Production of peptide leukotrienes in endotoxin shock

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Arachidonate metabolites are potent mediators generated in endotoxin shock. Following endotoxin administration (15 mg/kg) into unanesthetized rats, we found a rapid biliary secretion of peptide leukotrienes. Analysis of bile for peptide leukotrienes included organic solvent extractions, reversed phase-HPLC, radioimmunoassay (RIA), and spectrophotometry. The major immunoreactive endogenous leukotriene (LT) from bile was eluted between LTC_4 and LTD_4 in three chromatographic systems. It corresponded thereby to a biliary metabolite of injected LTC_4 and LTD_4 which in turn showed the ultraviolet spectrum of a peptide leukotriene. This demonstration of endotoxin-induced generation of peptide LTs in vivo was possible by sequential HPLC and RIA analyses in bile into which peptide LTs are eliminated from blood.

Leukotriene Endotoxin Bile Radioimmunoassay HPLC Eicosanoid

1. INTRODUCTION

Peptide leukotrienes LTC_4, LTD_4, LTE_4 and LTF_4 as well as the dihydroxylated LTB_4 are products of arachidonate metabolism formed via the 5-lipoxygenase pathway [1]. These compounds are mediators in anaphylactic and inflammatory reactions [1,2]. LTs have been demonstrated to elicit, on the other hand, many of the pathophysiologic symptoms [3–6] also observed in endotoxin shock, such as cardiac depression [7–9], augmentation of vascular permeability leading to tissue edema [10,11], and increased leukocyte adhesion to endothelial surfaces [12]. Endotoxins (LPS) are known to trigger the formation of cyclooxygenase [13–15] and 5-lipoxygenase products [16]. LT generation has been demonstrated in vitro to occur in several cell types including mononuclear phagocytes of various mammalian species after appropriate stimulation [2,17–20].

Inhibitor studies in mice have recently suggested a key role for peptide LTs in endotoxin shock [21,22]. Taking into consideration that the hepatobiliary secretion has been shown to be the main route of elimination for i.v. administered peptide LTs [23], we examined the secretion of endogenous peptide LTs into bile from endotoxin-treated rats. The evidence presented here is to our knowledge the first demonstration of peptide LT production induced by LPS in vivo.

Abbreviations: HPLC, high-performance liquid chromatography; HTMP, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; i.p., intraperitoneally; i.v., intravenously; LT, leukotriene; LPS, lipopolysaccharide or endotoxin; RIA, radioimmunoassay

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2. MATERIALS AND METHODS

2.1. *Materials*

Highly purified endotoxin from *S. minnesota* R595 was kindly supplied by Dr C Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, FRG. Sodium pentobarbital was from Serva, Heidelberg. HTMP from Sigma, St. Louis, MO. Tritiated LTC_4 and LTD_4 (40 Ci/mmol) as well as the LTC_4-RIA were obtained from New England Nuclear, Boston, MA. The unlabeled LTD_4 was generously provided by Dr V. Fiedler, Bayer AG, Wuppertal.

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2.2. *Animals*

Female Wistar rats were obtained from Voss, Tuttlingen, FRG. All animals were 12–14 weeks of age and were fed and unrestricted diet containing 20% protein, 38% carbohydrate, 4% fat, of which 26% were linoleic acid, 0.5% linolenic acid and 0.2% C_{20}–C_{22} fatty acids (Alma, Kempten, FRG).

2.3. *Bile sampling*

Bile collection was performed with the animals under light pentobarbital anesthesia (0.14 mmol/kg, i.p.) and subsequent bile duct cannulation. Bile collection in awake animals was performed as follows: a bilio-duodenal anastomosis tube was implanted 1 week before the experiment; for bile collection a loop of the anastomosis tubing (polyethylene, outer diameter 0.6 mm) was tapped subcutaneously. Bile was collected under argon into ice-cold methanol containing 1 mmol/l HTMP and brought to 80% methanol with methanol/1 mmol/l HTMP. After a minimum of 3 h at 0°C, these bile samples were centrifuged for 10 min at 8000 × *g* at 4°C. Aliquots of the deproteinized supernatants were either directly applied to RP-HPLC or subjected to an extraction for peptide LTs [24] prior to RP-HPLC. Before this extraction, methanolic bile samples were evaporated at 35°C under nitrogen.

2.4. *High-performance liquid chromatography*

RP-HPLC of LTs [25,26] was performed on a C_{18}(ODS)-Hypersil column (4.6 × 250 mm, 5 μm particles, Shandon Southern Products, Runcorn, England) using a Kontron apparatus with UV detection operating at 280 nm. The mobile phases were methanol/water/acetic acid (7:3:0.01, by vol.), pH 5.4 (solvent I); the same solvent containing 1 mmol/l EDTA (solvent II); acetonitrile/water/acetic acid (3:7:0.01, by vol.) pH 5.8 (solvent III); the pH values were adjusted with NH_4OH; the flow rates amounted to 1 ml/min. Aliquots of deproteinized or extracted bile samples were made 30% of methanol prior to RP-HPLC. RP-HPLC fractions for radioimmunologic analysis were immediately neutralized with KOH (2 mol/l) and stored at −20°C under argon for up to 3 days.

2.5. *LTC_4-radioimmunoassay (LTC_4-RIA)*

The RIA applied is reported to have the following cross-reactivities: 100% LTC_4, 55% LTD_4, 9% LTE_4, 10% LTC_4- and LTD_4-sulfone, 2% LTE_4-sulfone, 0.07% 5-HETE, 0.006% LTB_4, less than 0.005% other eicosanoids. The detection limit was at <0.03 pmol LTC_4. Aliquots of neutralized RP-HPLC fractions were evaporated to dryness at room temperature under reduced pressure and resuspended in assay buffer for subsequent radioimmunologic analysis; under the conditions employed no loss of immunoreactivity was observed.

3. RESULTS

3.1. *Peptide leukotrienes in bile of endotoxin-treated rats*

Intravenous injection of a high dose of endotoxin into anesthetized rats resulted in the production and hepatobiliary secretion of LTC_4-like immunoreactive material (fig.1). In bile samples extracted for peptide LTs, the major portion of RIA-detectable LT resided in a RP-HPLC fraction with a retention time of 1.3 ± 0.1 relative to LTC_4 in solvent I (fig.1). Minor amounts of endogenous

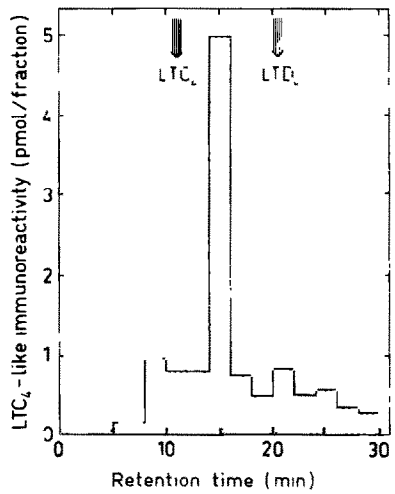

Fig.1. LTC_4-like immunoreactivity in HPLC-fractionated bile extract of endotoxin-treated rats. 1 h after endotoxin (from *S. minnesota* R595; 15 mg/kg, i.v.), bile was collected from anesthetized animals over a 1.5 h period. An aliquot of the deproteinized bile was selectively extracted for peptide LTs [24]; upon subsequent RP-HPLC (solvent II), fractions were analyzed by LTC_4-RIA. The dotted line at retention times shorter than 10 min indicates underestimation by RIA as high LT binding occurred in these fractions. Retention times of internal [^{3}H]LT-standards are indicated by arrows.

LTD_4 were detected in bile of rats during the first hour after LPS administration. When LPS was administered to awake animals, the biliary concentration of the endogenous peptide LT metabolite increased significantly within 30 min after LPS and returned to the basal level after 2 h (fig.2). Injection of 0.9% NaCl did not cause a significant change in the biliary concentration of the LTC_4-like immunoreactive material.

3.2. *Comparative HPLC-analyses of biliary leukotriene components*

The endogenous LTC_4-like immunoreactive material (figs.1 and 2) corresponded in its chromatographic properties to a biliary metabolite of i.v.

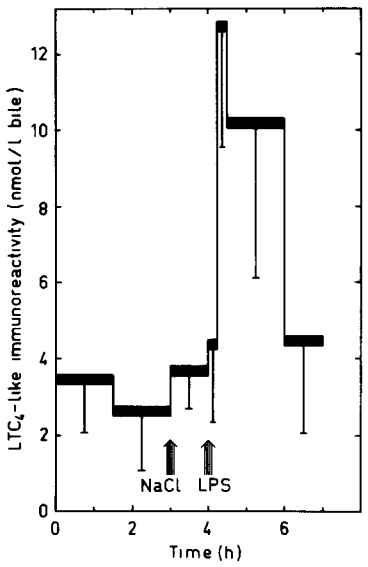

Fig.2. Production of peptide leukotrienes in endotoxin-treated rats. Bile was collected from unanesthetized animals by s.c. tapping of a bile fistula implanted 1 week prior to the experiment. Biliary LTC_4-like immunoreactivity before and after i.v. injection of NaCl and endotoxin (LPS, from *S. minnesota* R595; 15 mg/kg) was determined by RIA in fractions after RP-HPLC separation (solvent II) of deproteinized samples. The figure represents the major immunoreactive material in bile eluting with a retention time relative to LTC_4 of 1.3 ± 0.1 (see fig.1). Horizontal bars indicate concentrations in the respective bile sampling period. Mean values ± SE from 4–5 animals are given. Concentration of LTC_4-like immunoreactivity was significantly elevated above control ($P < 0.02$) 0.25–2 h after LPS as determined by student's *t*-test for paired observations.

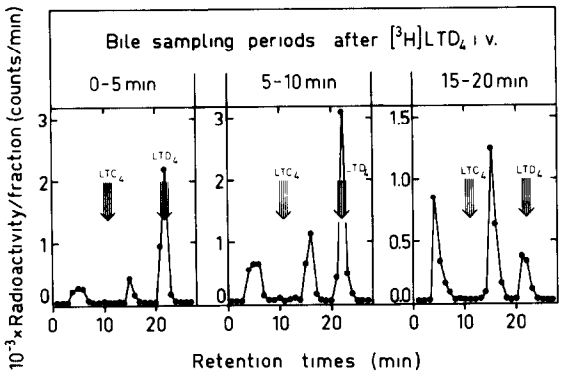

Fig.3. RP-HPLC analysis of rat bile after i.v. [^{3}H]LTD_4. Bile was collected from anesthetized animals during the indicated time periods following i.v. administration of tracer [^{3}H]LTD_4 (1 μCi/kg). Panels represent radioactivity in HPLC-fractions (solvent II) from 20 μl native bile after deproteinization; fractions of 1 ml were counted for ^{3}H radioactivity. Retention times of internal LT standards are indicated by arrows. Note that the relative amount of the metabolite eluting between LTC_4 and LTD_4 increased with time after i.v. [^{3}H]LTD_4 injection.

injected [^{3}H]LTD_4 (fig.3, solvent II), LTD_4 (fig.4, solvent I) and [^{3}H]LTC_4 (solvent II). Using HPLC solvent III we confirmed the identical chromatographic properties of the immunoreactive endogenous LT and the respective metabolite of [^{3}H]-LTC_4, [^{3}H]LTD_4, or LTD_4 (not shown). This metabolite represented a major fraction (about 30%) of the total biliary radioactivity over a 30 min period after i.v. injected [^{3}H]LTC_4 or [^{3}H]LTD_4; its relative amount in the respective bile samples increased with time (fig.3).

3.3. *Ultraviolet spectra of biliary leukotrienes*

Spectrophotometric analysis of bile components after LTD_4 (50 nmol/kg, i.v.) demonstrated that much of the injected LTD_4 appeared in bile unmetabolized (absorbance maximum, 280 nm; shoulders at 270 and about 290 nm) (fig.4, upper right panel). The ultraviolet spectrum of the more polar LTD_4 metabolite coeluting with the endogenous immunoreactive LTC_4-like material (fig.1) showed an intact peptide LT spectrum with the absorbance maximum at 280 nm and shoulders at 270 and about 290 nm (fig.4, upper left panel).

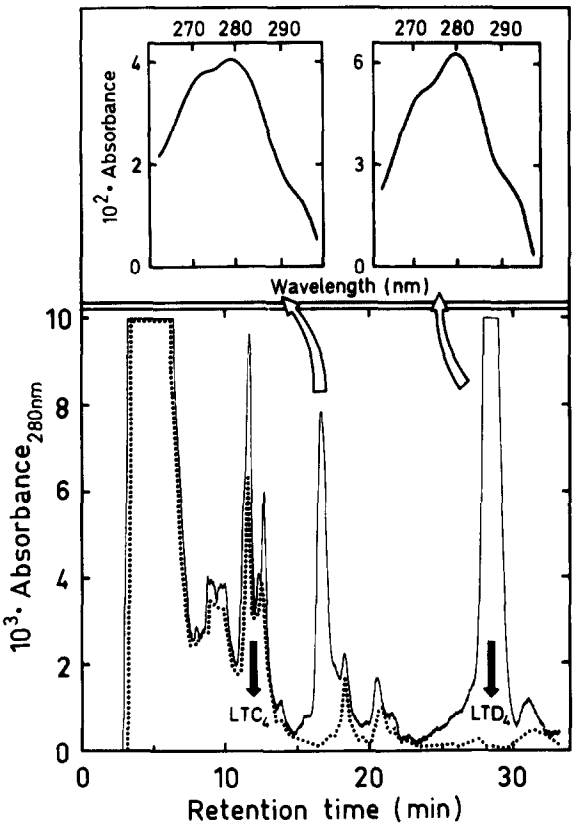

Fig.4. RP-HPLC profile and ultraviolet spectra of peptide leukotrienes in bile of rats after i.v. LTD_4. Bile was collected from anesthetized rats between 0 and 10 min after completion of LTD_4 administration (50 nmol/kg, i.v. within 5 min). Lower panel: RP-HPLC (solvent I) of a deproteinized bile aliquot corresponding to 50 μl native bile; striped arrows indicate retention times of [^{3}H]LT standards. Dotted line represents absorbance of control bile. Upper panels: Ultraviolet spectra of corresponding Rp-HPLC fractions (open arrows). The two HPLC fractions were neutralized, evaporated under nitrogen and their spectra recorded after resuspension in 50% methanol (v/v).

4. DISCUSSION

This study provides direct evidence for LPS-induced production of peptide LTs in vivo (figs 1 and 2). As judged from the biliary appearance of peptide LTs, LPS triggered a rapid and transient generation of peptide LTs that returned to basal levels within 2 h after LPS administration (fig.2). Under the experimental conditions described, only small amounts of LTD_4 were eliminated into bile of unanesthetized animals. Employing the HPLC-system containing acetonitrile as solvent, we excluded the appearance of biliary LTC_4 to any appreciable amount.

The major part of LPS-elicited peptide LTs in bile consisted of a metabolite eluting between LTC_4 and LTD_4 in 3 different HPLC systems. The indicated concentration of this peptide LT in bile is likely to be an underestimation as it was calculated assuming 100% cross-reactivity with the LTC_4 antibody. Taking further into account the impaired hepatobiliary LT elimination after LPS treatment [27], one may expect that distinctly higher amounts of peptide LTs were produced in the animals.

The structural identity of the main endogenous peptide LT found in bile after LPS awaits additional characterization. From the comparative analyses with injected LTs we conclude that the endogenous peptide LT is a metabolite of LTC_4 and LTD_4 (figs 3 and 4). Combined analysis of bile extracts using HPLC and RIA provides a novel approach to the determination of peptide LTs generated in vivo under various pathophysiological conditions.

NOTE ADDED IN PROOF

The endogenous peptide LT described in figs 1 and 2 has been identified as *N*-acetyl-LTE_4 by coelutions on HPLC with standard synthesized chemically by G. Weckbecker, Freiburg.

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