Uptake, production and metabolism of cysteinyl leukotrienes in the isolated perfused rat liver

Inhibition of leukotriene uptake by cyclosporine

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1. The isolated perfused rat liver efficiently takes up cysteinyl leukotrienes (LTs) C_4, D_4, E_4 and *N*-acetyl-LTE_4 from circulation. More than 70% of these cysteinyl LTs are excreted from liver into bile within 1 h of onset of a 5 min infusion, while about 5% remain in the liver. About 20% of infused *N*-acetyl-LTE_4 escapes hepatic first-pass extraction under our conditions. 2. Metabolites of LTC_4 appearing in bile within 20 min of the onset of infusion include mainly LTD_4 and *N*-acetyl-LTE_4, but also ω-hydroxy-*N*-acetyl-LTE_4 and ω-carboxy-*N*-acetyl-LTE_4. Metabolites generated from ω-carboxy-*N*-acetyl-LTE_4 by β-oxidation from the ω-end represent the major biliary LTs secreted at later times. 3. Stimulation of the isolated perfused liver by the combined infusion of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the Ca^{2+} ionophore A23187 results in a transient increase of endogenous cysteinyl LT production, which is independent of extrahepatic cells. 4. The immunosuppressive drug cyclosporine causes a dose-dependent inhibition of hepatobiliary cysteinyl LT excretion, probably by interference with the sinusoidal uptake system for cysteinyl LTs.

INTRODUCTION

The liver is recognized as a key organ in the elimination and deactivation of leukotrienes (LTs) *in vivo* [1–4]. These eicosanoids mediate various pathophysiological and physiological reactions such as circulatory and respiratory dysfunction, tissue trauma and inflammation [5–9]. During their short half-life in the circulation [10–13], cysteinyl LTs undergo rapid intravascular metabolism yielding LTD and LTE from parent LTC [10,14,15]. These cysteinyl LTs are rapidly and efficiently removed from the circulation, predominantly by the liver, and secreted into bile [16–20].

Local mediator actions of cysteinyl LTs on the liver *in vivo* contribute to intrahepatic cholestasis [1] and may play a role in experimental hepatitis [21,22]. Direct effects of LTC_4 and LTD_4 were recently demonstrated in the isolated perfused liver, such as increased glucose and lactate output [23–26], decreased oxygen uptake [25,26] and perfusion flow [23–26], increased portal pressure [25,26] and stimulated Ca^{2+} and K^+ release [25].

The contribution of the liver to cysteinyl LT uptake and metabolism has been studied in rats *in vivo*, mainly with LTC_4 [1,13,14,17–19,27,28], using hepatocytes and hepatoma cells *in vitro* [29,30], and recently also in the isolated perfused liver [24]. Therefore, the first objective of the present study was to monitor the distribution and metabolic pathways of LTC_4 as compared with its metabolites LTD_4, LTE_4 and *N*-acetyl-LTE_4 (LTE_4NAc) in the isolated perfused liver. We secondly sought to characterize the liver as a source of endogenous cysteinyl LTs, since rat liver Kupffer cells have recently been demonstrated *in vitro* as a hepatic site of such endogenous cysteinyl LT production [21,31,32]. Thirdly, hepatic transport of cysteinyl LTs was studied under the influence of the immunosuppressive drug cyclosporine. This compound, widely used in organ transplantation, was earlier shown to interfere with hepatocellular functions including bile acid transport [33–35], bile secretion [36,37] and bilirubin uptake [38]. Since cyclosporine itself is metabolized and eliminated mainly via the liver [39], we wondered whether cyclosporine also affected hepatic uptake and biliary elimination of cysteinyl LTs.

MATERIALS AND METHODS

Animals and materials

Male Wistar rats (200–250 g) were obtained from the Zentralinstitut für Versuchstiere (Hannover, Germany) and kept as described [18]. [14,15-^{3}H]LTC_4, [14,15-^{3}H]LTD_4, [14,15-^{3}H]LTE_4 [1.48 TBq/mmol (40 Ci/mmol) each] were purchased from New England Nuclear/DuPont (Boston, MA, U.S.A.), and [5,6,8,9,11,12,14,15-^{3}H]LTE_4 [3.7 TBq/mmol (100 Ci/mmol)] was from Amersham International (Little Chalfont, Bucks., U.K.). *N*-Acetyl-[14,15-^{3}H]LTE_4 and *N*-acetyl-[5,6,8,9,11,12,14,15-^{3}H]LTE_4 were synthesized from the appropriate radioactive LTE_4 precursor [19]. Reverse-phase h.p.l.c. separation [19] was used to purify and to control the purity of all LTs. Ketamine (Ketanest) was from Parke-Davis (Freiburg, Germany), xylazine (Rompun) from Bayer (Leverkusen, Germany), heparin (Liquemin) from Hoffmann–La Roche (Grenzach-Wyhlen, Germany), and cyclosporine (cyclosporine A, Sandimmun) from Sandoz (Basel, Switzerland). Cremo-

Abbreviations used: HTMP, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; LT, leukotriene; LTE_4NAc, *N*-acetyl-LTE_4; r.i.a., radioimmunoassay; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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phor EL [poly(oxyethylene)-40-ricinoleic acid], 12-*O*-tetradecanoylphorbol-13-acetate (TPA), sodium taurocholate and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (HTMP) were from Sigma (St. Louis, MO, U.S.A.). Bovine serum albumin (fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany) and A23187 was from Calbiochem (Frankfurt, Germany). All other chemicals were of reagent grade and from commercial sources. The antibody used in the radioimmunoassay (r.i.a.) for the quantitative determination of cysteinyl LTs was kindly provided by Dr. F. Kohen, Weizmann Institute of Science (Rehovot, Israel). Standards for ω-hydroxy-*N*-acetyl-LTE_4 and ω-carboxy-*N*-acetyl-LTE_4 were kindly provided by Dr. J. Rokach, Merck Frosst (Pointe-Claire Dorval, Canada).

Liver perfusion

The animals were anaesthetized by intraperitoneal injection of ketamine (80 mg/kg body wt.) and xylazine (12 mg/kg body wt.). After cannulation of the common bile duct with polyethylene tubing (inner diameter = 0.3 mm), the liver was perfused in a recirculating mode via the portal vein (antegrade direction) or via the hepatic vein (retrograde direction) in a 37 °C cabinet [40]. The perfusate medium consisted of a Krebs–Henseleit hydrogen carbonate buffer containing 5 mM-glucose, 2 mM-lactate and 0.2 mM-pyruvate equilibrated with O_2/CO_2 (19:1; v/v) and was enriched with 1% (w/v) bovine serum albumin and 20% (v/v) washed bovine erythrocytes [41]. In experiments on endogenous LT production, the perfusion medium contained in addition 2 mM-cysteine. Perfusions with cyclosporine or its vehicle were performed with erythrocyte-free medium in a recirculating mode after perfusing the liver essentially free of erythrocytes with about 600 ml of non-recirculated perfusion medium; total recirculating perfusate amounted to 100 ml. Perfusion pressure was constant at about 980 Pa (10 cm of water) with a flow rate of about 4 ml/min per g of liver under basal conditions. Flow was measured by fractionating the effluent. Sodium taurocholate was constantly infused at a rate of 15 μmol/h. After a 30 min equilibration period the experiment was started. Bile was collected continuously into ice-cold 90% (v/v) aqueous methanol containing 1 mM-HTMP/0.5 mM-EDTA, pH 7.4. After evaporation, [^{3}H]LTs were dissolved in saline containing 0.1% (w/v) bovine serum albumin and infused via the portal vein catheter for 5 min at a dose of 148 kBq/kg (4 μCi/kg), corresponding to 100 pmol/kg body wt. A23187 was dissolved in Me_2SO, diluted to 200 μl with 25 mM-glucose and infused within 1 min; TPA was infused similarly after diluting a stock solution in Me_2SO with 600 μl of saline. The vehicle of cyclosporine solutions consisted of a mixture of Cremophor EL and ethanol (66:33, v/v).

Sample preparation for leukotriene analysis

Effluent and liver were added during or at the end of the experiment respectively to ice-cold 90% (v/v) aqueous methanol containing 1 mM-HTMP/0.5 mM-EDTA, pH 7.4. The liver was homogenized at 0 °C in this methanolic solution. After a minimum of 3 h at −20 °C, all methanolic samples of effluent, bile and liver were centrifuged for 10 min at 10000 *g* at −10 °C; the liver tissue pellets were repeatedly extracted by this procedure. Aliquots of the supernatants were counted for radioactivity. The deproteinized supernatants were evaporated to dryness, resuspended in 30% (v/v) aqueous methanol and filtered (Millex HV_4; Millipore/Waters, Milford, U.S.A.) before reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. separation of leukotrienes

H.p.l.c. was performed on a C_{18}-Hypersil column (4.6 mm × 250 mm, 5 μm particles; Shandon, Runcorn, U.K.) with a C_{18}-precolumn (Millipore/Waters, Milford, U.S.A.). The mobile phase gradient (solvent system 1) consisted of 0.1% (v/v) acetic acid/1 mM-EDTA, pH 5.0 (adjusted with ammonium hydroxide) containing, at 0 min, no methanol, switching at 5 min to 40% (v/v) methanol and reaching 80% (v/v) methanol at 40 min. The flow rate was 1 ml/min. Continuous determination of ^{3}H in the h.p.l.c. eluent was performed with a liquid scintillation device (LB 506; Berthold, Wildbad, Germany) using Rialuma scintillation mixture (Baker Chemicals, Gross-Gerau, Germany). The isocratic mobile phase (solvent system 2) used for LT separation before r.i.a. consisted of methanol/water/acetic acid (65:35:0.1, by vol.), containing 1 mM-EDTA, pH 5.6 (adjusted with ammonium hydroxide).

Radioimmunoassay of leukotrienes

Evaporated h.p.l.c. fractions were submitted to r.i.a. as described [12]. The lower detection limit for LTE_4 was about 7 fmol, and with the antibody used the relative cross-reactivities of LTE_4, LTE_4NAc, LTD_4 and LTC_4 amounted to 100, 144, 160, and 219% respectively on a molar basis. Concentrations of cysteinyl LTs were calculated according to their individual cross-reactivities in the r.i.a. and corrected for the recovery of [^{3}H]LTs added to each sample prior to h.p.l.c. in amounts not interfering with the r.i.a.

RESULTS

Uptake and biliary excretion of cysteinyl leukotrienes

When livers were perfused via the portal vein (antegrade direction) in a recirculating mode, about 80% of infused cysteinyl LTs were extracted by the liver (Table 1). This efficient hepatic uptake showed no statistically significant differences between LTC_4 and its metabolites

Table 1. Distribution of radioactivity 1 h after infusion of [^{3}H]LTC_4 or its metabolites in isolated perfused rat liver

Livers were perfused in the antegrade direction with erythrocyte-containing medium. Bile was collected continuously during and after infusion of [^{3}H]LTC_4 or its metabolites [148 kBq (4 μCi)/kg body wt. each] within 5 min. Liver and perfusate were sampled 1 h after the onset of LT infusion as described (see the Materials and methods section). Data correspond to % of infused radioactivity recovered within 1 h in bile or detected at 1 h in liver and recirculating perfusate. Mean values ± S.D. from three or four perfused livers/group are given.

	Infused ^{3}H recovered (%)			
Infused leukotriene...	LTC_4	LTD_4	LTE_4	LTE_4NAc
Bile	71±8	79±7	70±5	70±4
Liver	6±1	5±3	6±2	3±1
Perfusate	23±8	16±7	24±7	27±4

LTD_4, LTE_4 and LTE_4NAc. Within 1 h of the onset of LT infusion, most of the LT radioactivity was excreted from liver into bile, while about 5% of the administered LTs still remained in the liver (Table 1). Depending on the cysteinyl LT infused, between 16 and 27% of the administered LT radioactivity was still circulating in the perfusate at 1 h (Table 1).

In separate perfusions, we studied hepatic first-pass extraction of [^{3}H]LTE_4NAc by collecting total liver effluent during and up to 2 min after LT infusion; after that time livers were perfused in a recirculating mode. Under these conditions, 18 ± 4% (mean $\pm$ S.D., $n = 3$) of the infused LT escaped first-pass extraction by the liver.

When livers were perfused in the retrograde direction via the hepatic vein, biliary ^{3}H excretion within 1 h of [^{3}H]LTE_4NAc infusion amounted to 57%, while the liver contained 5% and the perfusate 38% of the infused tracer at that time (mean values from two perfusions, individual values varying by less than 10% from the mean).

Hepatic metabolism of cysteinyl leukotrienes

LTD_4 was the predominant metabolite excreted in bile 10–20 min after the onset of a 5 min [^{3}H]LTC_4 infusion. Unmetabolized LTC_4 amounted to 20% of the radioactivity in this bile sample, and the remaining radioactivity consisted mainly of LTE_4NAc and ω-carboxy-LTE_4NAc (Fig. 1*a*). LT radioactivity in bile sampled within 60–80 min contained largely metabolites with retention times of 28, 23, and 20 min respectively (Fig. 1*b*); these metabolites probably correspond to ω-carboxy-19,20-dinor-LTE_4NAc, ω-carboxy-17,18,19,20-tetranor-14,15-dihydro-LTE_4NAc and ω-carboxy-17,18,19,20-tetranor-Δ^{13}-LTE_4NAc respectively, as judged from their chromatographic behaviour in an h.p.l.c. solvent system in which these β-oxidation products of ω-carboxy-LTE_4NAc were recently identified [42].

After infusion of [^{3}H]LTE_4, LT metabolites in bile sampled 0–10 min after onset of tracer infusion were mainly represented by LTE_4NAc (70%), but small amounts of ω-hydroxy-LTE_4NAc (4%) were detected in addition to ω-carboxy-LTE_4NAc (10%) and its β-oxidation products; unmetabolized LTE_4 could not be detected in bile (Fig. 2). LT radioactivity in bile collected 40–60 min after the onset of [^{3}H]LTE_4 infusion was accounted for mainly by the above-mentioned metabolites generated by β-oxidation of ω-carboxy-LTE_4NAc from the ω-end (Fig. 2*b*). No additional radioactive metabolites were detected in bile after infusion of *N*-acetyl-[5,6,8,9,11,12,14,15-^{3}H]LTE_4 as compared with those found after *N*-acetyl-[14,15-^{3}H]LTE_4 or [14,15-^{3}H]LTE_4 infusion (Fig. 2).

LT radioactivity detected in the liver at 1 h after [^{3}H]LTE_4 infusion consisted mainly of LTE_4NAc (90%), while the β-oxidation metabolites of ω-carboxy-LTE_4NAc and unmetabolized LTE_4 each represented 5%.

Endogenous production of cysteinyl leukotrienes in liver

LT production was monitored by measuring immunoreactive cysteinyl LTs after h.p.l.c. separation of bile. Biliary LTs were determined in samples collected before and up to 1 h after stimulation of the liver. In comparison with biliary cysteinyl LT concentrations from unstimulated control liver (Fig. 3*a*), increased amounts of

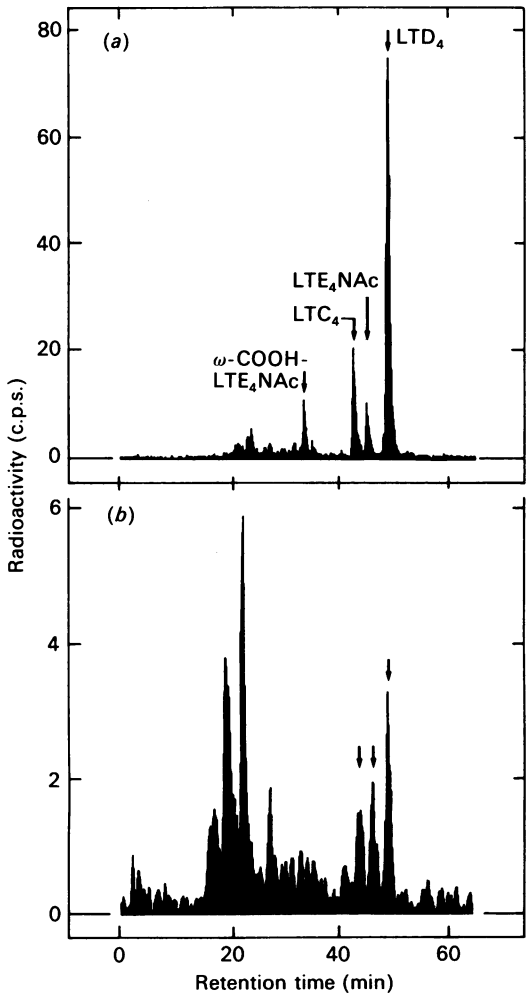

Fig. 1. Reverse-phase h.p.l.c. separation of [^{3}H]LTC_4 metabolites in bile from isolated perfused liver

Liver was perfused in the antegrade direction in a recirculating mode with erythrocyte-containing buffer. Bile was collected 10–20 min (*a*) and 60–80 min (*b*) after the onset of a 5 min [^{3}H]LTC_4 infusion. Samples were deproteinized and separated by reverse-phase h.p.l.c. using solvent system 1 as described in the Materials and methods section. Arrows indicate retention times of [^{3}H]LT standards.

cysteinyl LTs were generated and excreted into bile after stimulation of the liver by the combined infusion of the phorbol ester TPA (300 nmol/kg body wt.) and the Ca^{2+} ionophore A23187 (60 nmol/kg body wt.) (Fig. 3*b*). The biliary pattern of endogenous cysteinyl LTs demonstrated LTE_4NAc as the predominant metabolite, while varying but minor amounts of LTC_4 and LTD_4 were additionally found. Judged from biliary LT excretion, the transient increase in the production rate of cysteinyl LTs lasted for 20 min and reached up to 0.2 ± 0.1 pmol of cysteinyl LTs/10 min per g of liver (mean $\pm$ S.D., three perfusions). The observed production of cysteinyl LTs occurred to the same extent whether livers were perfused in a non-recirculating mode with erythrocyte-free buffer (Fig. 3) or in a recirculating mode using erythrocyte-containing buffer. Infusion of either TPA or A23187 alone did not cause an increased generation of cysteinyl LTs in liver.

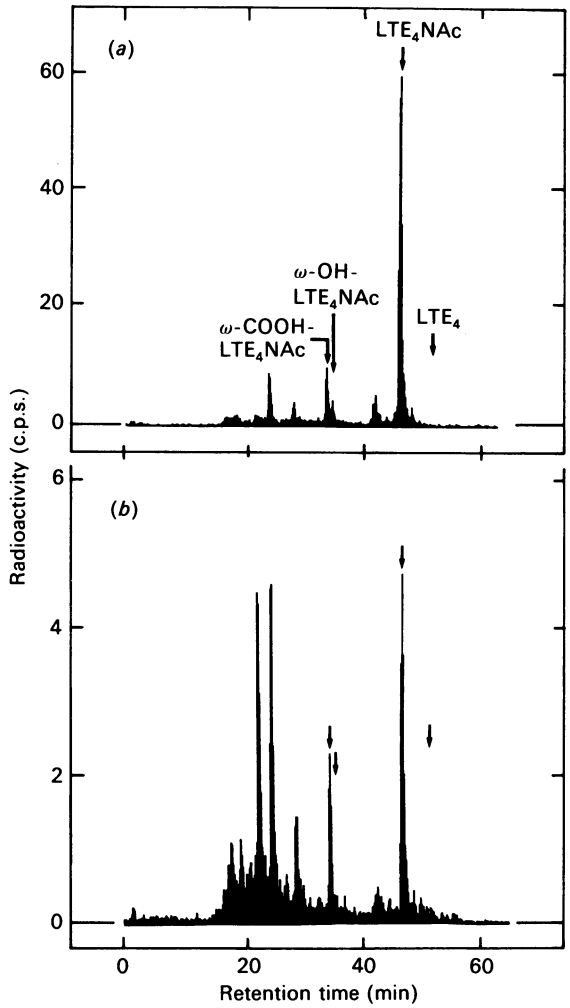

Fig. 2. Biliary excretion of [^{3}H]LTE$_4$ metabolites from isolated perfused rat liver

Liver was perfused in the antegrade direction in a recirculating mode with erythrocyte-containing buffer. Bile was collected 0–10 min (*a*) and 40–60 min (*b*) after onset of a 5 min [^{3}H]LTE$_4$ infusion and analysed after deproteinization by reverse-phase h.p.l.c. separation using solvent system 1 (see the Materials and methods section). Arrows indicate retention times of [^{3}H]LT standards.

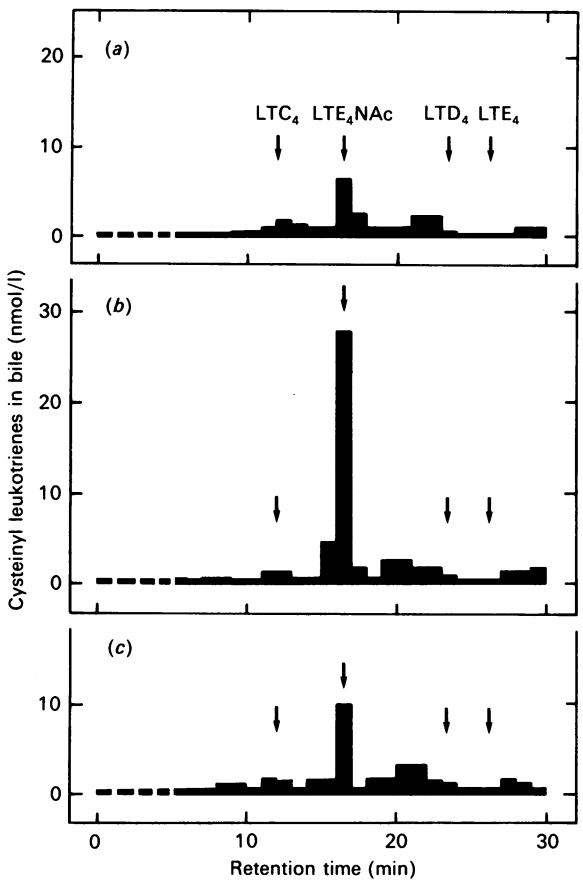

Fig. 3. Endogenous cysteinyl leukotriene production in isolated perfused liver stimulated by TPA and A23187

Liver was perfused in the antegrade direction in a non-recirculating mode with erythrocyte-free buffer. LTs were determined by r.i.a. after h.p.l.c. separation (solvent system 2) of bile sampled before and after a 1 min infusion of TPA (300 nmol/kg of body wt.) and A23187 (60 nmol/kg of body wt.). Bile was collected before (control, *a*), within 0–10 min of (*b*) or 10–20 min after (*c*) TPA/A23187 infusion. Arrows indicate retention times of internal [^{3}H]LT standards in these h.p.l.c. separations.

Inhibition of hepatobiliary leukotriene elimination by cyclosporine

The effect of cyclosporine on the uptake and biliary excretion of cysteinyl LTs was studied by adding the drug or its vehicle to the perfusate 30 min before [^{3}H]LTE$_4$ infusion into liver perfused in a recirculating mode. Cyclosporine pretreatment resulted in a dose-dependent reduction of biliary LT elimination (Table 2). At a dose of 20 mg/kg body wt., cyclosporine inhibited biliary LT excretion by about 80% as compared with vehicle pretreatment (Table 2). Concomitantly, bile flow was reduced by 42 ± 7% (means ± S.D., three perfusions) under these conditions compared with pre-cyclosporine values. Cyclosporine at a dose of 2 mg/kg body wt. still impaired LT excretion into bile by 66%, without, however, affecting bile flow at this dosage. When cyclosporine was given at 0.2 mg/kg body wt., biliary LT excretion was no longer significantly decreased (Table 2). LT radioactivity found in the liver at 1 h after [^{3}H]LTE$_4$ infusion was increased about 3-fold by cyclosporine (20 mg/kg body wt.) as compared with vehicle controls, while about 70% of the infused LT still circulated in the perfusate at that time (Table 2). This perfusate LT radioactivity of cyclosporine-treated livers consisted of unmetabolized LTE$_4$, whereas the presence of LTE$_4$ metabolites in liver tissue and bile was demonstrated, as in controls (Fig. 2).

DISCUSSION

The present investigation demonstrates and extends findings that the liver constitutes the main organ involved in the uptake of cysteinyl LTs from circulation, their metabolism and deactivation, and is also a potential site of endogenous cysteinyl LT generation. In line with studies *in vivo* [11–13,17–20], the isolated perfused liver removed LTC$_4$ and its metabolites LTD$_4$, LTE$_4$ and

Table 2. Inhibition of hepatobiliary cysteinyl LT uptake and excretion by cyclosporine in the isolated perfused rat liver

Livers were perfused in the antegrade direction in a recirculating mode with erythrocyte-free medium. Indicated dosages of cyclosporine or its vehicle (Cremophor EL/ethanol; 66:33, v/v) were added to the medium 30 min before a 5 min [^{3}H]LTE_4 infusion [148 kBq (4 μCi)/kg body wt.]. Data correspond to % of infused radioactivity excreted within 1 h into bile or detected at 1 h in liver and perfusate. Mean values ± S.D. from three or four perfusions/group are given. * indicates significant differences compared with the respective value for vehicle-treated livers ($P < 0.05$) according to Student's t test.

	Infused ^{3}H recovered (%)			
		Cyclosporine		
	Vehicle	20 mg/kg	2 mg/kg	0.2 mg/kg
Bile	82±8	15±1*	28±10*	71±8
Liver	6±1	16±4*	14± 5*	11±3*
Perfusate	12±8	69±4*	58± 7*	19±5

LTE_4NAc efficiently from the blood circulation and excreted them into bile after partial metabolism (Figs. 1 and 2; Table 1). The efficacy of the isolated perfused organ in extracting and eliminating cysteinyl LTs [24,43] is further demonstrated by our observation that only about 20% of infused LTE_4NAc escaped first-pass hepatic extraction under non-recirculating conditions. Biliary elimination of LTE_4NAc appears to be reduced when livers are perfused in retrograde direction; this may reflect either a reduced hepatic LT uptake in perivenous hepatic areas leading to their decreased biliary secretion, or an increased release of intrahepatic LT back into the circulation.

Metabolism of cysteinyl LTs in the liver includes intravascular formation of LTD_4 and LTE_4 [24] and proceeds in rat hepatocytes to LTE_4NAc [2,19,27] and its ω-oxidation products, ω-hydroxy-LTE_4NAc and ω-carboxy-LTE_4NAc [4,24,28,44–47]. While LTC_4 [24] and LTD_4 can pass from the circulation via hepatocytes into bile partially unmetabolized (Figs. 1 and 2), intrahepatic N-acetylation of LTE_4 is very active before biliary secretion so that LTE_4 is not detectable in bile after LTE_4 infusion (Fig. 2). Hepatocellular ω-oxidation of LTE_4NAc to ω-carboxy-LTE_4NAc via ω-hydroxy-LTE_4NAc also appears to occur rapidly as judged from the relative biliary amounts of these metabolites (Figs. 1 and 2). The subsequent shortening of the fatty acid backbone of cysteinyl LTs by β-oxidation from the ω-end [4,42,46,47] becomes apparent only in late bile samples (Figs. 1 and 2) and agrees with the earlier finding *in vivo* that these polar LT metabolites in bile increase in proportion with time [14,18].

The liver is not only a target organ for extracting, metabolizing and responding to eicosanoids [1–5,13–20, 22–28,43,47–54], but is in addition able to produce these potent mediators, as shown for prostanoids [31,52–58] and LTs [21,31,32]. Stimulation of endogenous LT production in the isolated perfused organ by the combined action of TPA and A23187 (Fig. 3) confirms this hepatic capacity and is in line with the described synergistic effect of these agents on LT production [59,60]. As with prostanoid formation in the isolated perfused liver [52–57], the enhanced hepatic LT generation was transient and independent of extrahepatic cells (Fig. 3). Since ω-oxidation metabolites of cysteinyl LTs escape our radioimmunological detection, the measured amounts of cysteinyl LTs may well be an underestimation of the originally producted LTs.

The inhibitory action of the immunosuppressive drug cyclosporine on hepatobiliary LT secretion (Table 2) can be dissociated from its cholestatic effect [36,37] as the former was also observed at a cyclosporine dose not affecting bile flow. Cyclosporine exerts its inhibitory action most likely on the sinusoidal uptake system for cysteinyl LTs, since the circulating extrahepatic LTE_4 remained unmetabolized, whereas LTs in liver and bile indicated normal intrahepatic LTE_4 metabolism under this condition. Cyclosporine is known to interfere with several hepatocellular transport systems located at the sinusoidal surface [33–35]. In addition, cyclosporine may also affect hepatocellular transport systems at the canalicular surface, as indicated by recent studies on bile acid excretion from hepatocytes [61]. This is in line with our observation that cyclosporine causes an up to 14-fold decrease in the bile/liver ratio of LT radioactivity as compared with controls (Table 2). The demonstrated action of cyclosporine on hepatic LT extraction in the isolated perfused organ was confirmed in preliminary studies *in vivo* (results not shown) and affects the major route of deactivation and elimination of these eicosanoids. A prolonged biological activity of circulating LTs due to cyclosporine may be critical to the affected organism under conditions of enhanced systemic production of these pro-inflammatory mediators, such as surgical trauma and bacterial infection [11,18].

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