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Metabolism of leukotriene A_4 by an enzyme in blood plasma: A possible leukotactic mechanism

(extracellular metabolism/leukotriene B_4/inflammation/neutrophils/diapedesis)

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ABSTRACT **Cell-free mammalian plasma transformed leukotriene A_4 into leukotriene B_4. This conversion originated from a soluble enzymatic activity. Heating at 56°C or digestion of plasma with a proteolytic enzyme eliminated formation of leukotriene B_4 but not other diastereomeric dihydroxyicosatetraenoic acids formed by nonenzymatic hydrolysis of leukotriene A_4. Plasma from several mammals, including guinea pigs, pigs, cows, sheep, rabbits, rats, dogs, and humans, exhibited a qualitatively similar activity. Maximal production of leukotriene B_4 occurred in guinea pig plasma near a pH of 7.6; metabolic capacity approached saturation at a substrate concentration of 10 μM. Leukotriene A_4 anion was transformed but not its methyl ester. The results suggest that conversion of leukotriene A_4 by an enzyme in blood plasma of mammals may provide local gradients of the chemotactic substance leukotriene B_4. High interfacial concentrations of leukotriene B_4 between the vascular endothelium and leukocytes could facilitate their diapedesis and accumulation at inflammatory sites.**

Leukotrienes (LT), a recently discovered class of naturally occurring lipids that originate from the oxidative metabolism of arachidonic acid, are involved in immediate hypersensitivity, inflammation, and respiratory disorders (1, 2). A labile epoxide, (5*S*)-5,6-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid, or LTA_4 (3), is a pivotal intermediate in the cellular enzymatic pathway that generates the biologically active leukotrienes designated LTB_4 [(5S,12*R*)-dihydroxy-6,14-*cis*,8,10-*trans*-icosatetraenoic acid] (4), LTC_4 [(5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-icosatetraenoic acid] (5), LTD_4 [(5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycine)-7,9-*trans*-11,14-*cis*-icosatetraenoic acid] (6, 7), and so forth (1). Because of its central role in the biosynthesis of leukotrienes, metabolic studies were initiated with intact LTA_4 rather than endogenous or exogenous arachidonic acid. This paper describes the metabolism of LTA_4 by cell-free plasma from mammals, especially guinea pigs.

MATERIALS AND METHODS

Materials. [14,15-^{3}H]LTA_4 lithium salt, 0.01–0.05 μCi/μg (1 Ci = 3.7×10^{10} Bq), and unlabeled LTA_4 lithium salt were prepared as described (8). The purity of both starting materials—LTA_4 methyl ester and [14,15-^{3}H]LTA_4 methyl ester (31 Ci/mmol; New England Nuclear)—and the products was evaluated by UV spectrophotometry and reversed-phase high-performance liquid chromatography (RP-HPLC) (9). Synthetic LTB_4 (Upjohn); SEP-PAK cartridges (Waters Associates); PD-10 gel filtration columns (Pharmacia); Centrifree ultrafiltration membranes (Amicon); glass-distilled solvents for HPLC (Rathburn Chemicals, Walkerburn, Scotland); methyl formate (Merck); and Pronase proteolytic enzyme (Calbiochem) were used as received.

Mammalian Plasma. Mammalian blood collected over heparin, 100 international units/ml, was centrifuged at 250 × *g* for 30 min at 25°C to sediment cells. Plasma was sterilized and small cells were excluded by passage through a 0.22-μm-pore Millex-HA filter (Millipore). For certain experiments plasma was (*i*) centrifuged at 100,000 × *g* for 60 min at 4°C, or (*ii*) fractionated on a PD-10 gel filtration column containing Sephadex G-25 medium (bed volume 9.1 ml, bed height 5 cm) equilibrated with 0.10 M pH 7.4 potassium phosphate buffer, or (*iii*) separated into macromolecule-free (<5%) and macromolecule-rich (>95%) fractions by ultrafiltration through a hydrophilic Amicon YMT membrane (M_r cutoff 10,000), or (*iv*) dialyzed against 0.10 M pH 7.4 phosphate buffer. Identical samples, heated at 56°C for 1 hr, were used as controls. Plasma (1.0 ml) diluted with 0.10 M pH 7.4 phosphate buffer (1.0 ml) was also subjected to digestion at 37°C for 6 hr with Pronase (5 mg).

Procedure. [^{3}H]LTA_4 lithium salt (1–15 μg) was added to plasma (1.0 ml) diluted with 0.10 M pH 7.4 phosphate buffer (1.0 ml). After 30 min at 37°C the solution was acidified to pH 3 with 1 M HCl. LTA_4 transformation products were isolated by percolating acidic, diluted samples (10 ml) through a C_{18} SEP-PAK cartridge. Adsorbed products were purified and concentrated by consecutive elution with 10 ml each of water, hexane, methyl formate, and methanol (10). The methyl formate fraction contained >95% of the adsorbed radioactive components; the solvent was evaporated under nitrogen and the residue was reconstituted in mobile phase (0.10 ml) and analyzed by HPLC as described (11). Products were detected by UV absorption at 270 nm and by liquid scintillation counting of tritium in the chromatographic fractions.

LTB_4 Bioassay. Bioassay for LTB_4 was performed on isolated guinea pig lung parenchymal strips suspended in a 3-ml perfusion chamber containing Tyrode's buffer under nonflow conditions as described (12).

Lysozyme Assay. Lysozyme was assayed turbidimetrically with suspensions of *Micrococcus lysodeikticus* (13).

Instrumental Analysis. Gas chromatography/mass spectrometry (GC/MS) was done on an LKB 9000 mass spectrometer equipped with a 2 m × 3 mm inside diameter column of OV-101, 1% wt/vol, on Chromosorb WHP, 100–120 mesh (Alltech, Deerfield, IL). The gas chromatograph was operated isothermally at 210°C with a helium flow of 20 ml/min. Ionization energy was 22.5 eV; trap current was 60 μA.

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Abbreviations: LT, leukotriene; RP-HPLC, reversed-phase high-performance liquid chromatography; SP-HPLC, straight-phase high-performance liquid chromatography; HPETE, hydroperoxyicosatetraenoic acid.

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RESULTS

RP-HPLC analysis detected, five UV-absorbing radioactive species (I–V) from guinea pig plasma incubated with [^{3}H]LTA_4 (Fig. 1). In 0.10 M pH 7.4 phosphate buffer or in buffer containing guinea pig albumin (40 mg/ml) only four UV-absorbing radioactive peaks (I, II, IV, and V) appeared; peak III was absent. Peaks I and II corresponded to a pair of diastereomeric 5,12-dihydroxyicosatetraenoic acids formed by nonenzymatic hydrolysis of LTA_4. The following properties were all identical to published values (11, 14) and to those of authentic standards of (5*S*,12*R*-dihydroxy-6,8,10-*trans*-14-*cis*-icosatetraenoic acid (peak I) and (5*S*,12*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-icosatetraenoic acid (peak II): their liquid chromatographic mobility; their UV spectra with triplet absorbance peaks at 259, 269, and 280 nm; the C value (relative retention time), 24.9, for their methyl ester trimethylsilyl ethers on an OV-101 gas chromatographic column; and their mass spectra with fragment ions at m/z (mass-to-charge ratio) = 129 (base peak), 191, 203, 217, 267, 293, 383, 404, 463, and 479. The UV, gas chromatographic, and mass spectral properties of the methyl esters isolated by straight-phase HPLC (SP-HPLC) were identical to those cited above.

Peaks IV and V corresponded to a pair of diastereomeric 5,6-dihydroxyicosatetraenoic acids, also formed by nonenzymatic hydrolysis of LTA_4. Their liquid chromatographic mobility, their UV spectra with triplet peaks at 263, 272, and 284 nm; the C values 24.0 (peak IV) and 23.9 (peak V) for their methyl ester trimethylsilyl ethers on an OV-101 column, and their mass spectra with prominent ions at m/z 171, 203 (base peak), 225, 291, 383, 404, 463, 479, and 494 were identical to published values (11, 14) and to those of standards of diastereomeric 5,6-dihydroxy-7,9,11,14-icosatetraenoic acid.

When [^{3}H]LTA_4 was incubated with guinea pig plasma, an additional UV-absorbing radioactive component, peak III, appeared. Its chromatographic mobility; its UV spectrum, with triplet peaks at 260, 270, and 281 nm; its C value, 23.6; and its mass spectrum with fragment ions at m/z = 129 (base peak), 167, 191, 203, 217, 229, 267, 293, 383, 404, 463, and 479 were identical to published values (11, 14) and to those obtained from synthetic LTB_4. After conversion to a methyl ester, peak III was homogeneous by SP-HPLC. It eluted coincident with synthetic LTB_4 methyl ester and contained no detectable (5*S*,12*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-icosatetraenoic acid. The UV, gas chromatographic, and mass spectral properties of the methyl ester isolated by SP-HPLC were identical to those cited above. Peak III was collected from several experiments to prepare a solution for comparative analysis by UV spectrophotometry and bioassay. On guinea pig lung parenchymal strips the sample had an indomethacin-sensitive contractile activity that was indistinguishable from that of synthetic LTB_4 (Fig. 2). Bioassay and UV spectrophotometry gave comparable values, 1.2 and 1.0 mM, respectively, for the LTB_4 concentrations in the samples.

Results were qualitatively equivalent for plasma from normal or sensitized guinea pigs. Analyses by either SP-HPLC or RP-HPLC were consistent. Each plasma examined metabolized LTA_4 into LTB_4, but not into (5*S*,12*S*)-dihydroxy-6,10-*trans*-8,14-*cis*-icosatetraenoic acid.

At an initial LTA_4 concentration of 5 μM, LTB_4 formation approached a maximum within 5 min (Fig. 3). A decline in the amounts of nonenzymatically produced 5,12-dihydroxyicosatetraenoic acids, with corresponding increases in LTB_4 formation, reflected the consumption of LTA_4 substrate. Under the conditions described, 8.2 ± 3.6% (mean ± SD, n = 8, range

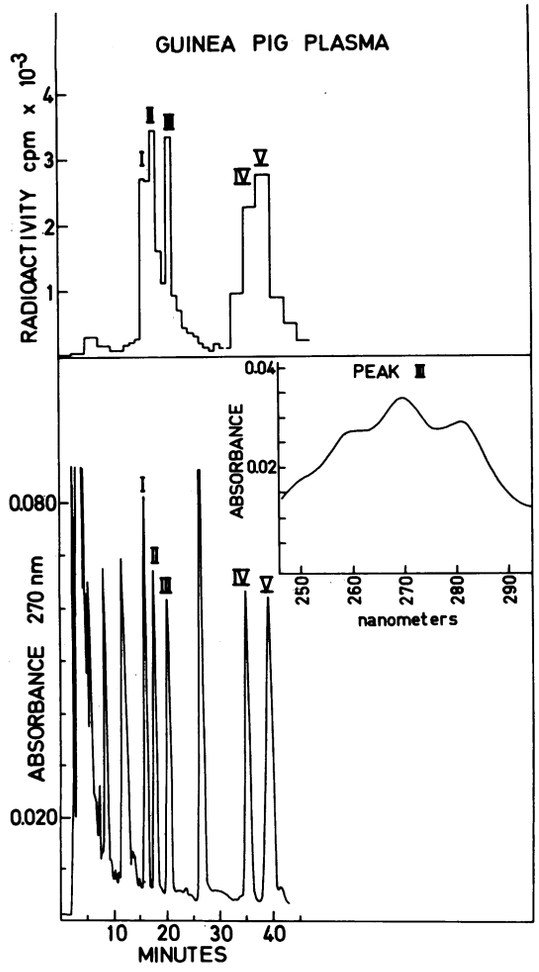

FIG. 1. RP-HPLC analysis of products formed during the incubation of 5 μM [^{3}H]LTA_4 with guinea pig plasma. Peaks not designated with roman numerals correspond to endogenous UV-absorbing components of plasma. The UV spectrum of peak III (*Inset*) had peaks at 260, 270, and 281 nm.

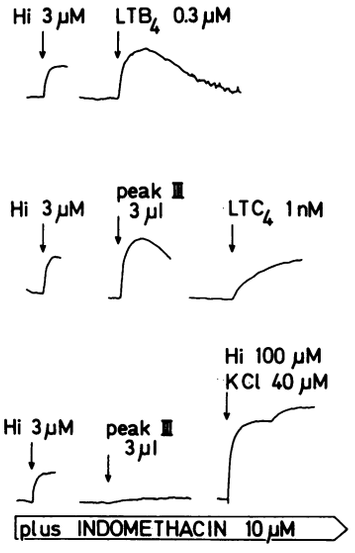

FIG. 2. Isometric contraction of guinea pig lung parenchymal strips in response to 3 μM histamine (Hi) and 0.30 μM LTB_4 standard (top traces) or to 3 μM histamine, 3 μl of peak III in buffer, and 1 nM LTC_4 (middle traces). Perfusion with buffer containing 10 μM indomethacin abolished the contractile response due to LTB_4 or peak III, but not histamine (bottom traces).

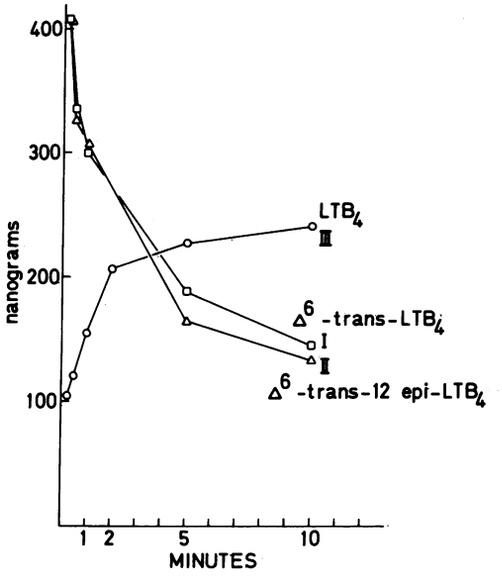

FIG. 3. Transformation kinetics of 5 μM LTA_4 into LTB_4 (III), Δ^6-*trans*-LTB_4 (I), and Δ^6-*trans*-12-epi-LTB_4 (II).

= 3.2–15.2%) of LTA_4 was transformed into LTB_4 by guinea pig plasma diluted 1:1 with 0.10 M pH 7.4 phosphate buffer. Activity, though detectable, diminished to <1% conversion at a 1:10 dilution; activity was undetectable at a 1:100 dilution. In contrast to the anionic form, the methyl ester of LTA_4 was not transformed into LTB_4 or LTB_4 methyl ester.

Heating guinea pig plasma at 56°C eliminated its ability to convert LTA_4 into LTB_4 (Fig. 4). Spontaneous decay varied among different samples. Plasma held at 4°C for 240 hr retained 50–80% of its activity; plasma samples incubated for 6 hr at 37°C retained 80–100% of their activity. The activity in plasma was characterized as a soluble, high molecular weight protein component in five ways: (*i*) guinea pig plasma (5 ml) dialyzed against 0.10 M pH 7.4 phosphate buffer (250 ml) for 24 hr at 4°C still converted LTA_4 into LTB_4. Heating at 56°C inactivated the dialyzed plasma. No activity was evident in the dialysate. The dialysis membrane retained substances with $M_r \geq 10{,}000$. (*ii*) A heat-sensitive activity remained in the macromolecule-enriched fraction after ultrafiltration through a hydrophilic membrane with a M_r cutoff of 10,000. No activity appeared in the protein-free-filtrate. (*iii*) Ultracentrifugation at 100,000 × *g* for 60 min did not sediment any active particulate fraction from guinea pig plasma. The supernatant retained a heat-sensitive activity. (*iv*) Activity eluted with the unretained high molecular weight fraction ($M_r \geq 10{,}000$) when guinea pig plasma (2 ml) was chromatographed on a PD-10 gel filtration column. (*v*) The activity in plasma was susceptible to proteolytic digestion. Incubation at 37°C for 6 hr with Pronase completely eliminated its capacity to form LTB_4. Control samples incubated under identical conditions without Pronase retained 95–100% of their enzymatic activity.

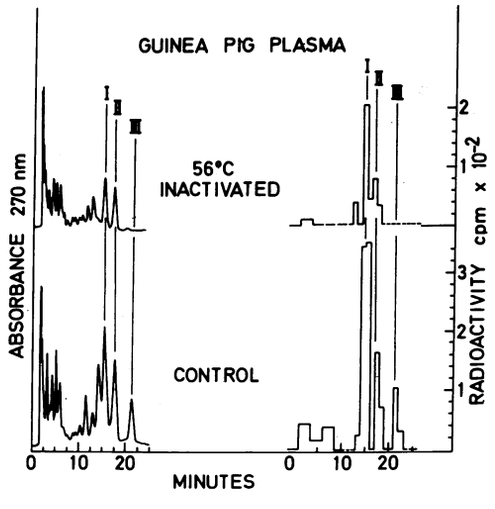

FIG. 4. Influence of 56°C heating on formation of LTB_4 (III) by guinea pig plasma. SP-HPLC analysis. I is Δ^6-*trans*-LTB_4; II is Δ^6-*trans*-12-epi-LTB_4.

Table 1. pH Dependence of LTB_4 formation

pH	Metabolite formed, nmol		
	I	II	LTB_4
6.5	1.12	1.08	0.48
7.1	1.13	1.01	0.48
7.6	0.70	0.70	1.16
8.5	0.90	0.81	0.89

Guinea pig plasma was diluted 1:1 with an appropriate 0.10 M phosphate buffer to obtain the pH tabulated. The initial LTA_4 concentration was 5 μM. Samples were treated as described and analyzed by RP-HPLC. I and II are Δ^6-*trans*-LTB_4 and Δ^6-*trans*-12-epi-LTB_4, respectively, formed by nonenzymatic decay of LTA_4.

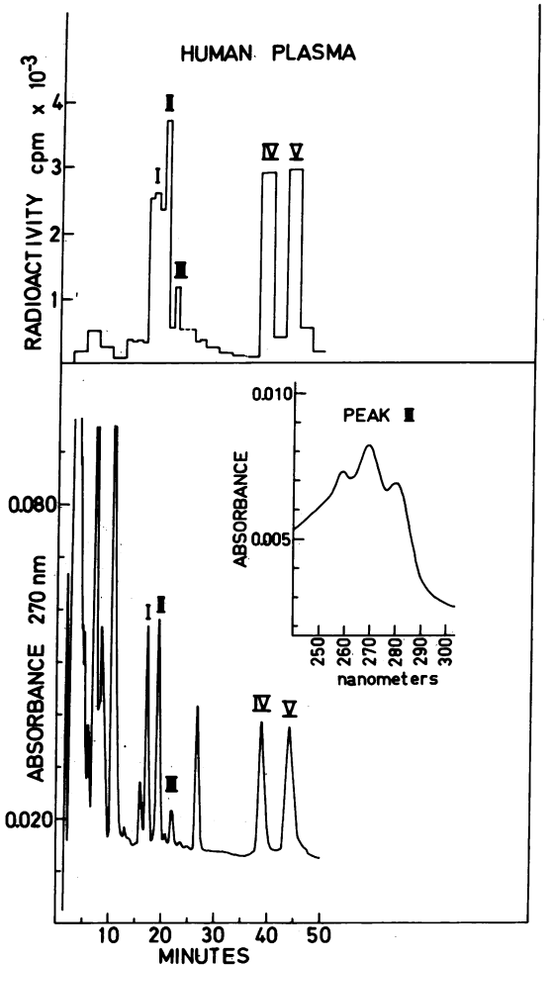

FIG. 5. RP-HPLC analysis of products formed from the incubation of 10 μM LTA_4 with plasma from a human female. The UV spectrum of peak III (*Inset*) had maxima at 260, 270, and 281 nm.

Table 2. Characteristics of plasma enzymatic activity that converts LTA_4 into LTB_4

1.	Nondialyzable, M_r >10,000
2.	Present in macromolecule fraction after ultrafiltration through membranes with M_r cutoff ≥10,000
3.	Not retained by Sephadex G-25 medium, activity present in protein fraction, M_r ≥10,000
4.	Activity destroyed by heating of plasma at 56°C
5.	Activity destroyed by digestion of plasma with Pronase
6.	Soluble component, not sedimented by centrifugation at 100,000 × *g* for 60 min
7.	pH-Dependent transformation with optimum pH 7.6 in guinea pig plasma
8.	Substrate specific for LTA_4 anion, not its methyl ester
9.	Saturable at substrate concentrations >10 μM
10.	Activity not correlated with leukocyte lysis

Conversion of LTA_4 to LTB_4 in guinea pig plasma was pH dependent, with an optimum near the physiological value (Table 1). The transforming capacity of guinea pig plasma approached saturation at an initial substrate concentration of 10 μM. For instance, LTB_4 production was 0.34, 0.59, 1.26, and 1.50 μM when substrate concentrations were 2.65, 5.3, 10.6, and 21.2 μM, respectively.

The LTA_4-metabolizing activity did not originate from leukocyte lysis during the isolation of plasma. Lysosomal enzyme activity was undetectable in most plasma samples and, compared to two samples with detectable lysozyme corresponding to 2% and 4% of the maximal available, there was no correlation between LTB_4 formation and leukocyte degranulation. Furthermore, when leukocytes from guinea pig blood (10 ml) were isolated and deliberately lysed, the cell-free supernatant containing as much as 10% of the available lysozyme was unable to transform LTA_4 into LTB_4.

A similar capacity to transform LTA_4 into LTB_4 was detectable in the plasma of other mammals, including the rat, rabbit, cow, dog, pig, sheep, and human. Fig. 5 depicts results from the RP-HPLC analysis for human plasma incubated with 10 μM LTA_4 lithium salt. In all cases, enzymatic activity was abolished by 56°C heating or by proteolytic digestion, and it was characterized as a soluble, high molecular weight component. The amount of LTB_4 formed differed among the species; relative production (mean ± SD) for several individual animals was 8.2 ± 3.6% (n = 8), 2.3 ± 0.5% (n = 3), 1.5 ± 0.4% (n = 4), and 1.2 ± 0.3% (n = 4), respectively for guinea pigs, pigs, cows, and sheep when plasma (1.0 ml) diluted with 0.10 M pH 7.4 phosphate buffer (1.0 ml) was incubated with 5 μM LTA_4 as described. For human plasma, concentrated 2× by ultrafiltration, LTB_4 production was 1.14 ± 0.40% (n = 6). Table 2 summarizes the characteristics of the plasma component that converted LTA_4 into LTB_4.

DISCUSSION

Our results indicate that cell-free mammalian blood plasma contains a component capable of converting LTA_4 into LTB_4, a chemokinetic, chemotactic, myotropic substance that induces neutrophil adhesion, aggregation, and degranulation (15–18). Heat inactivation, proteolytic digestion, dialysis, ultrafiltration, gel filtration, ultracentrifugation, pH dependence, substrate specificity, and saturability at micromolar substrate concentrations support the conclusion that LTB_4 formation is attributable to an enzymatic activity.

Leukotriene biosynthesis requires enzymes that are distributed among different subcellular compartments and it appears that their biosynthesis involves movement of intermediates between these compartments (19). In the case of LTB_4 formation the action of a soluble cytosolic hydrolase has been proposed to be the rate-limiting step (19). Consequently, intermediate substrates such as 5-hydroperoxyicosatetraenoic acid (5-HPETE) or LTA_4 must accumulate, decay via alternate enzymatic and

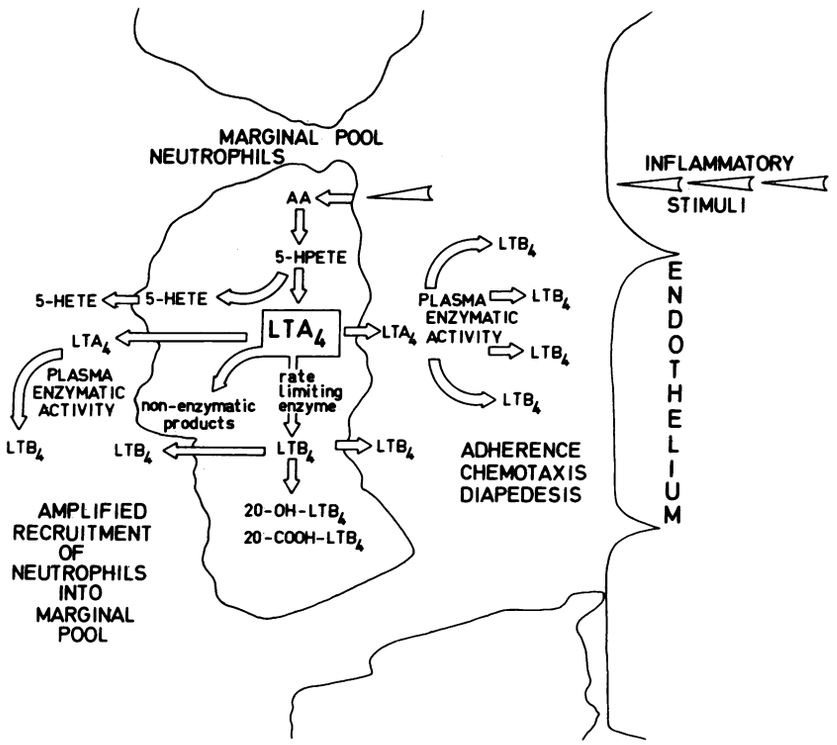

FIG. 6. Proposed scheme for $LTA_4 \rightarrow LTB_4$ plasma enzymatic activity involvement with neutrophil diapedesis and chemotaxis. AA, arachidonic acid; 5-HETE, 5-hydroxyicosatetraenoic acid.

nonenzymatic processes, or be released from cells, such as leukocytes. In the latter instance a plasma enzyme capable of converting extracellular LTA_4 seems reasonable from several perspectives. Physiologically, it could produce high interfacial concentrations of LTB_4 between vascular endothelial surfaces and leukocytes. Such local gradients of a highly chemotactic agent could facilitate adhesion of leukocytes to the endothelium and their diapedesis between cells with subsequent accumulation in the extravascular space. It is interesting to consider our results and their proposed significance in the context of known *in vivo* characteristics of granulocyte adherence (20). Namely, inflammatory stimuli increase the pool of marginating neutrophils; adherence involves no visible specifically adhesive substance between neutrophils and endothelium; adherence is a local phenomenon (displaced neutrophils do not adhere elsewhere and newly arriving neutrophils will adhere to vacant sites); and anticoagulants or fibrinolytic substances do not inhibit adherence, in contrast to corticosteroids, divalent cation chelators, or channel blockers (20). Furthermore, one or more nondialyzable, 56°C heat sensitive factors in plasma have a demonstrated ability to modulate granulocyte adherence *in vivo* (21). Leukocyte chemotaxis and diapedesis is a complex process that involves several molecular and cellular mechanisms (22), some of which remain unknown (23). Our results suggest that extracellular enzymatic transformation of LTA_4 into a potent chemotaxin, LTB_4, may be involved. Because plasma exudation is another prominent feature of inflammation and because LTB_4 induces not only neutrophil adhesion and locomotion but also aggregation, degranulation, and superoxide generation (15–18), the plasma enzymatic activity responsible for its production may play a general role in modulating the inflammatory response.

The physiological significance of our results depends upon cellular release of LTA_4, which must remain in an intact form, at least momentarily, for eventual presentation to and conversion by the enzymatic activity reported here. Several precedents indicate that this is reasonable. First, washed, viable neutrophil suspensions convert exogenously added LTA_4 into various leukotrienes *in vitro* (14, 24) and intact LTA_4 has been isolated from neutrophils stimulated by ionophore A23187 (25). These results indicate that LTA_4 can traverse cell membranes. Second, another plasma component, albumin, can stabilize LTA_4 to a significant degree *in vitro* (26). Third, transcellular metabolism occurs along the platelet/endothelium (27), platelet/neutrophil (28, 29), and the lymphocyte/monocyte axes (30). It is axiomatic that this related process, transcellular metabolism, involves release and transfer of substrate from a donor cell for assimilation and metabolism by a recipient cell. Labile substrates, such as prostaglandin endoperoxide, can undergo this process (27). Extracellular conversion, as described here for LTA_4, is an unusual enzymatic process in the arachidonic acid cascade. All previously described enzymatic transformations of LTA_4, or its counterparts, the prostaglandin endoperoxides, have originated exclusively from cell-associated microsomal (31–33) or cytosolic enzymes (34, 35).

Fig. 6 depicts our proposed scheme for one physiological role involving extracellular conversion of LTA_4 into LTB_4. This scheme is notable because it accommodates certain paradoxical aspects of diapedesis and inflammation such as the obligate presence of neutrophils to mediate LTB_4-induced increases in vascular permeability (36).

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