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TISSUE DISTRIBUTION OF LEUKOTRIENE A_4 HYDROLASE ACTIVITY IN GUINEA PIG

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SUMMARY: The distribution of the activity of leukotriene A_4 hydrolase, an enzyme catalyzing hydrolysis of the allylic epoxide leukotriene A_4 to the dihydroxy acid leukotriene B_4, was determined in various tissues of guinea pig by using the supernatant fraction (100,000 x g, 60 min) of the homogenates. The activity was ubiquitously distributed in all tissues examined, and the highest specific activity was found in small intestine, followed by lung, aorta, colon, and spleen, in this order. The specific activity in these tissues was higher than that of leukocytes. The physiological roles of this compound in these organs deserve reexamination in this context.

Leukotriene(LT)A_4 (5(S)-trans-5,6-oxido-7,9-trans-11,14-ciseicosatetraenoic acid), is a pivotal intermediate in the biosynthesis of other leukotrienes. This labile epoxide is formed from 5-HPETE (5(S)-hydroperoxy-6-trans-8,11,14-ciseicosatetraenoic acid), a 5-lipoxygenase product of arachidonic acid (1-3). LTA_4 can be conjugated with glutathione to give LTC_4, which is further metabolized to the additional cysteinyl-containing leukotrienes that together constitute the slow reacting substance of anaphylaxis (SRS-A)(4,5). Alternatively, LTA_4 is catalyzed by LTA_4 hydrolase forming LTB_4, which is the most potent chemotactic compound, and plays a significant role in inflammatory and allergic processes (4-6). LTA_4 hydrolase has been reported mostly in blood cells, such as human leukocytes (7), human erythro-

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Abbreviations: LT, leukotriene; PG, prostaglandin; SP-HPLC, straight phase high performance liquid chromatography; RP-HPLC, reversed phase high performance liquid chromatography.

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cytes (8), and rat neutrophils (9). Guinea pig liver and human liver (10), blood plasma (11) have also this enzyme activity. However, no studies have been available so far that demonstrate the intensive survey of the tissue distribution of this enzyme. In the present study, we quantitatively determined the LTA_4 hydrolase activity in various tissues of guinea pig and found that several tissues like small intestine, lung, or aorta have higher enzyme activity than leukocytes.

EXPERIMENTAL PROCEDURES

Materials. Methyl ester of LTA_4 was either synthesized by the method of Corey et al (12) or supplied from Takeda Pharmaceutical Co.(Osaka). LTB_4 was a kind gift from Ono Pharmaceutical Co.(Osaka). All other reagents were of analytical grade and used without further purification.

Purification of LTA_4. Methyl ester of LTA_4 was purified on straight phase high performance liquid chromatography (SP-HPLC). The column (Nucleosil 50-5, 1 x 30 cm) was eluted with hexane/ethyl acetate/triethylamine (100/1.2/0.4, v/v) at a flow rate of 3 ml/min. The column temperature was maintained at 4 °C to yield better resolution and recovery. Detailed procedures of purification will be published elsewhere. Prior to use, the lithium salt of LTA_4 was generated by saponification in a mixture of tetrahydrofuran and lithium hydroxide in water. After evaporation under a stream of nitrogen, it was dissolved in an ethanol-water mixture. The concentration of LTA_4 was calculated from UV absorption at 280 nm (ε = 40,000 (12)).

Preparation of enzyme sources. Three female guinea pigs (Hartley, body weight = 200-300 g) were used for one experiment. After sacrifying by exsanguination under anesthesia with sodium pentobarbital (50 mg/kg), the whole body was perfused with about 200 ml of cold 0.9% NaCl containing 5 mM EDTA via a cannula inserted into the right heart ventricle. Twelve organs (whole brain, spinal cord, lung, heart, liver, stomach, small intestine, colon, kidney, spleen, adrenal gland, and aorta) were quickly removed. Each organ of three guinea pigs was combined and kept on ice before homogenization in 3 volumes (v/w) of buffer A (50 mM potassium phosphate buffer, 0.15 M NaCl, 1 mM EDTA, pH 7.4) using a Physcotron blender homogenizer model NS-50 (Nichion, Chiba, Japan) five times at top speed each for 10 sec. Homogenates were centrifuged at 800 x g for 20 min. The supernatant thus obtained was recentrifuged at 10,000 x g for 20 min, followed by a final centrifugation at 100,000 x g for 60 min. The supernatant solutions were used as enzyme sources. All centrifugation were performed at 4 °C. For subcellular localization study, the pellet at each step was washed and resuspended in a defined volume of buffer A. Guinea pig blood was collected over EDTA (1 mg/ml) and centrifuged at 400 x g for 20 min to sediment cells. Plasma was recovered by decantation. Remaining blood cells were resuspended in buffer A to initial blood volume. Part of the blood cells were passed through cotton threads to remove leukocytes (13) (the efficiency of the removal was more than 95%), and used as an erythrocyte suspension. Leukocytes were isolated by dextran sedimentation and hypotonic hemolysis from another part of blood cells. They included 75% granulocytes, 20% lymphocytes, 5% other cells, and some platelets. Erythrocytes and leukocytes were resuspended in buffer A with concentrations of 6 x 10^9 cells/ml, and 3 x 10^7 cells/ml, respectively, and sonicated 3 x 10 sec with 1 min intervals in between using a Branson Sonifier model M12. After centrifugation at 10,000 x g for 20 min, the supernatants were recentrifuged at 100,000 x g for 60 min at 4 °C. The supernatant solutions and plasma were used as enzyme sources.

Assay of LTA_4 hydrolase. The previous method (7) was modified as follows. The standard reaction mixture contained 0.1 M Tris-HCl buffer, pH 7.8, and enzyme in a total volume of 0.1 ml. After preincubation at 37 °C for 1 min, LTA_4 (an ethanol-water solution, containing some lithium hydroxide) was added to give a final concentration of 80 μM. After 1 min incubation at 37 °C, the reaction was terminated by the addition of acidic methanol (0.2 ml, pH 3, adjusted with formic acid). Prostaglandin(PG) B_2 (600 pmol in 0.2 ml of methanol) was added as internal standard for HPLC analysis. It was kept at -20 °C for 30 min, followed by centrifugation at 10,000 x g for 5 min at 4 °C. The supernatant was removed and evaporated under a stream of nitrogen, reconstituted in solvent I, acetonitrile/methanol/water/acetic acid (300/100/300/0.6, v/v; containing 0.05% EDTA), and an aliquot (usually one-fifth) was analyzed for LTB_4 by reversed phase high performance liquid chromatograpy (RP-HPLC) in Shimadzu 6A systems. The column (TSK ODS-80TM, 5 μm, Toyosoda, Tokyo, 0.46 x 15 cm) was eluted with solvent I (1 ml/min), and the temperature was set at 35 °C. The absorbance at 270 nm was monitored, and the amount of LTB_4 formed was calculated from the peak-area ratio LTB_4/PGB_2 by using a data processing system (Shimadzu Chromatopac C-R3A). The efficiency of the extraction procedures for 600 pmol of PGB_2 and varying amounts of LTB_4 (60 to 1500 pmol) with lung enzyme (100,000 x g supernatant, 0.6 mg of protein) was constantly more than 95%. For each enzyme source, three incubations were carried out with different volumes of enzyme solutions. Linear correlations of the amounts of LTB_4 formed versus the amounts of the enzyme added were obtained (regression coefficients were usually more than 0.998). Specific activities were expressed as nmol of LTB_4 formed/mg of protein·min. Protein concentrations were determined by the method of Lowry et al. (14) with bovine serum albumin as standard.

Identification of the product. The material corresponding to the LTB_4-peak, obtained from several incubations, was treated with ethereal diazomethane and analyzed as methyl ester by SP-HPLC using a column (Nucleosil 50-5, 0.46 x 25 cm) with a solvent system of hexane/2-propanol/acetic acid (95/5/0.01, v/v) at a flow rate of 1.5 ml/min. Furthermore, after trimethylsililation with a mixture of hexamethyldisilazane and trimethylchlorosilane in pyridine, it was analyzed by a Hitachi M-80 gas chromatography mass spectrometer equipped with a packed column (2% OV-1) operated at 230 °C with helium as a carrier gas. The energy of the ionization was 70 eV. C-value (relative retention time) was calculated from retention times observed after chromatography of fatty acid methyl ester standards.

RESULTS

When homogenates of guinea pig lung or small intestine were subjected to differential centrifugation and the subcellular fractions were incubated with LTA_4, the activity of LTA_4 hydrolase was recovered predominantly in the 100,000 x g supernatant (Table 1) as in the cases of human leukocytes (7), rat neutrophils (9), and guinea pig liver (10).

When 100,000 x g supernatants from guinea pig lung were incubated with LTA_4, RP-HPLC analysis revealed three major peaks (I,II, and III) monitored at 270 nm. Peaks I and II cochromatographed with 6-trans-LTB_4 (peak I) and its 12-epimer (peak II), nonezymic hydrolysis products of LTA_4. While the area of peak III increased linearly depending on the enzyme amounts (Fig. 1a-c), it

Table 1. Subcellular localization of LTA_4 hydrolase activity in guinea pig lung and small intestine

	Lung			Small intestine		
	Total protein (mg)	Specific activity (nmol/mg·min)	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/mg·min)	Total activity (nmol/min)
Homogenate	40.3	0.63	25.4	42.0	1.05	44.1
10,000 x g pellet	5.7	0.24	1.4	4.7	0.35	1.6
100,000 x g pellet	8.0	0.11	0.9	8.0	0.14	1.1
100,000 x g supernatant	21.5	1.19	25.6	18.7	2.06	38.5

Lung (1 g) and small intestine (1 g) were homogenized in 3 volumes (v/w) of buffer A and subjected to centrifugations as indicated. Pellets were resuspended in buffer A. Aliquotes were incubated with 80 μM LTA_4 for 1 min, and assayed for LTB_4 production by RP-HPLC using PGB_2 as internal standard.

was almost absent when LTA_4 was incubated with heat-treated (90 ℃, 5 min) enzyme (Fig. 1d).

Peak III coeluted with a synthetic LTB_4 on RP-HPLC and exhibited an UV-spectrum with triplet peaks at 259, 269, and 280 nm. The methyl ester of peak

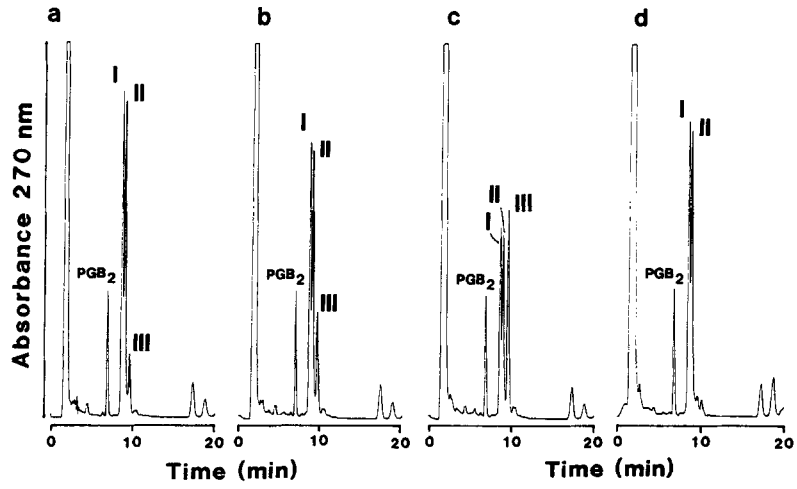

Fig. 1. RP-HPLC profiles of the products formed from the incubations of guinea pig lung enzyme with LTA_4. Twenty μl (a), 40 μl (b), 80 μl (c) of enzyme (100,000 x g supernatant, 7.3 mg protein/ml), and 80 μl of heat-treated enzyme (d) from guinea pig lung were incubated with 80 μM LTA_4 in 0.1 ml of 0.1 M Tris-HCl buffer, pH 7.8, at 37 ℃ for 1 min. After quenching the reactions with acidic methanol (pH 3, 0.2 ml) and addition of internal standard PGB_2 (600 pmol), aliquots were analyzed by RP-HPLC. The column (TSK ODS-80TM) was eluted with acetonitrile/methanol/water/acetic acid (300/100/300/0.6, v/v, containing 0.05% EDTA) at a flow rate of 1 ml/min. UV absorbance was monitored at 270 nm.

III had a retention time (20 min) identical to that of synthetic LTB_4 methyl ester on SP-HPLC. When analyzed by gas chromatography-mass spectrometry, the C-value of its methyl ester trimethylsilyl ether derivative was 23.6 on an OV-1 column, and the major fragment ions of the derivative were observed at m/z 129 (base peak), 167, 203, 217, 229, 267, 293, 383, 404, and 479, identical to the published values (15) and to those obtained from corresponding derivatives of synthetic LTB_4. These data showed that peak III corresponded to the enzymic product, LTB_4.

The time course and pH dependency of the enzymatic hydrolysis were studied using lung enzyme (100,000 x g, 60 min, supernatant). The amount of LTB_4 formed increased rapidly during the first seconds and leveled off at 5 min (Fig. 2). When pH in the incubation medium was varied (pH 5.0-11.0), the maximun formation of LTB_4 was found between pH 7.4 and pH 8.6.

Tissue distribution of LTA_4 hydrolase activity of guinea pig was shown in Table 2. The enzyme activity was ubiquitously distributed in all tissues examined. The specific activities were high in small intestine, lung, aorta, colon, and spleen, and relatively low in liver and spinal cord.

To exclude the possibility that these LTA_4 hydrolase activities in various guinea pig tissues originated from blood cells or plasma in tissues, the enzyme activities in plasma, erythrocytes, and leukocytes were determined. Plasma and the 100,000 x g supernatant solutions of erythrocytes had no de-

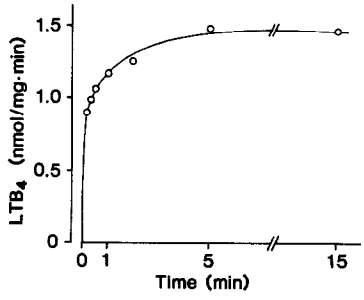

Fig. 2. Time course of enzymatic hydrolysis of LTA_4. Guinea pig lung enzyme (100,000 x g supernatant, 0.78 mg of protein) was incubated with 80 μM LTA_4 in the standard reaction mixture as described under "Experimental Procedures", and the reaction was quenched with acidic methanol at the times indicated. After addition of PGB_2 (600 pmol), aliquots were analyzed by RP-HPLC.

Table 2. Distribution of LTA_4 hydrolase activity in various tissues of guinea pig

Tissue	Specific activity (nmol/mg·min)	Tissue	Specific activity (nmol/mg·min)
Small intestine	1.59 ± 0.33	Kidney	0.63 ± 0.11
Lung	1.14 ± 0.14	Stomach	0.60 ± 0.17
Aorta	1.08 ± 0.25	Heart	0.56 ± 0.06
Colon	0.86 ± 0.17	Brain	0.47 ± 0.12
Spleen	0.72 ± 0.09	Spinal cord	0.41 ± 0.10
Adrenal gland	0.67 ± 0.06	Liver	0.28 ± 0.04

Values are expressed as mean ± S.D. of five experiments. Three guinea pigs were used in each experiment. Procedures of enzyme preparations (100,000 x g, 60 min, supernatant) and assay for LTA_4 hydrolase are described under "Experimental Procedures".

tectable activity. The specific activity of leukocytes including granulocytes, lymphocytes, and some platelets was 0.55 ± 0.13 nmol/mg·min (mean ± S.D. of four experiments), which was lower than those of most tissues examined. Therefore, the participation of blood contamination was negligible.

DISCUSSION

The major finding of the present study is that the LTA_4 hydrolase activity was not only located in leukocytes but was ubiquitously distributed in all tissues of guinea pig examined. Subcellular distribution, time course, and pH-dependence of the enzyme were very similar to those of the purified enzyme obtained from human leukocytes (7), or rat neutrophils (9). Epoxide hydrolases are proposed to be involved in the detoxification of potentially mutagenic and/or carcinogenic epoxides (16). Cytosolic epoxide hydrolases have been studied extensively in mammalian tissues (16-18). However, since these epoxide hydrolases mostly convert various non-allylic epoxides to vicinal diols (16-18), and could not convert allylic epoxide (LTA_4) to LTB_4 (T. Shimizu, unpublished data), the activity seen in the various tissues appears to be specific LTA_4 hydrolase. This suggested that these tissues can produce LTB_4, if a substrate LTA_4 is provided. It still remains unclear whether these tissues have all enzyme systems (5-lipoxygenase, LTA_4 synthase and LTA_4 hydrolase) intrinsically, and LTB_4 thus formed is tightly related to the specific function of each organ, or in inflammatory or allergic processes, circulating

leukocytes supply LTA_4 to these tissues which, thereby, augment the formation of LTB_4.

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