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LEUKOTRIENE A_4 HYDROLASE ACTIVITY OF HUMAN AIRWAY EPITHELIAL CELLS

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Human tracheal epithelial cells were incubated with LTA_4 and metabolic products were identified in extracted supernatants by high pressure liquid chromatography, ultraviolet spectroscopy, and gas chromatography-mass spectrometry. In the presence of epithelial cells, LTA_4 was converted to LTB_4, but not to LTC_4 or LTD_4. Maximum LTB_4 was released at an LTA_4 concentration of 3 μM and had occurred by 30 min. LTB_4 release was increased in the presence of albumin, but was not affected by extracellular calcium or A23187. This LTA_4 hydrolase activity had a slower time course and could not be clearly inactivated by repeated exposure to substrate as is the case for previously described LTA_4 hydrolase enzymes. This hydrolase appears to have novel biochemical characteristics.

The 5-lipoxygenase pathway of arachidonic acid metabolism is a potent source of pro-inflammatory mediators which affect inflammatory cell chemotaxis, adherence, and activation as well as influencing vascular permeability and smooth muscle tone (1). Leukotrienes are a family of oxygenated metabolites derived from this pathway via the unstable epoxide intermediate 5(S)5,6-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid or leukotriene A_4 (LTA_4). In humans, the distribution of the 5-lipoxygenase enzyme which metabolizes arachidonic acid to LTA_4 appears to be limited to inflammatory cells (1). LTA_4 can, however, be released intact by inflammatory cells (2) and can serve as an intermediate for further metabolism by non-inflammatory cells to the biologically active compounds LTB_4 (3-5) or LTC_4 (6-8). This intercellular exchange of LTA_4 has been a poorly recognized potential source of biologically active leukotrienes derived from cell to cell interactions.

Because products of the 5-lipoxygenase pathway have potent effects on airway function (9) and because inflammatory cells appear to play an important role in airway diseases (10), we

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Abbreviations: CMF-HBSS, calcium- and magnesium-free HBSS; DME/H16, Dulbecco's modified Eagle's Media with Ham's 16; HBSS, Hank's balanced salt solution; HPLC, high pressure liquid chromatography; LT, leukotriene; MEM, Minimal essential media; PG, prostaglandin.

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designed this investigation to determine whether human airway epithelial cells could metabolize exogenous LTA_4 into biologically active leukotrienes. We also questioned whether inflammatory cells could serve as a source of this intermediate.

Methods

Materials. HBSS, CMF-HBSS, MEM, FCS, and DME/H16 (Cell Culture Facility, University of California, San Francisco, CA). Dextran-500 and Ficoll-paque (Pharmacia Fine Chemicals Piscataway, N.J.). A23187 (Calbiochem-Behring Corp. LaJolla, CA). Redistilled-in-glass grade solvents (Burdick and Jackson Muskegon, MI). Cell culture plates (Costar Cambridge, MA). PGB_2 (Upjohn Kalamazoo, MI). Δ^6-trans-LTB_4, Δ^6-trans-12-epi-LTB_4, LTA_4-methyl ester, and LTB_4 (Biomol, Plymouth Meeting, PA). Dimethyl sulfoxide and endotoxin-free BSA (Sigma Chemical, St. Louis, MO). Human fibronectin (Collaborative Research, Bedford, MA). Bovine dermal collagen type I (Vitrogen®) (GIBCO, Grand Island, NY). N, O-*bis*(Trimethylsilyl)trifluoroacetamide (Pierce, Rockford, IL).

LTA_4 Hydrolysis. LTA_4 was prepared by hydrolysis of its methyl ester using a previously described technique (11). The resulting free LTA_4 was evaporated to dryness and resuspended in ethanol prior to its addition to cells.

Cell Preparations. For most experiments, transformed human tracheal epithelial cells were utilized. This cell line has been designated 9/HTEo$^-$ and has been previously characterized (12). These cells were grown to confluence on 100 mm tissue culture plastic dishes coated with collagen, fibronectin, and BSA. The cells were grown in MEM with 10% FCS. Human airway epithelial cells were also obtained from post-mortem tracheal specimens from patients dying without known airway pathology and acutely disaggregated by previously described techniques (13), placed in primary culture (13), and studied at confluence.

Human mononuclear cells were prepared by previously described techniques (14) and resuspended at 0.8 x 10^6 cells/ ml in HBSS prior to study.

Incubation Conditions. To identify products, 1 to 2 x 10^7 human tracheal epithelial cells were incubated with 3 μM LTA_4 in HBSS with 0.35% BSA, pH 7.4 for 30 min at 37°C. A dose-response curve was constructed to LTA_4 by incubating 9/HTEo$^-$ cells with 0.09 to 10 μM LTA_4 for 30 min at 37° C. The time course of metabolite generation was assessed by incubating 9/HTEo$^-$ cells with 3.0 μM LTA_4 for up to 4h. These cells were also incubated with LTA_4 in the absence of calcium or BSA. To determine the role of cell stimulation, 9/HTEo$^-$ cells were also challenged with the calcium ionophore A23187 in the presence or absence of added LTA_4. To determine if hydrolase activity could be inactivated by substrate, 9/HTEo- cells were incubated with 10 μM LTA_4 for 30 min, the supernatant collected and the cells were re-challenged with 10 μM LTA_4, twice. All three supernatants were analyzed for metabolic products. To evaluate the role of inflammatory cells as a source of LTA_4, 2.5 ml of mononuclear cell suspension was added to confluent epithelial cell cultures containing 1 to 2 x 10^7 cells. The cells were then co-incubated for 30 min and then stimulated with 1 μM A23187 for 30 min at 37°C. These incubations were compared to control incubations of mononuclear cells without epithelial cells which were also stimulated with A23187.

The final concentration of ethanol added to the media with LTA_4 did not exceed 0.1% for any experiment. A23187 was dissolved in dimethylsulfoxide and was added to media to give a final concentration of dimethylsufoxide of 0.1%.

Identification and Quantification of Metabolites. Immediately after experimental incubations, samples were collected and placed on ice. PGB_2 was added as an internal standard to correct for recovery of metabolites. Samples were acidified to pH 3.8 with 0.13 M phosphoric acid and then extracted by adding equal volumes of 2-propanol and chloroform (14). The percent recovery of PGB_2 and LT standards using this extraction ranged from 75-95%.

Extracts were dried under nitrogen and reconstituted in chromatography solvent for analysis by reversed phase-HPLC. The analysis was performed using a liquid chromatograph (Rainin Gradient HPLC System 34) fitted with a 4.6 mm x 10 cm column with 3 μm spherical octadecyldimethylsilane packing (Microsorb, Short-one, Rainin Instruments) that was developed at a flow rate of 1.0 ml/min using a gradient program. Solvent A consisted of methanol/ water/ phosphoric acid/ ammonium hydroxide (50:50:0.01:0.013, vol/vol), apparent pH 4.5. Solvent B consisted of methanol/ water/ phosphoric acid/ ammonium hydroxide (90:10:0.01:0.013, vol/vol),

apparent pH 5.8. The gradient was developed with 44 % solvent B from 0-12.5 min, and 100 % solvent B from 13-18 min. LTB_4 and peptide-containing LT were detected by their absorbance at 270 nm and 280 nm, respectively, using a Knauer UV detector (Berlin, West Germany) interfaced with a MacIntosh SE computer (Cupertino, CA) with real-time data acquisition and analysis software (Dynamax HPLC Method Manager, Rainin Instruments, Woburn,MA). Metabolites in the effluent from reversed phase-HPLC were collected separately, acidified to pH 3.5 with phosphoric acid and extracted with 1 volume of chloroform. Recoveries of PGB_2 and LT authentic standards using this extraction method ranged from 90-95%.

Isolated metabolites were converted to their methyl esters by treatment with ethereal diazomethane. The diazomethane was generated by the reaction of N-methyl-N'-nitro-N-nitroso guanidine with sodium hydroxide in a sealed generator (15). The methyl esters of metabolites were subjected to straight phase-HPLC using a 4.6 mm x 10 cm column with 3 μm spherical silica packing (Microsorb, Short-one, Rainin Instruments) developed at a flow rate of 2.0 ml/min with hexane/ 2-propanol (100:4 vol/vol).

Ultraviolet absorption spectra were obtained on peaks collected from reversed phase- and straight phase-HPLC in methanol using a scanning spectrophotometer (DU 70, Beckman Instruments, Palo Alto,CA). LTB_4 was quantitated by ultraviolet absorbance using a molar extinction coefficient of 50,000 at 270 nm.

The methyl esters of peaks of interest were converted to their trimethylsilylether derivatives by treatment with N, O-*bis*(Trimethylsilyl)trifluoroacetamide and 1.0% trimethylchlorosilane at 65° C for 30 min. The trimethylsilylether derivative of the methyl ester was analyzed by gas chromatography-mass spectrometry on a Kratos MS-25 mass spectrometer. Electron impact mass spectra were obtained using 70 eV ionizing voltage at 220° C.

Results

Identification of Metabolites. Reversed phase-HPLC analysis of extracts of media alone incubated with 3.0 μM LTA_4 in the absence of cells for 30 min revealed two peaks (Peak I and II) (Fig 1A) . When extracts of media from transformed and non-transformed tracheal epithelial cells incubated with 3.0 μM LTA_4 for 30 min were analyzed they consistently revealed three peaks of absorbance (Fig 1B). The retention times of peaks I and II corresponded exactly to the retention times of Δ^6-trans-LTB_4 (9.9 min) and Δ^6-trans-12-epi-LTB_4 (10.9 min), respectively. The presence of these two peaks was presumed to represent non-enzymatic hydrolysis of LTA_4 as previously shown (16). Peak III eluted exactly at the retention time of authentic LTB_4 (12.7 min). No peaks corresponding to the retention times of LTC_4 (7.7 min) or LTD_4 (19.7 min) were found in either sample. The ultraviolet absorption spectrum of peak III was characteristic of authentic LTB_4 with maximal absorption at 270 nm with shoulders at 260 and 281 nm (17). The methyl ester of peak III eluted as a single peak on straight phase-HPLC and corresponded exactly to the retention time of methylated authentic LTB_4 (8.0 min).

Gas chromatography-mass spectrometry analysis of the trimethylsilyl ether, methyl ester derivative of the compound contained in peak III was identical to that of the derivatized authentic standard, LTB_4, and previously published spectra (18). Mass spectra showed characteristic ions (*m/z*) of 479 (*M*-15, loss of $\cdot CH_3$), 404 (*M*-90, loss of trimethylsilanol), 383 (*M*-111, loss of $\cdot CH_2$-CH=CH-$(CH_2)_4$-CH_3), 293 (*M*-(111+90), 217, 203 (Me_3SiO+=CH-$(CH_2)_3$-$COOCH_3$) and (*M*-(111+180), 191, and 129.

Quantitative Characterization. When 9/HTEo⁻ cells were incubated with increasing quantities of freshly prepared LTA_4, from 0.09 to 10 μM for 30 min at 37° C maximum product

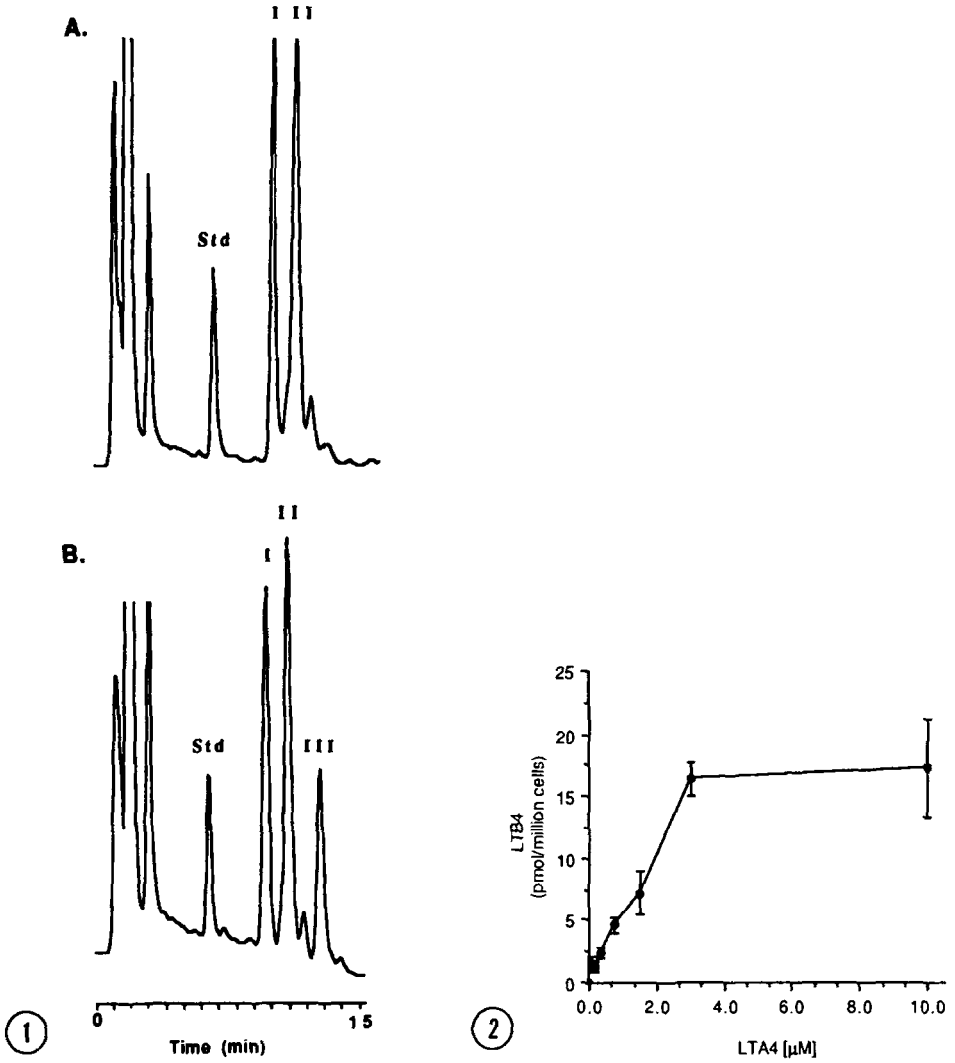
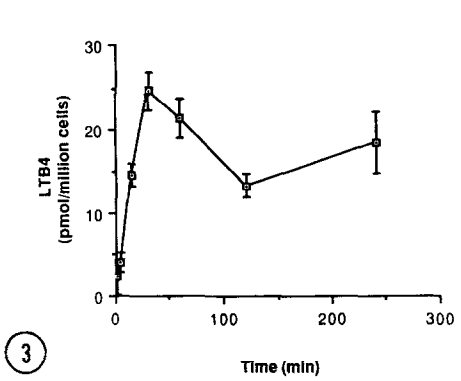


Figure 1. A) Reversed phase-HPLC chromatogram of an extract of HBSS incubated at 37°C with 3 μM LTA_4 for 30 min without cells (control). B) Reversed phase-HPLC chromatogram of a supernatant extract from 9/HTEo⁻ cells (1 x 10^7) incubated with 3 μM LTA_4 for 30 min. Both samples monitored at 270 nm. Peak I, Peak II, and Peak III corresponded to retention time of authentic Δ^6-trans-LTB_4, Δ^6-trans-12-epi-LTB_4, and LTB_4, respectively. Monitoring at 280 nm did not reveal evidence of LTC_4 or LTD_4.

Figure 2. Dose-Response Curve. 1-2 x 10^7 9/HTEo⁻ cells were incubated with up to 10 μM LTA_4 for 30 min and then extracted and analyzed as above. Maximum LTB_4 release was observed at or above 3 μM LTA_4. Similar results were obtained with primary cultures of non-transformed human tracheal epithelial cells.

(16.4 ± 1.3 pmol/10^6 cells)(mean ± sd) was detected at concentrations of LTA_4 at or above 3 μM (Fig 2). When the time-course of LTB_4 generation and release from 9/HTEo⁻ cells was examined, maximal LTB_4 release was observed at 30 min with a subsequent decrease in the amount of LTB_4 detectable in the media (Fig 3). Similar quantities of LTB_4 were detected when primary cultures of human tracheal epithelial cells were incubated with LTA_4 for 30 min (21.5 ± 8.9 pmol/10^6 cells). When 9/HTEo⁻ cells were incubated in media without added BSA, only 45.7 % (7.5 ± 1.1 pmol/10^6 cells) of the amount of LTB_4 formed in the presence of BSA was detected. However,



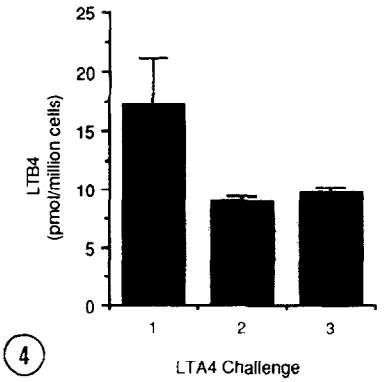


Figure 3. Time Course. 9/HTEo⁻ cells were incubated with 3 μM LTA_4 cells for up to 4h. Supernatants were then extracted and analyzed as above. Maximum LTB_4 release occured by 30 min.

Figure 4. Repeated Substrate Challenge. 9/HTEo⁻ cells were incubated with 10 μM LTA_4 for 30 min at 37° C. The supernatant was then collected and the cells were re-challenged with 10 μM LTA_4, twice. All three samples were examined for metabolic products.

no significant difference in the quantity of LTB_4 released was observed in the absence of calcium in the media and no increase was observed when the cells were stimulated with A23187 in the presence of LTA_4 (data not shown). Likewise, no 5-lipoxygenase products were detected when cells were stimulated with A23187 in the absence of LTA_4 (data not shown), as has been previously reported (19). When 9/HTEo⁻ cells were challenged three times with 10 μM LTA_4, the cells released LTB_4 with each successive challenge, although there was a decrease in product after the first challenge (Fig 4). When 9/HTEo⁻ cells were incubated with mononuclear cells for 30 min and then challenged with 1 μM A23187 and compared to mononuclear cells challenged with A23187 in the absence of epithelial cells, significantly greater quantities of LTB_4 were detected in supernatants (5.4 ± 0.5 vs 3.7 ± 0.2 pmol/10^6 mononuclear cells, respectively).

Discussion

We have demonstrated that human airway epithelial cells have the capacity to convert LTA_4, an unstable epoxide, to LTB_4. Because previous investigations have established that the generation of biologically active LTB_4 from LTA_4 requires a stereospecific hydrolase enzyme (16), we presume that our findings reflect the presence of a LTA_4 hydrolase in airway epithelial cells. We did not, however, find evidence of conversion of LTA_4 to LTC_4 and, thus, we have found no evidence for a glutathione-S-transferase activity specific for LTA_4 in airway epithelial cells. We have also demonstrated greater LTB_4 generation when inflammatory cells are incubated with epithelial cells which suggests that inflammatory cells are capable of providing LTA_4 as a substrate for conversion by the epithelial cell to LTB_4.

Only a small number of investigations have been reported regarding arachidonic acid metabolism in human airway epithelia; however, studies have established that this cell does not have a 5-lipoxygenase pathway, but, instead, has a potent 15-lipoxygenase pathway (19). We,

likewise, have not found 5-lipoxygenase activity in airway epithelial cells and, therefore, conclude that the airway epithelium is incapable of metabolizing arachidonic acid to LTA_4.

Although the 5-lipoxygenase enzyme appears to be limited to inflammatory cells (1), non-inflammatory cells -- devoid of 5-lipoxygenase --may contain enzymes capable of metabolizing LTA_4 to biologically active leukotrienes (3-8,20). Furthermore, a substantial body of experimental evidence has established that lipoxygenase metabolism can occur via exchange of the unstable intermediate, LTA_4, from inflammatory cells to non-inflammatory cells(21).

LTA_4 hydrolase has been purified and characterized from the neutrophil (22), whole human lung parenchyma (20) and human erythrocytes (23). The human neutrophil enzyme has a M_r of 69,140, consists of 610 amino acids, is cytosolic, has a K_m of 20-30 μM, a pH optimum of approximately 8, and is monomeric. The lung hydrolase, purified from whole lung appears to be the same as the neutrophil-derived enzyme as suggested by its n-terminal amino acid sequence, molecular weight, and similar biochemical characteristics. The cell of origin of this enzyme in the lung parencyhma is, however, unknown and could be derived from resident inflammatory cells. The LTA_4 hydrolase present in erythrocytes may be different based on preliminary purification data indicating a lower molecular weight of 54,000 (23). Nevertheless, features are shared by LTA_4 hydrolases purified, to date, from all sources and they are 1) inactivation by exposure to substrate ("suicide inactivation") (20,23,24) and 2) rapid deceleration of initial velocities after 1 min in all cases, whether in a whole cell or an enzyme preparation, and presumably due to suicide inactivation (23,24). In the present study we have not been able to convincingly, demonstrate suicide inactivation of this enzyme up to a substrate concentration of 10 μM and, consistent with this, we have found a slower time course than those previously described for LTA_4 hydrolase activity in intact neutrophils or erythrocytes. These preliminary differences may reflect differences in assay conditions and require confirmation with a purified LTA_4 hydrolase from human airway epithelial cells. These findings do, however, raise the question of whether the human airway epithelium has an LTA_4 hydrolase which is distinct from previously described enzymes.

The function and significance of an LTA_4 hydrolase in the airway epithelium responsible for the generation of the potent chemotactic lipid, LTB_4, is not clear. A number of functions are, however, possible including intracellular signaling, amplification of the amount of LTB_4 formed, and directed chemotaxis of inflammatory cells to the epithelium. If, on the other hand, this enzyme activity is not suicide-inactivated and has slower kinetics than that described for the neutrophil enzyme, the airway epithelium could provide a sustained source of LTB_4. Determination of the true function of this enzyme activity will require further investigation.

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