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THE CONVERSION OF LEUKOTRIENE C_4 TO ISOMERS OF LEUKOTRIENE B_4 BY HUMAN EOSINOPHIL PEROXIDASE

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The smooth muscle contractile and vasoactive mediator leukotriene C_4 (5(S)-hydroxy-6(R)-sulfido-glutathionyl-eicosatetraenoic acid; LTC_4) is converted by phorbol ester-stimulated human eosinophils to two isomers of leukotriene B_4, 5(S),12(R)-6,8,10 trans-14 cis-eicosatetraenoic acid (5(S),12(R)-"all-trans"-LTB_4) and 5(S),12(S)-"all-trans"-LTB_4, which are leukocyte chemotactic factors lacking the humoral functions of LTC_4. Optimal conversion of LTC_4 to the "all-trans" isomers of LTB_4 by intact eosinophils and soluble eosinophil peroxidase requires both H_2O_2 and halide ions. Oxidative metabolism of leukotrienes may represent an important regulatory function of eosinophils in hypersensitivity reactions.

INTRODUCTION

The 5-lipoxygenation of arachidonic acid in a variety of mammalian tissues and cells generates diverse mediators of hypersensitivity and inflammation (1-3). The products include 5(S)-hydroxy-6(R)-sulfido-glutathionyl-eicosa-tetraenoic acid (leukotriene C_4 or LTC_4) and 5(S)-hydroxy-6(R)-sulfido-cysteinyl-glycyl-eicosatetraenoic acid (leukotriene D_4 or LTD_4), which are potent smooth muscle contractile and vasoactive factors that constitute the slow-reacting substance of anaphylaxis (SRS-A) (4,5). In contrast, 5(S),12(R)-di-hydroxy-eicosa-6,14 cis-8,10 trans-tetraenoic acid (leukotriene B_4 or LTB_4) is the product which stimulates polymorphonuclear (PMN) leukocyte and lymphocyte functions (6,7). Isomers of LTB_4, including 5(S),12(R)-dihydroxy-eicosa-6,8,10 trans-14 cis-tetraenoic acid (5(S),12(R)-"all-trans"-LTB_4) and 5(S),12(S)-di-hydroxy-eicosa-6,8,10 trans-14 cis-tetraenoic acid (5(S),12(S)-"all-trans"-LTB_4), exert effects similar to those of LTB_4 on the leukocytic components of hypersensitivity reactions, but are far less potent (6). The possibility that some leukotrienes may be converted from one major functional class to

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the other now is suggested by the finding that eosinophils have the capacity to generate "all-trans" isomers of LTB_4 from LTC_4 by an apparently enzymatic peroxidation.

MATERIALS AND METHODS

Isolation of human eosinophils

Human eosinophils from four subjects with blood eosinophilia of 32-84% were purified by centrifugation of mixed leukocytes on density gradients of Metrizamide (Nyegaard, Gallard-Schlesinger Corp., Carle Place, NY) as described (8). Three ml portions of suspensions of 5-7 x 10^6 mixed leukocytes/ml in Hanks' solution (MA Bioproducts, Inc., Walkersville, MD) containing 0.1 g/100 ml of gelatin and 500 U/100 ml of DNase (Calbiochem-Behring Corp., La Jolla, CA) were layered on 12 ml linear gradients of 18-25 g/100 ml of Metrizamide in the same buffer and centrifuged at 1200 g for 45 min at 22°C. Eosinophils were recovered as the lowest band in the gradients and were washed and resuspended in Hanks' solution without or with 0.01 g/100 ml of ovalbumin (Miles Laboratories, Inc., Elkhart, IN). The purity of the eosinophils was 92% or greater and the viability was over 96%, as assessed by the exclusion of trypan blue dye.

Partial purification of human eosinophil peroxidase

Suspensions of 8 x 10^7 purified eosinophils/ml in 0.1 M sodium acetate containing 0.25% (v:v) Triton X-100 (pH 5.0) were sonified for 15 sec at 4°C (150 W, model 350, Branson Sonic Power Co., Danbury, CT). The sonicate was centrifuged at 20,000 g for 20 min at 4°C and the supernate was applied to a 1.9 cm x 70 cm column of Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, CA) that was equilibrated and developed with the sodium acetate-Triton X-100 buffer. The peroxidase activity eluted at 35%-42% of the bed volume. In order to determine peroxidase activity, 100 µl of a dilution of the preparation of enzyme were added to a 3.4 ml cuvette containing 2.9 ml of 0.02 M sodium acetate (pH 5.5) with 0.25 mM H_2O_2 and 0.02 mM guaiacol (Sigma Chemical Co., St. Louis, MO) and the optical density was measured continuously at 470 nm for 3 min at 22°C (9). The peroxidase activity was calculated employing an extinction coefficient for guaiacol of 2.66 x 10^4 M^{-1} cm^{-1} (10) and was expressed in terms of a unit defined as the oxidation of 1 µmole of guaiacol per min at 22°C.

Preparation of LTC_4, LTB_4 and other 5-lipoxygenase products

Radiolabeled LTC_4 was prepared by suspending 10^8 rat leukemic basophils in 3 ml of Tyrode's buffer containing 5 mM $CaCl_2$, 20 µM calcium ionophore A23187, 1 mM reduced glutathione, and 0.1 mM arachidonic acid (Supelco, Inc., Bellefonte, PA) with either [^{35}S]glutathione (16.8 Ci/mmol) or [^{3}H]arachidonic acid (5,6,8,9,11,12,14,15-^{3}H(N); 95 Ci/mmole) (New England Nuclear, Inc., Boston, MA) in place of the respective unlabeled reagent (11). After incubating the suspensions for 20 min at 37°C, the products were extracted and chromatographed on Amberlite XAD-8 without prior saponification (11). [^{35}S]LTC_4 and [^{3}H]LTC_4 were resolved from the other products by reverse-phase high performance liquid chromatography (HPLC) on a 10 µm ODS column (Ultrasphere, Altex Scientific Division of Beckman Instruments, Inc., Berkeley, CA) that was developed with methanol:water:glacial acetic acid (65:35:0.01, v:v) at a flow rate of 1 ml/min, utilizing a dual metered pump system fitted with a flow cell in a variable wavelength spectrophotometer (Hitachi Model 100-40, Altex Scientific, Berkeley, CA). LTB_4, 5(S),12(R)-di-hydroxy-6,8,10 trans-14 cis-eicosatetraenoic acid and 5(S),12(S)-di-hydroxy-6,8,10 trans-14 cis-eicosatetraenoic acid were generated by incubating human neutrophils with arachidonic acid and calcium ionophore A23187, and were purified by HPLC as described (6,11,12). The purified

compounds were derivatized and characterized by mass spectrometry (4,5,6,11,12). Synthetic LTC_4, LTD_4, and LTB_4 were supplied graciously by Dr. J. Rokach (Merck Frosst Canada, Inc., Montreal, Canada).

RESULTS

In preliminary experiments designed to examine the metabolism of LTC_4 by purified human eosinophils, an increase in the generation of 5,12-di-hydroxy-eicosatetraenoic acid isomers of LTB_4 was observed after the addition of LTC_4 when the eosinophils were stimulated with phorbol myristate acetate (PMA), but not with the chemotactic factors N-formyl-methionyl-leucyl-phenylalanine (10^{-8} M - 10^{-6} M) or C5a (10^{-9} M - 10^{-7} M). The use of [^{3}H]LTC_4, labeled in the fatty acid portion of the molecule, as the substrate facilitated an analysis of the products of metabolism. When stimulated by either PMA or sodium fluoride, at concentrations that evoke optimal oxidative activity, the eosinophils converted [^{3}H]LTC_4 to four distinct principles (Table I). The three metabolites which were less polar than LTC_4, co-chromatographed on reverse-phase HPLC with LTD_4, 5(S),12(R)-"all-trans"-LTB_4 and 5(S),12(S)-"all-trans"-LTB_4, of which the two di-hydroxy-eicosatetraenoic acids were the quantitatively predominant products. In similar experiments conducted with [^{35}S]LTC_4, none of the glutathione radioactivity was recovered with the two "all-trans" isomers of LTB_4, but the quantity of radioactivity in the more polar peak, designated as "x", was 7.2% and 8.5% of the total in two experiments in which the recovery of the fatty acid tritium label was 10.4% and 12.9%, respectively. Although this suggests that at least part of the peptide chain of LTC_4 was present in x, this principle was not identified.

The introduction of sodium azide, which inhibits eosinophil peroxidase, with PMA suppressed significantly the conversion of LTC_4 to the "all-trans" isomers of LTB_4 and x, without diminishing the conversion of LTC_4 to LTD_4. Catalase, which catalytically destroys H_2O_2, suppressed partially the conversion of LTC_4 to the "all-trans" isomers of LTB_4 and x, without altering the appearance of LTD_4, while boiled catalase had no significant effect.

The capacity of partially purified eosinophil peroxidase to metabolize LTB_4 in the presence of H_2O_2 and halide ions was studied in order to confirm

TABLE I

METABOLISM OF [^{3}H]LTC_4 BY HUMAN EOSINOPHILS

	5(S),12(R)-"all-trans"-LTB_4	5(S),12(S)-"all-trans"-LTB_4	x	LTD_4
	(percentage of initial radioactivity)			
Eos alone*	<0.5	<0.5	<0.5	2.8 ± 0.9
Eos + PMA	12.8 ± 2.3+	17.2 ± 3.4	10.7 ± 2.9	3.5 ± 1.2
Eos + F^-	7.8 ± 2.6	10.6 ± 3.3	6.7 ± 2.4	3.3 ± 1.5
Eos + PMA + sodium azide	2.5 ± 0.6	4.2 ± 1.8	2.9 ± 0.7	2.9 ± 1.0
Eos + PMA + catalase	5.6 ± 2.4	7.7 ± 2.9	5.6 ± 1.5	3.1 ± 0.8
Eos + PMA + boiled catalase	11.2 ± 3.8	15.5 ± 4.5	8.5 ± 3.4	3.4 ± 1.4

* 1×10^7 eosinophils, of over 92% purity, were incubated in 1 ml of Hanks' solution containing 0.01 g % of ovalbumin and 10^{-4} M NaI for 10 min at 37°C with 0.1 μCi of [^{3}H]LTC_4 and either buffer alone, 25 ng of phorbol myristate acetate (PMA), 20 mM sodium fluoride, 25 ng of PMA + 10^{-3} M sodium azide, 25 ng of PMA + 50 μg of catalase (2 times crystallized bovine liver catalase, Sigma Chemical Co., St. Louis, MO), or 25 ng of PMA + 50 μg of catalase that had been boiled at 100°C for 10 min. The incubation mixtures were extracted and the products resolved by sequential silicic acid chromatography and reverse-phase HPLC and quantified by the amount of radioactivity in the respective peaks.

+ Each value represents the mean ± S.D. of three experiments performed in duplicate with eosinophils from three different subjects. The values obtained with catalase and sodium azide for all compounds except LTD_4 were significantly lower than those with PMA alone at $p < 0.05$ and $p < 0.01$, respectively (two-sample t-test).

the peroxidatic mechanism and to obtain some products in quantities sufficient for structural analyses. Unlabeled LTC_4 was converted by the eosinophil peroxidase to the same three oxidative products as were elaborated by intact eosinophils, in a reaction that was dependent on time, the ratio of the peroxidase to LTC_4 and the presence of H_2O_2 and iodide (Fig. 1), but was not transformed to LTD_4. The ratio of the 5(S),12(S)-"all-trans"-LTB_4 to the 5(S),12(R)-"all-trans"-LTB_4 at 10 min was 1.47, 1.36, and 1.61 in three separate experiments. Mass spectrometric evaluation (6,12) of each of the products established the identity of the two 5,12-"all-trans" isomers of LTB_4.

DISCUSSION

The conversion of LTC_4 to two "all-trans" isomers of LTB_4 and a more polar product by intact eosinophils was dependent both on the enhanced generation of metabolites of oxygen and on peroxidase activity (Table I). Concentrations

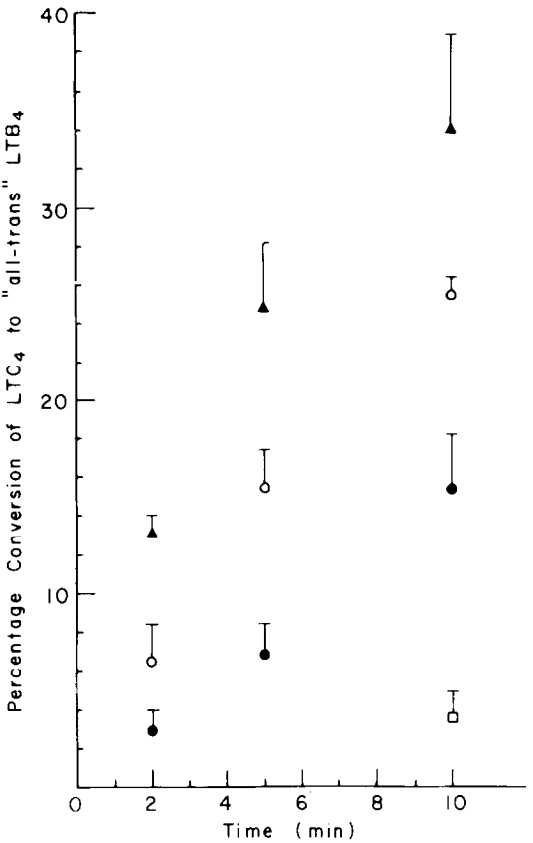

Figure 1. Time course of the conversion of LTC_4 to the two "all-trans" isomers of LTB_4 by eosinophil peroxidase. Each incubation mixture contained 3 µg of LTC_4 in 1 ml of 0.1 M NaCl-0.03 M sodium phosphate (pH 7.0) with 1 mM $MgSO_4$, 10^{-5} M NaI, 0.20 mM H_2O_2 and eosinophil peroxidase at a concentration of 200 mU/ml (●), 400 mU/ml (○), or 800 mU/ml (▲). Replicate mixtures with 400 mU/ml of eosinophil peroxidase lacked both NaI and H_2O_2 (□). After the incubation at 37°C, the mixtures were extracted and the products subjected to reverse-phase HPLC; the quantity of each product was determined from the area of the peak of optical density at 280 nm relative to that of known amounts of standard compounds. The points and brackets depict mean values ± S.D. (n = 3) for the sum of the quantities of the two "all-trans" LTB_4 isomers as a percentage of LTC_4.

of PMA and sodium fluoride which share the capacity to stimulate maximally the generation of H_2O_2, superoxide, and hydroxyl-radicals by eosinophils (13) similarly augmented the rates of conversion of LTC_4, while unstimulated eosinophils had no detectable activity. The inhibition of peroxidase activity by sodium azide prevented significantly the oxidative conversion of LTC_4. In contrast, the formation of LTD_4 from LTC_4, which requires only the cleavage of the peptide substituent, was neither enhanced by increases in oxidative metabolism nor diminished by the suppression of peroxidase activity in eosinophils (Table I) and was not achieved by the partially purified peroxidase.

The presence of H_2O_2 and a halide ion appeared to be required for maximal oxidative conversion of LTC_4 to the "all-trans" isomers of LTB_4 by eosinophil peroxidase (Fig. 1) and by intact eosinophils, where the addition of the H_2O_2 scavenger catalase significantly reduced the rate of appearance of both isomers of LTB_4 (Table I).

The conversion of LTC_4 to the isomers of LTB_4 requires considerable molecular rearrangement in addition to the removal of the 6(R)-glutathionyl group. The 7,9 trans-11 cis triene portion of LTC_4 is changed to a 6,8,10-trans arrangement in the LTB_4 isomers. A hydroxyl-group, which may be added initially at the 6-position, occupies the 12-position in the isomers of LTB_4, where the 12(S) conformation predominated over the 12(R) quantitatively (Table I). Although the nature of the more polar metabolite has not been defined, it is likely that it represents a form of LTC_4 in which the sulfido-group has been further oxidized, perhaps to a sulfone.

The peroxidative transformation of LTC_4 may be an important mechanism for the degradation of leukotrienes, as the resulting isomers of LTB_4 possess only approximately 1/300-1/100 the leukocyte chemotactic potency of LTB_4 and lack the humoral activities of LTC_4. This capacity of eosinophils to modify the expression of LTC_4 is the first example of a natural pathway for the regulation of leukotriene-mediated hypersensitivity responses.

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