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CONVERSION OF LEUKOTRIENE C_4 TO LEUKOTRIENE D_4 BY A CELL-SURFACE ENZYME OF RAT MACROPHAGES

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Leukotriene (LT) C_4-metabolizing enzyme was studied using rat leukocytes. Neutrophils and lymphocytes hardly metabolized LTC_4, whereas macrophages rapidly converted LTC_4 to LTD_4. The LTC_4-metabolizing enzyme of macrophages was present in the membrane fraction but not in the nuclear, granular and cytosol fractions. When macrophages were modified chemically with diazotized sulfanilic acid, a poorly permeant reagent which inactivates cell-surface enzymes selectively, the LTC_4-metabolizing activity of macrophages decreased significantly (>90%). These findings suggest that rat macrophages possess the LTC_4-metabolizing enzyme which converts LTC_4 to LTD_4, on the cell surface membrane.

Sulfidopeptide leukotrienes, leukotriene (LT) C_4, D_4 and E_4 are biologically active cysteinyl compounds which are produced from arachidonic acid via the lipoxygenase pathway, and are considered as mediators of inflmmation and allergy (1). Using renal enzymes, LTC_4 has been studied as transformed by γ-glutamyl transpeptidase (2) into LTD_4, which is further metabolized by dipeptidase (3) into LTE_4. In inflammed tissues, large numbers of leukocytes are present, and they are assumed to be involved in the metabolism of LTs at the site of inflammation. It has been recently found that macrophages possess the LTD_4-metabolizing dipeptidase activity on the cell-surface membrane, which is the highest among leukocytes including neutrophils and lymphocytes (4). However, there is little information on the LTC_4-metabolizing enzyme of leukocytes which catalyzes the conversion of LTC_4 to LTD_4. In this study, therefore, in order to elucidate the involvement of leukocytes in the metabolism of LTC_4, we have studied the LTC_4-metabolizing enzyme using rat leukocytes.

MATERIALS AND METHODS

Preparation of leukocytes

Resident peritoneal macrophages, glycogen-induced peritoneal neutrophils and blood lymphocytes were prepared from Sprague-Dawley rats as described

Abbreviations: LT, leukotriene; HPLC, high-pressure liquid chromatography; DSA, diazotized sulfanilic acid; buffer A, 137 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4; buffer B, 120 mM NaCl/4 mM KCl/25 mM Tris-HCl, pH 7.4.

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previously (5). The purity of isolated leukocytes examined by Wright-Giemsa staining was 90-95%.

Preparation of subcellular fractions from macrophages

Macrophages (4 x 10^6 cells/ml) suspended in 0.34 M sucrose were homogenized in a Teflon-glass homogenizer at 0°C for 20 min (6). The homogenate was centrifuged at 500g for 12 min, and the sedimented fraction was termed the nuclear fraction. The supernatant was centrifuged at 8,200g for 15 min, and the resultant pellet was termed the granular fraction. The supernatant was further centrifuged at 260,000g for 1 hr to yield the membrane fraction. The resultant supernatant was termed the cytosol fraction. All pellets were washed once with 0.34 M sucrose and resuspended in 0.34 M sucrose at 4 x 10^7 cell equivalents. The granular fraction was sonicated in ice for 2 min at 168 W(Supersonic vibrator, model UR-150P, Tominaga Works, Ltd., Tokyo) before use.

Modification of macrophages

Macrophages (10^7 cells/ml) were incubated with 2 mM DSA at 37°C for 5 min in buffer A as described eariler (6). Then, the cells were washed twice with ice-cold buffer B to stop the reaction and finally suspended in buffer B at 2 x 10^7 cells/ml. After sonication at 168 W for 2 min, the cell sonicate was assayed for enzyme activities. The cell viability examined by a Trypan blue dye exclusion test did not decrease by the modification and was >95%.

Enzyme assays

LTC_4-metabolizing activity was determined by incubating the cell sonicates with synthetic LTC_4 (240 pmol) at 37°C for up to 40 min in a total volume of 0.12 ml buffer B in the presence of 5 mM L-cysteine which inhibits the conversion of LTD_4 to LTE_4 by the LTD_4-metabolizing dipeptidase present in the leukocyte preparations, unless otherwise noted. The reaction was terminated by the addition of 0.18 ml acidified methanol (methanol:acetic acid=1000:1), followed by centrifugation at 8,300g for 10 min. Aliquots (100 μl) of the resulting supernatants were subjected to reverse phase HPLC for the analysis of LTC_4 and its metabolite(s) as described eariler (7), using a Finepak SIL C_{18} column (5 μm, 4.6 x 250 mm, Jasco-Japan Spectroscopic Co., Ltd., Tokyo) and a TRI ROTOR-VI pump (Jasco). The absorbance of the column effluent was monitored using a UVIDEC-100-VI spectrophotometer (Jasco) adjusted to 280 nm. The peak area was calculated using a Chromatocorder 11 (System Instruments Corp., Tokyo). The solvent system consisted of methanol/water/acetic acid (65:35:0.1, v/v) containing 0.05% EDTA, and was adjusted to pH 5.6 with ammonia. The flow rate was maintained at 1 ml/min. In some experiments, the bioactivities of the resulting supernatants were assayed using the isolated guinea-pig ileum (6). Alkaline phosphodiesterase I, glucose-6-phosphatase, β-N-acetyl-D-glucosaminidase, cytochrome oxidase and prolyl endopeptidase activities were assayed as described previously (6,8). One unit of activity is defined as the amount of enzyme necessary to split 1 μmol of substrate in 1 min under the condition used. Protein concentration was measured by the method of Lowry et al. (9), using bovine serum albumin as a standard.

Reagents

LTD_4 was purchased from Wako Pure Chemical Industries, LTd., Osaka. LTC_4 was a gift from Ono Pharmaceutical Co., Ltd., Osaka. DSA was prepared as outlined previously (6). Other reagents were of analytical grade.

RESULTS AND DISCUSSION

LTC_4 was incubated with rat sonicated leukocytes at 37°C for up to 40 min, and the supernatants of the reaction mixture were analyzed by reverse phase HPLC for LTC_4 and its metabolite(s). By the incubation with macrophages, the peak at 280 nm of LTC_4 with the retention time of 9.8 ± 0.1 min decreased and was quantifiably converted to a single new peak with the retention time of 16.0 ± 0.1 min and the absorption maximum at 280 nm with

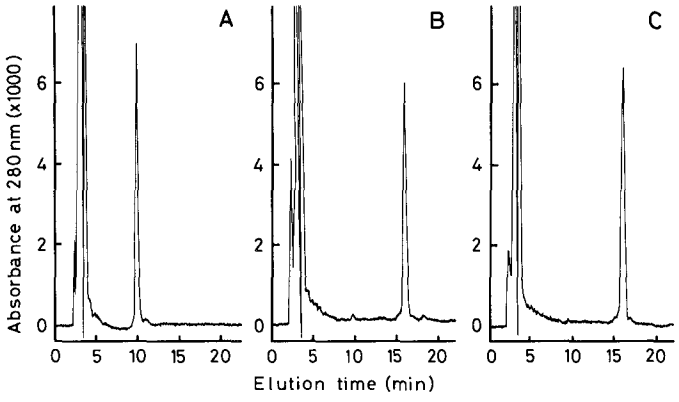

Fig. 1. Reverse phase HPLC elution profile of LTC_4 after incubation with macrophages. LTC_4 (240 pmol) was incubated with rat sonicated macrophages (1.2×10^6 cells) for 0 min (A) or 40 min (B) at 37°C in 0.12 ml buffer B. After the termination of the reaction and subsequent centrifugation, an aliquot of the resulting supernatant was subjected to HPLC. (C) authentic LTD_4.

the shoulders at 270 and 290 nm, corresponding to that of synthetic LTD_4 (Fig. 1). The peak of LTC_4 disappeared in a time dependent manner and was almost completely converted to the peak corresponding to LTD_4 after 40 min (Fig. 2A). The spasmogenic activity of LTC_4 increased to 357.5 ± 29.8% (n=3) of the initial activity after an incubation with macrophages for 40

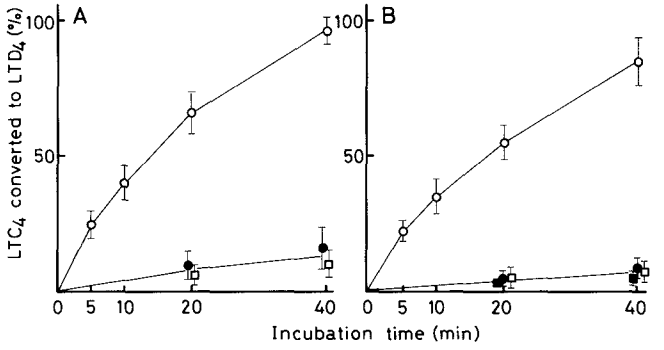

Fig. 2. Conversion of LTC_4 to LTD_4 by rat leukocytes or macrophage subcellular fractions. (A) LTC_4 (240 pmol) was incubated with each sonicated leukocyte fraction (1.2×10^6 cells) containing 84 ± 8 μg protein for neutrophils (●), 65 ± 7 μg protein for lymphocytes (□) and 96 ± 8 μg protein for macrophages (○), at 37°C for indicated time periods in 0.12 ml buffer B. (B) LTC_4 (240 pmol) was incubated with each macrophage subcellular fraction (1.2×10^6 cell equivalents) containing 15 ± 2 μg protein for nuclear fraction (■), 19 ± 1 μg protein for granular fraction (●), 25 ± 4 μg protein for cytosol fraction (□) and 25 ± 7 μg protein for membrane fraction (○). After the termination of the reaction and subsequent centrifugation, aliquots of the resulting supernatants were subjected to reverse phase HPLC and the amount of LTC_4 and LTD_4 was measured. The amount of LTC_4 converted to LTD_4 is expressed in terms of percent of the total LTC_4 and LTD_4 recovered, and given as the mean ± S.D. of 3 experiments. Total recovery of LTC_4 and LTD_4 after incubation with leukocytes was 93-100% relative to LTC_4 without incubation.

Table 1. Distribution of marker enzymes in subcellular fractions of macrophages

	Relative enzyme activity (%)			
	N	G	C	M
Alkaline phosphodiesterase I	7.1 ± 3.1	10.8 ± 2.1	2.7 ± 0.6	80.6 ± 4.6
Glucose-6-phosphatase	5.8 ± 2.4	16.2 ± 2.8	20.5 ± 6.4	57.7 ± 1.2
β-N-Acetyl-D-glucosaminidase	13.8 ± 4.5	70.2 ± 1.6	4.6 ± 0.7	11.5 ± 2.2
Cytochrome oxidase	14.8 ± 0.7	70.4 ± 1.5	3.1 ± 1.5	11.8 ± 3.6
Prolyl endopeptidase	2.2 ± 1.2	7.2 ± 1.8	81.2 ± 1.8	9.5 ± 2.3
Protein	18.3 ± 4.2	23.1 ± 3.1	29.7 ± 1.7	28.9 ± 5.7

Enzyme activities and protein concentrations in subcellular fractions are expressed in terms of percent of the total enzyme activities and protein recovered, respectively. Total recovery of the enzymes and protein was 90-110%. Total enzyme activities per 10^7 cells were 1154 ± 200 mU for alkaline phosphodiesterase I, 1.4 ± 0.2 mU for glucose-6-phosphatase, 18.6 ± 2.2 mU for β-N-acetyl-D-glucosaminidase, 0.92 ± 0.26 Δ_{550}/min for cytochrome oxidase and 5.1 ± 1.5 mU for prolyl endopeptidase. Total protein concentration was 803 ± 67 μg/10^7 cells. Values represent the mean ± S.D. of 3-4 experiments. N, nuclear fraction; G, granular fraction; C, cytosol fraction; M, membrane fraction.

min. This value was almost the same as that of the ratio of the spasmogenic activity of LTD_4 to LTC_4 on a molar basis (377.4 ± 63.5%, n=3). These results would indicate that rat macrophages possess the LTC_4-metabolizing enzyme which converts LTC_4 to LTD_4. On the other hand, rat neutrophils and lymphocytes hardly metabolized LTC_4 (Fig. 2A).

As macrophages possess the high LTC_4-metabolizing activity, the subcellular localization of the LTC_4-metabolizing enzyme was studied, using macrophages. Table 1 shows the distribution of marker enzymes among subcellular fractions. The bulk of alkaline phosphodiesterase I, a cell-surface marker and glucose-6-phosphatase, a microsomal marker, were recovered in the membrane fraction. The recoveries of β-N-acetyl-D-glucosaminidase, a lysosomal marker and cytochrome oxidase, a mitochondrial marker, were ca. 70% in the granular fraction. Prolyl endopeptidase, a cytosol marker, was enriched in the cytosol fraction. Fig. 2B shows the LTC_4-metabolizing activities in these subcellular fractions. The nuclear, granular and cytosol fractions hardly metabolized LTC_4, whereas the membrane fraction rapidly converted LTC_4 to LTD_4. These results indicate that the LTC_4-metabolizing enzyme is present in the membrane fraction but not in the nuclear, granular and cytosol fractions of macrophages.

The membrane fraction consisted of the cell-surface membrane and microsome (Table 1). Then, in order to examine which component, the cell-surface membrane or the microsome, possesses the LTC_4-metabolizing enzyme, macrophages were modified chemically with DSA, a pooly permeant reagent which inactivates cell-surface enzymes selectively (10). Alkaline phosphodiesterase I was inhibited to 15.4 ± 5.3% (n=3) of control by the modification. However, glucose-6-phosphatase, β-N-acetyl-D-glucosaminidase, cytochrome

oxidase and prolyl endopeptidase were hardly inhibited (92-100% of control), although these enzymes were completely inhibited by DSA if the cell sonicate was modified (data not shown). These results indicate that under our modification condition, DSA modified cell-surface enzyme selectively. When the LTC_4-metabolizing activity of modified macrophages was examined, the activity was inhibited 91.1 ± 4.5% (n=3) by the modification. These results would suggest that at least 90% of the LTC_4-metabolizing enzyme is located on the cell-surface of macrophages. In the case where most of the enzyme activity is present on the cell-surface membrane, intact cells are reported to show the same enzyme activity as sonicated cells (10,11). In this study, the LTC_4-metabolizing activity of intact macrophages (90 ± 22 $\mu U/10^7$ cells, n=3) was almost the same as that of sonicated macrophages (98 ± 24 $\mu U/10^7$ cells, n=3). These results seem to support the possibility that most of the LTC_4-metabolizing enzyme is present on the cell-surface membrane of macrophages.

The property of the LTC_4-metabolizing enzyme of macrophages was examined. The Km and Vmax values for the enzyme determined by Lineweaver-Burk analysis of data obtained at 5 substrate concentrations (0.25-4 μM) were 1.3 ± 0.3 μM and 100 ± 20 $\mu U/10^7$ cells (n=3), respectively. When the effect of various enzyme inhibitors on macrophage enzyme was studied, neither bestatin (100 μg/ml), captopril (100 μg/ml), ε-amino-n-caproic acid (3 mM), diisopropyl fluorophosphate (3 mM), N-ethylmaleimide (5 mM), o-phenanthroline (2 mM) nor cysteine (5 mM) affected the LTC_4-metabolizing activity. On the other hand, serine-borate complex (25 mM), a γ-glutamyl transpeptidase inhibitor (12,13), remarkably decreased the enzyme activity (8.6 ± 4.9% of control, n=3), suggesting that the LTC_4-metabolizing enzyme of macrophages is similar to γ-glutamyl transpeptidase.

We have recently found that macrophages have the LTD_4-metabolizing dipeptidase which converts LTD_4 to LTE_4, on the cell surface (4). In this study, we have clarified that macrophages also have the LTC_4-metabolizing enzyme which converts LTC_4 to LTD_4, on the cell surface. These findings suggest that among leukocytes macrophages may play an important role in the modulation of the inflammatory responses through the degradation of inflammatory mediators such as sulfidopeptide leukotrienes which have the ability to increase vascular permeability and constrict airway smooth muscles (1).

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