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Research Article

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Impaired Degradation of Leukotrienes in Patients with Peroxisome Deficiency Disorders

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Abstract

The degradation of leukotrienes by β-oxidation from the ω-end proceeds in peroxisomes (Jedlitschky et al. *J. Biol. Chem.* 1991. 266:24763–24772). Peroxisomal degradation of leukotrienes was studied in humans by analyses of endogenous leukotrienes in urines from eight patients with biochemically established peroxisome deficiency disorder and eight age- and sex-matched healthy infant controls. Leukotriene metabolites were separated by high-performance liquid chromatography, quantified by radioimmunoassays, and identified as well as quantified by gas chromatography-mass spectrometry.

Urinary leukotriene E_4 (LTE_4) and *N*-acetyl-LTE_4 excretions, relative to creatinine, were increased > 10-fold in the patients in comparison to healthy infants. The β-oxidation product ω-carboxy-tetranor-LTE_3 averaged 0.05 μmol/mol creatinine in the controls but was not detectable in the patients. However, ω-carboxy-LTE_4 (median 13.6 μmol/mol creatinine) was significantly increased in the patients' urine, whereas LTB_4 (median 0.07 μmol/mol creatinine) and ω-carboxy-LTB_4 were detected exclusively in the urines of the patients. These data indicate an impairment of the inactivation and degradation of both LTE_4 and LTB_4 in patients with peroxisomal deficiency.

The increased levels of the biologically active, proinflammatory mediators LTE_4 and LTB_4 might be of pathophysiological significance in peroxisome deficiency disorders. This is the first and so far only condition with a pronounced urinary excretion of ω-carboxy-LTE_4, ω-carboxy-LTB_4, and LTB_4. This impaired catabolism of leukotrienes and the altered pattern of metabolites may be of diagnostic value. These findings underline the essential role of peroxisomes in the catabolism of leukotrienes in humans. (*J. Clin. Invest.* 1993. 91:881–888.) Key words: leukotriene analysis • β-oxidation • peroxisomes • urinary leukotrienes • Zellweger syndrome

Introduction

Leukotrienes (LTs)[1] are potent lipid mediators derived from arachidonate in the 5-lipoxygenase pathway (1–4). LTB_4 is chemotactically active (5), whereas the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, increase microvascular permeability and induce smooth muscle contraction (1–4). In view of the role of LTs under various pathophysiological conditions (6), their metabolic inactivation and degradation is of major interest (7). LTC_4 and LTD_4 are rapidly metabolized in the blood circulation to LTE_4 (7–9). The liver represents the main organ for the uptake, metabolic inactivation, and biliary elimination of LTs and their metabolites (10–13). The metabolic inactivation of LTs is mediated by ω-oxidation and subsequent β-oxidation from the ω-end of LTB_4 (14–16), LTE_4, and *N*-acetyl-LTE_4 (17–21). Administration of radiolabeled LTC_4 and LTE_4 in human subjects leads to ω- and β-oxidation products which are excreted into bile and urine (22–24). After intravenous administration of [^{3}H]LTC_4 in the human and monkey [^{3}H]-LTE_4 is the main urinary metabolite (22, 23, 25, 26), whereas [^{3}H]LTB_4 was not detectable in urine after intravenous [^{3}H]-LTB_4 (27). Urinary LTE_4 has been proposed and used as the index metabolite for the systemic generation of cysteinyl LTs in humans (28–32).

The metabolism of LTE_4 and its compartmentation are summarized in Fig. 1.

The recognition that peroxisomes have a variety of physiological functions, together with the demonstration that they are absent or deficient in certain genetic diseases (33), has led to the identification of a group of peroxisome deficiency disorders, with the Zellweger (cerebrohepatorenal) syndrome as a well-defined example (34, 35). Clinically, the syndrome is associated with typical dysmorphic features, failure to thrive, hepatomegaly, renal cortical cysts, severe hypotonia, epileptic seizures, psychomotoric retardation, and premature death within the first year of life (36). Multiple peroxisomal biochemical processes are defective including β-oxidation of very long chain fatty acids (VLCFAs) (37), phytanic acid oxidation (38, 39), pipecolic acid oxidation (40), and plasmalogen (41) and bile acid (42, 43) biosynthesis.

Peroxisomes were recently identified as the site of LT β-oxidation from the ω-end (44) (Fig. 1). Whereas the cysteinyl LT ω-carboxy-*N*-acetyl-LTE_4 was exclusively β-oxidized in peroxisomes, ω-carboxy-LTB_4 was degraded both in isolated peroxisomes and mitochondria (44). Patients with disorders of peroxisome biogenesis offer a unique possibility to study the im-

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1. *Abbreviations used in this paper:* BSTFA, *N,O*-Bis(trimethylsilyl)trifluoroacetamide; CAD, collision-activated dissociation; CI, chemical ionization mass spectrometry; ECL, equivalent carbon chain length; GC-MS-MS, gas chromatography–mass spectrometry–mass spectrometry; HTMP, 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*(1)-oxyl; LT, leukotriene; MID, multiple ion detection; OTMS, trimethylsilyloxy; P^-, parent ion; PFB, pentafluorobenzyl; RIA, radioimmunoassay; RP-HPLC, reversed-phase high-performance liquid chromatography; TMS, trimethylsilyl; TMSOH, trimethylsilanol; VLCFAs, very long chain fatty acids.

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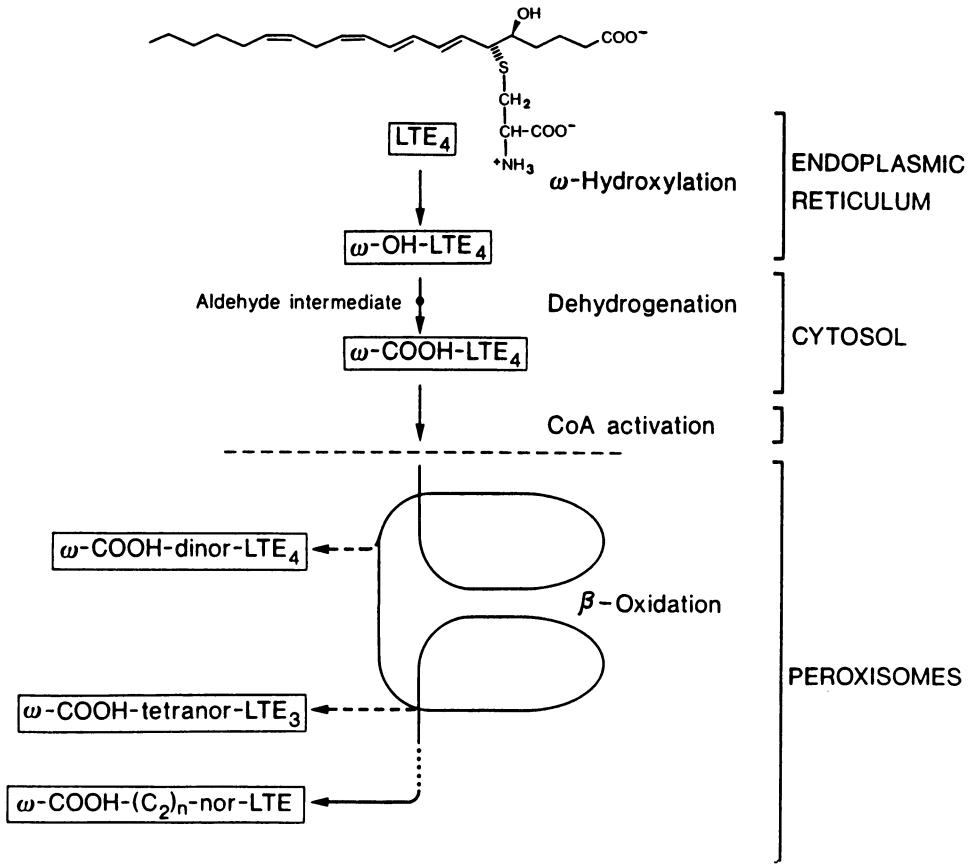

Figure 1. Structure of LTE_4, its degradation by ω-oxidation and subsequent β-oxidation, and the compartmentation of this pathway. ω-Hydroxylation of LTE_4 is catalyzed by microsomal LTE_4-20-monooxygenase (EC 1.14.13.14) (17) followed, particularly in hepatocytes, by two dehydrogenase-catalyzed reactions leading to ω-carboxy-LTE_4 (ω-COOH-LTE_4) (64); the ω-aldehyde intermediate is indicated. After activation at the ω-end by an acyl-CoA-synthetase, several peroxisomal β-oxidation cycles lead to a stepwise degradation of the LT from the ω-end (44). ω-Carboxy-$(C_2)_n$-nor-LTE indicates ω-carboxy-LTE_4 catabolites with < 16 carbon atoms in the dicarboxylate chain. LTB_4 is ω-oxidized and degraded by β-oxidation in reactions which are analogous (44, 65). The horizontal dashed line indicates the block in this degradation pathway in peroxisome deficiency disorders.

portance of peroxisomes in LT degradation in vivo. Since leukotrienes can be analyzed in human urine, it was the aim of this study to investigate the pattern of LT metabolites in urines from patients with peroxisome deficiency disorders. We demonstrate for the first time that these patients exhibit an impaired degradation and inactivation of cysteinyl LTs as well as LTB_4. These results may be of diagnostic value and possibly of pathophysiological interest.

Methods

Patients. Endogenous LT metabolite excretion was studied in eight patients with a peroxisome deficiency (Zellweger syndrome) and eight age- and sex-matched healthy infants. All patients exhibited the characteristic cranofacial and ocular abnormalities described for Zellweger syndrome (36). Convulsions were reported in all patients. There were no signs of cholestasis, liver failure, or impaired renal function. The diagnosis was established by specific biochemical analyses as described below.

Biochemical analyses for diagnosis. VLCFAs ($> C_{22}$) in plasma and fibroblasts (45) as well as plasma bile acid intermediates (46) were determined by gas chromatography. De novo plasmalogen biosynthesis in cultured fibroblasts was analyzed as described (47).

Chemicals. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*(1)-oxyl (HTMP), *N,N*-diisopropylethylamine, and butyryl cholinesterase (from horse serum, 500–1,000 U/mg) were obtained from Sigma Chemical Co., St. Louis, MO. LTC_4, LTD_4, LTE_4, LTB_4, ω-carboxy-LTB_4, and ω-carboxy-LTE_4 were purchased from Cascade Biochem. Ltd., University of Reading, UK. [^{3}H]LTC_4 (4.8 TBq/mmol), [^{3}H]-LTD_4 (1.5 TBq/mmol), [^{3}H]LTE_4 (4.8 TBq/mmol), and [^{3}H]LTB_4 (7.0 TBq/mmol) were from Du Pont-New England Nuclear, Boston, MA. ^{18}O-labeled water (95 at % ^{18}O) was from Preusser Chemie, Jülich, FRG. 2,3,4,5,6-Pentafluorobenzylbromide (PFB bromide) was obtained from Aldrich Chemical Co., Steinheim, FRG. *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethyl-chlorosilane was from Pierce Chemical Co., Rockford, IL. Rhodium (5% on activated alumina) was obtained from Fluka Chemie, Buchs, Switzerland.

Urine collection and leukotriene extraction. Urine was obtained either from spontaneous micturition or was collected from a catheter introduced into the urinary bladder. Immediately after collection, portions of urine were mixed with 2 vol of 90% (vol/vol) aqueous methanol of pH 8.5 containing 0.5 mM EDTA, 1 mM HTMP, and 20 mM $KHCO_3$, and stored at −80°C under argon (31). Directly before analysis, the samples were brought to room temperature and the appropriate internal standards (see below) were added. The samples were acidified to pH 4.5 by addition of 0.1 M HCl, mixed, and pumped slowly through activated Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA). The cartridges were washed with 50 ml of distilled H_2O and LTs were eluted with 5 ml of 90% aqueous methanol containing 1 mM HTMP and 0.5 mM EDTA. The eluates were evaporated to dryness under reduced pressure and resuspended in 30% ice-cold aqueous methanol (23).

An aliquot of each urine sample was screened (Combur^9 test, Boehringer Mannheim GmbH, Mannheim, FRG) to exclude the presence of pathological amounts of leukocytes, erythrocytes, and protein.

Reversed-phase high-performance liquid chromatography (RP-HPLC). Fractions containing distinct LTs were obtained by RP-HPLC on a C_{18} Hypersil column (4.6 × 250 mm, 5-μm particle size; Shandon, Runcorn, UK). Fractions containing LTC_4, LTD_4, LTE_4, and *N*-acetyl-LTE_4 were prepared by RP-HPLC using as mobile phase methanol/water (65:35, vol/vol) the aqueous part containing 0.1% acetic acid, 1 mM EDTA, and being adjusted to pH 5.6 by ammonium hydroxide (23). LTB_4 fractions were also prepared isocratically with an acetonitrile/water (38:62, vol/vol) system, the aqueous part showing the identical composition as described above. ω-Carboxy-tetranor-LTE_3, ω-carboxy-LTE_4, and ω-carboxy-LTB_4 were purified by a linear gradient of 0–80% methanol, 0.1% acetic acid, pH 5.0. For all separations,

a flow rate of 1 ml/min was used. For identification by its ultraviolet (UV) spectrum (see Fig. 2), ω-carboxy-LTE_4 was further purified by isocratic RP-HPLC using mobile phase methanol/water (52:48, vol/vol), containing 0.1% acetic acid, 1 mM EDTA, and adjusted to pH 5.0 with ammonium hydroxide (23). UV spectra were recorded online using a photodiode array detector type 990 (Waters Associates).

Preparation of ^3H-labeled analogues as internal standards for radioimmunoassays (RIAs). ^3H-labeled LTs were used as internal standards in the extraction/RP-HPLC/RIA procedure to correct for recovery losses and as competitive antigens in the RIA procedures. Not commercially available ^3H-analogues were prepared as follows: *N*-acetyl-[^3H]LTE_4 was synthesized from [^3H]LTE_4 as described previously (48). The product was purified by RP-HPLC. ω-Carboxy-tetranor-[^3H]LTE_3 was prepared by injecting *N*-acetyl-[5,6,8,9,11,12,14,15-$^3H_8$]LTE_4 intravenously into anaesthetized and bile duct-cannulated rats. Bile was collected continuously into ice-cold 90% aqueous methanol containing 1 mM HTMP and 0.5 mM EDTA, and aliquots were counted for radioactivity. Bile samples were evaporated to dryness and redissolved in 30% methanol. Separation of ω-carboxy-tetranor-*N*-acetyl-[^3H]LTE_3 was performed as described (44). Enzymatic deacetylation of this product to ω-carboxy-tetranor-[^3H]LTE_3 was performed by penicillin amidase (48) and the product was identified by co-chromatography with the synthetic standard (23, 44).

RIAs. For LT analysis by RIA, 20-ml aliquots of urine were spiked with 3,500 dpm each of the corresponding ^3H-labeled LTs. The RP-HPLC eluate was collected in 1-ml fractions, of which 300 μl was counted for calculation of the ^3H recovery and 700 μl was dried for the subsequent RIAs. These were performed as described (26, 49). Data were corrected for the recovery of the ^3H-labeled LTs added as internal standards. The lower detection limit for LTs was about 50 fmol. The monoclonal cysteinyl LT antibody was kindly donated by Dr. F. Kohen, The Weizmann Institute of Science (Rehovot, Israel). The molar crossreactivities at 50% binding of LTE_4, *N*-acetyl-LTE_4, LTD_4, and LTC_4 were 100%, 140%, 160%, and 210%, respectively. The LTB_4 antibody was kindly provided by Dr. A. W. Ford-Hutchinson (Merck Frosst, Pointe-Claire/Dorval, Quebec, Canada). Its molar cross-reactivities at 50% binding were as follows: LTB_4, 100%; cysteinyl LTs including 12(S)-hydroxyeicosatetraenoate, < 0.05%; 6-*trans*-LTB_4, 2.0%; 6-*trans*-12-epi-LTB_4, 0.6%; 20-hydroxy-LTB_4, 1.0%; ω-carboxy-LTB_4, 1.0% (data provided by Merck Frosst, Canada). Antisera to ω-carboxy-tetranor-LTE_3 were kindly provided by Dr. P. Tagari (Merck Frosst, Canada) and were purified from pooled sera of immunized rabbits as described (50).

Preparation of ^{18}O-labeled ω-carboxy-LTE_4 as internal standard for gas chromatography-mass spectrometry (GC-MS). 20 μg of synthetic ω-carboxy-LTE_4 was incubated with 500 μl of $H_2{}^{18}O$ for 12 h at 25°C in the presence of 300 U of butyryl cholinesterase. The incubation mixture was acidified to pH 3.5 with concentrated formic acid and extracted two times with 2 ml of ethyl acetate. The combined extracts were taken to dryness and redissolved in a methanol/ethanol (1:1, vol/vol) mixture. Determination of the ^{18}O-label distribution by negative ion chemical ionization mass spectrometry (CI-MS) of the hydrogenated and desulfurized pentafluorobenzyl (PFB)/trimethylsilyl (TMS) derivatives (24) revealed the following distribution: [$^{18}O_0$] = 1.0%, [$^{18}O_1$] = 12.3%, [$^{18}O_2$] = 84.3%, [$^{18}O_3$] = 2.4%. Mass spectrometric investigations of the derivatives prepared for CI-MS by electron impact MS showed that the ^{18}O incorporation occurred at the ω-end (as described in Results). The concentration of the [$^{18}O_2$]-ω-carboxy-LTE_4 solution was determined by stable isotope dilution with a solution of nonlabeled ω-carboxy-LTE_4, the concentration of which had been determined on the basis of its absorption at 280 nm and a molar absorptivity of ϵ = 40,000 $cm^{-1}M^{-1}$. The recovery of [$^{18}O_2$]-ω-carboxy-LTE_4 relative to the nonlabeled starting material was ~ 50%.

Quantification of ω-carboxy-LTE_4 by GC-MS. For the quantification of ω-carboxy-LTE_4 in urine, 220 ng of [$^{18}O_2$]-ω-carboxy-LTE_4 were added to a 20-ml urine sample. The sample was mixed and immediately worked up by solid-phase extraction and RP-HPLC as described above. The HPLC fractions containing endogenous urinary ω-carboxy-LTE_4 plus the internal standard were collected (1.5–2 ml) and without prior concentration subjected to the desulfurization/hydrogenation procedure catalyzed by Rh/Al_2O_3 to generate 5-hydroxy-1,20-eicosadioate exactly as described (24, 51). Then the samples were converted to their PFB esters by treatment with 100 μl of a 30% PFB bromide solution in acetonitrile plus 10 μl of *N,N*-diisopropylamine for 30 min at 30°C. After removal of the reagents under a stream of nitrogen, the samples were incubated for 60 min at 60°C with 50 μl of BSTFA to obtain the desulfurized and hydrogenated PFB/TMS derivatives. Aliquots thereof, corresponding to 1–2%, were then injected into a GC-MS system and analyzed by negative ion CI-MS. The analyses were performed on a double-focusing mass spectrometer type MAT 95 (Finnigan MAT, Bremen, FRG) directly interfaced to a HP-5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a temperature-programmable injection system (KAS-2, Gerstel, Mülheim, FRG). The GC column used was a OV-1 fused silica column (10-m length, 0.25-mm i.d., 0.1-μm film thickness, Macherey & Nagel, Düren, FRG). The GC temperature program started with an isothermal step of 1 min at 200°C, followed by a linear increase from 200 to 320°C at a rate of 15°C/min. Helium was used as carrier gas with a linear velocity of 1.4 m/s. The GC-MS interface temperature was adjusted to 280°C and the ion source to 220°C. Isobutane was used as reagent gas for negative ion CI-MS. Quantifications were performed in the multiple ion detection (MID) mode, where the ion signals at m/z 609.303 and 613.312 were monitored at a mass spectrometric resolution of ~ 2,500.

Identification of LTE_4, LTB_4, and ω-carboxy-LTB_4 by GC-MS-MS. Aliquots of urine were spiked with [^3H]LTE_4, [^3H]LTB_4, and ω-carboxy-[^3H]LTB_4. ω-Carboxy-[^3H]LTB_4 was prepared from [^3H]-LTB_4 using rat liver microsomes and cytosol (17, 52). The RP-HPLC fractions containing these standards were collected and evaporated under reduced pressure. The residues were redissolved in 100 μl methanol and treated individually for GC-MS-MS. Endogenous and synthetic LTE_4 was catalytically reduced and desulfurized to 5-hydroxy-1,20-eicosadioate applying the first step of the method described by Balazy and Murphy (51). Then, the samples were reacted with PFB bromide and BSTFA as described above to obtain the PFB-ester/TMS-ether derivatives. LTB_4 and ω-carboxy-LTB_4 were only reacted with PFB bromide and BSTFA. For identification, these samples were analyzed by GC-MS-MS on a triple-stage quadrupole mass spectrometer type TSQ 45 directly coupled to a gas chromatograph type 9611 (both Finnigan MAT, San Jose, CA) equipped with a fused silica OV-1 column (25-m length, 0.25-mm i.d., 0.25-μm film thickness, Macherey & Nagel). The GC temperature program started with an isothermal step of 2 min at 100°C, then the temperature was increased to 250°C at a rate of 25°C/min, followed by an increase to 320°C at a rate of 4°C/min. Injector, interface, and ion source were kept at 280°C, 300°C, and 130°C, respectively. Helium was used as GC carrier gas at a pressure of 55 kPa, and methane as reagent gas for negative ion CI. Collision-activated dissociation (CAD) was performed using argon at a pressure of 0.13 Pa at a collision energy of 10 eV for LTE_4 and LTB_4, and 14 eV for ω-carboxy-LTB_4.

Statistical analysis. Data are given as the median with the range in brackets. The Wilcoxon-Mann-Whitney test for the one-sided problem was used for statistical comparison between urinary LT metabolite concentrations from patients and normal subjects.

Results

Diagnosis of peroxisome deficiency disorder. The results of the diagnostic laboratory analyses are summarized in Table I. The VLCFA profiles in plasma were abnormal in all patients. In fibroblasts, the VLCFA profiles were abnormal in six patients; in the remaining two (patients 2 and 8), fibroblasts were not available. The same was observed for the de novo plasmalogen synthesis which was impaired in the six patients which could be analyzed. Abnormal intermediates of bile acid biosynthesis,

Table I. Biochemical Findings in Patients with Peroxisome Deficiency Disorders and Healthy Infant Controls

Patient	Plasma VLCFA			Abnormal bile acids in plasma	Fibroblast VLCFA		Fibroblast plasmalogen synthesis
	C26:0	C26:1	C26:0/C22:0		C26:0	C26:0/C22:0	
	mg/liter	*mg/liter*			*μg/mg protein*		*$^3H/^{14}C$ in alkenyl PE*
1	3.88	3.38	0.56	+	0.52	1.01	21.0
2	1.00	0.31	0.11	+	0.24	0.22	4.1
3	2.08	0.81	0.30	+	NA*	NA	NA
4	2.90	2.22	0.49	+	0.65	0.62	43.6
5	3.41	0.74	0.44	+	0.51	0.35	8.7
6	3.61	0.99	0.18	+	0.33	0.18	4.9
7	2.31	1.30	0.41	+	0.34	0.37	16.7
8	1.83	0.45	0.17	+	NA	NA	NA
Normal values	0.31 (110) [0.11–0.62]‡	< 0.05 (110)	0.01 (110) [0.001–0.020]	−	0.06 (60) [0.02–0.10]	0.03 (60) [0.02–0.05]	0.7 (59) [0.4–1.5]

* NA, not analyzed. ‡ Median with the number of individuals in parentheses and the 5–90% range in brackets.

such as 3α-, 7α-, 12α-trihydroxy-5β-cholestanoic acid, were detected in the plasma of all patients.

Quantification of urinary LTC_4 and LTD_4. LTC_4 and LTD_4 concentrations were below the detection limit in all urines in both groups.

Identification and quantification of urinary LTE_4. Urinary concentrations of LTE_4 were determined by RIA in controls and patients with peroxisome deficiency disorders as given in Table II. The median LTE_4/creatinine ratio for the patients was a factor of 11 in excess of the controls. In addition to the specificity of the RIA for LTE_4, its presence was confirmed by a mass spectrometric technique. Investigation of a HPLC fraction of urinary LTE_4 after derivatization to its desulfurized and hydrogenated PFB/TMS derivative by GC-MS-MS yielded a mass spectrum with the parent ion at m/z 399 (for $[M\text{-}PFB]^-$) and two characteristic fragment ions at m/z 309 (for $[M\text{-}PFB\text{-}TMSOH]^-$) and m/z 253 (for $[M\text{-}PFB\text{-}CH_3CH_2COOTMS]^-$). The mass spectrum and the GC retention time at which it was observed were identical for urinary and synthetic LTE_4.

Identification and quantification of urinary N-acetyl-LTE_4. Endogenous N-acetyl-LTE_4 was quantified by RIA (23, 31). After enzymatic deacetylation as described above the endogenous N-acetyl-LTE_4 co-chromatographed on RP-HPLC with synthetic LTE_4, thus providing additional evidence for its correct identification. As shown in Table II, the peroxisome deficiency patients showed an increased *N*-acetyl-LTE_4/creatinine ratio by a factor of 20.

Identification and quantification of ω-carboxy-LTE_4. A sample of 1 l of urine from patient 1 was concentrated in several steps by Sep-Pak C_{18} solid-phase extraction and then separated in a two-step HPLC procedure as described in Methods. As displayed in Fig. 2, UV detection at 280 nm showed the presence of a strongly absorbing compound with a RP-HPLC retention time of 23.0 min, being identical to the retention time of synthetic ω-carboxy-LTE_4.

Further, this compound showed the characteristic UV absorbance spectrum of a conjugated triene. Analysis of this HPLC fraction by GC-MS after derivatization into the desulfurized and hydrogenated PFB/TMS derivative gave an intense signal at m/z 609 at a retention time of 7 min 40 s. Calibration of the GC separation by a set of linear fatty acid methyl esters revealed the equivalence of this retention time to an equivalent carbon chain length (ECL) of 28.45. This ECL value reasonably fits to the investigated derivative of ω-carboxy-LTE_4 when compared to published data of homologous compounds (24). The negative ion CI mass spectrum of the urinary ω-carboxy-LTE_4 derivative showed an intense signal at m/z 609 (for $[M\text{-}PFB]^-$) as base peak and only a few minor

Table II. Endogenous LTs in the Urine of Normal Infants and Patients with Peroxisome Deficiency Disorders

	Normal infants	Peroxisome deficiency
	Concentration in urine	
	nM	
LTE_4	0.12 [0.04–0.20]	0.92 [0.67–1.65]
N-Acetyl-LTE_4	0.03 [0.003–0.040]	0.32 [0.05–0.77]
ω-Carboxy-LTE_4	0.3 [0.1–1.7]	16.1 [3.0–62.8]
ω-Carboxy-tetranor-LTE_3	0.11 [0.04–0.16]	< 0.01
LTB_4	< 0.01	0.16 [0.06–0.29]
	Leukotriene/creatinine ratio	
	μmol/mol	
LTE_4	0.06 [0.02–0.08]	0.68 [0.29–0.88]
N-Acetyl-LTE_4	0.01 [0.001–0.020]	0.20 [0.05–0.49]
ω-Carboxy-LTE_4	0.2 [0.1–0.3]	13.6 [2.9–53.7]
ω-Carboxy-tetranor-LTE_3	0.05 [0.02–0.10]	< 0.005
LTB_4	< 0.01	0.07 [0.03–0.22]

LTE_4, *N*-acetyl-LTE_4, ω-carboxy-tetranor-LTE_3, and LTB_4 were measured by RP-HPLC and subsequent RIA; ω-carboxy-LTE_4 was identified and quantified by GC-MS, as described in Methods. Analyses were performed in the urines of eight patients and eight healthy age- and sex-matched infant controls. Data are the median with the range in brackets. Each median in patients was significantly different from the respective median in infant controls ($P < 0.01$).

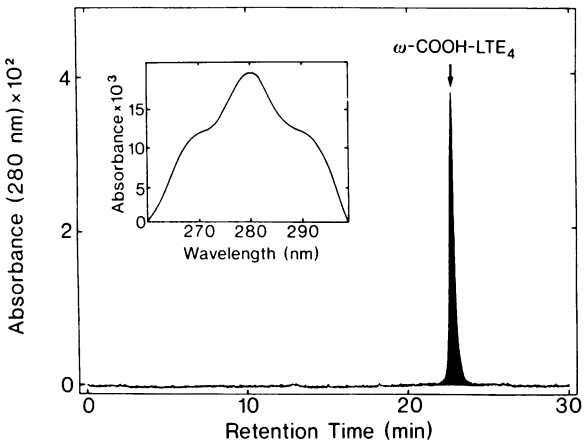

Figure 2. Detection and identification of endogenous ω-carboxy-LTE_4 (ω-COOH-LTE_4). A sample of 1 liter of urine of patient 1 with peroxisomal deficiency (Table I) was purified as described in Methods. The RP-HPLC run shown was performed with a 52% aqueous methanol mobile phase system. The peak with the retention time of 23 min co-eluted with synthetic ω-carboxy-LTE_4 as indicated by an arrow, and showed the characteristic UV absorbance spectrum of a conjugated triene (*inset*).

fragments, e.g., at m/z 519 (for [M-PFB-TMSOH]$^-$), and at m/z 535 (for [M-PFB-74]$^-$) with a relative abundance in the order of 10% of the base peak. For the [$^{18}O_2$]-ω-carboxy-LTE_4, these signals are shifted to 613, 523, and 539 as a result of the incorporation of two atoms of ^{18}O. The label distribution observed for the [$^{18}O_2$]-ω-carboxy-LTE_4 indicates an oxygen isotope exchange at a single carboxy group. To investigate the location of the label, the desulfurized/hydrogenated/PFB/TMS derivatives were investigated by electron impact MS. Investigation of the derivative of nonlabeled ω-carboxy-LTE_4 gave two intense fragment ions at m/z 369 and 523, representing the two complementary α-fission fragments at the OTMS-group at C-5 that contain the carboxy group at C-1 or C-20, respectively. In the corresponding analysis of [$^{18}O_2$]-ω-carboxy-LTE_4 these characteristic fragment ions were observed at m/z 369 and 527 showing the localization of the ^{18}O label at the ω-end.

The GC retention times of urinary, synthetic, and ^{18}O-labeled ω-carboxy-LTE_4 were identical within 1 s. Under the analytical conditions used, no back exchange of ^{18}O against ^{16}O was observed for the internal standard [$^{18}O_2$]-ω-carboxy-LTE_4. In addition, a calibration plot was established by addition of varying amounts of ω-carboxy-LTE_4 to a fixed amount of 6.75 ng of [$^{18}O_2$]-ω-carboxy-LTE_4. Seven mixtures were prepared and derivatized as described, and the ratio of m/z 609/m/z 613 ranging from 0.04 to 3.7 was determined by negative ion CI-MS. A plot of this ratio (y-axis) against the amount of ω-carboxy-LTE_4 added (x-axis) gave a straight line, with the regression equation $y = 0.017 + 0.1482x$ ($R = 1.00$).

Quantification of urinary ω-carboxy-LTE_4 levels by isotope dilution using [$^{18}O_2$]-ω-carboxy-LTE_4 as internal standard gave the data displayed in Table II. All quantitative determinations were performed in triplicate, and the average standard deviations observed were 1.5% and 10.5% for the samples of the patients and of the control subjects, respectively. To illustrate the procedure, Fig. 3 gives two negative ion CI mass spectra, showing the analysis of a urine sample of a control subject

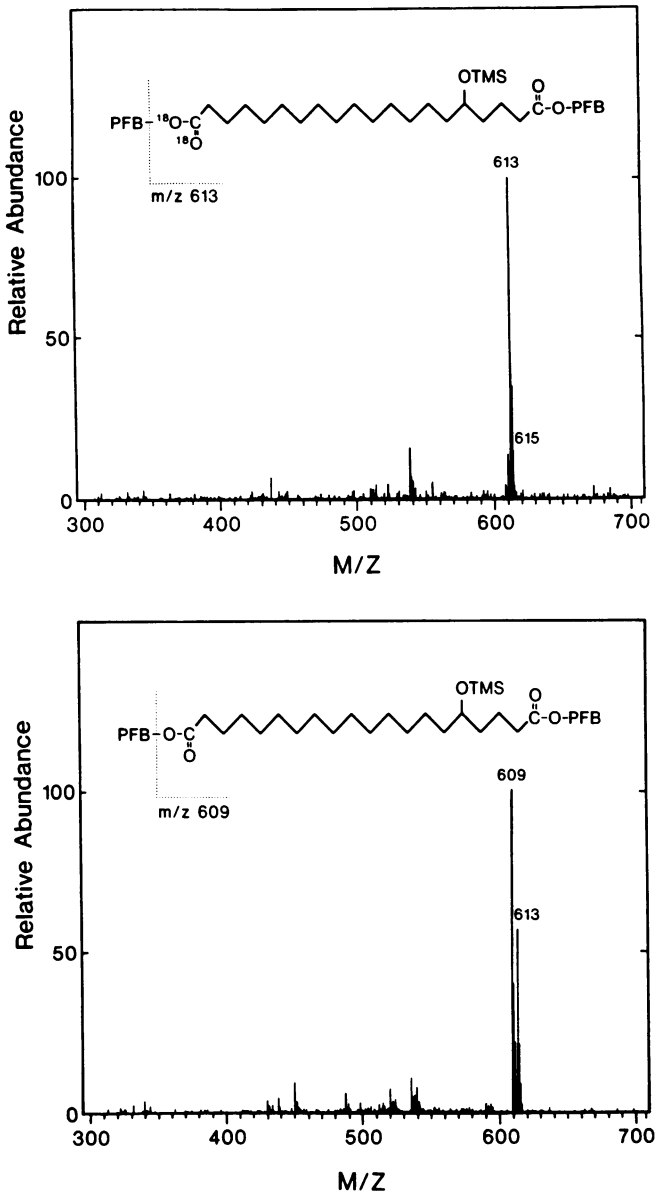

Figure 3. Mass spectrometric analysis of ω-carboxy-LTE_4 by negative ion chemical ionization. A 20-ml sample of urine was spiked with 220 ng of [$^{18}O_2$]ω-carboxy-LTE_4, and ω-carboxy-LTE_4 was separated by RP-HPLC, derivatized, and analyzed by GC-MS as described in Methods. *Upper panel:* control urine showing an intense signal for the ^{18}O-labeled internal standard at m/z 613; *lower panel:* urine of patient 7 (Table I) with a concentration of 38 nM of ω-carboxy-LTE_4 showing intense signals for sample and standard at m/z 609 and 613, respectively.

(*upper panel*) and the analysis of the urine of patient 7 (*lower panel*). The median of the ω-carboxy-LTE_4/creatinine ratio in the patients is a factor of 68 in excess of the corresponding control level.

Quantification of ω-carboxy-tetranor-LTE_3. As shown in Table II, the β-oxidation product of ω-carboxy-LTE_4, ω-carboxy-tetranor-LTE_3, was detectable by RIA exclusively in normal infants, but was below the detection limit in the patients.

Identification and quantification of LTB_4. LTB_4 could be identified by GC-MS-MS in urine samples of patients as shown in Fig. 4 (*upper panel*) but not of controls. The daughter ions at

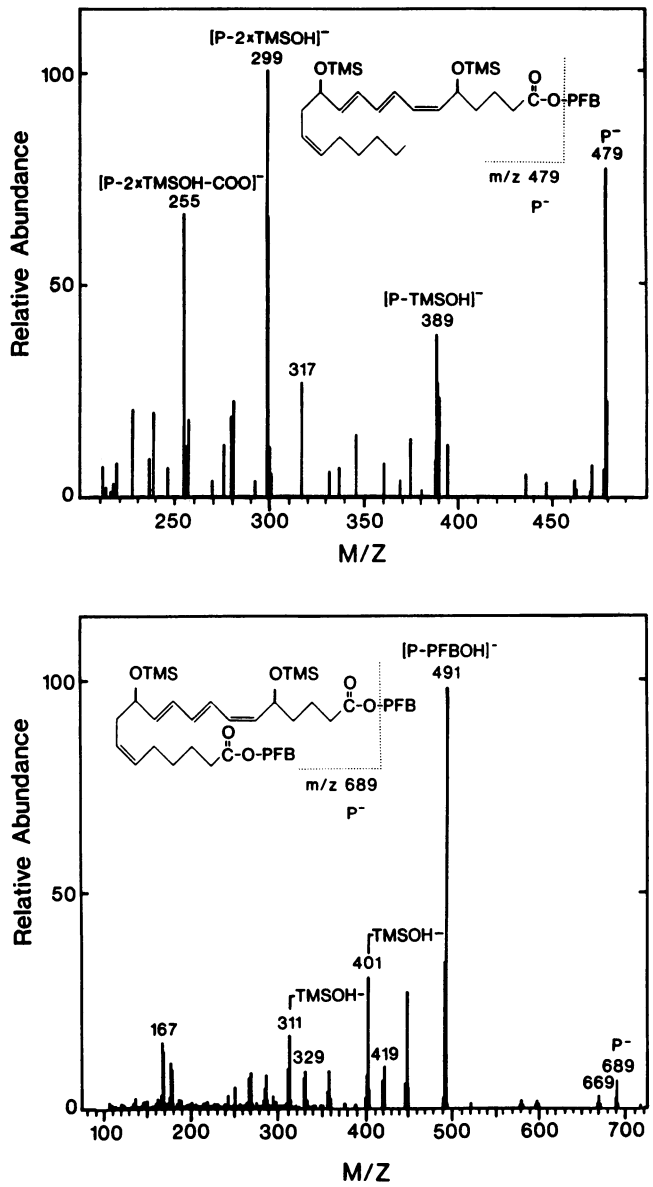

Figure 4. CAD mass spectra by GC-MS-MS analysis of the PFB ester/TMS ether derivatives of urinary LTB_4 (*upper panel*) and ω-carboxy-LTB_4 (*lower panel*) in patients with peroxisomal deficiency. The LT fractions were prepared as described in Methods. The mass spectra were generated by CAD of the corresponding $[M\text{-}PFB]^-$ (abbreviated as P^-) parent ions which represent the base peaks in the corresponding negative ion CI mass spectra. These ions are found at m/z 479 for LTB_4 and m/z 689 for ω-carboxy-LTB_4.

m/z 389, 299, and 255 in the mass spectrum of the HPLC fraction with the retention time of $[^3H]LTB_4$ (Fig. 4, *upper panel*) result from consecutive loss of one TMSOH group (m/z 389), two TMSOH groups (m/z 299), and two TMSOH groups plus CO_2 (m/z 255) by CAD from the parent ion at m/z 479. As shown in Table II, LTB_4 could be quantified by RIA only in urine samples of the patients, whereas it was below the detection limit in the corresponding samples of the controls.

Identification of ω-carboxy-LTB_4. In urine samples of all patients, the presence of ω-carboxy-LTB_4 was proven qualitatively by GC-MS-MS as shown in Fig. 4 (*lower panel*), whereas in all the samples of controls the corresponding signals were too weak to allow for identification. In the CAD mass spectrum of the PFB-TMS derivative from the HPLC peak with the retention time of tritiated ω-carboxy-LTB_4 (Fig. 4, *lower panel*), the most prominent daughter ion was m/z 491 which results from loss of PFB alcohol from the parent ion at m/z 689 ($[M\text{-}PFB]^-$). Other characteristic ions were observed at m/z 401 and 311 from consecutive loss of each TMSOH group from m/z 491 indicating two hydroxy groups.

Discussion

Peroxisome deficiency disorders, also termed disorders of peroxisome biogenesis, such as the Zellweger syndrome and neonatal adrenoleukodystrophy, are characterized by typical clinical features (35, 36) and by a specific set of biochemical abnormalities that include increased VLCFAs in plasma and fibroblasts (45), greatly elevated levels of intermediates of bile acid biosynthesis in plasma (46) and impaired de novo plasmalogen biosynthesis in fibroblasts (47). As shown in Table I, all these characteristic changes were found in the patients investigated in this study.

In vitro and in vivo studies have demonstrated that peroxisomes play a major role in the β-oxidation of LTs from the ω-end (44). In peroxisome deficiency one would expect, therefore, as a consequence a lack of β-oxidation products of LTs in urine. The degradation product of LTE_4 via β-oxidation, ω-carboxy-tetranor-LTE_3, has been identified as a urinary metabolite in the human (50). In the case of LTB_4, ω- and β-oxidation products have never been detected in urine (27) because of the extensive oxidative degradation of LTB_4 (16, 27). The mass spectra of the PFB-TMS derivatives of the compounds co-eluting with ^{18}O-labeled ω-carboxy-LTE_4 and ^3H-labeled ω-carboxy-LTB_4, and LTB_4, shown in Figs. 3 and 4, closely correspond to the ones obtained from the respective synthetic standards. This demonstrates unequivocally the presence of ω-carboxy-LTE_4, ω-carboxy-LTB_4, and LTB_4 in urine of patients with peroxisome deficiency. The present study shows an altered pattern of LT metabolites in urine which is consistent with an impairment of peroxisomal LT degradation. It is in accordance with the metabolic block indicated in Fig. 1 that ω-carboxy-LTE_4 was excreted in large amounts in urine of the patients (Table II). The presence of ω-carboxy-LTB_4 in patients' urine is of particular interest, because its degradation in isolated mitochondria has been shown to be an alternative route (44). In order to evaluate the importance of peroxisomes in humans with regard to the degradation of ω-carboxy-LTB_4, a normal mitochondrial function in these patients is a prerequisite. Abnormal structure and function of liver mitochondria have been described in some patients with Zellweger syndrome (33); although this is not a consistent finding in all patients (36). As shown in Table I, VLCFAs, which normally are oxidized in peroxisomes, accumulate in the patients. However, long chain fatty acids, which are mainly oxidized in mitochondria, did not accumulate. The latter results support the conclusion that the mitochondrial β-oxidation capacity was not impaired in our patients. In some of the patients listed in Table I, mitochondrial β-oxidation activity was assayed in cultured fibroblasts using $[1\text{-}^{14}C]$palmitate and found to be in the range of normal subjects. Degradation of ω-carboxy-LTB_4 in mitochondria, therefore, seems to be insufficient or not functioning in the patients. Furthermore, it is consistent with the lack of

peroxisomal β-oxidation in peroxisome deficiency (Fig. 1) that the β-oxidation product ω-carboxy-tetranor-LTE_3 was absent in all patient urines whereas this metabolite was detectable in normal infants, indicating an impairment of β-oxidation of ω-carboxy-LTE_4 in peroxisomal deficiency (Table II). Our results concerning impaired LT degradation and inactivation in peroxisomal deficiency together with the identification of peroxisomes as the site of LT degradation (44) contribute to the characterization of the functional role of peroxisomes in humans. In addition to the role of peroxisomes in the oxidation of VLCFAs (53) and bile acid precursors (43, 54–56), the organelle is involved in the chain-shortening of prostaglandins E_2 and $F_{2\alpha}$ from the C1-carboxy terminus (57–59) as well as in the degradation of dicarboxylic acids which are formed from long chain fatty acids via ω-oxidation (60).

As an unexpected finding, both LTE_4 and LTB_4 were excreted in increased amounts in the urine of patients (Table II). There are no data to suggest that ω-hydroxylation or conversion to ω-carboxy-LTE_4 and ω-carboxy-LTB_4 are impaired in peroxisome deficiency. However, lipids other than leukotrienes, which are normally degraded in peroxisomes, such as prostaglandins (57–59) and VLCFAs (53), are expected to accumulate at manifold higher concentrations than the LTs. Such compounds may interfere with hepatocellular uptake and/or one or more of the enzymes involved in LT catabolism by ω-hydroxylation, formation of the ω-carboxy derivative, and coenzyme A activation. At present, it cannot be decided whether competitive inhibition of one or more of these reactions leading from LTE_4 and LTB_4 to the respective substrates for peroxisomal β-oxidation is effective in peroxisome deficiency.

Urinary LTE_4 concentration may be elevated due to an increase in systemic cysteinyl LT production (28–32). In addition to LTE_4, the large amount of ω-carboxy-LTE_4 in urine suggests that there is an enhanced synthesis of cysteinyl LTs in the peroxisome deficient patients when compared to the matched control subjects. Impaired degradation and inactivation of LTs leading to nanomolar concentrations of these potent mediators (Table II) might be of pathophysiological significance in the course of the disease. LTE_4 is biologically still active, whereas the corresponding ω-oxidation product, ω-carboxy-LTE_4 and its β-oxidized catabolites are biologically inactive (21). Although LTE_4 is less active than LTD_4 and LTC_4 on a molar basis, it is still 10–100 times more active than histamine as a bronchoconstrictor in normal humans (61). Moreover, LTE_4 promotes plasma extravasation at low nanomolar concentration (62). Elevated concentrations of biologically active LTs might contribute to symptoms like vascular leakage, oedema, mucus secretion, and smooth muscle contraction (2–4, 6). ω-Oxidation of LTB_4 to ω-carboxy-LTB_4, which is associated with a loss of biological activity, has been observed in neutrophils (14, 15, 63) as well as in hepatocytes (16, 64). Furthermore, hepatocytes actively β-oxidize ω-carboxy-LTB_4 from the ω-end (16, 44, 65). [^{3}H]LTB_4 was almost completely degraded by ω-oxidation and subsequent β-oxidation from ω-end (27). Therefore, the presence of urinary ω-carboxy-LTB_4 found in all patients and the absence of this metabolite in normal infants indicate, analogous to ω-carboxy-LTE_4, an impairment of the degradation of this metabolite via β-oxidation in peroxisome deficiency. Moreover, the increased levels of LTB_4 (Table II), one of the most powerful chemotactic substances (5), might be of pathophysiological significance.

Whereas increased urinary LTE_4 and *N*-acetyl-LTE_4 occur under several pathophysiologic conditions (28–32), peroxisome deficiency is the first and so far only disorder with a pronounced urinary excretion of ω-carboxy-LTE_4, ω-carboxy-LTB_4, and LTB_4. The measurement of these metabolites, particularly in the absence of detectable amounts of urinary ω-carboxy-tetranor-LTE_3 (Table II) may contribute to the biochemical analyses for diagnosis of peroxisome deficiency disorders.

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