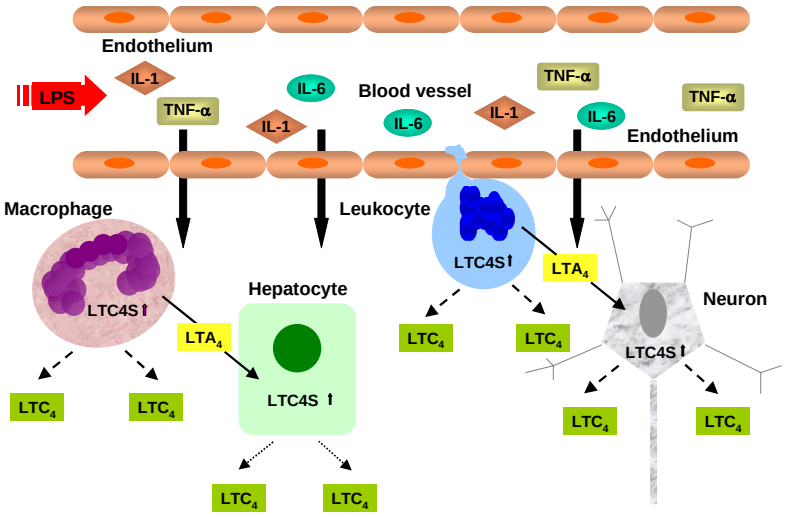

Figure 6: Model for increased cys-LT biosynthesis triggered by systemic inflammation. LPS induces up-regulation of pro-inflammatory cytokines, which in turn trigger the induction of yet undefined pathway(s) of LTC4S either in bone-marrow-derived cells or in certain parenchymal cell types.

Role of cysteinyl receptors in endothelial cells with emphasis on vascular inflammation

Though recognized more than 20 years ago for their effects on intact blood vessels [25], cys-LT have not been perceived as promoters of cardiovascular disease. Evidence regarding a role of cys-LT in vascular inflammation has only recently emerged from *in vitro* [42,61,67,112,139,142,149], morphological [180], and pharmacological studies [2,4,40,119,166,173] as well as data from genetically engineered mice [30,56]. Most *in vivo* effects of added cys-LT involve interactions of leukocytes and the endothelium [40]. Actions of cys-LT in blood vessels include coronary artery contraction [4,119], decline in left ventricular contractility [119], edema formation [40], blood pressure regulation [69], and leukocyte recruitment into the perivascular space [40]. Cys-LT promote P-selectin surface expression, von Willebrand factor secretion, and platelet-activating factor synthesis in

cultured endothelial cells [42,112,142] and stimulate arterial smooth muscle cell proliferation *in vitro* [112].

One of several target tissues of endogenously produced cys-LT in the blood vessel wall may be the endothelium due to its expression of $CysLT_2$. Indeed, our data clearly indicate that the $CysLT_2$ is the dominant receptor in HUVEC. Using quantitative RT-PCR, we found that HUVEC abundantly express $CysLT_2$ mRNA in vast excess (>500 to 4000-fold) of $CysLT_1$ mRNA. Moreover, Ca^{2+} fluxes elicited by cys-LT in these cells seem to emanate from perturbation of $CysLT_2$ rather than $CysLT_1$ as demonstrated by receptor agonist and antagonist studies. Challenge of HUVEC with BAYu9773, a partial $CysLT_2$ agonist, triggered Ca^{2+} mobilization. In addition, the biological response to receptor activation by the natural ligands LTC_4 and LTD_4 could be blocked by BAY u9773, which is also a dual CysLT antagonist, but not by the $CysLT_1$-selective antagonist MK-571.

To identify the genetic program induced by $CysLT_2$-dependent signalling in endothelial cells and by $CysLT_1$ activation of monocytes/macrophages, microarray expression analyses were carried out in HUVEC and in the monocytic cell line MonoMac6 after addition of LTD_4 [217]. In endothelial cells LTD_4-regulated genes were found to encode transcription factors, signalling molecules, an inhibitor of calcineurin signalling, chemokines, angiogenic factors, extracellular matrix-degrading molecules, adhesion molecules, and tissue factor as well as COX-2 [196]. Up-regulation of these pro-inflammatory targets may exacerbate chronic vascular inflammation by recruiting leukocytes into the arterial wall. By contrast, in human and mouse monocytic cell lines, LTD_4 did not affect MIP-2 but induced MIP-1α, a T cell chemoattractant [217], indicating that LTD_4 triggers different responses in a receptor and/or target cell-specific manner.

Recent studies using $CysLT_2$-deficient [17] and $CysLT_2$ transgenic mice [69] also suggest that cys-LT may trigger acute cardiovascular effects independent of transcript regulation. When $CysLT_2$ was deleted in mice the resulting phenotype showed decreased vascular permeability and attenuated bleomycin-induced lung fibrosis [17]. The latter finding may also be relevant to vascular fibroproliferative responses similar to that observed during atherosclerotic plaque formation and arterial wall remodelling [202]. In the endothelial cell-specific human $CysLT_2$ transgenic mouse model the resulting phenotype suggested a role of $CysLT_2$ in blood vessel permeability and indicated that this receptor participates in blood pressure regulation [69]. Further work is required to examine the actions of cys-LT on each of the cells that participate in vascular inflammation.

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