

15(S)-HETE modulates LTB₄ production and neutrophil chemotaxis in chronic bronchitis

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Profita, Mirella, Angelo Sala, Loredana Riccobono, Elisabetta Pace, Alessandra Paternò, Simona Zarini, Liboria Siena, Angela Mirabella, Giovanni Bon-signore, and Antonio M. Vignola. 15(S)-HETE modulates LTB₄ production and neutrophil chemotaxis in chronic bronchitis. *Am J Physiol Cell Physiol* 279: C1249–C1258, 2000.—We evaluated the levels of 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE] and the expression of 15-lipoxygenase (15-LO) mRNA in induced sputum obtained from 10 control and 15 chronic bronchitis subjects. 15(S)-HETE was evaluated by reverse phase high-performance liquid chromatography separation followed by specific RIA. 15-LO mRNA expression was determined by primed in situ labeling. The levels of both soluble and cell-associated 15(S)-HETE resulted significantly higher in chronic bronchitis than in control subjects. The percentage of cells expressing 15-LO mRNA was significantly higher in chronic bronchitis than in control subjects ($P < 0.01$). Double staining for specific cell type markers and 15-LO mRNA showed macrophages and neutrophils positive for 15-LO, whereas similar staining of peripheral blood neutrophils did not show evidence for 15-LO expression, suggesting that expression of 15-LO in neutrophils takes place on migration into the airways. Because 15(S)-HETE inversely correlated with the percentage of neutrophils in sputum of chronic bronchitis subjects, we studied the effect of 15(S)-HETE on leukotriene B₄ (LTB₄) production in vitro and evaluated the concentration of LTB₄ in induced sputum and the contribution of LTB₄ to the chemotactic activity of induced sputum samples ex vivo. The results obtained indicate that macrophages and neutrophils present within the airways of chronic bronchitis subjects express 15-LO mRNA; increased basal levels of 15(S)-HETE may contribute to modulate, through the inhibition of 5-lipoxygenase metabolites production, neutrophil infiltration and airway inflammation associated with chronic bronchitis.

5-lipoxygenase; 15-lipoxygenase

ARACHIDONIC ACID (AA) metabolites, such as leukotrienes and prostaglandins (29), play an important role among the mediators involved in the development of airway diseases. 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE], the product of AA metabolism through the

15-lipoxygenase (15-LO) pathway, represents the main AA metabolite synthesized by human lung and airways (9, 12). 15(S)-HETE is a potent mucosecretagogue in human airways (20) and prolongs the duration of airway obstruction during the early response (14), and 15-LO-dependent metabolites can exert several immunoregulatory functions that may be relevant in airway inflammation (33).

Several studies have documented that exogenous or endogenously generated 15(S)-HETE can be rapidly metabolized and/or reincorporated into cellular lipids (1, 4). Airway epithelial cells represent a significant source of 15(S)-HETE, and ozone exposure increases 15(S)-HETE production by, and reincorporation in, airway epithelial cells, leading to reduced synthesis of PGE₂, a cyclooxygenase metabolite of AA that possesses several anti-inflammatory activities in the airways (2). In addition, we recently found that reincorporation of 15(S)-HETE into phospholipids of pulmonary epithelial cells, as well as the expression of 15-LO, can be modulated by cytokines such as interleukin-4, suggesting that 15(S)-HETE production is regulated by the pulmonary microenvironment (25). The biological effect of 15(S)-HETE reincorporation into membrane phospholipids is largely unknown, but it seems that such a phenomenon may exert autocrine functions affecting the response of inflammatory cells to chemotactic stimuli (4), possibly through the modification of intracellular signal transduction pathways (16).

Although the role played by 15(S)-HETE has been extensively studied in asthma, its putative role in chronic bronchitis still remains largely unknown. In addition, despite previous in vitro evidence, the potential role played by 15(S)-HETE reincorporation in cells isolated from the airways of patients with chronic airway inflammation is still a matter of debate.

Therefore, the first aim of the present study was to compare soluble and cell-associated 15(S)-HETE levels in induced sputum samples obtained from normal and chronic bronchitis subjects. In addition, to identify

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(among those recovered in induced sputum samples) the cell(s) possibly contributing to the observed amounts of released or lipid-incorporated 15(S)-HETE, we evaluated the phenotype of cells bearing 15-LO mRNA. Moreover, we evaluated whether the increased 15(S)-HETE levels may influence airway inflammation. We investigated the ability of 15(S)-HETE to modulate the production of leukotriene B₄ (LTB₄), the most powerful neutrophil chemotactic AA metabolite, and therefore its ability to affect the migration of neutrophils into the human airways.

METHODS

Patients. The study was performed on two groups of subjects. The study groups included 10 control and 15 chronic bronchitis and/or chronic obstructive pulmonary disease (COPD) subjects. Chronic bronchitis and COPD (median age 68 yr, range 48–78 yr) were defined according to the criteria of the American Thoracic Society (3). Patients diagnosed as having COPD had a forced expiratory volume in 1 s (FEV₁) below 70% of that predicted and displayed a 10% or smaller increase in their FEV₁ after an inhaled dose of 200 µg of albuterol. Patients were excluded if they had a bronchial infection during the month preceding the study; no subject had received corticosteroids in any form during the 2 mo before the study. All chronic bronchitis patients who had routine chest X-rays and computed tomographic scans that showed obvious emphysema were excluded. Control subjects (median age 31 yr, range 25–43 yr) never suffered from asthma or chronic bronchitis, and they had not experienced any bronchial or respiratory tract infections during the month preceding the study. Control subjects were lifelong nonsmokers, and their pulmonary function was within the normal range. None of the control and chronic bronchitis subjects received aspirin during the 7 days before their evaluations.

The study was approved by the appropriate ethics committee, and patients gave written informed consent for participation.

Induced sputum production and sample processing. Each subject was submitted to spirometry before the beginning of the procedure. Induced sputum production and processing were carried out according to Fahy et al. (8) with slight modifications (35). Briefly, patients in a fasting condition were exposed, early in the morning, for 20 min to an aerosol of 3% hypertonic saline solution. The volume of the induced sputum, or saliva, was determined, and an equal volume of a dithiothreitol solution (0.1% in saline solution, vol/vol; Sigma Chemical, St Louis, MO) was added. Samples were then gently mixed with a vortex and placed in a water bath at 37°C for 15 min to ensure a complete homogenization. Samples were periodically removed from the water bath for further brief, gentle vortex mixing. The homogenized sputum and saliva were centrifuged at 800 g for 10 min at room temperature (RT) to separate the supernatants from the cell pellet. The supernatants were then aspirated and frozen at –80°C under argon atmosphere into siliconized glass centrifuge tubes for subsequent 15(S)-HETE analysis.

The cell pellet was resuspended in saline solution and filtered through a 70-µm nylon mesh. The eluate was centrifuged at 200 g for 5 min at RT, and the cell pellet was finally resuspended in PBS to a final concentration of 10⁶ cells/ml. Aliquots (10 µl) were used to assess cell viability by trypan blue exclusion, whereas differential cell counting was obtained from cytocentrifuged slides (Cytospin 2; Shandon In-

struments, Runcorn, UK) stained with Diff Quick (Merz-Dade, Duding, Switzerland). The slides were read blindly by two independent investigators who counted at least 400 cells per slide. The number of the squamous cells was subtracted from the total cell counts, and the differential cell counts were expressed as corrected percentage. A separate aliquot was centrifuged at 400 g for 10 min at 4°C, and the cellular pellet was resuspended in 1 ml of PBS and 1 ml of methanol and stored at –80°C under argon atmosphere into siliconized glass centrifuge tubes until analysis of cell-associated 15(S)-HETE.

Analysis of 15(S)-HETE in supernatants. The supernatants from induced sputum samples were acidified with 50 µl of formic acid. Extraction was carried out on octadecylsilyl (ODS) cartridges (Supelclean LC-18; Supelco, Bellefonte, PA) that were activated with 10 ml of ethanol and 10 ml of water. Supernatants were loaded onto cartridges, washed with 10 ml of water and 5 ml petroleum benzene, and eluted with 4 ml of ethyl acetate. The ethyl acetate fractions were collected, evaporated to dryness under a steady flow of nitrogen gas, and resuspended in 400 µl of acetonitrile:water (20:80, vol/vol). Reverse phase high-performance liquid chromatography (RP-HPLC) separation of 15(S)-HETE was carried out with the use of a Beckman System Gold Liquid Chromatograph (Beckman Analytical, Palo Alto, CA) equipped with an Ultrasphere ODS column (4.6 × 250 mm, 5 µm, Beckman Analytical). The column was eluted at a flow rate of 1 ml/min using a linear gradient from 20% solvent B (acetonitrile/acetic acid, 100/0.1, vol/vol) to 100% B over 18 min, with solvent A represented by water/acetic acid (100/0.1, vol/vol). Retention time of 15(S)-HETE standard was 21 ± 0.6 min, and it was checked daily with the use of radiolabeled 15(S)-HETE (182 Ci/mmol; NEN Products, Boston, MA). The fractions that corresponded to the retention time of 15(S)-HETE standard were collected, pooled, dried under vacuum, reconstituted with RIA buffer, and then assayed for 15(S)-HETE with a specific RIA kit (Advanced Magnetic, Framingham, MA) performed according to the manufacturer's protocol. Cross-reactivity with different eicosanoids, according to the manufacturer, was 5-HETE 0.1%; 12-HETE, 0.5%; 5,15-di-HETE and 8,15-di-HETE, 1%; we tested the cross-reactivity for 15(R)-HETE that resulted <5%. The lower limit of detection for the assay was 8.2 pg/tube. All measurements were made in duplicate, and the results were expressed in nanograms per corrected cell counts (10⁶ cells/ml).

Analysis of cell-associated 15(S)-HETE. The sputum cells' suspensions were extracted with 2 vol of 2-propanol containing 1.2% (vol/vol) acetic acid and chloroform (1:1, vol/vol) according to Conrad et al. (6). The resulting mixture was vortexed and centrifuged at 2,500 g for 15 min at RT. The upper and the intermediate phases were removed, whereas the lipids containing lower phases were dried under nitrogen and resuspended in 400 µl of methanol. An aliquot was treated with 100 µl of NaOH 2N and incubated at 60°C for 30 min under argon atmosphere. The solution was added with 100 µl of acetic acid 2N, and the final volume was adjusted to 800 µl with water. After centrifugation at 2,500 g for 10 min at RT, samples were analyzed for 15(S)-HETE by RP-HPLC separation followed by RIA as described above.

Purification of neutrophils from peripheral blood. Peripheral blood polymorphonuclear leukocytes were prepared from normal and chronic bronchitis subjects with the use of dextrane sedimentation and centrifugation over Ficoll cushions, as previously described (26).

Primed in situ labeling of 15-LO mRNA in induced sputum cells. To identify the cells expressing 15-LO, we performed primed in situ labeling (PRINS) for 15-LO mRNA, according

to Koch et al. (11) with minor modifications, as previously described (25).

At least four fields per slide were evaluated under light microscopy ($\times 400$ final magnification), and a minimum of 400 cells were counted. Results were expressed as the percentage of 15-LO mRNA-positive cells over the total.

To identify the phenotype of cells expressing 15-LO mRNA, PRINS was performed in induced sputum samples obtained from 10 control and 10 chronic bronchitis subjects after immunohistochemical characterization by using the immunoalkaline phosphatase-antialkaline phosphatase (APAAP) method according to Cordell et al. (7). The following monoclonal antibodies (MAb) were used: Dako macrophage (anti-CD68 EBM II; Dakopatts, Glostrup, Denmark; recognizing mononuclear phagocytes; dilution 1:100), Dako neutrophil elastase (anti-human neutrophil elastase cell line NP57, recognizing neutrophils; dilution 1:150), Dako primed T cells (anti-UCHL1, recognizing antigen-primed T cells; dilution 1:50), and the MAb anti-eosinophil (EG1) and anti-eosinophil cationic protein MAb (EG2) (Kabi Pharmacia, Uppsala, Sweden; dilution 1:5). The slides were read blindly by two independent investigators who counted at least 400 cells per slide. The evaluation of double immunostaining resulting from PRINS and immunohistochemistry was done using light microscopy at a final magnification of $\times 400$. Positive cells were characterized by a colocalization of the red and brown staining due to the APAAP and PRINS methods, respectively. Control slides were included in each staining session with the use of an irrelevant MAb (anti-smooth muscle; Ortho Pharmaceutical, Raritan, NJ).

Analysis of LTB_4 in supernatants. To understand whether, in chronic bronchitis, the inverse correlation between the levels of 15(S)-HETE and the percentage of neutrophils could be related to the levels of the potent neutrophil chemotactic factor LTB_4 , we measured LTB_4 in induced sputum samples obtained from chronic bronchitis subjects by RP-HPLC separation followed by quantitation using a commercial enzyme immunoassay (RIA), performed according to the manufacturer's directions (Amersham Pharmacia Biotech, Piscataway, NJ).

The effect of 15(S)-HETE on LTB_4 production was studied in vitro using isolated human neutrophils, challenged either with the calcium ionophore A-23187 (1 μ M, 10 min, 37°C) or with the formylated tripeptide formyl-methionine-leucine-phenylalanine (fMLP; 0.1 μ M, 10 min, 37°C) on preactivation with granulocyte/macrophage colony-stimulating factor (GM-CSF; 1 nM, 30 min, 37°C), as previously described (27, 28). To test the effect of phospholipid-incorporated 15(S)-HETE onto LTB_4 production, isolated human neutrophils (10^7 cells) were incubated with 15(S)-HETE (30 μ M) for 30 min and washed before GM-CSF priming and fMLP challenge.

LTB_4 and its ω -oxidized metabolites were analyzed by RP-HPLC coupled to diode-array UV detection as previously described (27, 28).

Neutrophil chemotaxis assay. To lend further support to the hypothesis that 15(S)-HETE may have a role in inhibiting neutrophil migration within the airways of chronic bronchitis subjects, we evaluated the ability of induced sputum samples from chronic bronchitis patients to induce neutrophil chemotaxis and correlated with the levels of 15(S)-HETE. We therefore selected induced sputum samples obtained from three subjects with 15(S)-HETE concentrations >8 ng/ 10^6 cells [high-15(S)-HETE producers: 50.5 (36–109) ng/ 10^6 cells] and from three subjects with 15(S)-HETE concentrations <8 ng/ 10^6 cells [low-15(S)-HETE producers: 2.2 (1.3–6.1) ng/ 10^6 cells]. Neutrophils obtained from the peripheral blood of healthy donors were resuspended at a concen-

tration of 1×10^6 /ml in PBS containing $CaCl_2$ (0.5 mM) and $MgCl_2$ (1 mM), and the supernatants from induced sputum of chronic bronchitis patients were tested for their chemotactic activity on neutrophils. In addition, to evaluate whether chemotactic activity of induced sputum samples related to the presence of LTB_4 , experiments were performed in the presence and absence of an LTB_4 receptor antagonist (10 μ M LY-223982; Eli Lilly, Basinstoke, UK) at 37°C. Chemotaxis was performed as previously described (23) using a 48-well microchemotaxis chamber (Neuro Probe; Costar, Cabin John, MD). Neutrophils were loaded into the upper well and the sputum supernatant diluted 1:4 was placed in the bottom chamber. The two wells were separated by a polycarbonate filter paper with a pore size of 3 μ m. The chamber was incubated at 37°C for 1 h. At the end of incubation, the filter was fixed, stained, and mounted on a glass microscope slide (observed at $\times 400$). Migration was assessed by counting the number of cells that had migrated beyond a certain depth into the filter. Each experimental condition was performed in duplicate, and three to four fields were assessed for cell migration. The number of cells migrating spontaneously (i.e., negative controls) were subtracted from all measurements before data analysis.

Statistical analysis. Results obtained from sputum samples were expressed as medians and 25 to 75 percentiles, and statistical analysis was performed with Mann-Whitney's *U*-test. The Spearman rank correlation was calculated to assess the correlation between data. Results obtained from isolated cell preparation were expressed as means and SE of *n* replication, and statistical analysis was performed with Tukey-Kramer's honestly significant difference test.

RESULTS

Demographic characteristics of patients. FEV₁ values of chronic bronchitis/COPD ranged from 23 to 98% of that predicted (median and percentiles: 75 and 62 to 90%). In the chronic bronchitis group, 4 of 15 patients had COPD because their FEV₁ values were $<70\%$. All patients had a normal total lung volume and diffusion capacity, excluding superimposing emphysema.

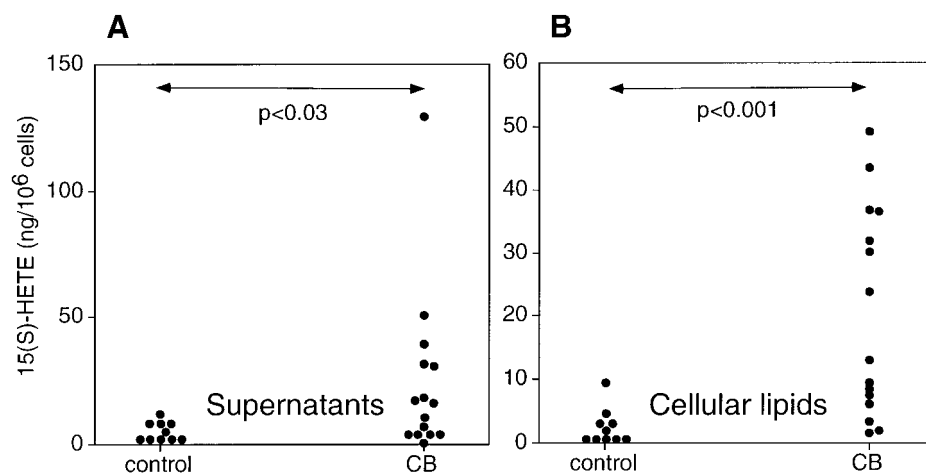
Total and differential cell counts in sputum and saliva. The percentage of squamous cells was not significantly different in sputum samples obtained from control subjects and chronic bronchitis patients (Table 1). The corrected median of total cell count resulted higher in chronic bronchitis patients (1.9, 0.9–3.8 10^6 cells/ml) than in control subjects (1.2, 1–1.8 10^6 cells/ml), although the difference was not statistically significant. The viability of sputum cells was 75% (range:

Table 1. Differential cell counts of induced sputum samples

	Control	Chronic Bronchitis	
Macrophages	81.8(80–89.4)	40.1(19.1–57.8)	$P < 0.001$
Neutrophils	16.5(10–23)	57.3(26.4–78.6)	$P < 0.01$
Lymphocytes	0(0–0.3)	0(0–0.5)	ns
Eosinophils	0	0.5(0–2.2)	$P < 0.001$
Epithelial cells	0(0–0.1)	2(0–7.2)	ns

Differential cell counts of induced sputum samples obtained from control and chronic bronchitis subjects. Data are expressed as medians (25–75 percentiles). Statistical analysis was performed using Mann-Whitney's *U*-test (ns, not significant).

Fig. 1. 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE] concentration in induced sputum obtained from control and chronic bronchitis patients. *A*: 15(S)-HETE concentration in supernatants. *B*: 15(S)-HETE concentration in cellular lipids. 15(S)-HETE was analyzed by specific RIA on reverse phase high-performance liquid chromatography (RP-HPLC) separation. 15(S)-HETE was released from cellular lipids by using base-catalyzed hydrolysis as described in METHODS. Results are expressed as ng/10⁶ cells. Statistical analysis was performed with the use of Mann-Whitney's *U*-test.



68–80%) in controls and 76% (range: 66–82%) in chronic bronchitis. No significant correlation between the cells' viability and 15(S)-HETE levels was observed in any group. The percentage of neutrophils was significantly higher in chronic bronchitis patients than in control subjects ($P < 0.01$). The percentage of eosinophils was also significantly higher in chronic bronchitis patients than in control subjects ($P < 0.001$). No differences were observed for lymphocytes and epithelial cells (Table 1).

Soluble and cell-associated 15(S)-HETE in induced sputum. 15(S)-HETE levels in the supernatants were significantly higher in induced sputum obtained from chronic bronchitis patients than in control subjects (Fig. 1A; $P < 0.03$ Mann-Whitney's *U*-test). To determine the possible role of 15(S)-HETE in cellular recruitment, the concentration of 15(S)-HETE was correlated with the number of macrophages and neutrophils of induced sputum. In chronic bronchitis patients, the levels of soluble 15(S)-HETE positively correlated with the percentage of macrophages ($P < 0.01$) and inversely correlated with the percentage of neutrophils (Fig. 2, A and B; $P < 0.01$). There was no significant difference between the levels of soluble 15(S)-HETE in induced sputum supernatants from chronic bronchitis

and COPD patients. 15(S)-HETE levels in saliva were not detectable.

The levels of cell-associated 15(S)-HETE, measured on base-catalyzed hydrolysis of cellular lipids, resulted significantly higher in induced sputum of chronic bronchitis patients than in sputum of normal subjects ($P < 0.001$, Mann-Whitney's *U*-test; Fig. 1B). In chronic bronchitis, the levels of cell-associated 15(S)-HETE inversely correlated with the percentage of neutrophils (Fig. 3A; $P < 0.02$). On the other hand, levels of cell-associated 15(S)-HETE directly correlated with the percentage of macrophages (Fig. 3B; $P < 0.03$). There was no significant difference between the levels of cell-associated 15(S)-HETE in chronic bronchitis and COPD patients. Neither soluble nor cell-associated 15(S)-HETE showed statistically significant correlation with the percentage of eosinophils. Neither soluble nor cell-associated 15(S)-HETE showed correlation with differential cell counts in control subjects. Cell-associated 15(S)-HETE levels in cell pellets from saliva were not detectable.

15-LO mRNA expression and immunohistochemistry. To evaluate whether cells recovered from induced sputum were actively expressing 15-LO, we performed PRINS for 15-LO mRNA on total cell populations re-

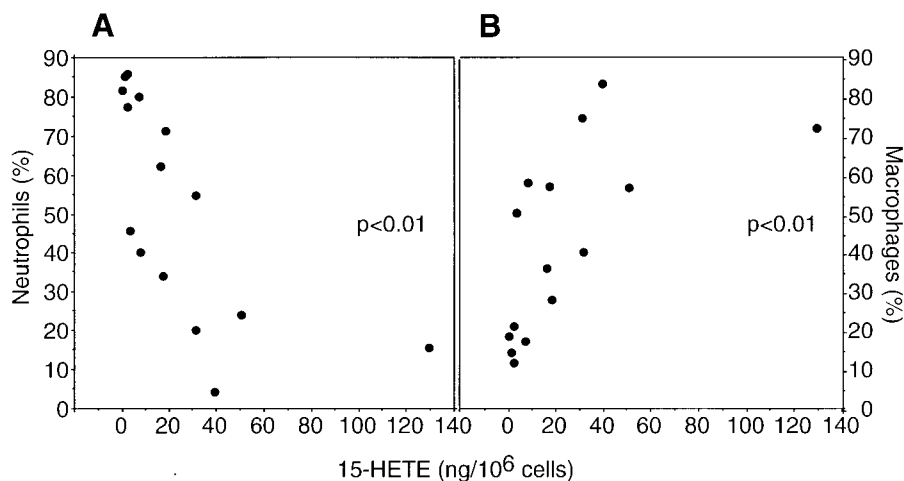


Fig. 2. Correlation between the concentration (expressed as ng/10⁶ cells) of 15(S)-HETE in supernatants and the percentage of neutrophils (A) and macrophages (B) in induced sputum samples obtained from chronic bronchitis patients. Statistical analysis was performed with the use of the Spearman rank test.

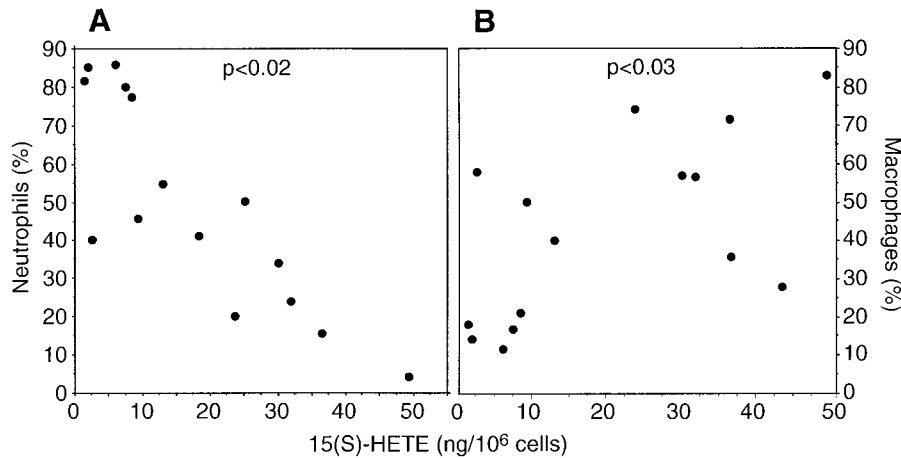


Fig. 3. Correlation between the concentration of 15(S)-HETE, esterified in cellular lipid, and the percentage of neutrophils (A) and macrophages (B) in induced sputum samples obtained from chronic bronchitis patients. Statistical analysis was performed with the use of the Spearman rank test.

covered from the two study groups. The percentage of positive cells in induced sputum obtained from chronic bronchitis (85%, range: 70–88%) (Fig. 4C) was significantly higher than in control subjects (13%, range: 0–20%; Fig. 4C and Table 2; $P < 0.01$, Mann-Whitney's *U*-test), but no significant correlation was observed between the 15(S)-HETE levels and the percentage of cells expressing 15-LO mRNA. To identify the phenotype of cells expressing 15-LO mRNA, we performed combined immunohistochemistry and in situ hybridization on cells recovered from induced sputum. Interestingly, the transcripts for 15-LO were localized not only in macrophages (Fig. 4D) but also in neutrophils (Fig. 4E), as identified through labeling with a MAb anti-CD68, specific for mononuclear phagocytes, and with a MAb anti-elastase, specific for neutrophils. Quantitative analysis of double-stained slides showed that macrophages represented >83% of the cells expressing 15-LO mRNA (Table 2). 15-LO mRNA was undetectable in squamous cells from induced sputum.

In agreement with previous results on the levels of 15-LO protein in blood polymorphonuclear leukocytes from normal subjects (19), 15-LO mRNA was undetectable in neutrophils purified from peripheral blood of normal subjects (data not shown), as well as from chronic bronchitis patients (Fig. 4F).

Analysis of LTB_4 in supernatants. To understand whether the inverse relationship between 15(S)-HETE levels and percentage of neutrophils involved modification of the chemotactic factor LTB_4 , we measured this mediator into sputum samples and found that LTB_4 inversely correlated with the observed values of 15(S)-HETE (Fig. 5; $P < 0.001$, Spearman rank test).

In vitro inhibitory effect of 15(S)-HETE on LTB_4 production. Exogenous 15(S)-HETE inhibited LTB_4 production elicited by the calcium ionophore A-23187 in isolated human neutrophils in a concentration-dependent fashion (Fig. 6A). When a more physiological stimulus was used (namely fMLP on GM-CSF priming), a nonsignificant increase in LTB_4 production was observed at concentrations of 15(S)-HETE lower than 1 μ M, whereas at 3 and 10 μ M, an inhibitory effect similar to that obtained in A-23187-challenged cells was observed (Fig. 6B).

Incubation of isolated neutrophils with 15(S)-HETE, resulting in incorporation into cell phospholipids, followed by washing of excess 15(S)-HETE, also resulted in a significant inhibition of LTB_4 production on GM-CSF priming and fMLP challenge ($-32 \pm 6.5\%$ vs. control, $n = 4$, $P < 0.02$).

Neutrophil chemotaxis assay. In chronic bronchitis subjects, sputum samples with concentrations of 15(S)-HETE <8 ng/10⁶ cells [low-15(S)-HETE producers] caused a greater neutrophil chemotaxis than samples with 15(S)-HETE concentrations >8 ng/10⁶ cells [high-15(S)-HETE producers] (Fig. 7A). In addition, preincubation with the LTB_4 receptor antagonist LY-223982 resulted in a greater inhibitory effect on neutrophil chemotaxis induced by sputum from low-15(S)-HETE producers than from high-15(S)-HETE producers (Fig. 7B).

DISCUSSION

The present results indicate that increased amounts of 15(S)-HETE are present in induced sputum obtained from chronic bronchitis patients compared with normal subjects. 15(S)-HETE is also found associated with cellular lipids, as an index of cellular incorporation of this AA metabolite. The increased levels of 15(S)-HETE observed are accompanied by a significant increase in the percentage of cells expressing 15-LO mRNA in chronic bronchitis patients. The concentration of either soluble or cell-associated 15(S)-HETE shows a negative correlation with the percentage of neutrophils in induced sputum in chronic bronchitis, suggesting a potential modulatory role of this mediator toward the migration of neutrophils into the airways. Expression of 15-LO mRNA takes place in neutrophils present in samples of induced sputum collected from chronic bronchitis subjects but not in peripheral blood neutrophils either from controls or from chronic bronchitis patients. Finally, we provide evidence that increased 15(S)-HETE levels can modulate the production of LTB_4 , resulting in a decreased neutrophil chemotaxis activity in sputum samples.

Limited amounts of data are available concerning values of 15(S)-HETE in human airways in vivo, and

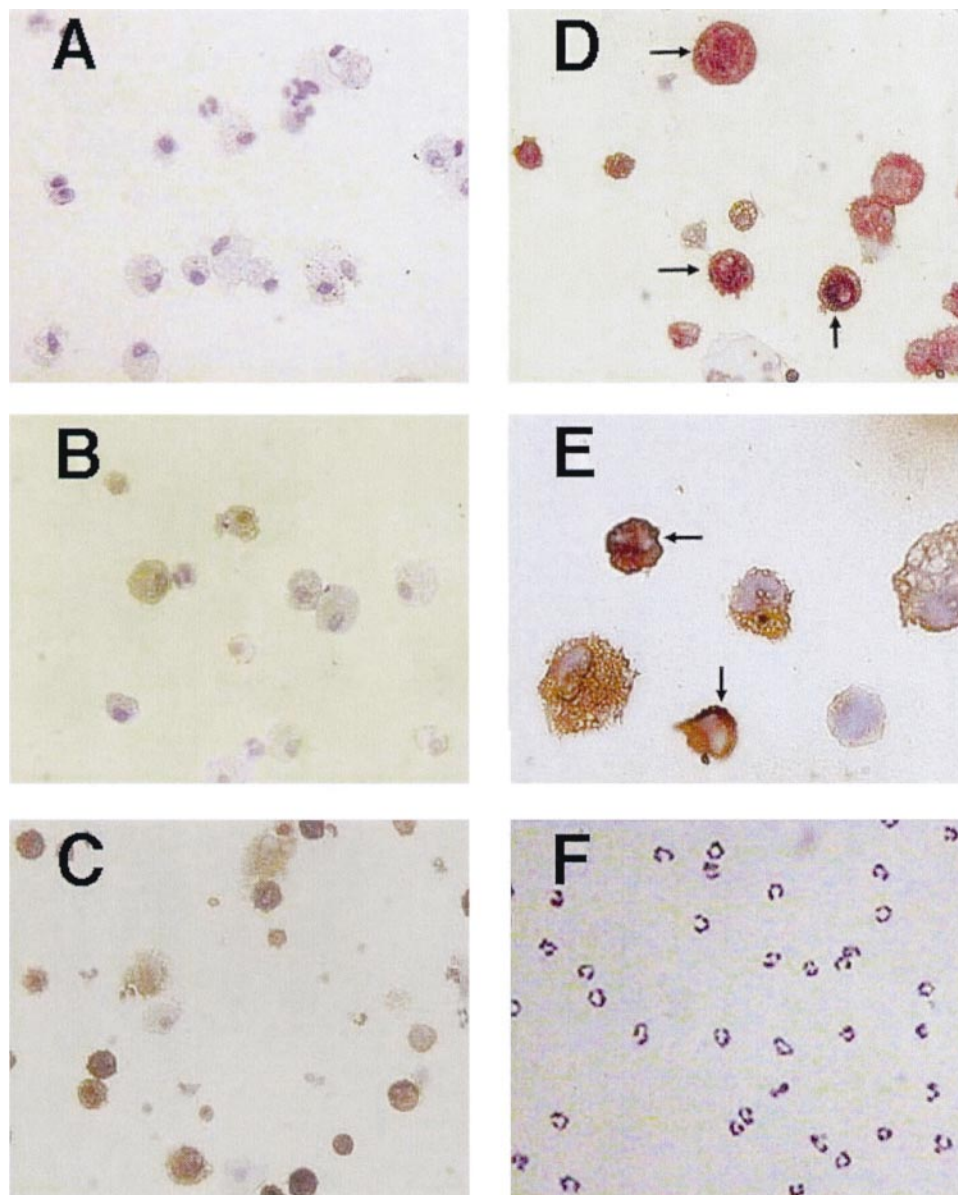


Fig. 4. *A*: induced sputum cells isolated from chronic bronchitis subjects stained for primed in situ labeling (PRINS), as described in METHODS, using a random sequence primer (negative control; original magnification, $\times 160$). *B*: expression of 15-lipoxygenase (15-LO) mRNA on cytospin preparations of cells isolated from induced sputum obtained from a normal subjects (original magnification, $\times 160$). 15-LO mRNA was stained using PRINS as described in METHODS. *C*: expression of 15-LO mRNA on cytospin preparations of cells isolated from induced sputum obtained from a chronic bronchitis subject (original magnification, $\times 160$). *D* and *E*: double staining of induced sputum cells obtained from a chronic bronchitis subject. Cells were labeled with an anti-CD68 monoclonal antibody (MAb; *D*) or with an anti-neutrophil elastase MAb (*E*) and then used to perform PRINS of 15-LO mRNA, as described in METHODS. The arrows indicate cells that are double positive for CD68 or for neutrophil elastase and 15-LO mRNA (original magnification, $\times 400$). *F*: expression of 15-LO mRNA on cytospin preparation of peripheral blood human neutrophils that were obtained from chronic bronchitis subjects (original magnification, $\times 160$).

most of these data refer to bronchial asthma. Increased levels of 15(S)-HETE were reported by Murray et al. (22) in bronchoalveolar lavage fluids from atopic asthmatic patients after specific challenge; on the other hand, no differences were observed after specific challenge in aspirin-intolerant subjects (30). For the first time, we provide evidence of highly significant differences in basal levels of this AA metabolite between normal subjects and subjects characterized by inflammation of the airways, such as chronic bronchitis.

15(S)-HETE can exert several biological actions that may be relevant in the pathogenesis or the control of airway inflammatory disorders. 15(S)-HETE has been shown to be a potent mucosecretagogue in human airways (20) and to possess chemotactic activity for neutrophils directly contributing to the recruitment of these cells in dog airways (10). Alternatively, it has been reported that 15(S)-HETE inhibits 5-lipoxygenase (5-LO) in neutrophils (24, 34), reducing the production of inflammatory mediators such as LTB_4 , and inhibits the changes in intracellular calcium concentrations induced by monocyte-derived neutrophil activating factor (31). The results of the present study support the hypothesis that in chronic bronchitis, 15(S)-HETE exerts an inhibitory rather than a stimulatory effect on neutrophilic inflammation. We indeed provide evidence that in chronic bronchitis, patient concentrations of free (soluble) 15(S)-HETE inversely correlate with the percentage of infiltrating neutrophils, as well as with the concentration of LTB_4 in induced sputum samples. In agreement with the latter observation, exogenous 15(S)-HETE inhibits LTB_4 production elicited in isolated human neutrophils by challenge with either A-23187 or fMLP after priming with GM-CSF.

The inverse correlation between concentrations of LTB_4 and concentrations of 15(S)-HETE was strength-

Table 2. Differential cell counts of cells expressing 15-LO mRNA in induced-sputum samples

	mRNA-Positive Cells, %		P
	Controls	Chronic bronchitis	
Total cells	13 (0–20)	85 (70–88)	< 0.001
Macrophages	3 (0–18)	70 (55.5–70)	< 0.01
Neutrophils	0 (0–0)	14 (10–15)	< 0.001
Lymphocytes	0 (0–1)	0 (0–1)	ns
Eosinophils	0 (0–0)	0 (0–0)	ns
Epithelial cells	0 (0–0.5)	0 (0–0)	ns

Differential cell counts of induced sputum samples obtained from control and chronic bronchitis subjects. 15-lipoxygenase (15-LO) mRNA was detected by primed in situ labeling. Data are expressed as medians (25–75 percentiles). Statistical analysis was performed using Mann-Whitney's *U*-test (ns, not significant).

ened by the neutrophil chemotactic activity observed in induced sputum samples from chronic bronchitis subjects: chemotactic activity was significantly higher in samples in which 15(S)-HETE concentration was lowest (<8 ng/10⁶ cells), compared with samples in which 15(S)-HETE concentration was higher than 8 ng/10⁶ cells. Furthermore, the chemotactic activity of induced sputum samples that showed low concentrations of 15(S)-HETE was significantly more sensitive to inhibition by an LTB₄ receptor antagonist, indicating the presence of higher concentrations of biologically active LTB₄.

In addition to the release, the incorporation of 15(S)-HETE into membrane phospholipids impairs the response of human polymorphonuclear neutrophil leukocytes to inflammatory stimuli, such as the formylated tripeptide fMLP (4), and inhibits the migration of neutrophils across cytokine-activated endothelium (32). In agreement with these data, the concentrations of 15(S)-HETE associated with cellular lipids in induced sputum samples from chronic bronchitis subjects inversely correlate with the percentage of infiltrating neutrophils. Experiments in vitro confirmed that incorporated 15(S)-HETE significantly inhibits LTB₄ pro-

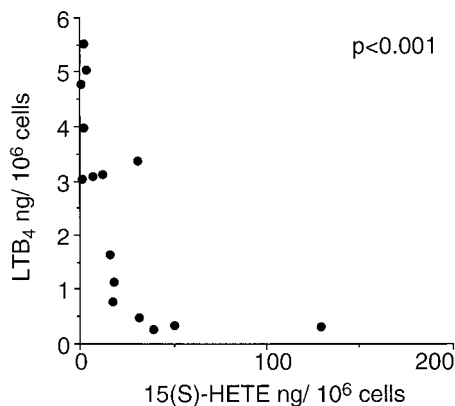


Fig. 5. Correlation between the levels of 15(S)-HETE and leukotriene B₄ (LTB₄) measured in the supernatants of induced sputum from chronic bronchitis subjects. 15(S)-HETE and LTB₄ were measured by specific RIA and enzyme immunoassay, respectively, on RP-HPLC separation. Statistical analysis was performed with the use of the Spearman rank test.

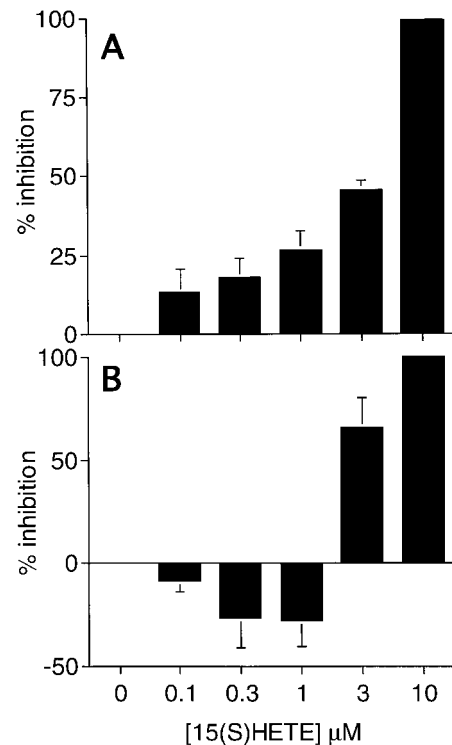
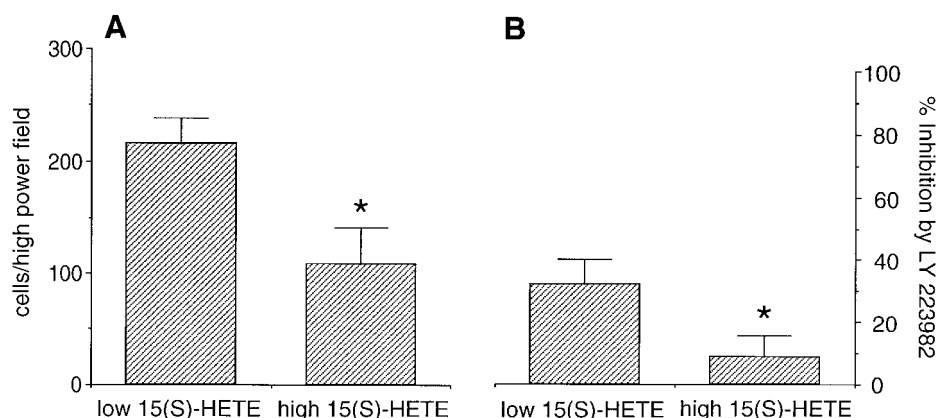


Fig. 6. Effect of exogenous 15(S)-HETE on the production of LTB₄ by isolated human neutrophils on challenge with the calcium ionophore A-23187 (A) or formyl-methionine-leucine-phenylalanine after priming with granulocyte/macrophage colony-stimulating factor (B). Results are expressed as percent inhibition of the LTB₄ production observed in control samples.

duction elicited in isolated human neutrophils by challenge with fMLP after priming with GM-CSF.

Eosinophils are known to express 15-LO and may represent a source of significant amounts of 15(S)-HETE. Differential count of cells present in induced sputum reveals that they only represent 1–2% of the cells recovered, suggesting that they do not play a major role in 15(S)-HETE production within the airways of chronic bronchitis patients. This is supported by the lack of a significant correlation between 15(S)-HETE and the relative number of eosinophils, whereas the strong positive correlation observed with macrophages proposes these cells as the most relevant with respect to 15(S)-HETE production. The main role played by macrophages may also explain why lower levels of 15(S)-HETE in induced sputum were observed in subjects with higher levels of neutrophils. In fact, when the differential count of cells that express 15-LO mRNA is taken into account, the ratio of macrophages vs. neutrophils that express 15-LO mRNA is, on the average, ~6:1. It is, therefore, likely that neutrophil-derived 15(S)-HETE does not contribute significantly. On the other hand, it is possible to speculate that neutrophils that express 15-LO may become substantially prevented from migrating into the airways, as suggested by the reduced chemotaxis of neutrophils that incorporate significant amounts of 15(S)-HETE into their membrane phospholipids (4).

Fig. 7. *A*: chemotaxis activity of supernatant fluids that were obtained from induced sputum of chronic bronchitis subjects showing 15(S)-HETE concentrations $<8 \text{ ng}/10^6$ cells [low-15(S)-HETE producers] and $>8 \text{ ng}/10^6$ cells [high-15(S)-HETE producers]. Results are expressed as the number of migrated neutrophils per high power field. *B*: effect of LY-223982 on neutrophil chemotaxis activity of induced sputum samples that were obtained from low- or high-15(S)-HETE producers. Results are expressed as percentage inhibition vs. control samples. Statistical analysis was performed with the use of Mann-Whitney's *U*-test. $*P < 0.05$.



Expression of 15-LO mRNA and protein has been reported in macrophages present in human atherosclerotic lesions, where it has been implicated in foam cell formation; 15-LO mRNA and 15-LO enzymatic activity has also been reported in human alveolar macrophages (17). With the use of *in situ* hybridization and immunohistochemistry, we observed 15-LO mRNA expression in human alveolar macrophages and in infiltrating neutrophils obtained from induced sputum of chronic bronchitis patients. The presence of 15-LO in neutrophils obtained from peripheral blood has been a subject of some controversy in the literature. Recently, when using immunochemical analysis of supernatants from highly purified neutrophils and eosinophils, one of us (A. Sala) confirmed that neutrophils do not express 15-LO (19). Primed *in situ* labeling for 15-LO mRNA coupled to immunohistochemistry confirms the absence of detectable transcripts for 15-LO in peripheral blood neutrophils obtained either from normal or chronic bronchitis subjects and indicates that expression of 15-LO is associated with induced migration of neutrophils into the airways. Studies are under way in our laboratories to identify the potential factor(s) responsible for such an effect. Expression of 15-LO by inflammatory cells within the airways may be relevant because 15-LO-derived products, in addition to 15(S)-HETE, can contribute to modulate the inflammatory response: lipoxin A_4 and lipoxin B_4 , AA metabolites arising from the sequential action of 5- and 15-LO, inhibit LTB_4 as well as fMLP-induced neutrophil chemotaxis (15).

No correlation between the overall percentage of cells that express 15-LO mRNA and 15(S)-HETE levels was observed, possibly reflecting the fact that mRNA expression, protein synthesis, and mediator formation/release, represent different steps, certainly linked but not strictly timewise correlated. Furthermore, we cannot rule out a significant contribution to the observed formation of 15(S)-HETE by airway epithelial cells or submucosal eosinophils. The final information provided by the increased number of cells that express 15-LO mRNA in chronic bronchitis is that an active process that results in increased formation of 15(S)-HETE is taking place.

A wide variability in supernatant and cell-associated 15(S)-HETE was observed among chronic bronchitis patients, but no significant differences, at least in terms of functional parameters such as $FEV_{1.0}$, could be detected between high- and low-15(S)-HETE-producers. Nevertheless, it would be possible to speculate that the decreased percentage of neutrophils present within the airways of patients with high levels of 15(S)-HETE may well be reflected in changes of the time course of the chronic inflammatory response characteristic of chronic bronchitis.

The ability of 15(S)-HETE to act as an immunoregulatory mediator for neutrophilic inflammation may play a central role in the pathogenesis of chronic bronchitis. Neutrophilic inflammation appears to be an important inflammatory component of the disease. Bronchoalveolar lavage studies carried out in patients with chronic bronchitis showed an increased number of neutrophils, compared with normal subjects, as well as increased levels of myeloperoxidase, a neutrophilic secretory protein (13, 21). The factors contributing to the recruitment of neutrophils in the bronchoalveolar lavage fluid is still a matter of discussion, but it has been demonstrated that cigarette smoking increases their numbers in the alveolar septum (18). Many factors control how neutrophils pass through the lungs, including the increased release of the neutrophil-chemotactic cytokines interleukin-8 and tumor necrosis factor- α , the unique and complex structure of the pulmonary capillary bed, the local hemodynamic factors, the neutrophil deformability, and neutrophil-endothelial interactions. As a consequence of these complex mechanisms, neutrophils from patients with chronic bronchitis have an increased chemotaxis, which suggests that they can be recruited more easily in the lungs (5) and represent a potential cause for increased proteolytic activity in the airways. Interestingly, although the mechanisms modulating neutrophil migration and activation in the lung have been extensively studied, little information is available with respect to mechanisms involved in the modulation of neutrophilic infiltration. The results of the present study may add important evidence, because they strongly suggest that increased 15(S)-HETE levels

may serve as a negative regulatory mechanism of neutrophil recruitment and activation in chronic bronchitis, modulating the synthesis of the chemotactic factor LTB₄.

In conclusion, analysis of 15(S)-HETE in supernatants [soluble 15(S)-HETE] from induced sputum samples provides evidence for significantly increased levels of this AA metabolite in inflamed airways of chronic bronchitis patients. Increased concentrations of 15(S)-HETE are also present in cellular lipids [cell-associated 15(S)-HETE] and may contribute to the potential effects of this mediator. A significant negative correlation between the concentrations of either soluble or cell-associated 15(S)-HETE and the percentage of neutrophils in chronic bronchitis patients suggests a role of 15(S)-HETE in modulating neutrophilic infiltration. Expression of 15(S)-HETE mRNA is observed in neutrophils present in induced sputum but not in neutrophils obtained from peripheral blood, suggesting that expression of 15-LO may take place on migration of neutrophils within the airways of chronic bronchitis patients. 15-LO expression and 15(S)-HETE production, as well as incorporation of this AA metabolite into cellular lipids, may represent important regulatory mechanisms that operate in pulmonary inflammation, inhibiting the production of 5-LO metabolites such as LTB₄, and, consequently, the infiltration of neutrophils within the airways of chronic bronchitis subjects.

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