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

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15(S)-HETE modulates LT(B₄) 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 (LT(B₄)) production in vitro and evaluated the concentration of LT(B₄) in induced sputum and the contribution of LT(B₄) 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...
increase in their FEV1 after an inhaled dose of 200 
below 70% of that predicted and displayed a 10% or smaller 
pation.

fee, and patients gave written informed consent for partici-

duction of the production of leukotriene B$_4$ (LTB$_4$), 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 sub-
jects. 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 (FEV$_1$) 
below 70% of that predicted and displayed a 10% or smaller 
increase in their FEV$_1$ 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 
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 commit-
tee, and patients gave written informed consent for participi-
ation.

**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. Sam-
ples were periodically removed from the water bath for fur-
ther 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 cen-
trifuge 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 cen-
trifuged at 200 g for 5 min at RT, and the cell pellet was finally 
resuspended in PBS to a final concentration of 10$^6$ cells/ml. 
Aliquots (10 µl) were used to assess cell viability by trypan 
blue exclusion, whereas differential cell counting was ob-
tained from cytocentrifuged slides (Cytospin 2; Shandon In-

struments, Runcorn, UK) stained with Diff Quick (Merz-
Dade, Dudingen, 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 sub-
tracted 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-asso-
ciated 15(S)-HETE.

**Analysis of 15(S)-HETE in supernatants.** The superna-
tants from induced sputum samples were acidified with 50 µl of 
formic acid. Extraction was carried out on octadecylsilyl 
(ODS) cartridges (Supelclean LC-18; Supelco, Bellafonte, 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 benzine, and eluted with 4 ml of 
ethyl acetate. The ethyl acetate fractions were collected, 
evaporated to dryness at 60°C, 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 
(Reckman Analytical, Palo Alto, CA) equipped with an Ultra-
sphere 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). Control 
tube containing 15(S)-HETE standard was 21 ± 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 
were collected for the retention time of 15(S)-HETE standard 
were pooled, dried under vacuum, reconstituted with RIA buffer, and then assayed for 15(S)-HETE with a 
specific RIA kit (Advanced Magnetic, Framingham, MA) per-
formed according to the manufacturer’s protocol. Cross-reac-
tivity with different eicosanoids, according to the manufac-
turer, 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 (10$^6$ cells/ml).

**Analysis of cell-associated 15(S)-HETE.** The sputum cells’ 
suspensions were extracted with 2 vol of 2-propanol contain-
ing 1.2% (vol/vol) acetic acid and chloroform (1:1, vol/vol) 
treatment of 1.2% (vol/vol) acetic acid (100/0.1, vol/vol), 
and then assayed for 15(S)-HETE with a 
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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 (10$^6$ cells/ml).

**Purification of neutrophils from peripheral blood.** Peripheral 
blood polymorphonuclear leukocytes were prepared from 
normal and chronic bronchitis subjects with the use of dex-
trane 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 (×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 control and chronic bronchitis subjects after immunohistochemical characterization by using the immunoglobulin-like antiphosphatase-antiphosphatase (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-eosinophilic (EG1) and anti-cytokine 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 ×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 LTB4 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 LTB4, we measured LTB4 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 LTB4 production was studied in vitro using isolated human neutrophils, challenged either with the calcium ionophore A-23187 (1 μM, 10 min, 37°C) or with the formylated tripeptide formyl-methionine-leucine-phenylalanine (fMLP; 0.1 μM, 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 LTB4 production, isolated human neutrophils (107 cells) were incubated with 15(S)-HETE (30 μM) for 30 min and washed before GM-CSF priming and fMLP challenge.

LTB4 and its α-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/106 cells [high-15(S)-HETE producers: 50.5 (36–109) ng/106 cells] and from three subjects with 15(S)-HETE concentrations <8 ng/106 cells [low-15(S)-HETE producers: 2.2 (1.3–6.1) ng/106 cells]. Neutrophils obtained from the peripheral blood of healthy donors were resuspended at a concentration of 1 × 106/ml in PBS containing CaCl2 (0.5 mM) and MgCl2 (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 LTB4, experiments were performed in the presence and absence of an LTB4 receptor antagonist (10 μM LY-229862; 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 μm. 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 ×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 ×106 cells/ml) than in control subjects (1.2, 1–1.8 ×106 cells/ml), although the difference was not statistically significant. The viability of sputum cells was 75% (range:

<table>
<thead>
<tr>
<th>Table 1. Differential cell counts of induced sputum samples</th>
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<tbody>
<tr>
<td>Control</td>
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</tr>
<tr>
<td>Macrophages</td>
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<tr>
<td>Neutrophils</td>
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<tr>
<td>Lymphocytes</td>
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<tr>
<td>Eosinophils</td>
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<td>Epithelial cells</td>
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</table>

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).
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-
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), 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 LT**\textsubscript{B}\textsubscript{4} in supernatants. To understand whether the inverse relationship between 15(S)-HETE levels and percentage of neutrophils involved modification of the chemotactic factor LT**\textsubscript{B}\textsubscript{4}, we measured this mediator into sputum samples and found that LT**\textsubscript{B}\textsubscript{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 LT**\textsubscript{B}\textsubscript{4} production. Exogenous 15(S)-HETE inhibited LT**\textsubscript{B}\textsubscript{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 LT**\textsubscript{B}\textsubscript{4} production was observed at concentrations of 15(S)-HETE lower than 1 \( \mu \text{M} \), whereas at 3 and 10 \( \mu \text{M} \), 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 LT**\textsubscript{B}\textsubscript{4} production on GM-CSF priming and fMLP challenge (–32 ± 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/10\(^6\) cells [low-15(S)-HETE producers] caused a greater neutrophil chemotaxis than samples with 15(S)-HETE concentrations >8 ng/10\(^6\) cells [high-15(S)-HETE producers] (Fig. 7A). In addition, preincubation with the LT**\textsubscript{B}\textsubscript{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 LT**\textsubscript{B}\textsubscript{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
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 LTB₄, 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 LTB₄ in induced sputum samples. In agreement with the latter observation, exogenous 15(S)-HETE inhibits LTB₄ 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 LTB₄ and concentrations of 15(S)-HETE was strength-
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/10^6 cells), compared with samples in which 15(S)-HETE concentration was higher than 8 ng/10^6 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 production elicited in isolated human neutrophils by 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.

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

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<thead>
<tr>
<th>mRNA-Positive Cells, %</th>
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<tbody>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Total cells</td>
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<tr>
<td>Macrophages</td>
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<td>Neutrophils</td>
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<td>Lymphocytes</td>
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<td>Eosinophils</td>
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<td>Epithelial cells</td>
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</table>

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).

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).
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 A4 and lipoxin B4, AA metabolites arising from the sequential action of 5- and 15-LO, inhibit LTB4, 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.
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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REFERENCES


