Biochimica et Biophysica Acta, 732 (1983) 69–74
Elsevier

BBA 71706

CHARACTERISTICS OF THE UPTAKE OF CYSTEINE-CONTAINING LEUKOTRIENES BY ISOLATED HEPATOCYTES

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(Received January 5th, 1983)

Key words: Leukotriene uptake; Energy dependence; (Hepatocyte)

Leukotrienes were transported into rat hepatocytes by a temperature- and energy-dependent mechanism. The uptake was saturable with high- and low-affinity sites (K_m values approx. 1 and 17 μM). Competition and kinetic experiments indicated that leukotrienes C_4, D_4 and E_4 were transported by a common mechanism. The maximal velocity of transport was about 50% higher for leukotrienes D_4 and E_4 than for leukotriene C_4. Leukotriene B_4, glutathione disulfide, and the glutathione-*S*-conjugate of acetaminophen did not interfere with the transport of leukotriene C into hepatocytes. This suggests that the process is specific for cysteine-containing leukotrienes. It is likely that the transport mechanism described here participates in biliary excretion of leukotrienes. This route was previously found to be a major one for elimination of leukotriene C_3 in mice and guinea-pigs.

Introduction

Slow-reacting substance of anaphylaxis was discovered in 1938 [1]. Its structure was recently elucidated and the new compounds were called leukotrienes [2,3]. Leukotriene C_4 (5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) is formed from arachidonic acid and glutathione. This compound is metabolized to leukotriene D_4, the corresponding 6-*S*-cysteinylglycine derivative, and leukotriene E_4, the corresponding 6-*S*-cysteine derivative [4,5]. Leukotrienes D and E are formed rapidly in vitro and in vivo from leukotriene C [6,7]. Leukotrienes having different numbers of double bonds in the fatty acid part, namely leukotriene C_3 (5-hydroxy-6-*S*-glutathionyl-7,9-*trans*-11-*cis*-eicosatrienoic acid) and leukotriene C_5 (5-hydroxy-6-*S*-glutathionyl-7,9-*trans*-11,14,17-*cis*-eicosapentaenoic acid [9]), have also been characterized [8,9].

Recent experiments have shown that in mice, intravenously injected ^{3}H-labeled leukotriene C_3 is rapidly taken up by the liver and excreted into the bile [10].

In this report, a transport mechanism for cysteine-containing leukotrienes in isolated hepatocytes is described. Part of the work has been published in a preliminary form [11].

Materials and Methods

Chemicals. Acetoaminophen was obtained from a local drugstore. β-Glucuronidase from bovine liver, type B-1, γ-glutamyl transpeptidase from hog kidney and sulfatase type H-1 from *Helix pomatia* were from Sigma Chemical Company.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide.

Other chemicals were purchased from local companies and were of at least reagent grade.

Leukotrienes. [5,6,8,9,11,12-^{3}H]Leukotriene C_3 was prepared as previously described [6]. [5,6,8,9,11,12,14,15-^{3}H]Leukotriene C_4 was prepared by analogous methods using 5,8,11,14-eicosatetraynoic acid as starting material. Leukotrienes D_3 and D_4 were obtained from leukotrienes C_3 and C_4 by treatment with γ-glutamyl transpeptidase [4]. Leukotrienes E_3 and E_4 were obtained by enzymatic degradation from leukotrienes C_3 and C_4 [5]. The products were purified by reverse-phase HPLC before use [6]. The specific radioactivity of leukotriene C_3 was 50 Ci/mmol and of leukotrienes C_4, D_4 and E_4 10 Ci/mmol. Synthetic leukotrienes B_4 (5(*S*)12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid), D_4 and E_4 were kindly provided by J. Rokach, Merck Frosst Canada, Inc.

Glutathione-S-conjugate of acetaminophen. The glutathione-*S*-conjugate of acetaminophen was prepared as follows. Isolated hepatocytes from phenobarbital-treated rats (80 mg/kg intraperitoneally for 3 days) were incubated in an SO_4^{2-}-free Krebs-Henseleit bicarbonate buffer (pH 7.4), supplemented with 25 mM Hepes/amino acid mixture [12]. The incubation mixture also contained 5 mM acetaminophen and 0.1 M acetone (to increase the synthesis of the glutathione-*S*-conjugate of acetaminophen [13]). After 2.5 h incubation at 37°C under carbogen (95% O_2 + 5% CO_2) gassing, cells were centrifuged at 3000 × *g* for 10 min. The supernatant was acidified with conc. HCl (10 μl/ml) and centrifuged again (1400 × *g*, 8 min). β-Glucuronidase (900 μU/ml) and sulfatase (100 μU/ml) were added to the supernatant, and the pH was adjusted to 5.0. The mixture was kept at 37°C for 16 h, and subsequently extracted with 3 vol. water-saturated diethyl ether four times. An aliquot of the extracted water phase was analyzed by HPLC [14], demonstrating that more than 90% of the acetaminophen was in the form of the glutathione-*S*-conjugate.

Isolation of hepatocytes. Male Sprague-Dawley rats (180–200 g) had free access to water and pelleted rat food. Isolated hepatocytes were prepared as previously described [15].

Incubations. Incubations were performed in rotating round-bottom flasks at 37°C under continuous gassing with carbogen unless otherwise stated. Hepatocytes (10^6 cells/ml) were preincubated for 15 min in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 25 mM Hepes and amino acid mixture [12]. To obtain partly ATP-depleted hepatocytes, 10 μM FCCP was added 10 min prior to the addition of leukotrienes [16]. This treatment decreased the cellular ATP content to about 20% of the original value. The ATP-depleted cells were fully viable after 30 min incubation with ^{3}H-labeled leukotriene C_3. The concentration of leukotrienes in the incubation mixture was 1 nM unless otherwise indicated.

To determine rates of uptake of ^{3}H-labeled leukotrienes, preheated (to 37°C) cell suspensions were transferred to small test tubes. After the addition of leukotrienes, the tubes (containing 1 ml) were kept at 37°C under carbogen gassing. Aliquots of 250 μl were taken out after 10 s and 1 min. The cells were separated from the supernatant by rapid centrifugation in a Beckman Microfuge for 5 s. The cells were washed once with ice-cold medium, recentrifuged and lysed with distilled water. The cellular radioactivity was counted in a Beckman liquid scintillation counter. The rate of uptake of leukotrienes into hepatocytes was linear for 1 min at 37°C. In order to correct for any non-saturable process, samples were removed after 10 s and 1 min. Saturable uptake was regarded as the difference between the value at 1 min and the value at 10 s, multiplied by 6/5 (to calculate uptake per min). Corrected values for high- and low-affinity uptake were obtained by iterative subtraction of the uncorrected values [17].

Results

Dependence on temperature and pH

Fig. 1 illustrates the effects of temperature, cellular ATP levels and pH on the uptake of ^{3}H-labeled leukotriene C_3 into isolated rat liver cells. The initial radioactivity increased rapidly and about 30% of the total radioactivity was associated with the cells after 15 min incubation. The influence of incubation temperature and cellular ATP content on the rate of uptake can be summarized as follows. At 28°C, the uptake rate was decreased by approx. 60% compared to the control at 37°C, at 20°C the final uptake was about 25%

of the control, and at 4°C almost no cellular accumulation of radioactivity occurred. 80% ATP-depletion of the cells also inhibited the transport of leukotriene C_3 by about 60%, suggesting that the uptake of leukotriene C_3 is due to an active, energy-requiring mechanism. No appreciable difference in the uptake of ^{3}H-labeled leukotriene C_3 was observed between pH 6.8, 7.4 and 8.0.

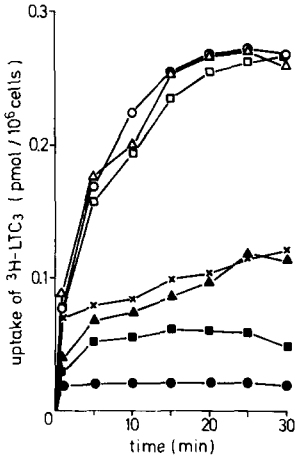

Fig. 1. Uptake of ^{3}H-labeled leukotriene C_3 (LTC_3) by isolated liver cells. Incubation temperature: 37°C; O——O, ▲——▲. 28°C; ■——■, 20°C; ●——●, 4°C (all at pH 7.4). □——□, pH 8.0, 37°C; △——△, pH 6.8, 37°C; ×——×, cells pretreated with 10 μM FCCP (37°C). The concentration of leukotrienes used was 1 nM.

The results of HPLC analyses of aliquots of cells or medium removed after 16 or 26 min of incubation are shown in Table I. The radioactivity in the medium was due mainly to unchanged leukotriene C_3, and only small amounts of ^{3}H-labeled leukotriene D_3 were detected. In the cells, unidentified polar metabolites eluting more quickly than leukotriene C_3 were observed in addition to leukotrienes C_3 and D_3.

Kinetic analyses.

Isolated hepatocytes took up ^{3}H-labeled leukotrienes D_4 and E_4 more rapidly than leukotriene C_4 (Fig. 2). The cellular concentrations of leukotrienes D_4 and E_4 were also higher at equilibrium than that of leukotriene C_4. Similar results were observed for leukotrienes C_3, D_3 and E_3 (Fig. 2).

Fig. 3 shows results of kinetic analyses of the uptake of leukotriene C_3. The double-reciprocal plots indicated that the uptake was biphasic. At lower concentrations (0.002–0.5 μM) the apparent K_m was 0.9 μM and the V_{max} was 0.07 nmol/10^6 cells per min. At concentrations from 0.5 to 8 μM, the apparent K_m was 19 μM and the V_{max}, 1.0 nmol/10^6 cells per min. After correction for the low-affinity uptake [18], the K_m at high affinity is 0.2 μM and the V_{max} 6 pmol/10^6 cells per min (Fig. 3).

Kinetic data for leukotrienes C_4, D_4 and E_4 in a range of substrate concentrations between 0.035 and 10 μM were also biphasic (Fig. 4). The kinetic constants for the uptake of leukotriene C_4 (Table II) were essentially the same as those determined

TABLE I

METABOLISM OF LEUKOTRIENE C_3 BY ISOLATED RAT HEPATOCYTES

The table shows the distribution of radioactivity during high performance liquid chromatographic (HPLC) analyses of samples. The values are given as percentages of the radioactivity recovered after HPLC.

Retention time [a]	Medium			Cell		
	0 min	16 min	26 min	0 min	16 min	26 min
Front					8	17
0.39					29.5	39
0.78		10				
1.00 (C_3)	100	83	82	100	58	42
1.60 (D_3)		7	18		4.5	2

[a] Retention times relative to leukotriene C_3.

for leukotriene C_3. The apparent K_m values for leukotriene D_4 and E_4 uptake were also similar to those observed for leukotrienes C_3 and C_4, whereas

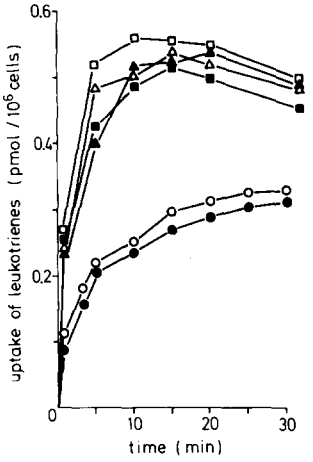

Fig. 2. Uptake of ^{3}H-labeled leukotrienes into isolated liver cells. ●——●, leukotriene C_4; ▲——▲, leukotriene D_4; ■——■, leukotriene E_4; ○——○, leukotriene C_3; △——△, leukotriene D_3; □——□, leukotriene E_3. Incubation temperature, 37°C. The concentration of leukotrienes used was 1 nM.

the V_{max} values were 30–60% higher than those for the C-type leukotrienes. This suggests that the hepatocellular uptake of leukotrienes D_4 and E_4 is more effective than the uptake of leukotriene C_4.

Specificity

Glutathione disulfide, and the glutathione-*S*-conjugate of acetaminophen, did not diminish the initial rate of uptake of ^{3}H-labeled leukotriene C_3 (Table III).

Leukotriene B_4 (10 μM) did not influence hepatocellular uptake of leukotriene C_4 (Table III), whereas at the same concentration, unlabeled leukotrienes C_4, D_4 and E_4 decreased the uptake of 5 nM ^{3}H-labeled leukotriene C_4. Leukotriene E_4 had the strongest inhibitory effect.

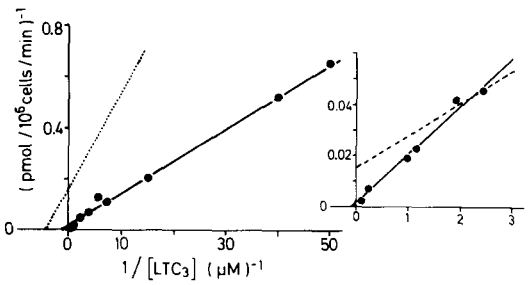

Fig. 3. Relationship between concentrations of leukotriene C_3 (LTC_3) and initial hepatocellular uptake. Left panel: Double-reciprocal plot at low concentrations. The dotted line shows corrected high-affinity uptake [17]. Right panel: Double-reciprocal plot at high concentrations. The broken line shows the high-affinity uptake. The data were obtained in three different experiments. Concentration range: 0.02–8.0 μM.

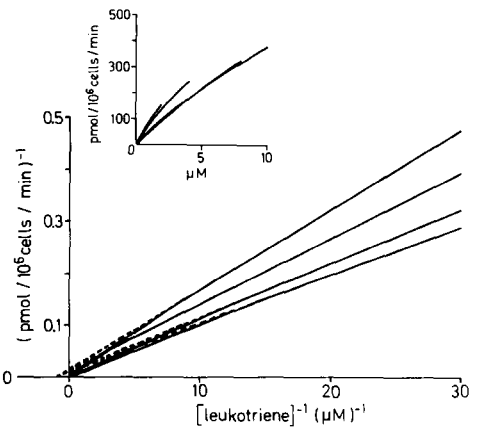

Fig. 4. Relationship between concentrations of leukotrienes and initial hepatocellular uptake. Inset: Initial uptake of leukotrienes E_4, D_4, C_3 and C_4 (from left to right). Concentration range: leukotriene C_3, 0.03–8 μM; leukotriene C_4, 0.035–10 μM; leukotriene D_4, 0.05–4 μM; leukotriene E_4, 0.05–2 μM. Main figure: double-reciprocal plots of the previous data. From top to bottom: leukotriene C_4, leukotriene C_3, leukotriene D_4 and leukotriene E_4. Data were obtained in 1–4 separate experiments.

TABLE II

KINETIC CONSTANTS FOR HEPTOCELLULAR UPTAKE OF LEUKOTRIENES

Concentrations used were for: leukotriene C_3, 0.002–8 μM; leukotriene C_4, 0.035–10μM; leukotriene D_4, 0.05–4 μM and for leukotriene E_4, 0.05–2 μM.

	K_{m1} (μM)	K_{m2} (μM)	V_{max} (nmol/10^6 cells per min)	V_{ma2} (nmol/10^6 cells per min)
Leukotriene C_3	0.9	19	0.07	1.0
Leukotriene C_4	1.1	17	0.07	1.0
Leukotriene D_4	0.9	17	0.09	1.3
Leukotriene E_4	1 0	16	0.11	1.4

TABLE III

EFFECT OF CHEMICALLY RELATED COMPOUNDS ON THE UPTAKE OF LEUKOTRIENE C INTO ISOLATED RAT HEPATOCYTES

Initial rate of uptake was measured as follows: [(uptake at 60 s)−(uptake at 10 s)]× $\frac{6}{5}$. Ratios are values relative to the initial uptake of leukotriene (LT) C_3 or C_4 alone. The rate of uptake is represented with mean ± S.D. GSSG, glutathione in oxidized form; AAP-GSH, Acetaminophen-*S*-glutathionyl conjugate.

Compound	Concentration	Uptake (pmol/10^6 cells)		Initial rate of uptake (pmols/min per 10^6 cells)	Ratio (%)
		10 s	60 s		
[^{3}H]LTC_3	1 nM (n = 9)	0.090 ± 0.007	0.141 ± 0.013	0.060 ± 0.017	100
GSSG	100 nM (n = 4)	0.092 ± 0.010	0.140 ± 0.009	0.058 ± 0.007	97
AAP-GSH	40 μM (n = 4)	0.098 ± 0.023	0.146 ± 0.018	0.058 ± 0.006	98
[^{3}H]LTC_4	5 nM (n = 7)	0.448 ± 0.018	0.652 ± 0.012	0.245 ± 0.030	100
LTB_4	10 μM (n = 2)	0.454	0.669	0.257	105
LTC_4	10 μM (n = 2)	0.430	0.597	0.201	82
LTD_4	10 μM (n = 2)	0.447	0.597	0.179	74
LTE_4	10 μM (n = 2)	0.429	0.556	0.153	62

Reversibility

Fig. 5 illustrates the release of tritium from hepatocytes preexposed to ^{3}H-labeled leukotriene C_3. A marked loss of radioactivity in the cells occurred during the washing procedure. Subsequently, a rapid decrease of cellular radioactivity was observed during the initial phase of incubation, and within 15 min, about 60% of the radioactivity had disappeared from the cells at 37°C. The decrease of cellular radioactivity at 20°C was even more rapid than at 37°C, suggesting that reuptake influenced the latter data and that the release of tritium occurs by passive diffusion rather than active transport in the model system of isolated hepatocytes. At 4°C, the rate of release was intermediate between the rates at 37°C and 20°C.

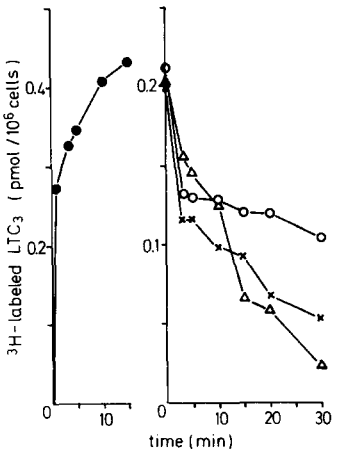

Fig. 5. Release of radioactivity from heptocytes preincubated with 2 nM of ^{3}H-labeled leukotriene C_3 (LTC_3) for 15 min at 37°C. After centrifuging and washing with fresh medium at 4°C, equal numbers of cells were resuspended in fresh medium at different temperatures (37°C (○), 20°C (△), and 4°C (×)) and incubated for varying times at the same temperatures prior to determining the radioactivity in cells and media.

Discussion

Isolated rat hepatocytes take up radioactivity during incubations with ^{3}H-labeled leukotrienes C_4, D_4 and E_4. The uptake is probably related to a previously described hepatic elimination of leukotriene C_3 from blood in guinea-pigs and mice [8,9]. The transport process is specific, saturable and sensitive to changes in temperature and cellular ATP levels. This suggests that it is mediated by an active transport system for cysteine-containing leukotrienes.

As shown in Fig. 3, double-reciprocal plots of the initial rate of uptake of leukotriene C_3 versus substrate concentration were biphasic. The high-affinity and low-capacity phase probably represents active transport, whereas the lower-affinity and higher-capacity phase may be due to binding to the cell surface without translocation across the

plasma membrane [18]. Since the specific radioactivity of leukotrienes C_4, D_4 and E_4 was lower than that of leukotriene C_3, the kinetics for the uptake of these substances were estimated from a narrower range of concentrations. Nevertheless, double-reciprocal plots of the initial rates versus concentration of the four-series leukotrienes were also biphasic (Fig. 4). The K_m values calculated for the high- and low-affinity processes were similar for leukotrienes C_3, C_4, D_4 and E_4, whereas the V_{max} values for leukotrienes D_4 and E_4 were higher than those for leukotrienes C_3 and C_4 in both the high- and low-affinity reactions (Table II).

The effects of some chemically related compounds on the initial uptake of ^{3}H-labeled leukotrienes C are shown in Table III. Neither glutathione disulfide nor the glutathione-*S*-conjugate of acetaminophen (40 μM) affected the uptake of 1 nM ^{3}H-labeled leukotriene C_3. Glutathione disulfide and the glutathione-*S*-conjugate of acetaminophen are not taken up by rat hepatocytes (Ref. 19 and Ormstad, K., unpublished data).

Leukotriene B_4 contains a conjugated triene structure but lacks the amino acid part of leukotrienes C, D and E. At a concentration of 10 μM, it had no effect on the uptake of leukotriene C_4, whereas both leukotriene D_4 and leukotriene E_4 at the same concentration inhibited the uptake.

In summary, the results presented above suggest that cysteine-containing leukotrienes are taken up by hepatocytes by a shared mechanism. Leukotriene E_4 is taken up most effectively, and is followed by leukotrienes D_4 and C_4. Competition experiments indicate that the transport system is specific for cysteine-containing leukotrienes and that elimination of either the fatty acid or the amino acid part of these molecules reduces or prevents interaction with the system. The transport system for leukotrienes of the C, D and E types described in this report is probably involved in the rapid elimination of these compounds from the circulation which occurs in vivo [7,10], and impairment of the function of this system may potentiate the biological effects of leukotrienes.

Acknowledgements

The authors are grateful to Dr. J. Rokach, Merck Frosst Canada Inc. for kindly supplying leukotrienes B_4, D_4 and E_4, to Professor Sten Orrenius in the Department of Forensic Medicine for critical discussions, and to Ms. Lilleba Bohman for technical assistance. This work was supported by grants from the Swedish Medical Research Council (03X-5914 and 03P-6396).

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