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METABOLISM OF LIPOXINS A_4 AND B_4 AND OF THEIR ALL-TRANS ISOMERS BY HUMAN LEUKOCYTES AND RAT LIVER MICROSOMES

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Abstract : Incubation of lipoxin A_4 (LXA_4) either with human leukocytes or with rat liver microsomes in the presence of NADPH very selectively led to a more polar metabolite retaining the conjugated tetraenic structure of LXA_4. Lipoxin B_4 (LXB_4) underwent a very similar metabolism into a more polar metabolite, whereas the all-trans isomers of LXA_4 and LXB_4 were selectively transformed by the same biological systems into metabolites derived from the reduction of one of the double bonds of the conjugated tetraene moiety of the starting compounds. Microsomal metabolism of LXA_4 and LXB_4 was NADPH-dependent and strongly inhibited by CO and miconazole indicating the involvement of cytochrome P-450 monooxygenase enzymes. Striking similarities between the metabolism of lipoxins and that of leukotriene B_4 (LTB_4) suggest that LXA_4 and LXB_4 are mainly hydroxylated, on ω or ω-1 position, by human leukocytes and rat and human liver microsomes, whereas their all-trans isomers are mainly reduced into conjugated trienic compounds.

Lipoxins are endogenous compounds derived from the arachidonic acid cascade which are formed through the conjunction of the 5- and 15-lipoxygenase pathways and contain three hydroxy groups and a fully conjugated tetraene system (1-3). They are biologically important compounds which are implicated in the stimulation of human neutrophils, the modulation of immunological activities of lymphocytes and in processes involving inter- and intra-cellular exchanges (4).

Although lipoxins have been detected in body fluids and various tissues (5), nothing is presently known on their metabolism. The metabolic fate of a related but simpler compound (compare formula of Scheme 1 and Fig.1 and 2) derived from the arachidonic acid cascade, LTB_4, has been recently determined. The main transformation of LTB_4 in human PMNL and liver microsomes is its ω-hydroxylation, catalyzed by a cytochrome P-450 enzyme (6-10), whereas that of its all-trans isomer is the reduction of its conjugated triene moiety into a diene moiety (11) (Scheme 1).

This communication describes first preliminary results on the metabolism of lipoxins A_4 and B_4 by human PMNL and liver microsomes, which indicate a rather selective transformation of such complex molecules and suggest that they are mainly hydroxylated in a manner similar to LTB_4 . They also indicate that their all-trans isomers are reduced at the level of their polyene moiety, in a manner similar to all-trans LTB_4.

Abbreviations : **LXA_4**, lipoxin A_4 or 5(S), 6(R), 15(S)-trihydroxy- 7(E), 9(E), 11(Z), 13(E) -eicosatetraenoic acid; **LXB_4**, lipoxin B_4 or 5(S), 14(R), 15(S)-trihydroxy- 6(E), 8(Z), 10(E), 12(E) eicosatetraenoic acid ; **all-trans LXA_4**, Δ^{11} trans-LXA_4 ; **all-trans LXB_4**, Δ^8 trans-LXB_4 ; **LTB_4** , leukotriene B_4 ; **all-trans LTB_4**, Δ^6 trans-LTB_4 **PGB_2**, prostaglandin B_2 ; **PMNL**, polymorphonuclear leukocytes ; **PB**, phenobarbital, **PBS**, phosphate buffered saline solution ; **RP-HPLC**, reverse-phase high pressure liquid chromatography.

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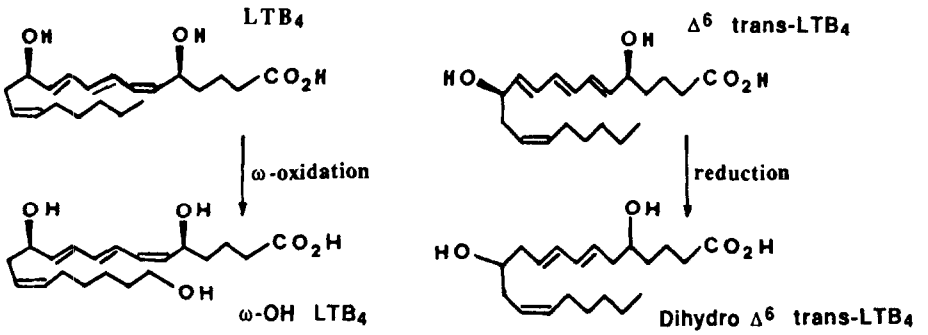

Scheme 1 . Metabolism of LTB_4 and of Δ^6 trans-LTB_4 by human leukocytes.

MATERIALS AND METHODS

Chemicals : LXA_4, LXB_4 , Δ^{11} trans-LXA_4, Δ^8 trans-LXB_4, LTB_4 and Δ^6 trans-LTB_4 were synthesized in our laboratory by J.C. Depezay and his collaborators according to published procedures (12,13). 20-hydroxy-LTB_4 was kindly provided by Dr J. Rokach (Merck-Frosst, Pointe-Claire, Canada). PGB_2 and lauric, linoleic and arachidonic acids were from Sigma, NADPH, NADH, NADP, G6P and G6PDH from Boehringer, and miconazole and metyrapone from Janssen. Other compounds were obtained from sources as indicated : *clofibrate (Fluka), phenobarbital (Merck) and SKF 525A (Smith, Kline and French Laboratories). All other* compounds and solvents used were of the highest purity commercially available.

Preparation of human PMNL: Human circulating leukocytes were prepared from blood collected on heparinized tubes, followed by dextran sedimentation, Ficoll gradient separation and hypotonic lysis, as described by Boyüm (14). The cells were resuspended in free calcium PBS at a concentration of 4 x 10^7 cells per ml.

Preparation of rat liver microsomes : Male Sprague-Dawley rats (200-250 g) were provided laboratory chow and water ad libitum. After 10 days of adaptation, animals were treated either with clofibrate (500 mg/kg, in corn oil, i.p. for 4 days) or with PB (80 mg/kg , in 0.9% saline, i.p. for 4 days). The respective controls were treated with the vehicle alone. Microsomes were prepared as reported (15) and stored at -80°C before use. Protein concentrations were determined by the method of Lowry (16) with bovine serum albumin as standard. Cytochrome P-450 contents were determined by the method of Omura and Sato (17).

Incubation procedures : 10^7 human leukocytes in 0.5 ml (final volume) PBS buffer containing 2.5 mM Ca^{++} and Mg^{++} were preincubated 2 min at 37°C and treated with 1 µM LX or with 1 µM LTB_4. Reactions were stopped by the addition of 0.5 ml methanol containing 100 ng PGB_2 as an internal standard. The suspensions were frozen 2h at -30°C, centrifugated at 3000 rpm for 20 min at 4°C and the supernatants were then directly analyzed by RP-HPLC.

Rat liver microsomes (0.4 mg protein) in 0.4 ml (final volume) 0.1M phosphate buffer, pH 7.4, containing G6P (5 mM), NADP (0.5 mM) and 1 µM LX or 1 µM LTB_4 were preincubated 2 min at 37°C and the reactions were initiated by addition of G6PDH (1 unit per ml, complete system). Fatty acids or cytochrome P-450 inhibitors were added before preincubations. Incubations were stopped, treated and analyzed as in the case of incubations using human leukocytes.

Reverse-Phase HPLC analyses : Separations of LX and of LTB_4 metabolites were carried out using a Kontron solvent delivery system and a Spectra-Focus (Spectra-Physics) high speed scanning UV spectrophotometer. The stationary phase was a column (250 X 4.6 mm) of ODS-2 silica (5 µm, Nucleosil, Societe Française Chromato-Colonnes). The mobile phase was a gradient between water : phosphoric acid (100 : 0.005) and acetonitrile as follows : 0 min, 15% CH_3CN ; 5 min, linear gradient to 100% CH_3CN in 35 min. Flow rate was 1 ml/min. Quantifications were done by comparison with PGB_2 as an internal standard *using calibration curves obtained with each starting compound and assuming that the tetraenic products* formed from LXA_4 and LXB_4 have the same ε values at 302 nm as LXA_4 and LXB_4. Quantitation of the trienic products derived from all-trans LXA_4 and LXB_4 was done by assuming that they have ε values at 270 nm identical to that of all-trans LTB_4.

RESULTS

The **metabolism of LXA_4 by human leukocytes** was followed by RP-HPLC at 302 nm. As shown on Fig.1, almost only one metabolite appeared. Its formation was linear as a function of time at least for 20 min. Its formation was clearly correlated to the consumption of LXA_4. From its retention time, this metabolite

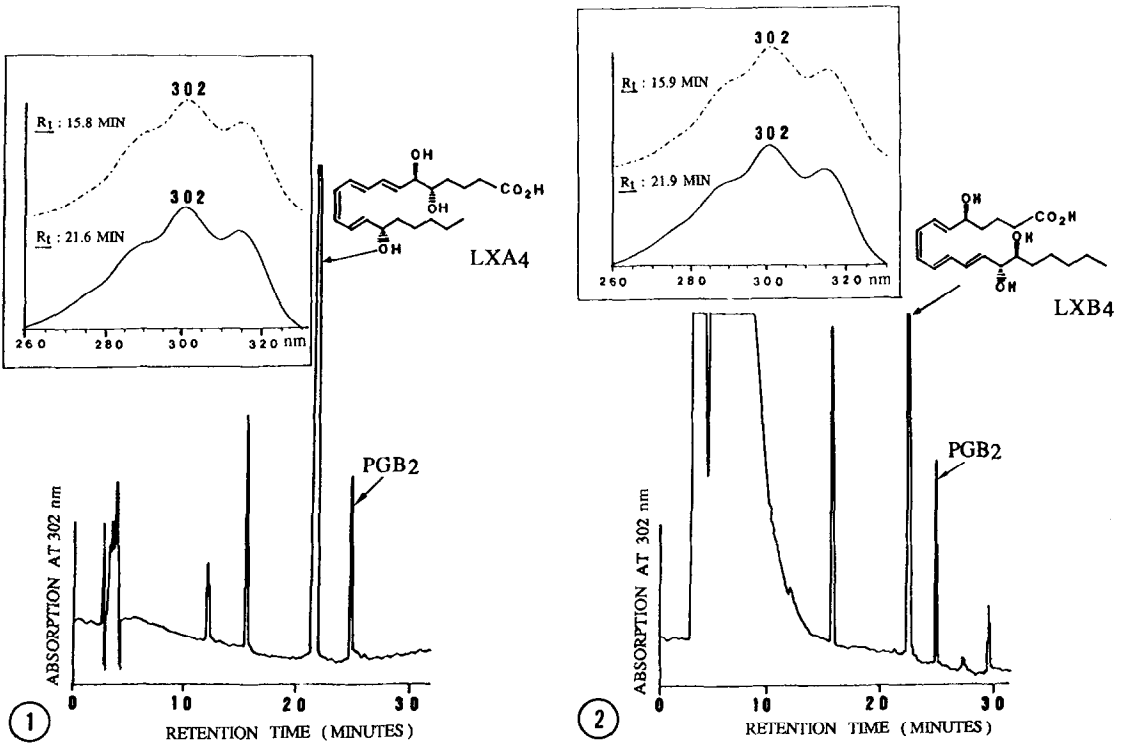

Figure 1 . **Reverse-phase HPLC analysis of LXA_4 incubated 10 min with human leukocytes** (incubation and analysis conditions in Materials and Methods). **Insert:** UV spectrum of LXA_4 (retention time : 21.6 min) and of its metabolite (dashed line, retention time : 15.8 min). Peaks at 13 min were also observed in control incubations of human leukocytes without LXA_4.

Figure 2 . **Reverse-phase HPLC analysis of LXB_4 incubated 10 min with PB-treated rat liver microsomes and NADPH** (incubation and analysis conditions in Materials and Methods). **Insert** : UV spectrum of LXB_4 (retention time : 21.9 min) and of its metabolite (dashed line, retention time : 15.9 min). Peaks around 30 min were also observed in control incubations without LXB_4.

appeared more polar than LXA_4, and its UV spectrum was identical to that of LXA_4 indicating that it retained the tetraene moiety of this lipoxin. Similar analyses of the reaction mixture at different wavelengths (between 230 and 350 nm) showed almost identical HPLC patterns with no indication of formation of other metabolites. A similar result was obtained for the **metabolism of LXA_4 by PB-treated rat liver microsomes** in the presence of either NADPH or a NADPH-generating system. Actually, the HPLC patterns of such incubations were very similar to that of Fig.1, with the almost exclusive formation of one compound co-eluted with the LXA_4 metabolite derived from leukocytes incubations and exhibiting an identical UV-spectrum. Formation of this metabolite was linearly dependent on the incubation time and microsomal protein concentration, and exhibited typical saturation kinetics (K_m around 20 μM).

Similar results were obtained for the **metabolism of LXB_4 by human leukocytes and rat liver microsomes,** with the formation of only one metabolite, more polar than LXB_4 (Fig.2) but exhibiting a UV spectrum identical to that of LXB_4, and a concommitant disappearence of starting LXB_4. It is noteworthy that HPLC analyses of incubates from LXA_4 and LXB_4 by using various wavelengths and various eluting solvents or gradients always showed the formation of only one metabolite for each lipoxin.

Incubation of the **all-trans isomer of LXA_4 by human leukocytes** led to a major metabolite which exhibited a RP-HPLC retention time, and presumably a polarity, very similar to the starting compound, but a

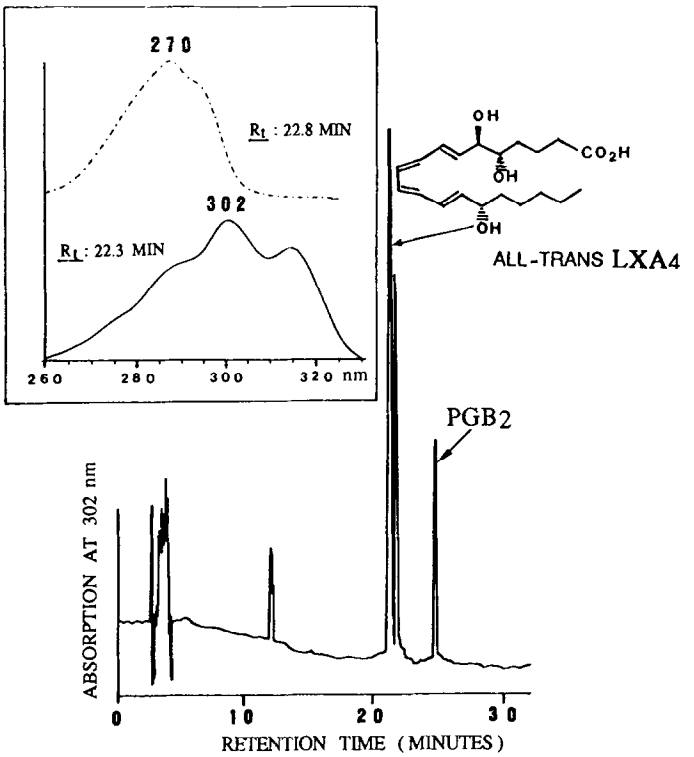

Figure 3 . Reverse-phase HPLC analysis of all trans-LXA_4 incubated 10 min with human leukocytes (incubation and analysis conditions in Materiels and Methods). **Insert** : UV spectrum of all-trans LXA_4 (retention time : 22.3 min) and of its metabolite (dashed line, retention time : 22.8 min). Peaks at 13 min were also observed in incubations of human leukocytes without all-trans LXA_4.

very different UV spectrum (Fig.3). This spectrum involving a peak at 270 nm was very similar to that of all-trans LTB_4 and characteristic of a conjugated triene moiety (18). Metabolism of all-trans LXA_4 by PB-treated rat liver microsomes also led to the major formation of this trienic metabolite. Interestingly, the **metabolism of all-trans LXB_4** either by human leukocytes or rat liver microsomes, under identical conditions, also resulted in the appearance of almost only one compound as polar as all-trans LXB_4 and characterized by a UV spectrum identical to that of the metabolite of all-trans LXA_4 (data not shown).

Effects of various factors on the microsomal metabolism of lipoxins.

As shown in Table 1, the formation of metabolites derived from LXA_4 and LXB_4 by rat liver microsomes seemed to be catalyzed by cytochrome P-450-dependent monooxygenases. This was clearly shown by the absolute requirement of NADPH, the cofactor of these monooxygenases, and by the dramatic inhibitory effects of classical cytochrome P-450 inhibitors such as CO and miconazole. Accordingly, boiled microsomes and the liver cytosolic fraction which do not contain active cytochromes P-450 were completely inactive.

On the contrary, the microsomal metabolism of the all-trans isomers of LXA_4 and LXB_4 was only weakly affected by miconazole and not at all inhibited by CO.

Interestingly enough, the ω-hydroxylation of LTB_4 and the metabolism of LXA_4 and LXB_4 by rat liver microsomes were similarly affected by the different studied factors (Table 1). However, LTB_4 and lauric acid (100 μM) failed to affect the microsomal metabolism of LXA_4 and LXB_4, and the pretreatment of rats by known inducers of hepatic cytochromes P-450 , PB or clofibrate, did not modify this metabolism.

Table 1 . Effects of various factors on the metabolism of lipoxins and of their all-trans isomers and on the ω-hydroxylation of LTB_4 by rat liver microsomes

Substrates / Systems	LXA_4	LXB_4	Δ^{11} trans-LXA_4	Δ^{8} trans-LXB_4	LTB_4
	Rate of metabolite formation (% of control)				
complete system [a]	100	100	100	100	100
boiled microsomes [b]	0	0	0	0	0
cytosol [c]	0	0	0	0	0
- NADPH	0	0	11	7	0
+ CO	4	0	113	115	8
+ miconazole (100μM)	4	0	60	93	8
+ LTB_4 (100μM)	105	104	67	67	-
+ lauric acid (100μM)	73	86	83	95	-
untreated rats [d]	91	98	96	80	104
clofibrate-treated rats [d]	78	84	72	48	108

a) Microsomes from PB-treated rat liver (2.5 nmol P-450 per mg protein) in aerated buffer in the presence of either NADPH or a NADPH-generating system (see Materials and Methods). Activities obtained (in pmol metabolite formed per min and mg protein) with 1μM substrate : 12 ± 3, 4 ± 1, 16 ± 2, 13 ±4 and 95 ± 10 respectively for LXA_4, LXB_4, trans-LXA_4, trans-LXB_4 and LTB_4. b) Microsomes preteated 10 min at 95°C. c) Using 1 mg protein per ml of rat liver cytosolic fraction instead of microsomes. d) microsomes from control or clofibrate-treated rats instead of microsomes from PB-treated rats.

Finally, it is noteworthy that human liver and rat kidney microsomes showed a behaviour similar to rat liver microsomes toward LXA_4 and LXB_4, although with lower activities (data not shown).

DISCUSSION

The metabolism of LXA_4 and LXB_4 by human leukocytes and liver microsomes leads to their very selective transformation into a more polar derivative. Several characteristics of their microsomal transformation -i.e. the requirement of NADPH and the inhibitory effects of CO and miconazole- indicate that their metabolites are formed by a cytochrome P-450-catalyzed oxidation. As these characteristics are similar to those of the microsomal ω-hydroxylation of LTB_4, which has a structure related to lipoxins, and as the difference between the HPLC retention times of lipoxins and their metabolite on one side, and of LTB_4 and 20-hydroxy-LTB_4 on the other side, are very similar, it is likely that lipoxin metabolites are formed by an hydroxylation of LXA_4 and LXB_4. An ω-(or ω-1) hydroxylation would be consistent with the UV spectra of the metabolites which show that the conjugated tetraene structure of lipoxins remains inact. However, further studies, based on ^1H NMR and mass spectroscopy for instance and which are presently difficult because of the low amounts of metabolites produced, are required before to conclude on the complete metabolite structure. Whatever their structures may be, the LXA_4 and LXB_4 metabolites are formed by a cytochrome P-450-dependent oxidation, and the corresponding cytochrome(s) P-450 is (are) present, as the LTB_4-ω-hydroxylase, in several tissues (human leukocytes, rat liver and kidney, human liver) and is not inducible by PB or clofibrate (10).

The strong similarity observed between the metabolism of lipoxins and LTB_4 was also found for their all-trans isomers. Our results show that, contrary to LXA_4 and LXB_4 which are oxidized to a more polar metabolite, their all-trans isomers are mainly reduced at the level of their conjugated tetraene moiety with formation of trienic metabolites (UV spectra) having a polarity almost identical to that of the starting compounds. This situation is very close to that of all-trans LTB_4 which, contrary to LTB_4 which is mainly ω-hydroxylated, was found to be mainly reduced at the level of its triene structure into a diene metabolite by human leukocytes (11).

Thus, it seems that the cis or trans stereochemistry of one double bond of the conjugated polyenic moiety of the isomers of LXA_4 and LXB_4 as well as of LTB_4, plays a key role in determining their main metabolic pathway (hydroxylation or reduction) in leukocytes and liver microsomes. The structure and biological activities of the observed metabolites of lipoxins, and the physiological importance of these metabolic transformations remain to be determined.

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