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INVESTIGATIONS OF THE BIOLOGICAL ACTIVITY OF LEUKOTRIENE A_4 IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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An investigation was undertaken to compare the responses of human neutrophils to the epoxide leukotriene A_4 with those elicited by its stable product leukotriene B_4 under identical IN VITRO conditions. LTA_4 evokes neutrophil responses similar in nature to those induced by LTB_4 but at much higher concentrations. Evidence suggests that LTA_4 is important primarily for its role as an intermediate rather than for inherent activity.

Among the products of the arachidonic acid cascade are several unstable compounds which nevertheless function as biological mediators. The prostaglandin endoperoxides, thromboxane A_2 and prostacyclin have chemical half lives of a few minutes or less and yet exert potent activity on platelet function (1). In leukocytes, the unstable epoxide leukotriene A_4 is the immediate precursor of the potent mediators LTB_4 and LTC_4. In aqueous solution at pH 7.4 and 37°C, LTA_4[1] has a half life of only a few seconds (2). However, in the presence of physiological concentrations of albumin, LTA_4 is more stable and exhibits a half life of several minutes (3).

In the present study we have studied LTA_4 as a significant mediator of human neutrophil functions independent of its enzymatic conversion to LTB_4. We chose to test the potency of LTA_4 as a stimulus for neutrophil responses (aggregation, superoxide anion production) which were rapid in onset and for which comparisons could readily be made to LTB_4. In addition to

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[1] Abbreviations: BSA, bovine serum albumin; FMLP, n-formyl-met-leu-phe; LTA_4, LTB_4, LTC_4, leukotrienes A_4, B_4, C_4; SOD, superoxide dismutase.

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evaluation of LTA_4 as a primary neutrophil stimulus, we also tested the ability of LTA_4 to modulate these same neutrophil responses evoked by other agents (FMLP, LTB_4) as it is becoming increasingly clear that lipoxygenase products may function in this manner (4,5).

MATERIALS AND METHODS

Ferricytochrome c (Type III), FMLP, SOD, and BSA were purchased from Sigma chemical Company, St. Louis, Missouri. Hank's balanced salt solution (HBSS) was obtained from the Grand Island Biological Company, Grand Island, NY.

LTB_4 was kindly provided by J. Rokach, Merck Frosst Canada, Inc. Quebec, Canada.

LTA_4, methyl ester was obtained as a gift from the Upjohn Company. The stock solution was dissolved in diethylether:triethylamine:n- hexane (15/1/84) and stored at -78°C. Aliquots of the stock were converted to the lithium salt prior to its use by the following procedure. A solution of freshly distilled tetrahydroturan/H_2O (40:1) was gassed with argon for thirty minutes. A 100 µl portion of this solution was added to an argon filled reaction vial with 2 µl of 1N LiOH and 1-2 µl (50-100µg) of the LTA_4 stock. The mixture was left at room temperature for 24-48 hours with occasional vortexing, necessitated by the tendency to settle into a separate phase. The course of the reaction was monitored by adding 1 µl to 50 µl of 50 mM KH_2PO_4 (pH 4.5), mixing and immediately injecting onto a Bio Rad Reverse Phase HPLC column (BioSil ODS-5S,250 mm x 4 mm). Elution was performed with Methanol:H_2O: glacial acetic acid (80/20/.01), monitoring by U.V. detection at 280nM. The ratio of the amount of the two 5,12 all trans diols (hydrolysis products of LTA_4) to the two methyl esters of these compounds served as an indicator of production of free LTA_4. Once hydrolysis was complete, the tetrahydrofuran solution was carefully evaporated and ethanol added immediately upon reaching dryness. LTA_4 was then quantitated by U.V. analysis (in ethanol using ε=40,000 at 279 nM). The lithium salt was sorted at -78°C and used within 5 days of preparation.

Neutrophil suspensions: Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers as described.

Neutrophil superoxide release. Superoxide (O_2^-) was assayed by measuring the O_2^--dependent (SOD-inhibitable) reduction of ferricytochrome c, as previously described (6). All individual experiments were performed in triplicate and repeated the given number of times for statistical evaluations. Neutrophils (7.5×10^6 cells/0.5 ml) were incubated for 10 minutes prior to the addition of a test compound or its solvent control. The cells were then incubated with the lipid for two minutes and pipetted into cytochrome c-solutions (final concentration 80 µM) and the cell suspension (1.5×10^6 cells/ml) was incubated with FMLP (10^{-7}M) for 10 minutes at 37°C. The cells were then pelleted by centrifugation and the extent of cytochrome c reduction in each supernatant was determined by the change of absorbance at 550 nM (Gilford 250 recording spectrophotometer).

Neutrophil Aggregometry. Neutrophil aggregation was measured via a Payton aggregometer (7). The recorder was calibrated at maximal light transmission using HBSS only. heights of aggregatory waves were measured five minutes following the addition of stimuli and recorded in mm.

Addition of Test Lipids: LTA_4 (in ethanol)was added directly to 50 mg/ml BSA solutions. Dilutions were made with additional 50 mg/ml BSA solutions. Dilutions were made with additional 50 mg/ml BSA solution to provide the correct level of LTA_4 in 250 µl aliquots (superoxide release experiments) or in 5-25 µl aliquots (neutrophil aggregations). The final concentration of ethanol was less than 0.2%. Controls containing ethanol in 50 mg/ml BSA were

run concommitantly. LTB_4 was treated similarly except that its solvent (methanol) was evaporated to dryness before sonication in 50 mg/ml BSA.

RESULTS

Hydrolysis of LTA_4 methyl ester to the unesterified compound was monitored by measuring the ratio of amount of the two 5,12 all trans diols (hydrolysis products of LTA_4) to the two methyl esters of these compounds as described in the Methods section. A typical HPLC profile before and after hydrolysis is depicted in Figure 1 along with an accompanying u.v. spectrum of free LTA_4 (λ_{max}=279). Hydrolysis of the LTA_4 methyl ester group was achieved in 24-48 hours without loss of the characteristic triene chromophore. LTA_4 was dissolved in a saline solution with 50 mg/ml BSA before addition to neutrophil suspensions because such BSA concentrations greatly extend the half life of LTA_4 in aqueous solution (3).

We have recently demonstrated that LTB_4 (10^{-9}M) is a potent enhancer of superoxide anion production initiated by the chemotactic peptide FMLP (4).

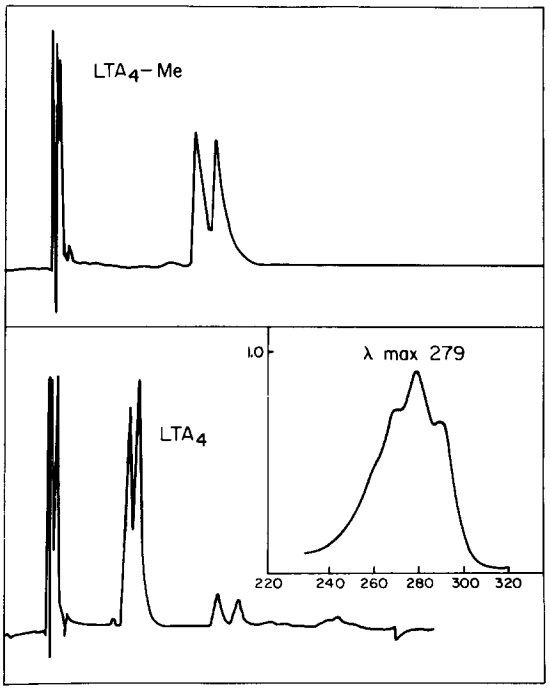

Fig. 1. Reverse phase HPLC chromatograms of 5,12-diols (non-enzymatic hydrolysis products) of either LTA_4-methyl ester (upper panel) or LTA_4 (lower panel) along with a u.v. spectrum (insert) taken of LTA_4.

LTB_4 is, however, a relatively weak primary stimulus for superoxide anion formation, requiring micromolar levels to achieve a response (8). We tested LTA_4 as both a potential modulator of the FMLP oxidative response and as a primary stimulus. LTA_4 enhanced superoxide anion production by FMLP-stimulated human neutrophils at 10^{-7}M concentrations, although it was ineffective at this concentration as a superoxide anion stimulus in cells that were not exposed to FMLP (Figure 2). Lower levels of LTA_4 (10^{-8} and10^{-9}M) were far less effective in modulating the FMLP oxidative response (20% enhancement at 10^{-8}M). in some experiments, neutrophils were exposed to LTA_4 and added immediately to the superoxide assay medium with FMLP without the usual two minute preincubation. Under these conditions enhancement by LTA_4 could not be demonstrated. The possibility that non-enzymatic hydrolysis products of LTA_4 were producing the observed activity was addressed by incubating the leukotriene in cell-free medium for periods of up to 30 minutes. A time-dependent reduction of the enhancing potency of LTA_4 to less than 50% of that seen with unincubated solutions we observed when the compound was tested as a modulator of FMLP-induced superoxide formation (data not shown).

LTA_4 initiated a substantial aggregatory response in the concentration range of 10-400 nM. The responses (which were quite variable with different

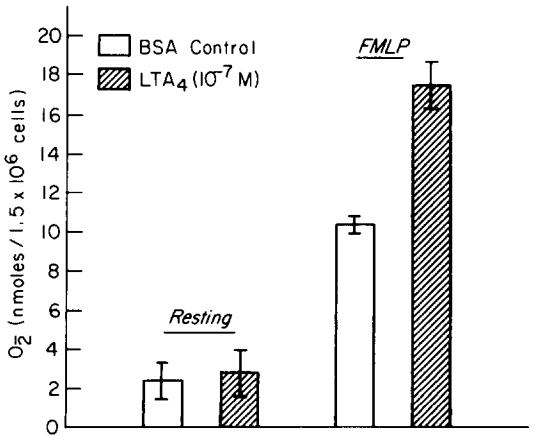

Fig. 2. Effect of LTA_4 (10^{-7}M) on resting and FMLP-induced superoxide formation. Brackets denote the standard deviation of four experiments. *P < .001.

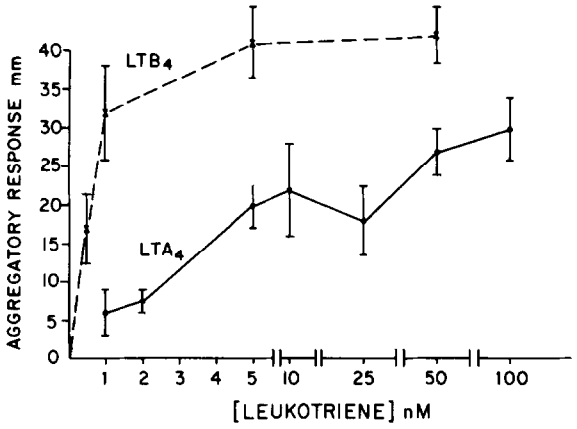

Fig. 3. Neutrophil aggregatory responses to LTA_4 and LTB_4. Brackets denote the standard deviations for eight experiments.

donors) were similar in magnitude to those elicited by LTB_4, although the latter exhibited this potency at a 50-fold lower concentration (Figure 3). The aggregatory wave induced by LTA_4 was not as steep as LTB_4 and commenced after a 15-20 second lag period which was not seen with LTB_4. Preincubations of neutrophils with LTA_4 did not substantially influence subsequent aggregatory responses initiated by FMLP, although responses provoked by LTB_4 (50nM) were greatly inhibited (Figure 4).

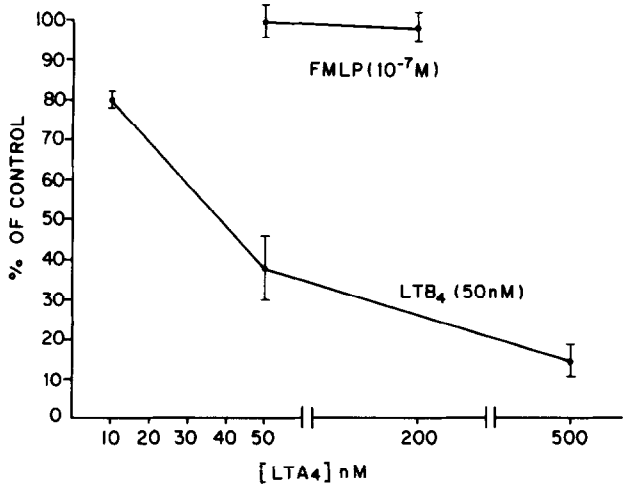

Fig. 4. Effect of pre-exposure of neutrophils to LTA_4 on subsequent aggregatory responses to LTB_4 (50nM) or FMLP (10^{-7}M).

DISCUSSION

Exposure of human neutrophils to LTA_4 resulted in responses (aggregation and enhancement of FMLP-evoked superoxide anion production) which were qualitatively similar to those induced by LTB_4, although much higher concentrations of the epoxide were required for equivalent responses. Thus, although LTA_4 is unstable and is bound to albumin, it is still able to interact with the cells. O'Flaherty et al reported similar relative effective concentrations for neutrophil degranulation (9). In our experiments we obtained several lines of evidence to suggest that the neutrophil responses were due to conversion of LTA_4 to LTB_4 by the cells rather than to weak agonist activity of the exoxide itself. First, prior exposure of neutrophils to LTA_4 caused the desensitization of aggregator responses to LTB_4. Secondly, neither leukotriene influenced aggregation in response to FMLP. thirdly, a lag period was observed with LTA_4-initiated aggregation which was not observed with LTB_4. The latter finding is compatible with time being required for production of effective concentrations of LTB_4 or other leukotriene metabolites. Conversion of LTA_4 to LTB_4 by human neutrophils in vitro has been previously reported using 75-100 micromolar levels of substrate (10). We have similarly observed the production of nanomolar levels of LTB_4 in HPLC profiles of extracts obtained from incubation of LTA_4 with neutrophils (data not shown).

Sirois et al. (11) have recently reported that LTA_4 is almost as potent a myotropic substance on the guinea pig lung paranchyma as LTD_4 and that the biological activity required biotransformation of LTA_4 to LTB_4, LTC_4 and LTD_4. LTA_4 exhibited lesser potencies on trachea nad was inactive on the longitudinal muscle of giunea pig ileum, responses correlated with the degree of biotransformation of LTA_4.

It, therefore appears that LTB_4 is the more potent mediator of neutrophil function and that LTA_4 is not analogous to other unstable intermediates (prostacyclin, thromboxanes) which are far more active than their stable products.

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