STIMULATION OF HUMAN LEUKOCYTE DEGRANULATION BY LEUKOTRIENE B_4 AND ITS ω-OXIDIZED METABOLITES

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1. Introduction

Leukotriene (LT)B_4 has been indicated to play an important role in inflammatory reactions such as leukocyte chemotaxis [1,2] and adhesion to post-capillary venules [3]. In [4–6] LTB_4 was suggested to be a weak stimulator of polymorphonuclear leukocyte (PMNL) degranulation. However, it is now clear that previous purification procedures for biosynthetically prepared LTB_4 were inadequate. The newly discovered LTB_4 isomer, 5(*S*),12(*S*)-DHETE, co-chromatographs with LTB_4 in the reverse phase high-pressure liquid chromatography (HPLC) systems commonly used [7]. As a consequence, earlier studies probably tested preparations which contained a mixture of both isomers with the non-leukotriene product predominating. Therefore, we have investigated the role of LTB_4, 5(*S*),12(*S*)-DHETE and their ω-oxidation products (fig.1) [8] in leukocyte degranulation in vitro. In addition, the activity of the non-enzymatically formed LTB_4 isomers was investigated.

This study shows that LTB_4-stimulated degranulation at 10^{-8} M; the ω-oxidation products of LTB_4 were less potent while 5(*S*),12(*S*)-DHETE had no activity even at 2×10^{-6} M. Furthermore, the latter compound inhibited enzyme release induced by LTB_4.

Abbreviations: 5(*S*),12(*S*)-DHETE, 5(*S*),12(*S*),dihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; fMLP, *N*-formyl-methionyl–leucyl–phenylalanine; LTB_4, 5(*S*), 12(*R*)-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-eicosatetraenoic acid; 20-OH-LTB_4, 5(*S*),12(*R*),20-trihydroxy-6-*cis*,8,10-*trans*, 14-*cis*-eicosatetraenoic acid; 20-COOH-LTB_4, 5(*S*),12(*R*)-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-eicosatetraen-1,20-dioic acid; 5(*S*),12(*S*),20-THETE, 5(*S*),12(*S*),20-trihydroxy-6-*trans*, 8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid

2. Materials and methods

Cytochalasin B, phenolphthalein glucuronic acid, and dried *Micrococcus lysodeikticus* were purchased from Sigma (St Louis MO). Dextran T-500 was obtained from Pharmacia Fine Chemicals (Uppsala) and fMLP was a product of UCB SA (Brussels).

2.1. *Cell preparation and incubations*

Purified polymorphonuclear leukocytes were prepared from leukocyte concentrates of human peripheral blood (Karolinska Hospital, Stockholm) by dextran sedimentation followed by hypotonic lysis of residual erythrocytes [9]. The cells were diluted to 5×10^6 cells/ml in Dulbecco's phosphate-buffered saline (pH 7.4). Aliquots (1–2 ml) of the cell suspension were preincubated for 5 min with cytochalasin B (5 μg/ml in DMSO 1 μg/ml) in plastic vessels at 37°C. Test substances were added as free acids in either ethanol, methanol or DMSO. At the levels used, these solvents had no effect on spontaneous release. Incubations were terminated after 5 min by transfer to an ice bath followed by rapid centrifugation. The cell-free supernatants were subsequently assayed for the presence of lysozyme, β-glucuronidase and lactic acid dehydrogenase (LDH).

2.2. *Enzyme assays*

β-Glucuronidase was measured by a modification of the procedure in [10]. Supernatant (0.2 ml) was added to 2 ml acetate buffer (0.1 M, pH 4.6) followed by 0.1 ml phenolphthalein glucuronic acid (0.01 M, pH 7.0) as substrate. The reaction was terminated after 18 h at 37°C by the addition of 5 ml glycine buffer (0.2 M in 0.2 M NaCl, pH 10.4). The absorb-

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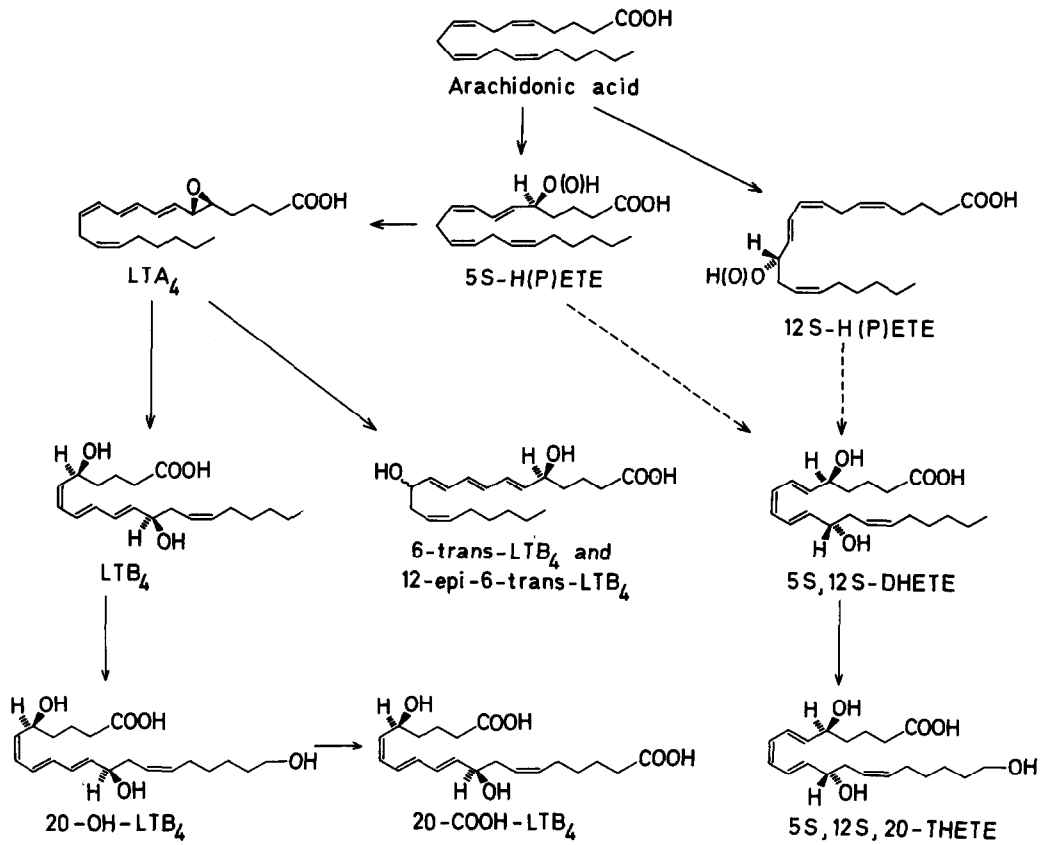

Fig.1. Products of arachidonic acid metabolism by human polymorphonuclear leukocytes: (---►) proposed conversions.

ance of each sample was determined at 540 nm *vs* a glycine buffer blank.

Lysozyme was determined by a turbidimetric assay [11] based on the ability of this enzyme to lyse suspensions of *Micrococcus lysodeikticus*. Supernatant (0.2 ml) was added to 0.8 ml *M. lysodeikticus* suspension (0.03 mg/ml in 0.1 M phosphate buffer, pH 7.0) and the fall in absorbance was monitored at 450 nm in a Cary 219 recording spectrophotometer.

LDH was monitored according to [12] and never significantly exceeded control levels.

The obtained enzyme release was corrected for spontaneous release and then calculated as a percentage of the total cellular enzyme content. This was determined by assaying the 10 000 × *g* supernatant of sonicated cell suspensions.

2.3. *Leukotriene preparation*

LTB_4, 5(*S*),12(*S*)-DHETE [7], 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 [13] and 20-OH-LTB_4 [8] were prepared as reported. 20-COOH-LTB_4 was prepared by a further purification of the major polar fraction in [8]. This fraction was subjected to reverse-phase HPLC using methanol/water/acetic acid (50:50:0.01, by vol.) followed by rechromatography eluting with methanol/water/acetic acid (45:55:0.01, by vol.). The purity of this compound was verified on straight-phase HPLC [8] after conversion to the dimethyl ester.

3. Results

A series of arachidonic acid metabolites was tested for the ability to induce degranulation in cytochalasin B-treated human PMNL in vitro. The compounds studied were LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5(*S*),12(*S*)-DHETE and 5(*S*),12(*S*),20-THETE. LTB_4 and its metabolites, 20-OH-LTB_4 and 20-COOH-LTB_4, induced the release of both lysozyme and β-glucuronidase (fig.2). 6-*trans*-

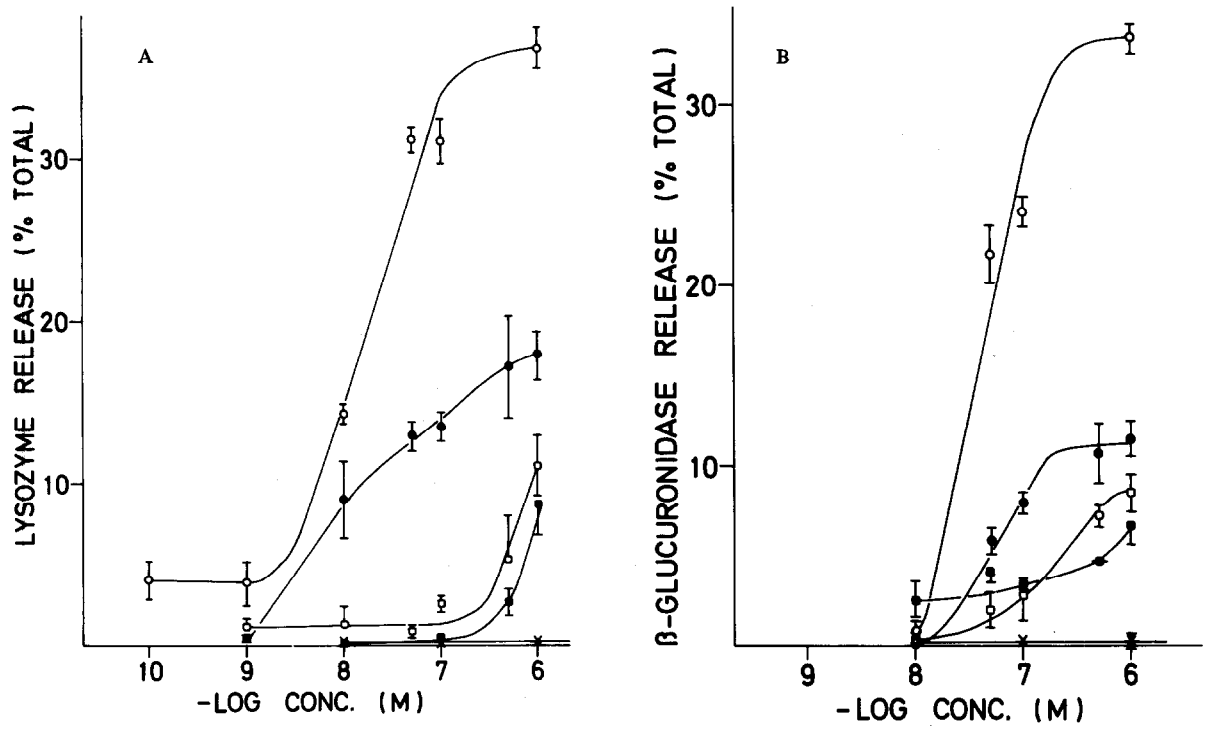

Fig.2. The release of granular enzymes from human polymorphonuclear leukocytes stimulated with varying concentrations of LTB_4 (•), 20-OH-LTB_4 (□), 20-COOH-LTB_4 (■), 5(*S*),12(*S*)-DHETE (×) and fMLP (○). Error bars denote standard errors of the mean of triplicate observations: (A) lysozyme; (B) β-glucuronidase. The curves representing fMLP-induced release are presented for comparison to other studies in which this agent is frequently used. Although only a few points are plotted, the shape of the curve is supported by data in [14,15].

LTB_4, 12-epi-6-*trans*-LTB_4, 5(*S*),12(*S*)-DHETE and 5(*S*),12(*S*),20-THETE were all unable to induce enzyme release. LTB_4 was found to yield a half-maximal release of lysozyme at 3.5×10^{-8} M (mean of 4 expt). 20-OH-LTB_4 and 20-COOH-LTB_4 were able to stimulate enzyme release equal to half the LTB_4 maximum only at levels 10–100-times higher (on a molar basis). At 5×10^{-7} M, the LTB_4-induced lysozyme release was 17.2%. When added at the same molar concentration, 20-OH-LTB_4 and 20-COOH-LTB_4 caused 5.3% and 2.6% release, respectively.

The activity of LTB_4 was compared to that of the chemotactic peptide, fMLP. Maximal enzyme release induced by LTB_4 was 35–50% of the enzyme released by fMLP.

5(*S*),12(*S*)-DHETE has been found to make up a large (60–75%) fraction of the 'LTB_4-peak' isolated from reverse-phase HPLC [7]. Therefore, the possibility that the two isomers interacted to alter the activity of LTB_4 alone was tested. 5(*S*),12(*S*)-DHETE, at 10^{-6} M, inhibited half-maximal LTB_4 stimulation of lysozyme release by 42% ($p < 0.001$). Furthermore, this concentration of 5(*S*),12(*S*)-DHETE had no effect on the release of lysozyme induced by fMLP.

4. Discussion

Several groups have investigated the enzyme-releasing effects of LTB_4 on peripheral leukocytes. LTB_4 was found to induce degranulation of cytochalasin B-treated human peripheral neutrophils but this occurred at a half-maximal dose of $\geqslant 10^{-6}$ M [4,6]. Earlier biosynthetic LTB_4 preparations obtained from human leukocyte suspensions contained a non-leukotriene isomer, 5(*S*),12(*S*)-DHETE [7]. Although the ratio of these two conjugated trienes is variable, 5(*S*),12(*S*)-DHETE can make up considerably >50% of the ultraviolet light-absorbing material eluting in the 'LTB_4-peak' from reverse-phase HPLC. These

isomers can now be separated using straight-phase HPLC [7]. It was therefore of interest to re-evaluate the biological activity of LTB_4, 5(*S*),12(*S*)-DHETE and the mixture of the 2 compounds.

LTB_4 was found to be a potent inducer of enzyme release in human polymorphonuclear leukocytes. When added at as low as 10^{-8} M, LTB_4 caused the release of significant amounts of lysozyme. The ω-oxidized metabolites, 20-OH-LTB_4 and 20-COOH-LTB_4 were much less potent, indicating that ω-oxidation may be a first step of biological inactivation of LTB_4.

The maximal amount of enzyme released by LTB_4 was always lower than the amount released from fMLP-stimulated cells. Thus, LTB_4 is roughly 50% as efficacious as the chemotactic peptide (fig.2). This difference does not seem to be due to a selective discharge of one granule-type since markers for both azurophil (lysozyme) and specific granules (β-glucuronidase) yielded similar results. Instead, this observation suggests that different receptor populations may be involved.

5(*S*),12(*S*)-DHETE did not stimulate enzyme release. 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 5(*S*), 12(*S*),20-THETE were also inactive in this respect. This shows that LTB_4-induced degranulation is a highly stereospecific effect. Similar observations have been made for LTB_4-induced chemotaxis [2]. However, at high relative concentrations, 5(*S*),12(*S*)-DHETE can interfere with LTB_4 activity. This competition, presumably at a cell-surface receptor, results in an observed inhibition of LTB_4-induced enzyme release. Previous studies, therefore, probably underestimated the potency of LTB_4 in two important ways:

(i) The concentration of ultraviolet light-absorbing material included large amounts of 5(*S*),12(*S*)-DHETE, which was biologically inactive;

(ii) This compound acted as a competitive antagonist of the LTB_4-induced enzyme releasing activity.

This report indicates that LTB_4 is sufficiently potent to play an important role in leukocyte degranulation in vivo. Further studies to clarify this role are in progress.

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