

15-Lipoxygenase expression and 15(S)-hydroxyeicoisatetraenoic acid release and reincorporation in induced sputum of asthmatic subjects

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Background: Recent evidence shows that 15(S)-hydroxyeicoisatetraenoic acid (15[S]-HETE) can be released and rapidly reincorporated into cellular lipids. These mechanisms exert several immunoregulatory functions that may be relevant in airway inflammation.

Objective: Our purpose was to evaluate the levels of both soluble and cell-associated 15(S)-HETE and to examine 15-lipoxygenase (15-LO) messenger RNA (mRNA) expression in sputum samples obtained from 10 control and 18 asthmatic subjects.

Methods: Levels of 15(S)-HETE were measured by reverse-phase HPLC separation followed by RIA in supernatants and in cell membrane-extracted phospholipids after acid hydrolysis. 15-LO mRNA was evaluated by primed in situ hybridization (PRINS). Combined immunocytochemistry and PRINS was used to identify the phenotype of cells bearing 15-LO transcripts.

Results: Levels of both soluble and cell-associated 15(S)-HETE were higher in asthmatic than in control subjects ($P < .0001$). The percentage of cells expressing 15-LO mRNA was higher in asthmatic than in control subjects ($P < .01$). On double staining for specific cell-type markers and 15-LO mRNA, macrophages were the major source for 15-LO.

Conclusion: This study shows that the induced sputum technique allows the evaluation of 15-LO activity and that soluble, cell-associated 15(S)-HETE and 15-LO levels are higher in asthmatic than in control subjects. In addition, this study indicates that, in induced sputum, airway macrophages are the major source of 15(S)-HETE in asthma. (*J Allergy Clin Immunol* 2000;105:711-6.)

Key words: 15(S)-HETE, asthma, airway macrophages, induced sputum

The release of arachidonic acid (AA) metabolites, such as leukotrienes and PGs¹ by airway cells plays an

Abbreviations used

AA:	Arachidonic acid
APAAP:	Alkaline phosphatase-antialkaline phosphatase
15-LO:	15-Lipoxygenase
mRNA:	Messenger RNA
PI:	Phosphatidyl inositol
PRINS:	Primed in situ labeling
RT:	room temperature
15(S)-HETE:	15(S)-hydroxyeicoisatetraenoic acid

important role in the pathogenesis of bronchial asthma. 15(S)-hydroxyeicoisatetraenoic acid (15[S]-HETE), represents the main AA metabolite synthesized by human trachea,² human bronchi,³ and human parenchyma⁴ and can be produced by several cells such as macrophages,⁵ eosinophils,⁶ and airway epithelial cells.⁷ 15(S)-HETE arises from the metabolism of AA through the 15-lipoxygenase (15-LO) pathway, whereas its stereoisomer 15(R)-HETE has recently been described as the main product of aspirin-treated cyclo-oxygenase-2 isoenzyme, as well as one of the metabolites of cytochrome-P450 mono-oxygenases. Little is known about the potential biologic activities of 15(R)-HETE, whereas there is now substantial evidence that 15(S)-HETE can play an important role in the pathophysiologic mechanisms of asthma, being able to increase mucus secretion in human airways,⁸ to prolong the duration of airway obstruction during the early response,⁹ contributing to several immunoregulatory functions that may be relevant in airway inflammation¹⁰ associated with the disease. Although most of these biologic activities depend on the release of 15(S)-HETE and its subsequent interaction with specific target cells, it is now increasingly evident that some immunoregulatory functions of this mediator are also related to its reincorporation into cellular lipids.

Several studies have indeed documented this phenomenon, showing that exogenous or endogenously generated 15(S)-HETE can be rapidly metabolized or reincorporated into cellular lipids either spontaneously or after stimulation.¹¹⁻¹⁴ Of particular interest is the evidence that 15(S)-HETE incorporation can be modulated by the external microenvironment. We recently found that the reincorporation of 15(S)-HETE into phospholipids of

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pulmonary epithelial cells, as well as the expression of 15-LO, can be modulated by cytokines such as IL-4, a cytokine playing a major role in the pathogenesis of airway inflammation in asthma.¹⁵ In addition, ozone exposure has also been found to increase 15(S)-HETE production and reincorporation in airway epithelial cells, leading to reduced synthesis of PGE₂, a cyclo-oxygenase metabolite of AA possessing several anti-inflammatory activities in the airways.¹⁶ 15(S)-HETE reincorporation into membrane phospholipids seems therefore to exert autocrine functions, affecting the response of inflammatory cells to chemotactic stimuli,¹¹ possibly through the modification of intracellular signal transduction pathways.¹⁷ It is therefore likely that 15(S)-HETE reincorporation may play a major role in the regulation of cell activation and on the release of inflammatory mediators.

In the current study we evaluated the levels of both soluble and cell-associated 15(S)-HETE in induced sputum samples obtained from 10 control subjects and 21 asthmatic subjects. In addition, to evaluate whether cells recovered from induced sputum were actively expressing 15-LO, we studied 15-LO messenger RNA (mRNA). Finally, to identify the phenotype of cells expressing 15-LO mRNA, we performed combined immunohistochemistry and *in situ* hybridization on cells recovered from induced sputum.

METHODS

Patients

The study was performed on 2 groups of subjects: 10 control subjects and 18 patients with asthma. Asthmatic subjects were 21 to 72 years old (median and percentiles: 43, and 30 to 55 years, respectively). Asthma was diagnosed on the basis of criteria previously described in detail.¹⁸ None of the subjects was a current or previous smoker. Patients were excluded from the study if they had undergone a severe exacerbation of asthma requiring hospitalization during the month preceding the study. Inhaled corticosteroids or oral corticosteroids were withdrawn for at least 2 months before commencement of the study, whereas the use of nedocromil sodium or cromoglycate was stopped for at least 2 weeks and theophylline in the previous 48 hours.

Control subjects were 25 to 43 years old (age median and percentiles: 31 and 29 to 37 years, respectively) and were selected for the study. None of these subjects had ever had asthma. They had not had any bronchial or respiratory tract infection during the month preceding the study. All subjects were lifelong nonsmokers, and their pulmonary function was within the normal range.

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

Induced sputum production and sample processing

Each subject underwent spirometry before the beginning of the procedure. Induced sputum production and processing were carried out according to Fahy et al¹⁹ and Maestrelli et al²⁰ with slight modifications.²¹ The homogenized sputum and saliva were centrifuged at 800g for 10 minutes 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, as previously described.¹⁵ With use of synthetic 15(S)-HETE standards,

we previously found that sputum processing does not affect 15(S)-HETE levels. The average recovery estimated with use of radioactive 15(S)-HETE ranged from 70% to 85%.

The cell pellet was resuspended in saline solution and filtered through a 70-μm nylon mesh. The eluate was centrifuged at 200g for 5 minutes at RT, and the cell pellet was finally resuspended in PBS to a final concentration of 106 cells per milliliter. Ten microliter aliquots were used to assess cell viability by Trypan blue exclusion, whereas differential cell counting was obtained from cytocentrifuged slides (Cytospin 2, Shandon Instruments, Runcorn, UK) stained with Diff-Quik (Merz-Dade, Duding, Switzerland). The slides were read blindly by 2 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 400g for 10 minutes 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 cell-associated 15(S)-HETE

The sputum cell suspensions were extracted with 2 volumes of 2-propanol containing 1.2% (vol/vol) acetic acid and chloroform (1:1, vol/vol) according to Conrad et al.²² The resulting mixture was vortexed and centrifuged at 2500g for 15 minutes at RT. The upper and the intermediate phases were removed, whereas the lipids containing lower phase were dried under nitrogen and resuspended in 400 μL of methanol. An aliquot was treated with 100 μL of sodium hydroxide 2N and incubated at 60°C for 30 minutes under argon atmosphere.²³ 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 2500g for 10 minutes at RT, samples were analyzed for 15(S)-HETE by reverse-phase HPLC separation, followed by RIA as described.¹⁵

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 al²⁴ with minor modifications.¹⁵

At least 4 fields per slide were evaluated under light microscopy (×400 final magnification). Results were expressed as the percentage of 15-LO mRNA-positive cells over the total.

Identification of 15-LO bearing cells

To identify the phenotype of cells expressing 15-LO mRNA, PRINS was performed after immunohistochemical characterization with use of the immunoalkaline phosphatase-antialkaline phosphatase (APAAP) method, according to Cordell et al²⁵; the following mAbs were used: mAb anti-CD68 (EBM II, Dakopatts, Glostrup, Denmark; dilution 1:100), mAb antineutrophil elastase (antihuman neutrophil elastase cell line NP 57, dilution 1:150), mAb antieosinophil (EG1) and mAb antieosinophil cationic protein (EG2) (Kabi Pharmacia, Uppsala, Sweden; dilution 1:5),²⁶ mAb anti-antigen-primed T cells (anti-UCHL1, Dako; dilution 1:50).²⁷ Control slides were included in each staining session with an irrelevant mAb (anti-smooth muscle, Ortho Pharmaceutical, Raritan, NJ).

Statistical analysis

Results are expressed as medians and 25th to 75th percentiles. Statistical analysis was performed with the Mann-Whitney *U* test to assess differences between groups. Spearman's rank correlation was calculated to assess the correlation between data.

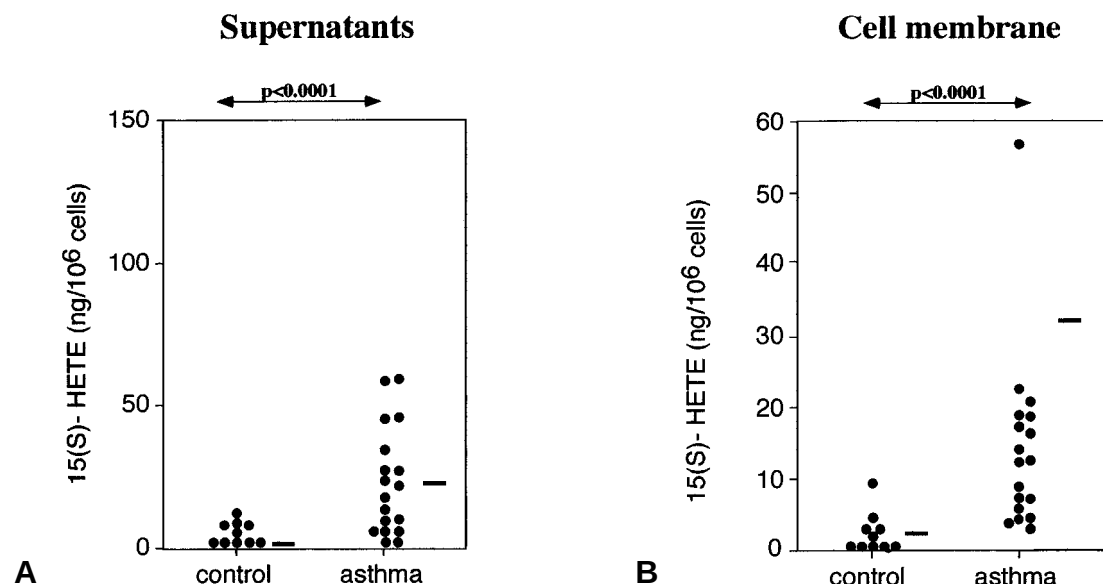


FIG 1. 15(S)-HETE concentration in induced sputum obtained from control and asthmatic patients. **A**, 15(S)-HETE concentration in supernatants. **B**, 15(S)-HETE concentration in cellular lipids. 15(S)-HETE was analyzed by specific RIA after reverse-phase HPLC separation. 15(S)-HETE was released from cellular lipid by use of base-catalyzed hydrolysis as described in Methods. Results are expressed as nanograms per 10^6 cells. The line represents the median value for each data set. Statistical analysis was performed with the Mann-Whitney *U* test.

RESULTS

Demographic characteristics of patients

The median ages of patients with asthma and of control subjects were similar. FEV₁ values in asthmatic patients ranged from 49% to 131% of predicted values (median, 25th-75th percentiles: 85.5, 77%-91% of predicted).

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 asthmatic patients (Table I). The corrected median total cell counts was similar for control subjects (1.2, 1-1.8 10^6 cells/mL) and asthmatic patients (0.9, 0.6-1.7 10^6 cells/mL). The viability of sputum cells was 75% (range 68% to 80%) in controls and 74% (range 62% to 83%) in asthma. No significant correlation between cell viability and 15(S)-HETE levels was observed in any group. The percentage of neutrophils was significantly higher in asthmatic than in control subjects ($P < .02$). The percentage of eosinophils was significantly higher in asthma than in control subjects ($P < .0001$). No differences were observed for lymphocytes and epithelial cells (Table I).

In saliva, the median percentage of the total cell count was not significantly different in controls (0.8, 0-2 10^6 cells/mL) and asthmatics (1, 0-3 10^6 cells/mL).

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 asthmatic

TABLE I. Differential cell counts of induced sputum samples

Cell type	Control	Asthma	Statistical significance
Macrophages	81.8 (80-89.4)	52 (40.5-71.6)	$P < .0005$
Neutrophils	16.5 (10-23)	42.8 (21.7-48.8)	$P < .02$
Lymphocytes	0 (0-0.3)	0 (0-0.6)	NS
Eosinophils	0	2.7 (2.1-4.6)	$P < .0001$
Bronchial epithelial cells	0 (0-0.1)	0 (0-2.6)	NS

Data are expressed as medians (25th-75th percentiles). Statistical analysis was performed with the Mann-Whitney *U* test to assess differences between groups. NS, Not significant.

ic (median and 25th-75th percentiles: 17.5, 11.8-17.5) than in control subjects (median and 25th-75th percentiles: 3, 1.9-8.1) ($P < .0001$, Mann-Whitney *U* test) (Fig 1). 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, were found to be significantly higher in induced sputum of asthmatic (median and 25th-75th percentiles: 32, 22-50) than in sputum of healthy subjects (median and 25th-75th percentiles: 3.5, 0-13) ($P < .0001$ Mann-Whitney *U* test) (Fig 1). Cell-associated 15(S)-HETE levels in cell pellet from saliva were not detectable.

In asthma, the level of soluble 15(S)-HETE was significantly correlated with the percentage of macrophages ($p = 0.6$, $P < .03$, Spearman rank test) (Fig 2). By contrast, soluble 15(S)-HETE was not correlated with eosinophils and neutrophils percentages.

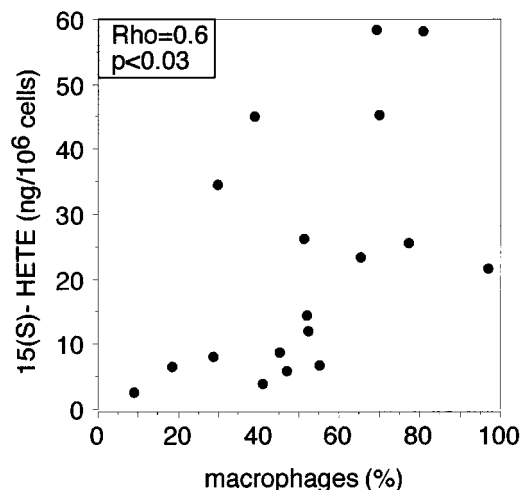


FIG 2. Correlation between concentration (expressed as ng/10⁶ cells) of 15(S)-HETE in supernatants and percentage of macrophages in induced sputum samples obtained from asthmatic patients. Statistical analysis was performed with the Spearman rank test.

TABLE II. Percentage of 15-LO mRNA-positive cells in induced sputum samples

	Controls	Asthma	Statistical significance (control/asthma)
mRNA-positive cells	15 (5-18)	71 (68-78)	$P < .01$
Macrophages (%)	13 (0-20.5)	70 (44.5-77)	$P < .01$
Neutrophils (%)	0 (0-0.25)	1 (1-2)	NS
Lymphocytes (%)	0 (0-0.1)	1 (0-1)	NS
Eosinophils (%)	0 (0-0)	0 (0-0)	NS
Epithelial cells (%)	0 (0-0.5)	0 (0-0)	NS

NS, Not significant.

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 recovered from the 2 study groups. The percentage of positive cells in induced sputum obtained from asthmatic patients were significantly higher than in control subjects ($P < .01$, Mann Whitney *U* test) (Table II, Fig 3). To identify the phenotype of cells expressing 15-LO mRNA, we performed combined immunohistochemistry and in situ hybridization on cells recovered from induced sputum. In asthma a higher percentage of macrophages expressed transcripts for 15-LO than in controls ($P < .01$, Mann Whitney *U* test) (Fig 3), as identified through labeling with an mAb anti-CD68. In both asthmatic and control subjects, the percentage of neutrophils, T lymphocytes, and EG-1- or EG-2-positive eosinophils expressing 15-LO mRNA was very low and not statistically different.

DISCUSSION

The current study shows that the levels of 15(S)-HETE measured in induced sputum samples obtained from asthmatic subjects are higher than in control subjects and that airway cells isolated from sputum samples of asthmatic subjects are characterized by an increased ability to reincorporate 15(S)-HETE into cellular lipids, an index of cellular esterification of this AA metabolite.¹⁵ In addition, in asthma the increased levels of 15(S)-HETE are accompanied by a higher percentage of cells expressing 15-LO mRNA. Finally, in asthma the concentration of soluble 15(S)-HETE significantly correlated with the percentage of macrophages, which also appeared to be the major 15-LO mRNA-bearing cells.

Limited amounts of data are available concerning values of 15(S)-HETE in human airways in vivo. Increased levels of 15(S)-HETE were reported by Murray et al²⁸ 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.²⁹ For the first time we provide evidence of highly significant differences in basal levels of this AA metabolite in the induced sputum obtained from asthmatic and control subjects.

The release of 15(S)-HETE can exert several biologic actions that may be relevant in the pathogenesis or in the control of airway inflammatory disorders. 15(S)-HETE has been shown to be a potent mucosecretagogue in human airway⁸ and to possess chemotactic activity for neutrophils directly contributing to the recruitment of these cells in dog airways.³⁰ It has also been found that 15(S)-HETE can prolong the duration of airway obstruction during the early allergic response, suggesting that it may increase the release of mediators from mast cells or potentiate the effects of other mediators on airway smooth muscle.⁹

An additional mechanism by which 15(S)-HETE can also influence the functional characteristics of airway cells is its cellular reincorporation by esterification into phospholipids of the cell membrane. Indeed, it has been shown that 15(S)-HETE can be rapidly metabolized or incorporated into phosphatidyl inositol (PI) of the cell membrane of human tracheal epithelial cells.^{13,31} In addition, the exposure of human tracheal epithelial cells to ozone, the major oxidant in photochemical air pollution, increases 15(S)-HETE production as well as its esterification into phospholipids of airway epithelial cells.¹⁶

In the current study we show for the first time that 15(S)-HETE reincorporation can be observed in airway cells isolated from induced sputum samples and that such a process is greater in asthmatic than in control subjects. To better understand the biologic relevance of this phenomenon and its potential consequences, additional in vitro and ex vivo studies are needed. However, it is possible to hypothesize that the reincorporation of 15(S)-HETE into phospholipids of the cell membrane can modulate the cellular response to specific stimuli.^{13,15,31} The

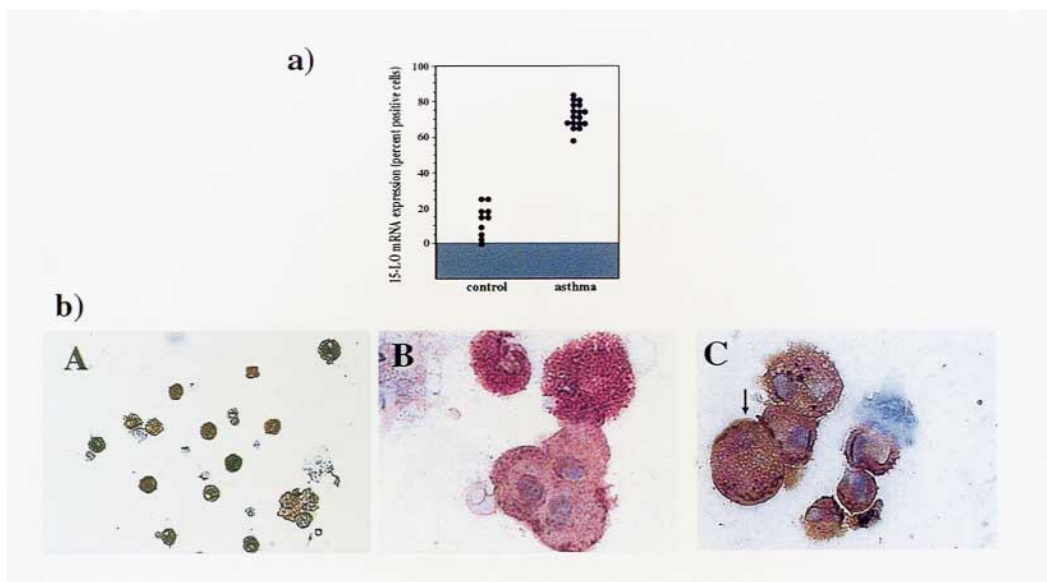


FIG 3. **a**, Expression of 15-LO mRNA by cells isolated from induced sputum of control and asthmatic subjects. **b**, Expression of 15-LO mRNA on cytospin preparations of cells isolated from induced sputum obtained from asthmatic subject. **A**, Airway cells expressing 15-LO mRNA, identified with PRINS technique. (Original magnification $\times 160$.) **B**, Macrophages identified by immunocytochemistry (APAAP) with use of an mAb anti-CD68. (Original magnification $\times 400$.) **C**, Airway macrophages expressing 15-LO mRNA, identified combining PRINS and APAAP techniques (arrow). (Original magnification $\times 400$.) Brown staining is to the PRINS technique. Red staining is the result of the APAAP technique.

presence of 15-HETE in PI has been shown to reduce its subsequent phosphorylation into PIP and PIP₂, decreasing the formation of second messengers such as diacylglycerol and inositol triphosphate.¹⁴ In addition, the reuptake of released 15-HETE may contribute to decrease the pericellular concentration of this proinflammatory mediator, promoting the resolution of inflammatory processes.

15(S)-HETE esterified into cellular phospholipids has been shown to modulate the receptor-mediated activation of human neutrophils³² and to decrease the ability to generate leukotriene B₄ and to cause a marked reduction in agonist-induced cell aggregation.¹¹ Moreover, the incorporation of 15-HETE into cellular phospholipids of airway epithelial cells has been found to reduce the synthesis of PGE₂, a different AA metabolite possessing several anti-inflammatory activities.¹⁶ Therefore it is likely that the reuptake of 15(S)-HETE by airway cells may represent an important mechanism regulating the development of airway inflammation.^{13,16} Recent evidence has indeed shown that 15(S)-HETE can influence the functional activation of airway macrophages, leading to increased production of 5,15-diHETE and lipoxins by these cells,²³ 2 mediators that are considered as markers of exogenous 15(S)-HETE metabolism.³³

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.³⁴ In addition, 15-LO mRNA and 15-LO enzymatic activity has also been reported in human alveolar

macrophages.⁵ Although the evidence about the functional properties of airway macrophages isolated from induced sputum are still limited, the role of these cells has been extensively studied in asthma with use of bronchoalveolar lavage. Most studies have shown an increased activation of airway macrophages in asthma,³⁵⁻³⁹ and it has been consistently shown that airway macrophages release spontaneously or after activation increased levels of various AA metabolites, including 15-HETE.^{35,40-43}

The results of the current study confirm and extend this evidence, demonstrating that macrophages isolated from induced sputum are a major source of 15(S)-HETE in the airways of asthmatic subjects. With use of in situ hybridization and immunohistochemistry, we indeed observed that in sputum samples from asthmatic subjects most of the cells bearing transcripts for 15-LO mRNA were alveolar macrophages.

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 asthmatic 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 on several airway cells. 15-LO expression and 15(S)-HETE production, as well as incorporation of this AA metabolite into cellular lipids, may represent important regulatory mechanisms operating in airway inflammation.

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