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Generation of Eicosanoids from 15(S)-Hydroxyeicosatetraenoic Acid in Blood Monocytes from Steroid-dependent Asthmatic Patients

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ABSTRACT. The aim of this study was to investigate eicosanoid metabolism by human peripheral blood monocytes (PBM) from steroid-dependent asthmatic patients as compared to control subjects and untreated asthmatic patients. Eicosanoid biosynthesis by PBM isolated from venous blood using Percoll gradient centrifugation was evaluated following stimulation of 5×10^6 cells with calcium ionophore A23187, with or without exogenous 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), and analyzed by reverse phase high performance liquid chromatography (RP-HPLC). Without 15(S)-HETE, PBM synthesized leukotriene B_4 (LTB_4) only (40 ± 12 ng and 59 ± 11 ng for untreated and steroid-dependent asthmatics, respectively). In the presence of 15(S)-HETE, PBM produced six-fold smaller amounts of leukotriene B_4 ($P < 0.0001$). They also released 5(S),15(S)-dihydroxyeicosatetraenoic acid (5(S),15(S)-diHETE) in similar amounts for all the populations, whereas low amounts of lipoxins (LXs) were produced by PBM from asthmatics only (2.7 ± 0.7 ng and 4.6 ± 2.8 ng for untreated and steroid-dependent asthmatics, respectively). Moreover, PBM were also able to release an unknown compound containing conjugated triene chromophore. Cells from steroid-dependent asthmatic patients synthesized this unknown metabolite in higher amounts than controls and untreated asthmatics (133 ± 18 ng vs 52 ± 19 ng and 68 ± 15 ng, respectively, $P < 0.02$).This work shows for the first time that human PBM are able to metabolize 15(S)-HETE and lead to lipoxins and to an unknown metabolite, with the amounts of the latter being enhanced by long-term corticosteroid treatment. BIOCHEM PHARMACOL **56**;4:535–541, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. monocytes; asthma; corticosteroids; leukotriene B_4; 15(S)-HETE; lipoxins

AA† metabolites from the 5-LO pathway, such as HETEs, LTs and LXs, are associated with bronchial asthma [1–3]. These products have been detected *in vivo* in bronchoalveolar lavage fluid [4–8] and are biosynthesized *in vitro* by isolated phagocytic cells such as human alveolar macrophages and neutrophils after nonspecific stimulations and incubations [9] both in the presence or absence of exogenous 15(S)-HETE [10]. Cells obtained from asthmatic patients produce higher amounts of LTs and LXs than those from control subjects [11–14].

The synthesis of lipid mediators is initiated by phospholipase A2, which selectively releases AA from cell membranes. AA is then converted into 5(S)-HPETE, leukotriene A_4 and LTB_4 by successive actions of 5-LO. Cysteinyl LTs synthesis requires the action of leukotriene C_4 synthase on leukotriene A_4, in the same cell type or by transcellular metabolism after leukotriene A_4 release [15]. LX formation is the result of transcellular metabolism and the consequent involvement of at least two LO [10, 16], or they may be formed by the action of only one LO following specific activation [17, 18].

We have previously studied LT production by alveolar macrophages and blood neutrophils, assuming that peripheral blood cells were activated before trafficking to the airways. The phospholipid fatty acid composition of human monocyte preparations shows a 23.5 ± 0.9% level for AA [19], whereas the amounts of AA in macrophages and peripheral blood neutrophils are 15.7 ± 4.1% and 9.5 ± 1.5% of the total fatty acid amounts, respectively (Damon M and Chavis C, unpublished observations). Although the amounts of AA in PBM are greater than those found in other phagocytic cells, alveolar macrophages were described to have greater amounts of 5-LO enzyme than monocytes [20]. Thus, we studied eicosanoid production to investigate whether a 5-LO activity is displayed by PBM and modulated by diseases and treatments. The enzyme activity was evaluated by analyzing the structure and levels of the eicosanoids produced *in vitro* after cellular stimula-

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† *Abbreviations*: AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; 15(S)-HETE, 15(S)-hydroxyeicosatetraenoic acid; 5(S),15(S)-diHETE, 5(S), 15(S)-dihydroxy-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; LTB_4, 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; LXs, lipoxines; PBM, peripheral blood monocytes; RP-HPLC, reverse phase high performance liquid chromatography.

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TABLE 1. Characteristics of healthy subjects and asthmatic patients

	Controls	Untreated Asthmatics	Steroid-dependent Asthmatics
Number	9	18	29
Age (years)	28 ± 5	52 ± 4	55 ± 2
Steroid Dose*	—	—	18 (2.5 to 80)
FEV_1†	100 ± 2	74 ± 6	72 ± 3

*Oral prednisolone doses are expressed as mg/day.

†FEV_1 is forced expiratory volume in 1 sec, expressed as % of the predicted value.

Data are expressed as mean values ± SEM, except for oral prednisolone doses which are expressed as averages, with minimum and maximum doses.

tion by Ca^{2+} ionophore A23187 with or without 15(S)-HETE.

Glucocorticoids are commonly used in the treatment of inflammatory diseases, particularly in severe asthma. They exert a variety of effects on inflammatory cells, depending on the mode of administration and duration of the treatment. Because there are no studies reporting the effect of long-term treatment by glucocorticoids on monocyte lipoxygenase activities, we assessed this effect in PBM obtained from control subjects, untreated and steroid-dependent asthmatic patients.

MATERIALS AND METHODS

Reagents

Culture materials, nutritive medium and fetal calf serum (FCS) were obtained from Life Technologies. All solvents were of HPLC grade and obtained from Farmitalia. Calcium ionophore A23187 was purchased from Sigma. LTB_4, 5(S),15(S)-diHETE, 8(S),15(S)-diHETE, LXs, 5, 12, and 15(S)-HETE were from Cayman Chemical Co (Ann Arbor, MI). Zileuton, a 5-LO inhibitor, was a generous gift from Dr. James V. MacArdle (SmithKline Beecham).

Selection of Subjects

The study included 9 controls (healthy subjects), 18 untreated asthmatic patients, and 29 steroid-dependent asthmatic patients. None of the subjects were smokers. Asthma was defined according to the American Thoracic Society as previously described in detail [21]. Forced expiratory volume in 1-sec values of asthmatics ranged from 53 to 102% of the predicted values for untreated asthmatics and from 45 to 117% of the predicted values for the steroid-dependent ones. These patients were included on the basis of their long-term treatment (at least 1 year with oral steroid [prednisolone] and inhaled steroid (beclomethasone) and their continued requirement for oral steroids (Table 1). The most recent dose of steroid was administered the day before the experiment.

PBM Preparation

Cells were isolated and purified from heparinized (20 U/mL) venous blood (20 mL) by centrifugation of samples at 400 *g* for 20 min at 20°, over a discontinuous Percoll gradient [22]. PBM were aspirated from the upper layer, added to an equal volume of saline and then centrifuged at 400 *g* for 10 min, whereas granulocytes remained in the under layer. The pellet was resuspended in 10 mL of a solution of $NH_4Cl/Tris/K_2CO_3$ (0.130 M/0.01 M/0.016 M) (pH 7.4) to lyse contaminating erythrocytes. After centrifugation, PBM were resuspended in RPMI medium 1640 and counted using neutral red stain. 5×10^6 cells were incubated in Petri dishes with 10% FCS in a humidified atmosphere of 95% air and 5% CO_2, and allowed to adhere for 2 hr. The culture medium was then removed and the PBM covered with 1 mL of PBS (pH 7.4) containing $CaCl_2$ and $MgCl_2$ (final concentrations 2×10^{-3} M and 0.5×10^{-3} M, respectively). The cells were prewarmed at 37° for 5 min prior to stimulation with calcium ionophore. The purity of PBM, evaluated using May Grünwald staining on fixed cell preparations obtained by cytocentrifugation, was always greater than 95%. Viability determined by exclusion of Trypan blue stain was greater than 90%.

Stimulation Procedures

A23187 (final concentration 5×10^{-6} M) was added to the cell monolayer, with or without 15(S)-HETE (final concentration 3×10^{-6} M). All incubations were carried out for 30 min at 37° at the end of which PBM were scraped into their incubation medium and added to the same volume of cold methanol in order to stop reactions and extract the metabolites. Cells and supernatants were stored at −20° until analysis of mediators. Seven experiments were carried out on cells prewarmed to 37° in the presence of zileuton (5×10^{-6} M) as inhibitor of 5-LO.

Metabolite Identification and Quantification

After thawing and centrifugation, the supernatants of the stored samples were immediately subjected to RP-HPLC analysis as previously described [13].

LT, LX and unknown product detection. RP-HPLC was carried out on a Lichrospher 100 RP-18 column (150 mM × 3.9 mM, 5 μm particles; Merck). LXs, LTs, 5(S),15(S)-diHETE, 8(S),15(S)-diHETE and the unknown product were eluted with methanol/water/acetic acid (65: 35: 0.1, v/v/v, pH 5.6) as the mobile phase, at a flow rate of 0.5 mL/min for 15 min and 1 mL/min thereafter. The products were detected by simultaneously monitoring wavelengths of 302 nm, 270 nm and 246 nm, corresponding to the λ_{max} of conjugated tetraenes, trienes and dienes, respectively.

Identification and quantification. AA metabolites were identified using co-chromatography with synthetic standards and UV spectroscopy in the stop-flow mode. They

were quantified using the external standard method, on the basis of molecular extinction coefficients of 55,000, 35,000 and 28,000 for conjugated tetraenes, trienes and dienes, respectively. The sensitivity threshold was 0.2 ng at 302 nm and 0.5 to 1 ng at the other wavelengths.

Statistical Analysis

Results are presented as means ± SEM ng of product per 5×10^6 cells. Statistical differences were determined using the Mann-Whitney *U*-test for unpaired samples (controls: N = 9, untreated asthmatics N = 18, steroid-dependent asthmatic patients: N = 29). Significance level was set at $P < 0.05$.

RESULTS

Activities of lipoxygenase enzymes in human PBM were investigated using conditions of stimulation by calcium ionophore A23187. Experiments were performed in the presence or absence of exogenous 15(S)-HETE on the cells from 9 controls, 18 untreated and 29 steroid-dependent asthmatic patients.

Eicosanoid Biosynthesis

Stimulation of human PBM by A23187 alone generated a conjugated triene detected at 280 nm, co-eluting with LTB_4. There was no evidence of any metabolite such as 20-hydroxy LTB_4 or Δ6-trans isomers of LTB_4. Exposure of PBM to A23187 in the presence of 15(S)-HETE resulted in the generation of products containing conjugated triene and tetraene chromophores. The metabolites were detected simultaneously at 246, 280 and 302 nm in the same sample injection. The product detected at 246 nm co-eluted with 5(S),15(S)-diHETE. The profile recorded at 280 nm (Fig. 1) showed two peaks with retention times of 22.3 and 23.9 min, corresponding to LTB_4 and the unknown product, respectively. The UV spectrum of the unknown product was characteristic of a conjugated triene, with maximum absorption at 272 nm and shoulders at 260 and 286 nm. This metabolite did not co-elute with 5,6-diHETEs nor with 8(S),15(S)-diHETE. The trihydroxy derivatives containing conjugated tetraene, detected at 302 nm, co-eluted with natural LXs and their all-trans isomers.

The eicosanoid pattern obtained after *in vitro* stimulation of PBM differed according to the mode of activation. Figures 2A and B show the amounts of eicosanoids synthesized by PBM from untreated and steroid-dependent asthmatics, respectively, stimulated by A23187 with and without 15(S)-HETE. When the cells were incubated in the presence of 15(S)-HETE, the amounts of LTB_4 were significantly lower: 6 ± 2 vs 40 ± 12 ng/5×10^6 cells, $P < 0.05$, for untreated asthmatics and 14 ± 4 vs 59 ± 11 ng/5×10^6 cells, $P < 0.001$, for steroid-dependent asthmatics. The PBM generated an unknown product: 68 ± 15 ng/5×10^6 cells for untreated asthmatics and 133 ± 18 ng/5×10^6 cells for steroid-dependent asthmatics. They also transformed exogenously added 15(S)-HETE into dihydroxy derivatives, 5(S),15(S)-diHETE: 88 ± 14 ng and 102 ± 21 ng/5×10^6 cells, for untreated and steroid-dependent asthmatics, respectively. The PBM generated low amounts of LXs: 4.5 ± 2.8 ng and 2.7 ± 0.7 ng/5×10^6 cells for untreated and steroid-dependent asthmatics, respectively. When the cells of the three populations were incubated under the same conditions, but in the presence of zileuton, none of the above metabolites was detected. The unknown product was only generated when PBM were incubated with 15(S)-HETE.

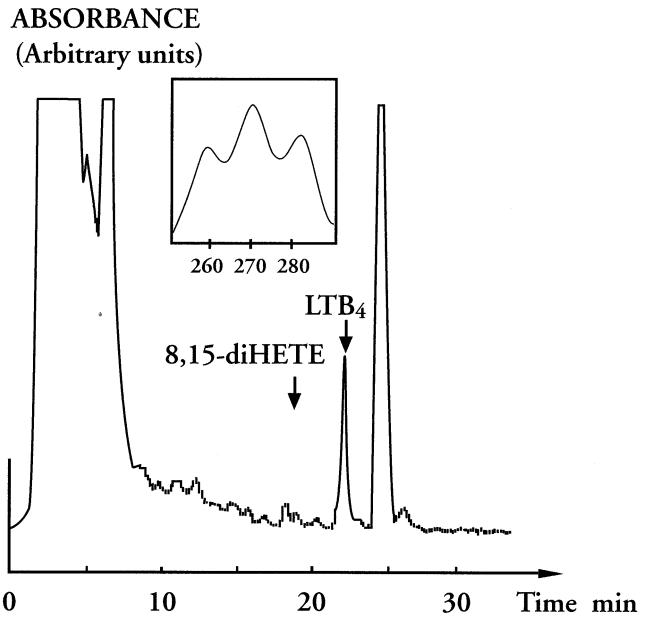

FIG. 1. RP-HPLC profile of arachidonic acid metabolites synthesized by human PBM, recorded at 280 nm. 5×10^6 cells/mL were incubated with calcium ionophore A23187 (5×10^{-6} M for 30 min at 37°), in the presence of 15(S)-HETE (3×10^{-6} M). Sample aliquots were directly analyzed by RP-HPLC using a Lichrospher 100 RP-18 column, the eluting solvent being methanol/water/acetic acid (65:35:0.1, v/v/v) adjusted to pH 5.6, at a flow rate of 0.5 mL/min for 15 min and 1 mL/min from 15 to 65 min. Inset: stop-flow UV spectrum of the unknown product (λ_{max} at 272 nm with shoulders at 260 and 286 nm). Arrow indicates elution position of authentic LTB_4. The chromatogram shown is from one representative experiment (cells from a steroid-dependent asthmatic) and similar results were obtained with repeat experiments.

Eicosanoid Production in Asthma

LTB_4 levels released after PBM incubations with A23187 alone were much greater in cells from steroid-dependent asthmatics (59 ± 11 ng/5×10^6 cells) as compared to controls and untreated asthmatics (6 ± 5 and 40 ± 12, respectively), but significant differences were observed between steroid-dependent asthmatics and controls only ($P < 0.01$). Figure 3 shows eicosanoid levels released when PBM were incubated with 15(S)-HETE. LTB_4 amounts were higher in cells from steroid-dependent asthmatics

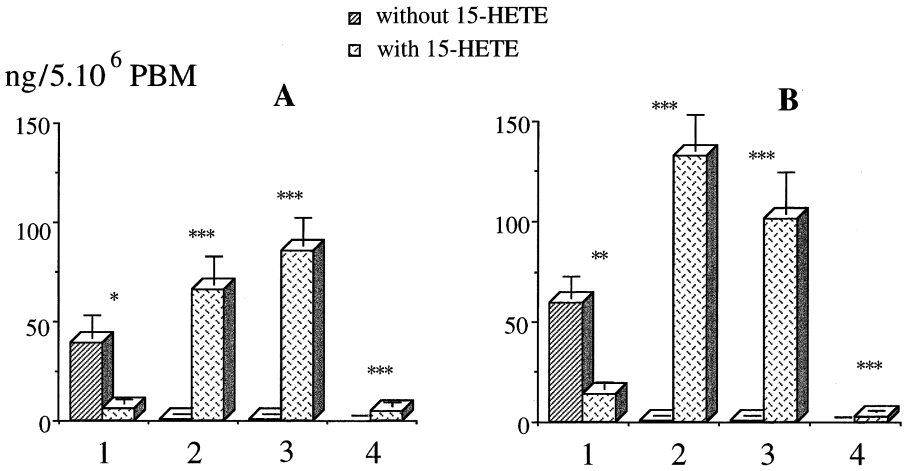

FIG. 2. Eicosanoids generated by PBM stimulated by A23187 with and without 15(S)-HETE, under the conditions described in the legend to Fig. 1. (1) LTB4, (2) unknown product, (3) 5,15-diHETE, and (4) LXs. The total amounts of biosynthesized metabolites were determined by HPLC as outlined in Methods. They were quantified using the external standard method (comparison of the peak area to standard curves obtained under the same conditions of analysis on the basis of molecular extinction coefficients of 55,000, 35,000 and 28,000 for conjugated tetraenes, trienes and dienes, respectively). Results given as means ± SEM are presented as ng/5 × 10^6 cells. LTB_4 levels were significantly lower in PBM stimulated in the presence of 15(S)-HETE. In contrast, the unknown product, LXs and 5,15-diHETE were only detected when PBM were incubated in the presence of 15(S)-HETE. (A) PBM from untreated asthmatic patients (N = 18). (B) PBM from steroid-dependent asthmatic patients (N = 29). Similar profiles were obtained for controls. Significant differences were observed according to the mode of incubation: *$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$.

than in the other populations, and LX amounts were higher in PBM from untreated asthmatic patients than in cells from steroid-dependent asthmatics, but the differences were not significant (Fig. 3A). 5,15-diHETE levels were nearly identical in the cells from the three populations (81 ± 16, 88 ± 14 and 102 ± 21 ng/5 × 10^6 cells). In contrast, the unknown product was synthesized in significantly greater amounts (133 ± 18 ng vs 52 ± 9 ng and 68 ± 15 ng/5 × 10^6 cells for controls and untreated asthmatics, respectively, $P < 0.02$) (Fig. 3B).

DISCUSSION

The aim of our study was to investigate whether human PBM were able to generate LXs when incubated in the presence of 15(S)-HETE. We investigated the generation

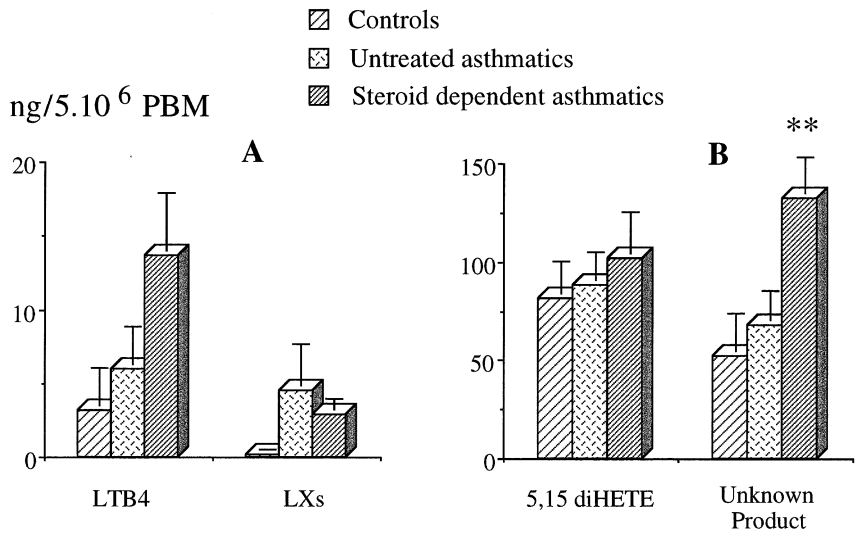

FIG. 3. LXs and the unknown product generated by A23187 stimulated PBM in the presence of 15(S)-HETE. PBM were incubated with A23187 (5 × 10^{-6} M) for 30 min at 37° with 15(S)-HETE (3 × 10^{-6} M) under the conditions described in the legend to Fig. 1. Results given as means ± SEM are presented as ng/5 × 10^6 cells for the number of subjects in each population. (A) products whose levels are lower than 20 ng/5 × 10^6 cells. (B) products whose levels are higher than 50 ng/5 × 10^6 cells. The levels of the unknown metabolite were significantly higher in PBM from steroid-dependent asthmatics (N = 29) as compared to control cells (N = 9) and to untreated asthmatics (N = 18): **$P < 0.02$ (Mann–Whitney *U*-test). No significant difference was observed for other eicosanoids.

of eicosanoids from A23187-stimulated human PBM under two different culture conditions, with or without exogenous precursor. Human PBM were isolated and purified by adherence to avoid any contaminating cells and thus eliminate any possible eicosanoid synthesis resulting from transcellular metabolism with granular leukocytes. RP-HPLC coupled to a multiwavelength spectrophotometer detector was used to resolve and monitor products containing conjugated trienes and tetraenes. Aliquots of samples were injected directly into an isocratic RP-HPLC system, and the analysis procedure did not involve solid phase extraction, concentration or derivatization. The samples were simply subjected to deproteinization and centrifugation, thus minimizing double-bond isomerization, loss and deterioration of eicosanoids. Metabolites were characterized by their retention times and UV chromophores by spectra in the stop-flow mode.

In agreement with previous reports [23–25] and a more recent work [26], we found that A23187-stimulated human PBM were able to release LTB_4 only, and we were unable to detect any amounts of 20-hydroxy metabolites or Δ6-trans isomers, thus showing the absence of contaminating granulocytes. The levels of LTB_4 were lower than the amounts of LTB_4 released from human neutrophils or alveolar macrophages. Nevertheless, we were able to assess the presence of 5-LO [20, 26] in human monocytes from controls, untreated or steroid-dependent asthmatics, since the action of zileuton inhibited LTB_4 release.

We report for the first time that human PBM were able to metabolize 15(S)-HETE into 5,15-diHETE and LXs, and produce another compound whose structure remains to be elucidated. Moreover, zileuton inhibited the release of all these metabolites. Taken together, these results suggest that the biosynthesis of 5,15-diHETE and the unknown product may be dependent on a 5-LO activity displayed by the PBM. When incubated with 15(S)-HETE, PBM released lower levels of LTB_4 after stimulation with ionophore A23187. These results show that 15(S)-HETE was not only a 5-LO substrate but also acted as a 5-LO inhibitor, as reported in other works for different cell types [27], mainly by the switching-over of substrate utilization [28].

It is commonly accepted that in the presence of glucocorticoids, 5-LO activity is reduced. The amounts of LTB_4 arising from endogenous AA and metabolites from exogenous 15(S)-HETE, except LXs, were higher in PBM from steroid-dependent asthmatic patients as compared to those from untreated patients and control subjects, but significant differences were observed in the case of the unknown product only. Because glucocorticosteroids have anti-inflammatory effects, and hence are thought to reduce inflammatory mediator production, this result is unexpected.

The role of glucocorticoids in the regulation of 5-LO activity is uncertain. The effects of glucocorticoids on the metabolism of AA have been studied in a great variety of cell types, but the results are very controversial, depending on the cell type and the mode of administration of glucocorticoids. Studies performed *in vitro* did not show a significant effect on LTB_4 release by human blood neutrophils [29] and monocytes [30], whereas specific eicosanoid synthesis inhibition and lipocortin-1 induction by glucocorticoids were reported for macrophages [31]. In contrast, a recent study reported increased expression of 5-LO in blood monocytes from healthy volunteers cultured in the presence of dexamethasone [32]. Studies performed *ex vivo* show that short-term treatment with prednisolone did not reduce eicosanoid release from stimulated or unstimulated whole blood from rheumatoid patients [33], whereas pulse glucocorticosteroid therapy inhibited whole blood LTB_4 biosynthesis [34], but enhanced leukotriene A_4 biosynthesis by human neutrophils [35]. In contrast, a single oral dose of prednisone inhibited LTB_4 released by alveolar macrophages from asthmatic patients with nocturnal asthma but not control subjects [36]. Taken together, all these studies show that human alveolar macrophages are the most likely cell targets for glucocorticoid-induced eicosanoid inhibition and that this inhibition was related to the inflammatory state of the cells. Although blood monocytes are the precursors of alveolar macrophages, leukotriene biosynthesis was not affected by short-term *in vitro* [37] and *ex vivo* glucocorticoid action [38]. Very few studies are reported in the literature concerning the effect of long-term treatment with corticosteroids. Long-term inhaled corticosteroids decreased leukotriene C_4 levels in bronchoalveolar lavage [39] but long-term oral corticotherapy was associated with increased leukotriene E4 urine excretion [40] and inhibition of leukotriene synthesis by blood neutrophils [38]. This study shows an effect of long-term corticotherapy on the unknown metabolite, specifically biosynthesized by the monocytes from 15(S)-HETE. Because 15(S)-HETE is reported to have anti-inflammatory effects [28, 41, 42], the increased amounts of its metabolite biosynthesis after corticotherapy may attest to the antiinflammatory properties of glucocorticoids by the existence of an upregulation of 5-LO activity, simultaneously with a down-regulation of the glucocorticoid receptor gene as previously described in short-term treatment [43].

This work demonstrates that the use of 15(S)-HETE as an exogenous substrate is a useful approach for assessing the activation of PBM lipoxygenase activities. 15(S)-HETE is known to be a major metabolite of AA in human lungs [44]. Human airway 15-LO is expressed in epithelium [45] and its activity is induced in epithelial cells by airway inflammatory disease [46, 47]. Moreover, in numerous diseases, vascular endothelial cells are now considered to be active inflammatory cells rather than a biologic barrier to body fluids [48], and are able to release 15-HETE [49, 50]. Therefore, the enhanced LO activation of blood monocytes from steroid-dependent asthmatics may reflect *in vivo* priming by cytokines released from nearby cells: the transformation of 15(S)-HETE could be considered as a model of transcellular metabolism either between leukocytes and epithelial cells at the site of inflammation or between monocytes and vascular endothelium as a result of the inflammatory process amplification.

LXs are potential endogenous modulators of PMN trafficking in vascular events [51], and the eicosanoids coming from the 15-LO pathway, such as 15-HPETE and LXA_4, have anti-inflammatory properties [52–54]. The clinical improvement in asthma by glucocorticoids might result in part from the utilisation of an endogenous anti-inflammatory mediator by phagocytic cells. It would be of interest to proceed further with this work to elucidate the structure of the unknown product and coculture PBM with epithelial and endothelial cells to prove that transcellular metabolism leads to the unknown product and to evaluate the impact of cytokines and adhesion molecules on its production.

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