Synthesis and metabolism of leukotrienes in γ-glutamyl transpeptidase deficiency

Ertan Mayatepek,[1,*] Jürgen G. Okun,[†] Thomas Meissner,[*] Birgit Assmann,[*] Judith Hammond,[§] Johannes Zschocke,[**] and Wolf-Dieter Lehmann[††]

Department of General Pediatrics,* University Children's Hospital, Düsseldorf, Germany; Department of General Pediatrics,† University Children's Hospital, Division of Metabolic and Endocrine Diseases, Heidelberg, Germany; NSW Biochemical Genetics Service,§ Royal Alexandra Hospital for Children, Sydney, Australia; Institute of Human Genetics,** University of Heidelberg, Heidelberg, Germany; and Central Spectroscopy Unit,†† German Cancer Research Institute, Heidelberg, Germany

Abstract Leukotrienes (LTs) are active lipid mediators derived in the 5-lipoxygenase pathway. LTC_4, the primary cysteinyl LT, is cleaved by γ-glutamyl transpeptidase (GGT), resulting in LTD_4. We studied the synthesis and metabolism of LTs in three patients with GGT deficiency. LTs were analyzed in urine, plasma, and monocytes after HPLC separation by enzyme immunoassays, radioactivity detection, and electrospray tandem mass spectrometry. Analysis of LTs in urine revealed increased concentrations of LTC_4 (12.8–17.9 nmol/mol creatinine; controls, <0.005 nmol/mol creatinine), whereas LTE_4 was below the detection limit (<0.005 nmol/mol creatinine; controls, 32.2 ± 8.6 nmol/mol creatinine). In plasma of one patient, LTC_4 was found to be increased (17.3 ng/ml; controls, 9.6 ± 0.4 ng/ml), whereas LTD_4 and LTE_4 were below the detection limit (<0.005 ng/ml). LTB_4 was found within normal ranges. In contrast to controls, the synthesis of LTD_4 and LTE_4 in stimulated monocytes was below the detection limit (<0.1 ng/10^6 cells; controls, 37.1 ± 4.8 cells and 39.4 ± 5.6 ng/10^6 cells, respectively). The formation of [^{3}H]LTD_4 from [^{3}H]LTC_4 in monocytes was completely deficient (<0.1%; controls, 85 ± 7%). Our data demonstrate a complete deficiency of LTD_4 biosynthesis in patients with a genetic deficiency of GGT. GGT deficiency represents a new inborn error of cysteinyl LT synthesis and provides a unique model in which to study the pathobiological coherence of LT and glutathione metabolism.—Mayatepek, E., J. G. Okun, T. Meissner, B. Assmann, J. Hammond, J. Zschocke, and W-D. Lehmann. **Synthesis and metabolism of leukotrienes in γ-glutamyl transpeptidase deficiency. *J. Lipid Res.* 2004.** 45: **900–904.**

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Leukotrienes (LTs) constitute a group of biologically highly active lipid mediators derived from 20-carbon polyunsaturated fatty acids, predominantly arachidonic acid via the 5-lipoxygenase pathway (1–3). They include the cysteinyl LTs LTC_4, LTD_4, and LTE_4, representing biologically active constituents of the long-known "slow-reacting substance of anaphylaxis" and the dihydroxyeicosatetraenoate LTB_4.

The biosynthesis of LTs is limited to a few types of human cells, including mast cells, eosinophils, basophils, and macrophages. The synthesis of LTs is initiated by cell activation with the release of arachidonic acid from membrane phospholipid by the action of cytosolic phospholipase A_2. Arachidonic acid then binds to the 5-lipoxygenase-activating protein and is presented to 5-lipoxygenase (4). Calcium-dependent activation of 5-lipoxygenase converts arachidonate via 5-hydroperoxyeicosatetraenoate to 5,6-epoxide LTA_4, which is unstable and is catalyzed to LTB_4 (5, 6). Alternatively, the conjugation of LTA_4 with glutathione at carbon 6 is mediated by LTC_4 synthase, resulting in the formation of LTC_4, the primary cysteinyl LT (7). LTC_4 is known to be cleaved by γ-glutamyl transpeptidase (GGT), which removes the glutamyl moiety to form LTD_4 (1). LTC_4 conversion to LTD_4 has long been thought to be mediated solely by GGT. The cleavage of glycine from LTD_4 yields LTE_4 (8).

Some years ago, a human gene was cloned that appeared to direct the cleavage of LTC_4. This enzyme was termed γ-glutamyl transpeptidase-related (GGT-rel) (9). GGT-rel shares an overall 40% amino acid sequence identity with human GGT and is capable of cleaving the γ-glutamyl linkage of LTC_4, but it is unable to hydrolyze synthetic substrates that are commonly used to assay GGT. GGT-rel is not expressed in the mouse (9).

Recently, mice deficient in GGT were developed and used in LT metabolism studies (10–12). These studies un-

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[1] To whom correspondence should be addressed.
e-mail: mayatepek@uni-duesseldorf.de

expectedly revealed that GGT-deficient mice are competent to metabolize LTC_4 as a result of the presence of an additional LTC_4/LTD_4-converting enzyme, named γ-glutamyl leukotrienase (GGL) (10).

At present, there have been five patients reported with GGT deficiency (13–16). These patients have increased glutathione concentrations in plasma and urine, but their cellular levels are normal. In addition to glutathionuria, these patients have increased levels of γ-glutamylcysteine and cysteine. Decreased activity of GGT can be demonstrated in leukocytes or cultured skin fibroblasts, but not in erythrocytes, which also lack this enzyme under normal conditions. The clinical relevance of the condition is not known; patients with variable central nervous system (CNS) symptoms as well as asymptomatic patients have been recognized (17). GGT deficiency appears to be transmitted as an autosomal recessive trait, and the gene family for GGT has been mapped to chromosome 22q11.2-q12.1.

In this paper, we report the results of our studies on LT synthesis and metabolism in patients with GGT deficiency, in whom we demonstrate a defect in the conversion of the parent compound LTC_4 to LTD_4.

MATERIALS AND METHODS

Patients

Three different patients (14, 16) with GGT deficiency were investigated. Detailed clinical and biochemical findings of these patients are shown in **Table 1**. All patients exhibited glutathionuria and significantly decreased activity of GGT in cultured skin fibroblasts.

Analysis of LTs in plasma and urine

Urine was obtained from all patients by spontaneous micturition, screened for the presence of pathobiological constituents, and mixed with two volumes of 90% (v/v) aqueous methanol, pH 8.5, containing 0.5 mmol/l edetic acid, 1 mmol 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*(1)-oxyl (Sigma Chemical Co., St. Louis, MO), and 20 mmol/l $KHCO_3$. $[^3H]LTC_4$, $[^3H]LTD_4$, $[^3H]LTE_4$, and $[^3H]LTB_4$ were added as internal standards to the plasma (patient 1) and urine samples and acidified to pH 4.5 by the addition of 0.1 mol/l HCl. LT concentrations were measured by enzyme immunoassay after extraction on Sep-Pak cartridges and reversed-phase high-pressure liquid chromatography purification with an acetonitrile-water (38:62, v/v) system. Specific antibodies (Cayman) have been described in detail (18).

Analysis of LTs in stimulated monocytes

Monocytes from peripheral blood of patient 1 were isolated as previously described (19). Of the 2×10^6 adherent mononuclear cells per plate, 94% were monocytes as identified by their structure after staining with safranin or Giemsa. Monocyte monolayers were activated with calcium ionophore A23187 (final concentration, 10 μmol/l; Sigma Chemical Co.) for 15 min at 37°C (19).

For studies of the inhibitory activity of LTD_4 synthesis in the plasma of patients with GGT deficiency, monocytes were isolated from healthy controls and stimulated with calcium ionophore A23187 as described above in the presence or absence of plasma from patient 1.

For the measurement of different LTs, $[^3H]LTC_4$, $[^3H]LTD_4$, $[^3H]LTE_4$, and $[^3H]LTB_4$ were added to the cell supernatants as internal standards. LT content was assessed by enzyme immunoassays after extraction on Sep-Pak cartridges and reversed-phase high-pressure liquid chromatography purification as described in detail (20).

Measurement of $[^3H]LTD_4$ formation from $[^3H]LTC_4$ in monocytes

$[^3H]LTC_4$ (Du Pont-New England Nuclear) was added to isolated monocytes, and incubations were carried out as described (21). After centrifugation and evaporation to dryness, the residue was taken up in isopropanol, acidified to pH 3 with 5 mol/l formic acid, and extracted with diethyl ether. After separation and addition of 10 mmol/l NH_4OH, the sample was dried and the residue was adjusted to pH 9 by NH_4OH. The mixture was extracted on Sep-Pak cartridges, and analysis was done by reversed-phase high-pressure liquid chromatography as described (19). The eluent was monitored for radioactivity using a Raytest radioactivity detector (Raytest, Straubenhardt, Germany). Quantification of radioactivity was carried out by collection of fractions from the high-pressure liquid chromatography analysis in a Beckman multipurpose scintillation counter (LS 6500; Beckman Instruments, Fullerton, CA). Results are expressed as percentage capacity to form $[^3H]LTD_4$ from $[^3H]LTC_4$.

Electrospray tandem mass spectrometry

Electrospray mass spectra were recorded in the negative ion mode using a triple quadrupole instrument type TSQ 7000 (Finnigan, San Jose, CA) equipped with a nanoelectrospray ionization source (EMBL, Heidelberg, Germany). Spray capillaries were made in house using a micropipette puller type 87 B (Sutter Instruments). Conductivity of the capillaries was achieved by sputtering a thin film of gold onto the surface. The spray was started by applying a voltage of approximately −500 V. Tandem mass

TABLE 1. Clinical and biochemical findings in patients with γ-glutamyl transpeptidase deficiency

Findings	Patient 1	Patient 2	Patient 3
Age (years)	39	20	21
Clinical symptoms	Mental retardation, severe behavior problems, psychiatric symptoms	Prader-Willi syndrome, strabismus, easy bruising, poor coordination, dysmorphic features	Seizures, abnormal electroencephalogram, asthma, easy bruising
Glutathionuria	Present	Present	Present
Intracellular glutathione	Normal	Normal	Normal
γ-Glutamyl transpeptidase activity in cultured fibroblasts (mU/mg protein)[a]	<0.03	<0.03	<0.03

[a] Controls, 1.54 (0.53–7.78) mU/mg protein (median and range; n = 6).

TABLE 2. Endogenous urinary leukotrienes

Leukotriene	Patient 1	Patient 2	Patient 3	Controls[a]
		nmol/mol creatinine		
LTC_4	12.8	15.7	17.9	<0.005
LTE_4	<0.005	<0.005	<0.005	32.2 ± 8.6

[a] For controls, n = 30 (mean ± SD).

spectrometry was performed using argon as a collision gas at a nominal pressure of 2.5 mTorr in the collision cell. Scan time was 3 s per scan. Single-stage spectra represent as average of 10 scans, and tandem mass spectra represent the average of 50 scans. The HPLC fractions were reduced to approximately one-third of their original volume under a stream of nitrogen and then lyophilized completely. The residue was redissolved in 40 µl of methanol, and 5 µl thereof was transferred into a spray capillary.

RESULTS

Analysis of LTs in urine of all three patients with GGT deficiency revealed increased concentrations of LTC_4, which is usually not detectable in human urine, in all three patients (**Table 2**). Conversely, LTE_4, which is the major urinary leukotriene metabolite in humans, was below the detection limit in all three patients.

Identification of LTC_4 in the urine of the patients with GGT deficiency was confirmed by tandem mass spectrometry. The single-stage mass spectra of the HPLC fractions isolated from the urine samples of all patients contained a signal at *m/z* 624, the *m/z* value for the [M-H] ion of LTC_4. To confirm the identification of this signal as LTC_4, a precursor ion scan for *m/z* 272 was performed, and the resulting spectra showed the ion at *m/z* 624 as the most abundant signal. The precursor ion scan for *m/z* 272 was selected because this fragment has been reported as an abundant fragment ion of LTC_4 generated by fission of the sulfur bridge, with retention of the sulfur atom at the fatty acid part and charge retention at the glutathionyl part, using fast atom bombardment (22) or electrospray ionization (23), as indicated in **Fig. 1**.

Complete product ion spectra were recorded for the signals at *m/z* 624, as observed in the HPLC fractions from the patient urine samples, and compared with the corresponding spectrum of synthetic LTC_4. These product ion spectra are presented in **Fig. 2**. The product ion spectrum of LTC_4 (Fig. 2A) is characterized mainly by fragment ions

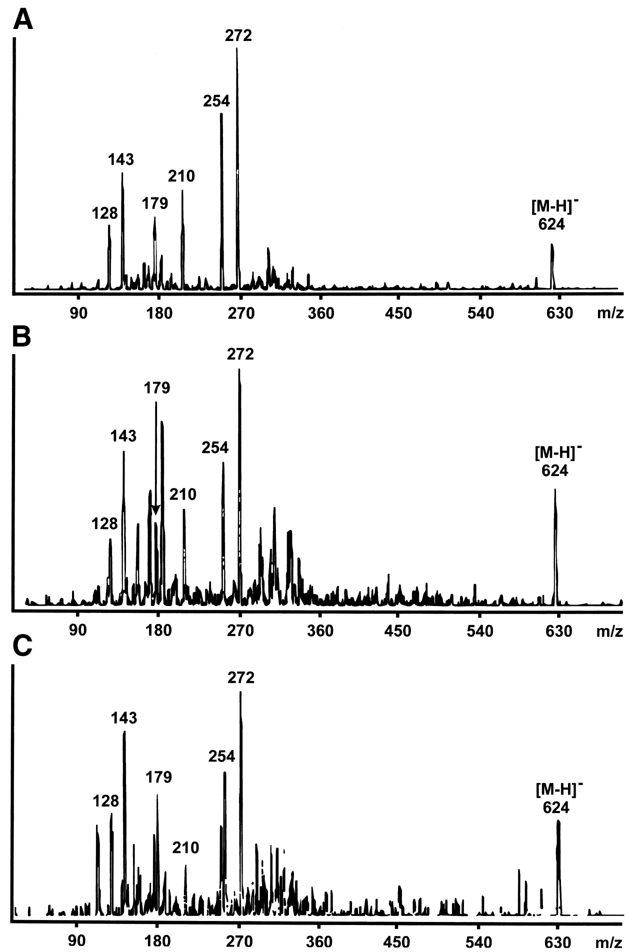

Fig. 2. Negative ion nanoelectrospray ionization product spectra of *m/z* 624 from different samples. A: LTC_4 standard. B: LTC_4 fraction isolated from urine of patient 1. C: LTC_4 fraction isolated from urine of patient 2.

originating from the glutathione part of the molecule (22). All major fragment ions observed in this product ion spectrum at *m/z* 128, 143, 179, 210, 254, and 272 are also found with similar relative abundance in the corresponding spectra obtained from the samples of the patients (Fig. 2B, C). These spectra contain a few additional ion signals compared with the reference spectrum shown in Fig. 2A, indicating the additional presence of some minor isobaric contamination at *m/z* 624 in the spectra of the HPLC fractions prepared from urine.

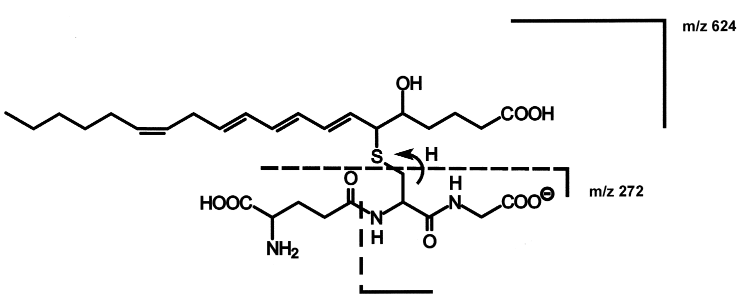

Fig. 1. Structure of the cysteinyl leukotriene LTC_4 and the main points of cleavage. Formation of the two most intense fragment ions at *m/z* 143 and *m/z* 272 from the $[M-H]^-$ ion of LTC_4 at *m/z* 624.

TABLE 3. Leukotrienes in plasma

Leukotriene	Patient 1	Controls[a]
	ng/ml	
LTB_4	10.8	10.4 ± 0.6
LTC_4	17.3	9.6 ± 0.4
LTD_4	<0.005	12.8 ± 0.8
LTE_4	<0.005	13.2 ± 0.8

[a] For controls, n = 30 (mean ± SD).

In plasma of the patients with GGT deficiency, LTC_4 was increased compared with control values, whereas LTD_4 and LTE_4 were below the detection limit (**Table 3**). LTB_4 was found within normal ranges.

In contrast to the control samples, the synthesis of LTD_4 as well as LTE_4 in stimulated monocytes was below the detection limit, whereas the formation of LTC_4 was subsequently increased (**Table 4**). The synthesis of LTB_4 in stimulated monocytes was within normal ranges. The formation of $[^3H]LTD_4$ from $[^3H]LTC_4$ in monocytes was completely deficient (Table 4).

The synthesis of LTB_4 and LTD_4 in stimulated monocytes of healthy controls (n = 10; LTB_4, 50.6 ± 4.8 ng/10^6 monocytes; LTD_4, 33.9 ± 3.9 ng/10^6 monocytes) was not reduced when incubated with plasma of a patient with GGT deficiency (LTB_4, 49.2 ± 4.4 ng/10^6 monocytes; LTD_4, 34.3 ± 3.7 ng/10^6 monocytes). These results indicate that there was no inhibitory activity of LTD_4 synthesis in the plasma of patients with GGT deficiency.

DISCUSSION

The results of our study demonstrate a complete deficiency of LTD_4 biosynthesis in patients with a genetic deficiency of GGT. Patients displayed an abnormal profile of LTs in urine, with the complete absence of LTE_4, the index metabolite for cysteinyl LT generation in humans (2). Highly increased concentrations of LTC_4 in the urine of GGT-deficient patients were confirmed by tandem mass spectrometry. To date, LTC_4 has not been reported to be present in human urine under physiological or pathophysiological conditions. Analysis of patient plasma revealed a corresponding abnormal profile, with increased concentrations of LTC_4 and absence of LTD_4 as well as LTE_4, whereas LTB_4 synthesis was not affected. Incubation studies with stimulated monocytes from healthy controls with plasma from a GGT-deficient patient excluded the presence of an inhibitory activity of LTD_4 synthesis in the plasma of affected patients. Finally, functional experiments with monocytes clearly showed that the formation of LTD_4 is completely deficient in patients with GGT deficiency.

TABLE 4. Formation of leukotrienes in monocytes

Leukotriene	Patient 1	Controls[a]
	ng/10⁶ cells	
After calcium ionophore A23187		
LTB_4 (ng/10^6 cells)	52.1	47.2 ± 5.6
LTC_4 (ng/10^6 cells)	65.8	32.8 ± 3.6
LTD_4 (ng/10^6 cells)	<0.1	37.1 ± 4.8
LTE_4 (ng/10^6 cells)	<0.1	39.4 ± 5.6
$[^3H]LTD_4$ formation (%)		
Monocytes	<0.1	85 ± 7

[a] For controls, n = 30 (mean ± SD).

Three of the five known patients with GGT deficiency were ascertained by urinary screening for amino acid defects in mentally retarded individuals, revealing glutathionuria. These patients had variable CNS symptoms, although two siblings with complete GGT deficiency showed no signs of severe CNS dysfunction (17). Our results clearly indicate that there are serious abnormalities in cysteinyl LT synthesis in each of the three investigated patients. It seems possible that the metabolic defect, either excessive LTC_4 or more likely lack of LTD_4 and LTE_4, may contribute to some or even all of the observed symptoms. In accordance, another disorder of cysteinyl LT metabolism, LTC_4 synthesis deficiency, has been found to be associated with a fatal developmental syndrome, including severe muscular hypotonia, psychomotor retardation, failure to thrive, and microcephaly (24, 25).

Some years ago, a human γ-glutamyl-cleaving enzyme related to but distinct from GGT was identified (9). In vitro studies indicated that this protein, named GGT-rel, has at least a minor capacity to convert LTC_4 to LTD_4 (9, 10), and it was suggested that GGT could no longer be considered the only enzyme capable of cleaving the γ-glutamyl linkage of LTC_4. Little is known about the tissue distribution of different enzymes with GGT function. We found a complete absence of LTD_4 biosynthesis in monocytes of patients with GGT deficiency as well as corresponding biochemical findings in blood and urine. Assuming that GGT deficiency in the investigated patients is caused by a recessive single gene defect, our results indicate that GGT is the only enzyme capable of converting LTC_4 to LTD_4 in the human tissues/body fluids studied. Alternatively, "GGT deficiency" in our patients would need to be caused by a lack of more than one enzyme, which would be difficult to reconcile with the apparent lack of clinical symptoms in some affected individuals.

Recently, mice deficient in GGT have been developed (11, 12). These mice are small and grow slowly. They fail to mature sexually, develop cataracts, and begin to die at ~12 weeks of age. At the time of these studies, it was thought that GGT was the only enzyme responsible for converting LTC_4 to LTD_4, and it was expected that GGT-deficient mice would be unable to catalyze this reaction. However, it was subsequently shown that these mice have substantial conversion of LTC_4 to LTD_4, facilitated by another enzyme named GGL (10). It was hypothesized that GGL and GGT-rel may represent the human and mouse counterparts of the same enzyme, because GGT-rel is not found in the mouse. If this were the case, different tissue distributions of GGT-rel in humans and GGL in mice would be expected. No mice have been reported that are deficient in both GGT and GGL.

In conclusion, our results show that the synthesis of LTD_4 is deficient in patients with GGT deficiency, leading

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to highly increased LTC_4 and reduced or absent LTD_4 as well as LTE_4 in urine, plasma, and blood cells. GGT deficiency thus represents the second known inborn error of cysteinyl LT synthesis. The challenge of understanding the pathways of LT and glutathione metabolism in humans, including the pathophysiology of conditions of impaired LT biosynthesis, will be substantial. GGT deficiency provides a unique model in which to study this important pathobiological coherence.

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