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5-15-DIHETE AND LIPOXINS GENERATED BY NEUTROPHILS FROM ENDOGENOUS ARACHIDONIC ACID AS ASTHMA BIOMARKERS

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SUMMARY: 5-Lipoxygenase (5-LO) activation of human blood polymorphonuclear cells (PMN) from healthy subjects (HS) and from asthmatic patients (AP) was investigated comparing their respective capacities to produce lipoxins, 5,15-dihydroxyeicosatetraenoic acid (5,15-diHETE) and leukotrienes, under *in vitro* stimulation by ionophore A23187. PMN from AP were able to generate higher leukotriene levels from endogenous sources than PMN from HS. Moreover they produced 5,15-diHETE (from 50 to 280ng / 10^7 cells) and lipoxins (from 1 to 30ng / 10^7 cells), in a linear manner, whereas in the same experimental conditions no detectable amounts of these compounds appeared in PMN from HS. The enhanced 5-LO activation of blood PMN may reflect transcellular signalisation priming indicating that lipoxins and 5,15-diHETE could be much more specific inflammatory state biomarkers than leukotriene B_4.

The generation of arachidonic acid metabolites by lipoxygenase-catalyzed reactions is associated with the activation of a wide range of human cell types that are involved in both physiologic and pathophysiologic events (1). Human blood neutrophils which are able to biosynthesize eicosanoids from the 5-lipoxygenase pathway (5-LO) (2) are specifically involved in inflammatory diseases such as asthma (3). Previous studies demonstrated that polymorphonuclear cells (PMN) from patients with inflammatory diseases produced higher

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Abbreviations: 5-LO, 5-lipoxygenase; HETE, hydroxyeicoatetraenoic acid; LTB_4, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid; 5,15-diHETE, 5, 15-dihydroxy-eicosatetraenoic acid; LXs, Lipoxines; LXA_4, (5S, 6R, 14S)-trihydroxy-7, 9, 13-trans-11-cis-eicosatetraenoic acid; 6S-LXA_4, (5S, 6S, 15S)-trihydrox-7, 9, 13-trans-11-cis-eicosatetraenoic acid; 11-trans-LXA_4, (5S, 6R, 15S)-trihydroxy-7,9, 11,13-trans-eicosatetraenoic acid; 6S-11-trans-LXA_4, (5S 6S, 15S) -trihydroxy-7, 9, 11, 13-trans-eicosatetraenoic acid; 7-cis-11-trans-LXA_4, (5S, 6R, 15S)-trihydroxy-9,11,13-trans-7-cis eicosatetraenoic acid; LXB_4, (5S, 14R, 15S)-trihydroxy-6, 10, 12-trans-8-cis-eicosatetraenoic acid; 8-trans-LXB_4, (5S, 14R, 15S)-trihydroxy-6, 8, 10, 12-trans-eicosatetraenoic acid; 14S-8-trans-LXB_4, (5S, 14S, 15S)-trihydroxy-6, 8, 10, 12-trans-eicosatetraenoic acid; PMN, polymorphonuclear cells; AP, asthmatic patients; HS, healthy subjects; RP-HPLC, reverse-phase high-performance liquid chromatography; PBS, Dulbecco's phosphate buffer saline .

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levels of LTB4 than the cells from healthy volunteers (4) and that human neutrophils from donnors with peripheral blood eosinophilia were able to generate 5,15-diHETE (5).

This high level of 5-LO activity may occur as a result of specific cellular activation due to cellular cooperation with environmental blood cells. It is now well established that transcellular cooperations lead to lipoxin biosynthesis (6) as a result of interactions between several lipoxygenases. Since PMN are able to respond to various stimuli by the synthesis of arachidonic acid derived mediators involved in acute and chronic inflammatory processes, the aim of this study was to investigate whether 5,15-diHETE and LXs could be considered as specific biomarkers of a pathophysiological state.

The PMN activation via the 5-lipoxygenase pathway was estimated by analysis of the structure and level of eicosanoids produced after cell stimulation by the Ca^{++} ionophore (A23187) without any exogenous precursor.

MATERIAL AND METHODS

Materials. Culture materials and nutritive medium came from Flow Laboratories (France). Leukotrienes, HETEs, 5,15-diHETE and lipoxins (LXA_4, 6S-LXA_4 and LXB_4), were from Cayman Chemical Company (Ann Arbor MI, USA). 8-trans-LXB_4, 14S-8-trans LXB_4, 11-trans LXA_4, 6S-11-trans-LXA_4 were generous gifts of Dr C.B. Pickett from Merck Frosst Montreal (Canada).

Selection of subjects. The study included 7 healthy subjects (HS) and 25 stable asthmatic patients (AP). All subjects were nonsmokers and none of them were on medication. Theophylline and beta-agonists were stopped 2 days prior to the study; nonsteroidal antiinflammatory drugs (NSAID) and corticosteroids had been discontinued for at least 8 days. These subjects did not present hypereosinophilia and their FEV1 was always up to 70% from predicted values.

Human neutrophil isolation. Human PMN were isolated and purified from heparinized (20 U/ml) venous blood (50 ml) by centrifugation of samples over discontinuous Percoll gradient (4). After purification and lysing contaminating erythrocytes, the PMN were resuspended int PBS (pH 7.4) containing $CaCl_2$ and $MgCl_2$ (final concentration $2x10^{-3}$ M and $0.5x10^{-3}$ M,respectively). Purity of the PMN, evaluated by microscope observation after cytocentrifugation and May Grünwald staining, was greater than 95% without contaminating platelets and erythrocytes. The preparations, greater than 85% viable as measured by trypan blue exclusion, were adjusted to 10^7 cells/1 ml and the cell suspensions were prewarmed at 37°C for 5 min in a water bath before ionophore stimulation.

Stimulation procedures. Ionophore A 23187 (final concentration $5x10^{-6}$M) was added to the cell suspension in ethanol so that the alcohol concentration did not exceed 0.1% and the incubation was continued for 5 min. At the end of the stimulation the reaction was stopped by adding the same volume of ice-cold methanol to the suspension and the samples were stored at -20°C before centrifugation for further analysis.

Metabolite identification and quantification. The stored samples were directly investigated by reverse phase high performance liquid chromatography (RP-HPLC) analysis (Waters model). Detection was monitored with a programmable multiwavelength detector model 490 (Waters).

a) *Dihydroxy and trihydroxy eicosatetraenoic acid analysis*: RP- HPLC was carried out on a Lichrospher 100 RP-18 column (150 mm x 3.9 mm, 5µ particles. Merck). Eicosanoids were eluted with methanol/water/acetic acid (65:35:0.1, v/v/v, pH 5.6) containing 0.5% EDTA as the mobile phase, at a flow rate of 0.5 ml/min for 15 min and 1 ml/min from 15 to 65 min; they were detected by simultaneously monitoring the following wavelengths, 302 nm, 270 nm and 246 nm, corresponding to conjugated tetraenes, trienes and dienes, respectively.

b) *Detection and quantification*: Arachidonic acid metabolites were identified using co-chromatography with synthetic standards and UV spectroscopy in stop-flow mode. Their

amounts were determined by external standardisation. The sensitivity threshold was 0.2 ng at 302 nm and 0.5 to 1 ng at the other wavelengths.

c) *Statistical analysis*: The results presented as ng per 10^7 cells were expressed as mean ± SEM. Statistical differences were determined using Student's t test for unpaired samples (HS: n = 7 and AP: n = 25). The results were considered significant when $p < 0.05$.

RESULTS

The 5-lipoxygenase (5-LO) activity in human PMN was investigated via lipoxin and 5,15-diHETE synthesis under stimulation conditions by the Ca^{++} ionophore A23187 which is known to generate products containing conjugated diene detected at 237 nm, and conjugated triene detected at 280 nm. Detection at 237 nm showed the presence of 5-HETE as usually described for neutrophils but failed to reveal any detectable amounts of 15-HETE, 12-HETE or HHT (data not shown). Fig. 1A reports the analysis at 280 nm of 20-OH LTB_4, the two Δ6-trans-LTB_4 isomers generated from non enzymatic leukotriene A_4 opening and LTB_4, with retention times of 6.80, 20.50, 20.22 and 24.70 min respectively. No detectable amounts of LTC_4 were observed in AP or in HS. Simultaneous detection at 246 nm usually showed the same eicosanoid pattern with characteristic equal peak areas for the two Δ6-trans-LTB_4 isomers since the molecular extinction coefficients are identical for all the LTB_4 metabolite containing conjugated triene chromophore. However the AP cell analysis detected a new peak at 22.40 min with the same retention time as that of the second LTB_4 isomer (Fig. 1B). This product coeluted with authentic 5,15-diHETE and showed the same UV spectrum

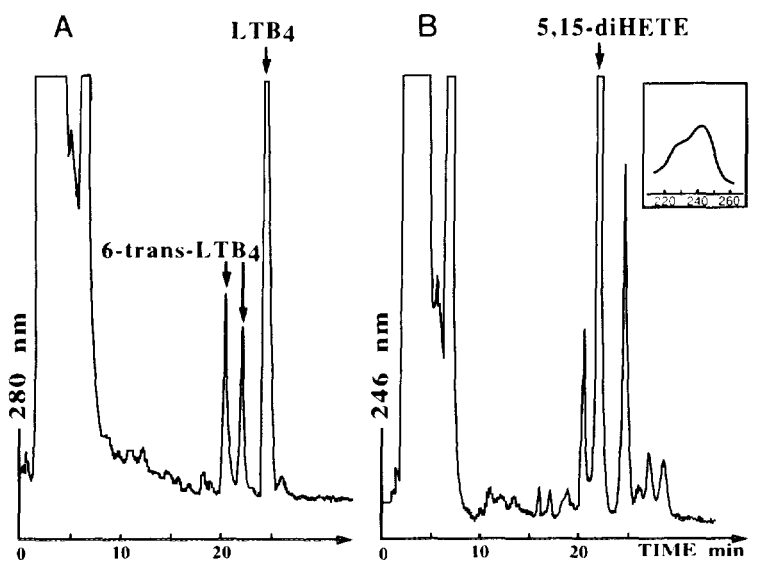

Fig. 1. RP-HPLC profiles of dihydroxy arachidonic acid metabolites obtained with human PMN (10^7 cells / ml) from HS and AP incubated with calcium ionophore A23187 (5μM) at 37°C for 30 min. Chromatography was carried out with eluting solvent MeOH / H2O / AcOH (65 / 35 / 0.1 v / v / v) adjusted to pH 5.6. **A**: Recording at 280 nm. **B**: Recording at 246 nm: *inset*: stop-flow UV spectrum of 5,15-diHETE which was detected in PMN from AP only. *Arrows* indicate elution positions of authentic standards.

as the reference standard, with a maximum at 246 nm and a shoulder at 235 nm characteristic of a double conjugated diene (5).

Simultaneous detection at 302 nm, which usually does not reveale any metabolites in these analysis conditions, showed several peaks which UV spectra gave a triplet of absorption bands at 288, 302 and 316 nm characteristic of the conjugated tetraene system of the lipoxin structure (Fig. 2 insert). This pattern was observed only in the analysis of PMN from AP. Fig. 2 displays an expansion of the lipoxin region monitored at 302 nm. The metabolite stereochemistries were assigned to the respective peaks by several successive chromatographic superimpositions with synthetic standards according to the method described in a previous work (7). The peak with a retention time of 9.30 min coeluted with 14S-8-trans LXB_4 and 8-trans LXB_4 isomers which were not resolved in this mobile phase. The peak at 10.10 min coeluted with the natural LXB_4, the peak at 11.40 min coeluted with 11-trans-LXA_4 and 6S-11-trans-LXA_4 isomers, the peak at 12.60 min with the natural LXA_4 and the peak at 14.80 min with 6S-LXA_4. .

Fig. 3 reports the amounts of all metabolites generated by the 2 cell populations. As described in previous works (4,8,9), PMN from AP released into the culture medium higher levels of 20-OH LTB_4, LTB_4 and LTB_4 isomers (203.5 ± 9.1, 173.1 ± 9.1 and 144.8 ± 6.3 ng / 10^7 cells respectively) than PMN from HS (139.4 ± 12.8, 153.7 ± 12.8 and 113.2 ± 6.8 ng / 10^7 cells respectively) with significant differences for 20-OH LTB_4 and Δ6-trans LTB_4 ($p < 0.005$ and 0.001 respectively) and without any detectable formation of LTC_4. Besides the leukotrienes generated by both cell populations, PMN from AP were able to biosynthesize high amounts of 5,15-diHETE (135.4 ± 14.2 ng 10^7 cells) as well as lipoxins (6.1 ± 1.0 ng /

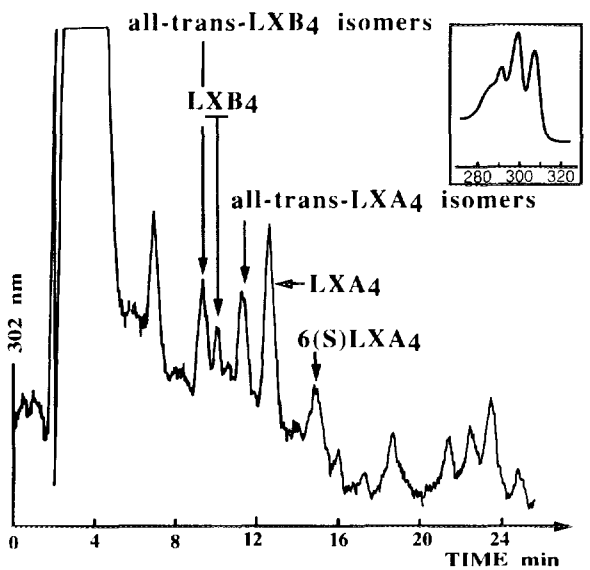

Fig.2. Expansion of the RP-HPLC chromatograms recorded at 302 nm under the conditions described in Fig.1 and obtained with the PMN from AP only. Inset: stop-flow UV spectrum of LXA_4.

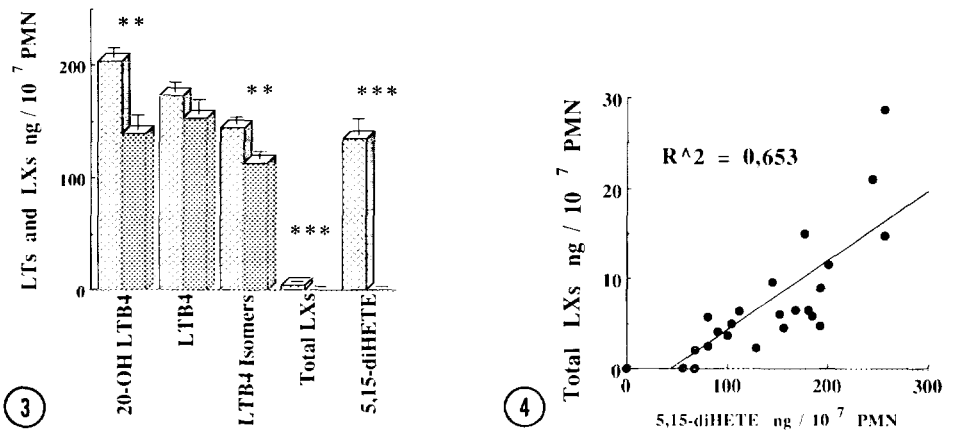

Fig.3. Di and trihydroxy products generated from endogenous sources by A23187 stimulated PMN (at 37°C for 5 min). The total amounts of biosynthesized metabolites were determined by HPLC as outlined in the Material and Methods. Hatched bars = AP and filled bars = HS. Results presented as ng per 10^7 cells were expressed as mean ± SEM from the number of subjects in each population (AP n = 25 and HS n = 7). Significant differences were observed (except for LTB4): ** $p < 0.05$ *** $p < 0.001$.

Fig.4. Linear relationship between LXs and 5,15-diHETE levels generated by PMN from AP stimulated by ionophore A23187 (5.10^{-6} µM for 5 min at 37°C). Data are expressed as ng / 10^7 cells.

10^7 cells). Moreover the metabolites were released according to a linear relationship between LX and 5,15-diHETE levels as reported in Fig.4, with correlation coefficients R^2: 0.653.

DISCUSSION

We investigated the generation of di and trihydroxyeicosatetraenoic acids by A 23187-stimulated human PMN incubated in the absence of any exogenous precursor. Human blood PMN were isolated and purified to avoid any contaminating cells such as erythrocytes and platelets, thus eliminating the possibility of eicosanoid synthesis resulting from transcellular metabolism. RP-HPLC coupled to a multiwavelength spectrophotometer detector was useful for resolving and monitoring products containing conjugated triene and tetraene. Aliquots of samples were directly injected into an isocratic RP-HPLC system and the analysis procedure did not involve solid phase extraction, concentration or derivatization. The samples only had to be deproteinized and centrifuged, thus minimizing double-bond isomerization, loss and deterioration of eicosanoids. UV chromophores were characterized by their retention times and spectra in the stop flow mode.

The aim of our study was to demonstrate, via the 5-LO pathway, human PMN hyperactivation consecutive to lung pathology, by measuring the cell capacity to generate lipoxins from endogenous arachidonic acid. According to our previous results (4,10), human neutrophils from AP were able to biosynthesize from their endogenous arachidonic acid pool the same products as PMN from HS, LTB_4 and its metabolites, but at higher levels. In contrast to alveolar macrophages (7), PMN from AP generated 2 additional series of products

that were not biosynthesized by PMN from HS: dihydroxy derivatives containing conjugated dienes identified to 5,15-diHETE and trihydroxy derivatives containing conjugated tetraene identified, as LXB_4-trans isomers, LXA_4-trans isomers, 6S-LXA_4 and both natural lipoxins LXA_4 and LXB_4. Moreover there was a linear relationship between the dihydroxy acid and the total LXs amounts.

The new class of hydroxy-tetraenes was first isolated and characterized by Serhan et al.(11,12) and then described using various incubation systems (6,13,14). Substantial amounts of lipoxins were produced by A 23187 stimulated cells. The synthesis of lipoxins generated by human neutrophils (15,16) incubated with 15-HETE, by nasal polyps (17) and permeabilized human platelets (18) incubated with LTA_4, all required the addition of exogenous substrate. In contrast, the synthesis of lipoxins generated by eosinophil-enriched suspensions (19), or human cell-cell coincubations such as platelet-granulocytes (20), platelet-GM-CSF primed neutrophils (21, did not require any exogenous addition since the substrate was provided by one cell type to be transformed by the other via the mechanism of transcellular metabolism.

This is the first time that the simultaneous generation of 5,15-diHETE and lipoxins by human PMN has been reported without exogenous addition of a precursor or coincubation with an other cell type. Our results on lipoxin generation from endogenous sources cannot be explained by a dual cell system involving platelet contamination: in fact the platelet / leukocyte ratio required to generate the same lipoxin level ranged from 50 to 100 as reported in the literature (20): this ratio is higher than the ratio that would exist if adherent platelets remained during the purification gradient. Our analysis of experiments with AP PMN preparations did not show the presence of LTC4, in contrast to the results reported by Maclouf et al. (22) for mixed platelet / neutrophil suspensions, nor 12-HETE which would have indicated the presence of platelets. Moreover no high 5,15-diHETE levels were observed when PMN were stimulated in the presence of various quantities of platelets in the absence of 15-HETE (data not shown). This suggested that 5,15-diHETE and lipoxins were generated *in vitro* by the asthmatic patient blood PMN from their own endogenous arachidonic acid. The *in vitro* 5,15-diHETE and lipoxin generation obtained in this study reflected specific PMN activation induced by the bronchial disease and demonstrated these eicosanoids as inflammatory state biomarkers which are much more specific than LTB_4.

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