LEUKOTRIENE B_4 – A STEREOSPECIFIC STIMULATOR FOR RELEASE OF LYSOSOMAL ENZYMES FROM NEUTROPHILS

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1. Introduction

Polymorphonuclear (PMN) neutrophil granulocytes react with increased adherence, chemotaxis, degranulation, oxidative metabolism and bacterial killing after contact with substances generated in an inflammatory or infected site, e.g., the C5a-fragment and bacterial products, such as fMLP. Although critical for host defence, these mediators are also responsible for part of the tissue damage characterizing inflammation because of release of PMN lysosomal enzymes and formation of toxic oxygen radicals [1–4].

A novel group of mediators of inflammation are called leukotrienes [5]. These are formed within PMNs after stimulation with fMLP or the calcium ionophore A 23187 by a lipoxygenase-dependent oxygenation of arachidonic acid. The generated epoxide leukotriene A_4 (LTA_4) is unstable and converted to leukotriene B_4 (LTB_4, 5,12-dihydroxyeicosatetraenoic acid) by enzymatic hydrolysis. Two other 5,12-dihydroxy isomers (compounds I and II) can also be formed by a non-enzymatic hydrolysis of LTA_4. Further, leukotriene C_4 (LTC_4), is formed from LTA_4 by adding glutathione, and can be metabolized to LTD_4 by elimination of its γ-glutamyl residue. LTC_4 and LTD_4 have been identified in SRS-A from various sources, being potent bronchoconstrictors and increasing permeability of the microvasculature [6].

LTB_4 stimulates neutrophil migration (in vitro and in vivo), aggregation and, to a lesser degree, degranulation [7–10]. Also, the lipoxygenase product 5-hydroxyeicosatetraenoic acid (5-HETE) has been reported to elicit a chemotactic response and cause release of lysosomal enzymes [11]. This report concerns further investigations of the effects on the release of lysosomal enzymes from PMNs by LTB_4, its isomers compounds I and II, LTC_4 and 5-HETE, documenting the stereospecificity of LTB_4 and the only partial dependence of cytochalasin B for this reaction.

2. Methods

2.1. *Leukocyte preparation*

Erythrocytes in heparinized (10 IU heparin – without preservative/ml blood) human venous blood, obtained in sterile plastic tubes (Nunc, Denmark) were sedimented in 4.5% dextran T-500 at room temperature for 45 min [12]. The leukocyte-rich supernatant was centrifuged at 500 × *g* for 6 min. The cellular pellet was washed twice in heparinized 0.9% saline (1 IU heparin/ml saline). The leukocytes were resuspended, to make an appropriate neutrophil concentration, in Hank's balanced salt solution (HBSS) containing 0.1% gelatin.

2.2. *Leukotrienes*

LTB_4, compounds I and II and LTC_4 was obtained after incubation of PMNs with arachidonic acid (Nu-Chek Prep., Elysian MN) and ionophore A 23187 (Eli Lilly, Indianapolis IN). The incubate was extracted and purified by high-pressure chromatography [13].

2.3. *Determination of enzyme release*

This was performed with a modification of the assay in [14]. A leukocyte suspension (0.5 ml), containing 2×10^9 PMN/l, was preincubated with 2.5 μg cytochalasin B, dissolved in dimetylsulphoxide (DMSO, Sigma, St Louis MO at a final concentration of 0.5%), for 15 min at 37°C. Leukotrienes, or 5-HETE, dissolved in ethanol (at a final concentration of 1%), or fMLP, dissolved in HBSS, were added and

incubated with the leukocytes for an additional 15 min at 37°C. In some experiments with leukotrienes cytochalasin B was omitted and leukocytes were incubated with LTs only for 15 min. The reaction was stopped by lowering the temperature rapidly to +4°C. The cells were sedimented by centrifugation at 200 × *g* for 5 min and the cell-free supernatants were collected. These were assayed for the activity of β-glucuronidase by measuring the release of phenolphtalein after 24 h incubation with phenolphtaleinglucuronic acid as substrate at 37°C [15] and of lysozyme by measuring the lysis of *Micrococcus lysodeikticus* with the micrococcus plate method [16]. Human lysozyme (State Bacteriological Lab.) was used as a standard at pH 7.5. To assess the unspecific leakage of cytoplasmic enzymes, lactate dehydrogenas (LDH), and its isoenzymes 1–5, were measured [17].

The stimulated net release of enzymes was expressed as % total cellular enzyme content, determined after lysis of PMNs with 0.2% Triton X-100 (Sigma).

Neither LTs, 5-HETE, fMLP, cytochalasin B or ethanol, at these concentrations, influenced the assays of lysozyme or β-glucuronidase. Controls were incubated with solvent only. At the concentrations used neither cytochalasin B or ethanol, nor their combinations, caused any release of enzymes compared with controls containing only leukocytes (mean 7.1% for β-glucuronidase and 5.6% for lysozyme). Experiments with cytochalasin B treated PMNs had shown that fMLP at 10^{-5} M caused a mean net release of 15.3% of the total cellular content of β-glucuronidase (being ~99.3 U/10^6 PMN) and 18.2% for lysozyme (~2.4 μg/10^6 PMN), both being completed within a few minutes of incubation without any net release of LDH.

The standard error (expressed as % of the mean) for the differences between 50 double determinations is 4.7% for β-glucuronidase and 3.0% for lysozyme.

3. Results

LTB_4 caused a significant release of lysozyme and β-glucuronidase from cytochalasin B-treated PMNs at 10^{-5} M, but not at lower concentrations (fig.1). From PMNs not treated with cytochalasin B LTB_4 at 10^{-5} M also released small amounts of lysozyme but not of β-glucuronidase. Similarly, compound II at 10^{-5} M caused a small release of lysozyme from cytochalasin B-treated neutrophils but not of β-glucuronidase. However, neither compound I, LTC_4 nor 5-HETE

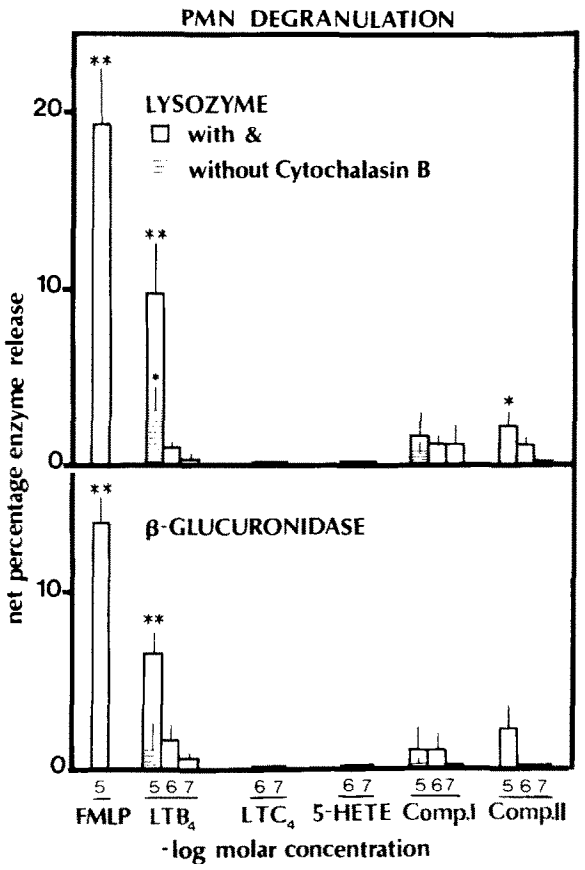

Fig.1. The release of lysozyme and β-glucuronidase from neutrophils after stimulation with leukotrienes, 5-HETE or fMLP, with or without pretreatment with cytochalasin B. The results are expressed as means and SE values for the net percentage of enzyme release from PMNs from duplicate determinations from 3 different subjects: * $p < 0.05$; ** $p < 0.01$ (Wilcoxon's signed rank test).

stimulated a net secretion of any of the enzymes. The release of both enzymes from cytochalasin B-treated PMNs by LTB_4 at 10^{-5} M was ~1/2 of that noted after stimulation with fMLP at the same concentration. However, in contrast to LTB_4, fMLP did not induce any net secretion of enzymes from PMNs not pretreated with cytochalasin B. The LTs or 5-HETE did not induce any net release of LDH.

4. Discussion

Previous observations have linked arachidonic acid and its oxygenated metabolites to degranulation of neutrophils. Thus, arachidonic acid caused a release

of lysozyme [18] and eicosatetraynoic acid (ETYA) caused a dose-dependent decrease of PMN degranulation induced by the ionophore A 23187 or fMLP [18,19]. This indicates that oxygenation of arachidonic acid is essential for the enzyme release of human PMNs to these stimuli since ETYA inhibits both the lipoxygenase and cyclooxygenase pathways. Further, only high concentrations of indomethacin (shown to inhibit mainly cyclo-oxygenase and to a lesser extent lipoxygenase) also hampered part of the secretary effect of fMLP, whereas lower concentrations (inhibiting only the cyclo-oxygenase) did not affect enzyme release [19]. These findings are compatible with a role of lipoxygenase products for neutrophil degranulation.

When lipoxygenase metabolites of arachidonic acid in PMNs (5-HETE, LTC_4, LTB_4 and compounds I and II) were studied, LTB_4 was found to induce a release of lysosomal enzymes in cells treated with cytochalasin B, but also in untreated cells, a response which differs from fMLP, the latter being completely dependent on cytochalasin B. The reason for this discrepancy is unknown. The response of cytochalasin treated PMNs to LTB_4 was 1/2 the magnitude of that for fMLP at the same concentration. Interestingly, the secretary effect of LTB_4 occurs at a 10-fold higher molar concentration than the concentrations for optimal stimulation of neutrophil chemotaxis and adherence [20]. This agrees with other observations concerning fMLP and LTB_4 [8,14,21]. Here, only one of the non-enzymatic isomers of LTB_4 (compound II) exhibited a weak activity and finally, 5-HETE and LTC_4 did not show any significant effects on the release of enzymes. The stereospecificity in the response to LTB_4 provides support for a physiological role for LTB_4 as a secretagogue, possibly released by activated neutrophils. However, it has been hypothesized that LTB_4 is formed mainly in order to attract more neutrophils to an inflammatory area [7,20] since LTB_4 affects PMN migration and adherence at lower concentrations and with responses comparable with fMLP and C5a. Compared with fMLP, LTB_4 caused only a small extrusion of lysosomal enzymes as shown here, and did not affect production of oxygen radicals [20]. The clinical significances of these findings remains to be determined.

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