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Comparative Analysis of Isolated Human Bronchi Contraction and Biosynthesis of Cysteinyl Leukotrienes Using a Direct 5-Lipoxygenase Inhibitor

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ABSTRACT. Quantitation of cysteinyl leukotriene production and smooth muscle contraction upon immunological challenge of isolated human bronchi was evaluated. Analysis of picomole amounts of leukotriene C_4, D_4, and E_4 was achieved using HPLC separation and enzyme immunoassay quantitative determination. The aim of the study was to correlate the contraction of airway smooth muscle and cysteinyl leukotriene production with and without 5-lipoxygenase inhibition. In human isolated bronchial tissue treated with indomethacin and pyrilamine to make their contractile responses leukotriene dependent only, the novel 5-lipoxygenase inhibitor 5,6-Dihydroxy-2-(*N*,*N*-Dimethylhydrazino)-1,2,3,4-tetrahydro-naphtalene bromide (CHF 1909) caused a concentration-dependent inhibition of the immunologically induced contraction, showing an IC_{50} value of 13 ± 2.2 μM (mean ± CV). At the concentration of 30 μM, this compound caused more than 90% inhibition of the maximal bronchoconstriction *in vitro*, and inhibited cysteinyl leukotriene production by 90% as well. Contemporary measurement of immunologically induced contraction and production of cysteinyl leukotrienes in isolated human bronchi provided a direct correlation between smooth muscle contraction and synthesis of leukotriene C_4, D_4, and E_4. BIOCHEM PHARMACOL **54**;4:437–442, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. isolated human bronchi; immunological challenge; cysteinyl leukotrienes; 5-lipoxygenase inhibition; HPLC, enzyme immunoassay

The enzyme 5-lipoxygenase [EC 1-13-11-34], largely expressed in the human bronchial tissue, catalyzes the conversion of arachidonic acid, released from membrane phospholipids, to the allylic epoxide leukotriene A_4 (LTA_4).§ This unstable intermediate can be converted into leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4), both involved in different inflammatory reactions [1]. LTB_4 is a potent neutrophilic chemotactic agent and contributes to the recruitment of polymorphonuclear leukocytes (PMNL) at the site of inflammation. LTC_4, as well as its metabolites leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4), is able to induce profound modifications of vascular permeability through direct action on endothelial cells, and is among the most potent bronchoconstricting agents tested in humans [2–5].

In light of their potential involvement in the pathophysiology of bronchial asthma, a number of pharmaceutical companies have developed research programs targeted at discovering 5-lipoxygenase inhibitors as well as receptor antagonists of cysteinyl leukotrienes [6, 7]. A model of choice for the development of these drugs is represented by the preparation of isolated human bronchi where contraction of the smooth muscle is elicited by immunological challenge of resident bronchial mast cells. These contractions in human isolated bronchi are associated with cysteinyl leukotriene LTC_4, LTD_4, and LTE_4 (cys-LT) formation and represent a model of early asthmatic reactions *in vitro* [8].

Taking advantage of a sophisticated and sensitive analytical approach, coupling HPLC separation and enzyme immunoassay of all cys-LT, we studied the activation of 5-lipoxygenase pathway in isolated human bronchial strips undergoing immunological challenge, with contemporary measurement of variation in isometric tension. Inhibition of 5-lipoxygenase product formation, namely cys-LT, was associated with significant inhibition of leukotriene-dependent smooth muscle contraction.

MATERIALS AND METHODS

Chemicals and Reagents

Human IgE antibody was purchased from Kirkegaard & Perry Labs Inc., Gaithersburg, MD. Acetylcholine and

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§ *Abbreviations:* cys-LT, cysteinyl leukotrienes; CHF1909, 5,6-Dihydroxy-2-(*N*,*N*-Dimethylhydrazino)-1,2,3,4-tetrahydro-naphtalene bromide; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; LTE_4, leukotriene E_4; EIA, enzyme immunoassay; RP-HPLC, reverse phase-high pressure liquid chromatography.

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pyrilamine were from Sigma Chemical Co. (St. Louis, MO). Eicosanoids, EIA tracers and antibodies were purchased from Cayman Chemical Co. (Ann Arbor, MI). Tritium labeled leukotrienes were obtained from NEN-Life Science Products (Boston, MA). HPLC-grade solvents and chemicals were obtained from Merck (Darmstadt, Germany). Type I "plus" water was obtained using a MilliQ Plus water purifier (Millipore, Molsheim, France), fed with double distilled water. Compound LY-171883 was generously provided by Dr. M. Niedenthal (Eli Lilly, Indianapolis, IN), and compound SKF 104353 by Dr. H. Sarau (SK&F Laboratories, King of Prussia, PA). 5,6-Dihydroxy-2-(*N*,*N*-dimethylhydrazino)-1,2,3,4-tetrahydro-naphtalene bromide (CHF 1909) was synthesized in the Chemistry Department of Chiesi Farmaceutici (Parma, Italy).

Immunological Challenge of Isolated Human Bronchi

Human bronchi with an inner caliber of 2–3 mm were isolated following surgery for bronchial carcinoma or bronchiectasis; a fine polyethylene catheter was gently slipped through the full length of the bronchus, which was dissected free of parenchyma and blood vessels and spiraled to obtain a strip (2–3 mm wide and 2–3 cm long). Several strips were prepared and suspended under an isometric resting tension of 500 mg at 37° in a Tyrode's solution gassed with 95% O_2–5% CO_2, and medicated with 10 μM indomethacin and 0.3 μM pyrilamine to make the antigen-induced contractile response dependent on the activity of cys-LT only. From a single bronchus 2 or more strips were usually prepared, one of which was used as control while the others were treated with various concentrations of CHF 1909.

The preparations were allowed to equilibrate for 60 min and the bath medium was replaced every 15 min with fresh solution to attain a steady baseline tone. After this period, a cumulative concentration–response (10–300 μM) curve to acetylcholine (ACh) was induced to test tissue contractility. After extensive rinsing of the preparations and recovery of the basal tone, bronchial strips were exposed to different concentrations of CHF 1909 or blank solution for 30 min and subsequently challenged with cumulative additions of a specific polyclonal antihuman IgE antibody (0.01–1.0 μg/mL protein). When the maximal antigen concentration plateau was reached, selective receptor antagonists of LTD_4 (LY 171883 or SKF 104353; 10 μM) were added. Incubating medium was then withdrawn, added with internal standards (15,000 to 25,000 dpm of [^{3}H]-LTC_4, [^{3}H]-LTD_4, [^{3}H]-LTE_4 (specific activity 39 Ci/mmol) and stored at −80°C until analysis. The contraction elicited by the anti-human IgE antibodies was expressed as a percentage of a maximal tension developed by acetylcholine 300 μM.

Metabolism of Exogenous LTC_4 by Isolated Human Bronchi

Metabolism of exogenous LTC_4 was studied in isolated human bronchi set up for isometric recording as described. [^{3}H]-LTC_4 (100,000 dpm) was added at the beginning of an immunologically induced contraction experiment. After development of the final contractile plateau, the whole incubation medium was rapidly extracted using a Supelclean LC18 solid phase cartridge (Supelco, Bellafonte, PA) and tritium radio-labeled cys-LT eluted with 2 mL of methanol:water (90:10, v:v). The dried extract was reconstituted and analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC) coupled with diode array-UV detection. UV absorbance was monitored at 280 and 235 nm, and full UV spectra (210–340 nm) acquired at a rate of 0.5 Hz. A multilinear gradient from solvent A (methanol/acetonitrile/water/acetic acid 10/10/80/0.02, v/v/v/v, pH 5.5 with ammonium hydroxide) to solvent B (methanol/acetonitrile 50/50, v/v) at a flow rate of 1 mL/min was used to elute a 4 × 250 mm column (RP-18 endcapped Superspher, 5 μm, Merck, Darmstadt, Germany). Solvent B was increased to 35% over 6 min, to 65% over 25 min, and to 100% over 3 min. The HPLC effluent was diverted to a radioactivity HPLC monitor (Ramona, Raytest, Straubenhardt, Germany), equipped with a liquid scintillation cell (340 μL), after mixing with 3 vol of scintillation cocktail FloScint IV (Packard, Groningen, The Netherlands). Radioactive data were acquired and analyzed using the Gina® software (Raytest).

Identification and Assay of Cysteinyl Leukotrienes

Cysteinyl leukotrienes were identified and quantitated by RP-HPLC followed by a specific enzyme immunoassay (EIA) as previously published [9], with minor modifications.

Briefly, after thawing, samples were added with methanol (1:1 vol.) to precipitate proteins and centrifuged at 3500 × *g* for 10 min at 4°C. Supernatant was diluted with ultra pure water to obtain a methanol concentration of 25% and extracted on Supelclean LC18 as described.

The methanolic fraction was dried, reconstituted in 100 μL methanol and 50 μL water, and injected into an HPLC system (Beckman Analytical, San Ramon, CA) through a Rheodyne injector Mod. 7125 (Rheodyne, Cotati, CA). Isocratic elution was performed using methanol:water:acetic acid, 65:35:0.02 (apparent pH adjusted to 5.5 by addition of diluted ammonium hydroxide) at the flow rate of 1 mL/min (column: Beckman Ultrasphere® ODS, 25 cm × 4.6 mm, 5 μm particle size, Beckman Analytical).

Fractions (1 mL) were obtained using a fraction collector Mod. 201 (Gilson, Villiers Le Bel, France); 200 μL volume was used to measure radioactivity by liquid scintillation after addition of 5 mL of the scintillation cocktail Ultima gold (Packard).

Fractions corresponding to peaks of radioactivity as well as neighbouring fractions were evaporated and reconstituted in 800 μL of EIA buffer (0.1 M phosphate buffer pH 7.4 containing 0.4 M NaCl, 1 mM EDTA, 0.01% BSA, and 0.07% NaN_3). Serial dilutions of fraction corresponding to the peaks of radioactivity were performed using a MicroLab

M (Hamilton, Bonaduz, Switzerland). The amount of leukotriene present in each fraction or dilution was then evaluated by EIA with acetylcholine esterase bound to LTE_4 or LTC_4 as tracer [10, 11]. This assay was performed on 96-well microplates (immunoplate mod. 96F I, Nunc, Denmark), using a Titertek apparatus (Flow Laboratories, Helsinki, Finland), including the Microplate Washer S8/12, the Autodrop, and the Multiskan MC equipped with a 414 nm filter.

Leukotriene standard or biological sample, enzymatic tracer, and specific antibody were added at a volume of 50 μL each to give a final incubation volume of 150 μL. Two different antibodies were used: one specific for LTC_4 and the second for LTE_4. The former, showing a crossreactivity of 80% with LTD_4, was also used to quantitate the fractions corresponding to LTD_4.

The concentrations of biological samples tested were estimated by LTC_4, LTD_4, and LTE_4 standard curves ranging between 15 pg/mL and 2 ng/mL.

The results, corrected for recovery and internal standard added, were expressed as pmol of cys-LT per g of wet weight of the single bronchial strip.

Data Analysis

In all experiments, differences between control and treated groups were analyzed for statistical significance using a one-way analysis of variance (ANOVA) and Student's *t*-test (two-tailed) for unpaired samples. A value of $P < 0.05$ was accepted as significant. Results are expressed as mean ± standard error of the mean of *n* observations.

Anti-IgE concentration–response curves as well as anti-IgE concentration–inhibition curves (in presence of CHF 1909) were evaluated using MacAllfit 1.0 QB [12]; paired-data inhibitions at the different anti-IgE concentrations were averaged and used for the extrapolation of a CHF 1909 concentration–inhibition curve. The resulting IC_{50} was expressed as mean ± coefficient of variation (CV).

Statistical analysis of HPLC-cys-LT values was performed by introducing, for samples where cys-LT were undetectable, a value corresponding to the limit of detection of the method divided by two.

RESULTS

Stimulation of normal human bronchi *in vitro*, using a specific polyclonal antihuman IgE antibody, caused a sustained and long-lasting contraction proportional to the concentration of anti-human IgE antibody used [13].

Cumulative concentration–effect curves were observed using 0.01 to 1 μg protein/mL; bronchial contraction (Fig. 1—upper tracing) developed with a delay and a slope typical of a *slow-reacting* substance, and reached a maximum corresponding to 80–110% of the maximal contraction elicited by acetylcholine 300 μM.

The increased bronchomotor tone obtained by immunological challenge was persistent even after repeated washout, but was completely resolved by the treatment with specific

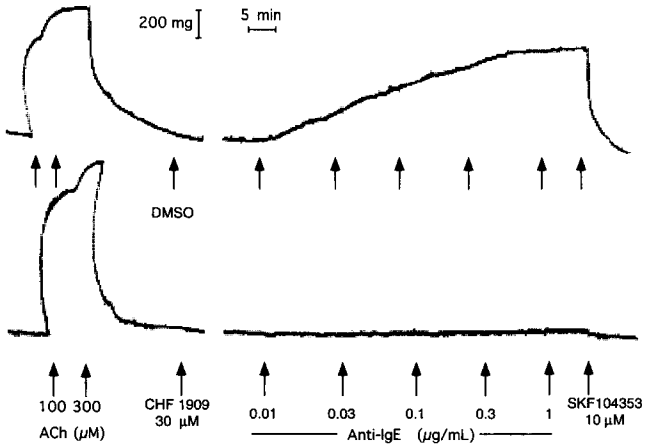

FIG. 1. Isometric tension recording from immunologically challenged isolated human bronchi in presence of indomethacin (10^{-5} M) and pyrilamine (3×10^{-7} M). Upper trace: control bronchus. Lower trace: bronchus pretreated with CHF 1909 30 μM.

LTD_4 receptor antagonists such as LY171883 (10 μM) or SKF104353 (10 μM) [14, 15]. This observation supports the specificity of mechanisms and mediators involved in the functional modification observed. It is important to note that the specificity of the model was the result of the use of the antihistamine pyrilamine and of the cyclooxygenase inhibitor indomethacin in the incubating medium.

Pretreatment of bronchial fragments with CHF 1909 (3–30 μM) caused a concentration-related inhibition of the antigen-induced contractile response, resulting in a rightward shift of the antigen concentration–response curves, with an estimated IC_{50} of 13 ± 2.2 μM (Fig. 2). Pretreatment of human bronchial strips with CHF 1909 at the concentration of 30 μM caused more than 90% inhibition of the maximal contraction elicited by the anti-human IgE antibody (Fig. 1—lower tracing, Fig. 2).

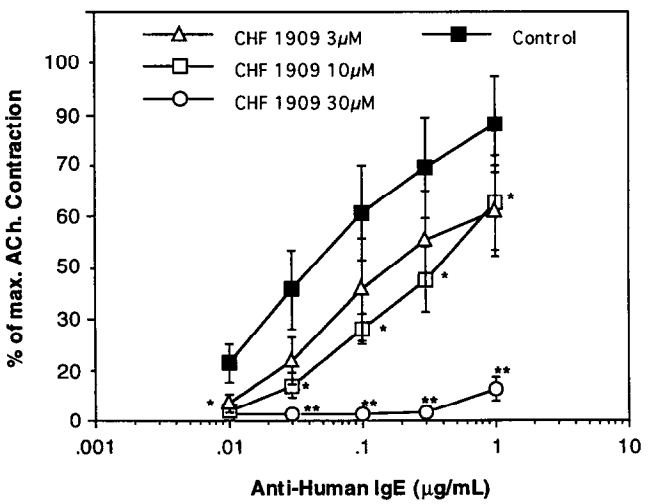

FIG. 2. Concentration-related inhibition by CHF 1909 of the contractions elicited by challenge with increasing amounts of anti-human IgE antibodies, in isolated human bronchi. Values are mean ± SEM ($n = 4–6$). *$P < 0.05$; **$P < 0.01$.

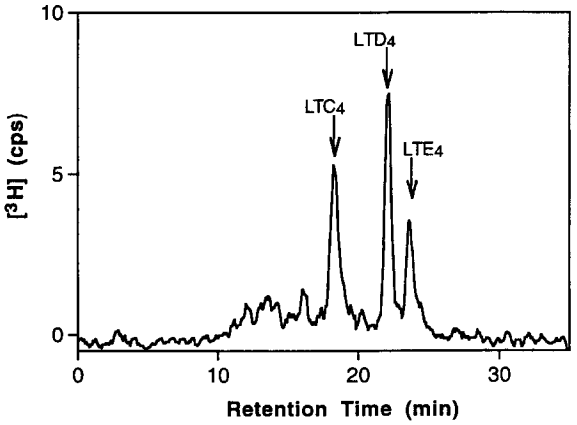

FIG. 3. Radioactive profile of RP-HPLC analysis of incubation medium from immunologically challenged, isolated human bronchus. [^{3}H]-LTC_4 (100,000 dpm) was added at the same time as the first concentration of antihuman IgE antibody, and incubated for approximately 1 hr. Arrows indicate the retention time of authentic tritium-labeled compounds.

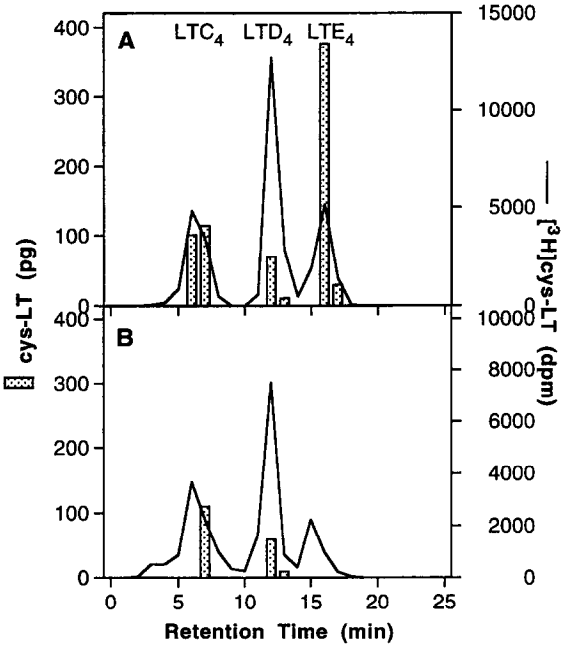

FIG. 4. HPLC-EIA analysis of cys-LT from incubates of immunologically challenged, isolated human bronchi. (A) Control human bronchus. (B) Human bronchus pretreated with CHF 1909 (30 μM).

Metabolism of exogenous radiolabeled LTC_4 by isolated human bronchi during challenge with anti-human IgE antibody showed that at the end of observation period, all three cysteinyl leukotrienes could be detected in the incubation medium (Fig. 3).

In agreement with these data, HPLC-EIA analysis of incubation media from control bronchial strips showed the presence of variable amounts of LTC_4, LTD_4, and LTE_4 (Table 1, Fig. 4). Cys-LT-immunoreactive material was eluted from the RP-HPLC column with a slight delay with respect to radioactive standards, according to the described isotopic effect of tritium substitution (Fig. 4) [16]. This observation, together with the parallelism between dilutions of standard and LT-immunoreactive material (data not shown), further contributed to the identification of LT present in HPLC fractions. Pretreatment of the isolated human bronchi with CHF 1909, 30 μM resulted in a significant suppression of cysteinyl leukotriene production (Table 1).

DISCUSSION

Cys-LT are known to be among the most potent bronchocontracting agents tested in human, and a possible role of cys-LT as mediators of bronchoconstriction in atopic and aspirin-sensitive asthma has been proposed [4, 17–19]. Human lung parenchyma, as well as human lung purified mast cells, are able to synthesize substantial amounts of cys-LT upon immunological challenge *in vitro* [20, 21]. Production of cys-LT associated with immunologically induced bronchoconstriction has been shown using bronchoalveolar lavages [22, 23] and urinary LTE_4 as a marker of 5-lipoxygenase activation *in vivo* [24, 25].

We used a specific anti-human IgE antibody to challenge isolated human bronchi preparations, evaluating the contractile response and the cys-LT production induced by the immunological challenge. Human lung tissue preparations were treated with indomethacin and pyrilamine in order to make their contractile responses substantially leukotriene dependent [8].

The use of polyclonal antibodies against human IgE that are normally localized on the bronchial mast-cell surface

TABLE 1. Analysis of cysteinyl leukotrienes in incubation media from immunologically challenged isolated human bronchi in presence or absence of CHF 1909 30 μM.

Treatment	LTC_4 (pmol)	LTD_4 (pmol)	LTE_4 (pmol)	Total LTs	pmol/g tissue	Mean ± SE
—	0.359	0.163	0.924	1.446	5.784	
—	<0.025	0.486	1.51	2.008	7.448	6.33 ± 0.56
—	<0.025	0.562	0.899	1.473	5.752	
CHF 1909 30 μM	0.176	0.139	<0.03	0.330	1.162	
CHF 1909 30 μM	<0.025	<0.025	<0.03	<0.08	<0.283	0.68 ± 0.30*
CHF 1909 30 μM	<0.025	0.188	<0.03	0.215	0.737	

* $P < 0.001$.

enables one to stimulate tissue specimen without preliminary sensitization, mimicking the early phase of the asthmatic reaction to a specific antigen. This is a substantial advantage if compared to the calcium ionophore A-23187, which represents a nonspecific and nonphysiological challenge widely used throughout the pharmacological characterization of 5-lipoxygenase inhibitors.

Previous studies have considered lung tissue cysteinyl leukotriene production and contraction of isolated human bronchi; nevertheless, these studies used human lung parenchyma as a model of immunologically induced formation of cys-LT, while evaluating immunologically induced contraction of airway tissue in isolated human bronchi [13, 26]. Taking advantage of a sophisticated approach to the analysis of trace amounts of cysteinyl leukotrienes, concomitant measurement of LT production, and contraction of isolated human bronchi is reported here for the first time: contraction of the human bronchi preparation is associated with the presence of LTC_4, LTD_4, and LTE_4 in incubation media. The importance of measuring all three cysteinyl leukotrienes was clearly pointed out by a preliminary study on the metabolism of exogenous radiolabeled LTC_4 by the isolated human bronchi. Significant amounts of radiolabeled LTC_4, LTD_4, and LTE_4 were indeed found after a time required for completion of a concentration–response curve of anti-human IgE antibody.

To test the effect of 5-lipoxygenase inhibition on the parameters studied, we used a novel compound synthesized by the Chemistry Department of Chiesi Farmaceutici (Parma, Italy) and shown to directly inhibit the 5-LO enzyme in RBL-1 cell lysates (Civelli M, unpublished data).

The results obtained showed a significant activity of compound CHF 1909 in modulating the leukotriene-dependent contractile response of isolated human bronchi to immunological challenge *in vitro*. This activity correlated well with the inhibitory action of CHF 1909 on the enzyme 5-lipoxygenase, as shown by the quantitative analysis of cys-LT present in the incubation media: pretreatment with CHF 1909, 30 μM, resulted in a significant inhibition of cys-LT production, as indicated by the amounts of LTC_4, LTD_4, and LTE_4, together with suppression of the contractile response (over 90% inhibition for both activities).

In conclusion, we performed simultaneous measurement of cys-LT and immunologically induced contraction in isolated human bronchi. Inhibition of the 5-LO pathway of arachidonic acid metabolism, evaluated at the same time of the contractile response, is able to prevent the contraction of isolated human airways in response to IgE-mediated activation of bronchial mast cells. The method described may represent a model of choice for the development of 5-lipoxygenase inhibitors targeted to asthma therapy.

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