14,15-Dehydroleukotriene A_4: a specific substrate for leukotriene C_4 synthase

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We studied the metabolism of 14,15-dehydro-leukotriene A_4 (14,15-dehydro-LTA_4) by human platelet leukotriene C_4 (LTC_4) synthase and polymorphonuclear leucocyte (PMNL) leukotriene A_4 (LTA_4) hydrolase. Metabolites were separated and identified using reversed-phase HPLC coupled to diode-array UV detection. Human platelets metabolize 14,15-dehydro-LTA_4 to 14,15-dehydro-LTC_4 with apparent kinetics identical with authentic LTA_4. Metabolism to 14,15-dehydro-LTC_4 is inhibited by MK-886, a reported LTC_4 synthase inhibitor in human platelets, with a potency comparable with that shown by LTA_4. In contrast, neither human red-blood-cell lysates nor human PMNL enzymically convert 14,15-dehydro-LTA_4 into 14,15-dehydro-leukotriene B_4. Minor amounts of 14,15-dehydro-LTC_4, observed in some PMNL preparations, result from variable eosinophil contamination, as confirmed using highly purified neutrophil and eosinophil-enriched preparations. In addition, 14,15-dehydro-LTA_4 irreversibly inhibits PMNL LTA_4 hydrolase with an IC_{50} of 0.73 μM. The geometry of the methyl terminus of LTA_4 does not influence the metabolism by human platelet LTC_4 synthase. The double bond at C-14,15 is essential for the catalytic activity of LTA_4 hydrolase but not for binding to this enzyme.

INTRODUCTION

Leukotrienes (LTs), namely leukotriene B_4 (LTB_4) and cysteinyl leukotrienes leukotriene C_4 (LTC_4), leukotriene D_4 and leukotriene E_4, represent a family of potent, biologically active compounds. They are synthesized from the unstable epoxy intermediate leukotriene A_4 (LTA_4) through the action of two enzymes: LTA_4 hydrolase, which catalyses the formation of LTB_4, and LTC_4 synthase, which conjugates glutathione and LTA_4, giving rise to LTC_4 [1]. Synthesis of LTB_4 and LTC_4 can be carried out either within the same cell, which also contains the enzyme 5-lipoxygenase, catalysing the conversion of arachidonic acid into LTA_4, or in different cells. This phenomenon, whereby a cell is able to transfer the unstable intermediate LTA_4 to a different cell, which carries out the final conversion into LTB_4 or LTC_4, has been termed 'transcellular biosynthetic pathway of leukotrienes'. A number of examples supporting the importance of this pathway for LT biosynthesis have been reported over the last decade [2–4].

LTA_4 hydrolase and LTC_4 synthase may represent potential independent targets for drug activity, and both enzymes have been cloned and sequenced [5–7]. Studies on the mechanism-based inactivation of the LTA_4 hydrolase by LTA_4 led to the identification of a potential site for irreversible binding of LTA_4 on LTA_4 hydrolase [8].

Several analogues of LTA_4 have been used by different groups to test the influence of structural modifications on the enzymic activity of LTA_4 hydrolase or LTC_4 synthase [9–12]. We report on a novel LTA_4 analogue, tested both on human platelet LTC_4 synthase and on human erythrocyte and polymorphonuclear leucocyte (PMNL) LTA_4 hydrolase.

MATERIALS AND METHODS

Human blood (40 ml) was withdrawn from healthy donors that had not taken medications for at least 1 week; it was collected into a 50 ml polypropylene centrifuge tube containing 5.7 ml of ACD (41 mM citric acid/100 mM sodium citrate/136 mM glucose) and carefully mixed. After centrifugation for 20 min at room temperature (RT) and 200 ***g***, platelet-rich plasma was removed, re-acidified with 1 vol. of ACD and centrifuged for 15 min at RT and 1000 ***g***. Pelleted platelets were resuspended with 5 ml of washing buffer (36 mM citric acid/5 mM glucose/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/103 mM NaCl, pH 6.5) containing 0.4 % (w/v) BSA and prostaglandin E_1 (final concentration 100 nM), according to Patscheke [13]. Platelets were further centrifuged for 15 min at RT and 400 ***g***, and finally resuspended in PBS without Ca^{2+} and Mg^{2+} (PBS^-).

To sediment erythrocytes, the residual blood was diluted with 1 vol. of saline [0.9 % (w/v) NaCl] and 0.5 vol. of dextran T-500 [6 % (w/v) in saline]. The upper layer was centrifuged for 20 min at RT and 200 ***g***, and the pellet subjected to erythrocyte lysis by gentle resuspension in 1 vol. of NaCl solution (0.2 %, w/v), followed by dilution with 1 vol. of a balancing solution [1.6 % (w/v) NaCl/0.2 % (w/v) sucrose].

PMNL were separated by centrifugation on Ficoll cushions ($d = 1.077$) for 30 min at RT and 400 ***g***. PMNL were then washed twice with PBS^- and resuspended in PBS^- at a concentration of approx. 10^7 cell/ml.

Purified neutrophils and eosinophil-enriched cell preparations were obtained according to Koenderman et al. [14]. Briefly, PMNL were exposed to *N*-formylmethionyl-leucyl-phenylalanine (10^{-8} M) for 10 min at 37 °C in PBS^-, in order to decrease the density of neutrophils, and then centrifuged over a discontinuous gradient of 1.082 and 1.100 g/ml Percoll. Neutrophils were recovered at the upper interface while the lower interface collected an enriched eosinophil preparation (10–25 % eosinophils, as assessed on cytocentrifugates stained with Giemsa). Platelets and PMNL were counted in a Burker chamber. The viability of PMNL was assessed by Trypan Blue dye exclusion, and was > 90 % throughout the experiments.

Erythrocytes from dextran sedimentation were diluted with sterile, 0.9 % (w/v) NaCl and centrifuged twice at RT and 200 ***g***,

Abbreviations used: PMNL, polymorphonuclear leukocytes; LT, leukotriene; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; RT, room temperature; PBS^-, PBS without Ca^{2+} and Mg^{2+}; RP-HPLC, reversed-phase HPLC.

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discarding the supernatant. Red blood cells were lysed, adding an equal volume of distilled water for 20 s, and centrifuged at 4 °C and 100000 ***g***. The supernatant was removed, filtered through a 0.45-μm Millipore filter and tested for LTA_4 hydrolase activity [15].

LTA_4 and 14,15-dehydro-LTA_4 free acids were obtained through base-catalysed hydrolysis of corresponding methyl esters. Briefly, LTA_4 and 14,15-dehydro-LTA_4 methyl esters were dried under a stream of nitrogen and reconstituted in ice-cold acetone/0.25 M NaOH (4:1, v/v); hydrolysis was performed at room temperature for 60 min and the free acids obtained were added to human platelets (10^8 cells), to PMNL (10^7 cells) or to red-blood-cell lysates (5 mg of protein) at a final concentration of 0.1–10 μM. The purity of methyl esters was checked by normal-phase HPLC, using cyclohexane/ethyl acetate/triethylamine (99:0.5:1, by vol.) to isocratically elute a Lichrospher Si-100 column (4 mm × 250 mm, 5 μm; Merck) at a flow rate of 1 ml/min. Free acids were analysed for purity by reversed-phase HPLC (RP-HPLC) using acetonitrile/0.01 M borate buffer, pH 10 (4:6, v/v) to isocratically elute an Ultrasphere RP-18 column (4 mm × 250 mm, 5 μm; Beckman Analytical, Palo Alto, CA, U.S.A.) at a flow rate of 1 ml/min [16].

UV absorbance was monitored at 280 nm and full UV spectra (240–340 nm) acquired at a scan rate of 0.5 Hz, using a diode-array UV detector (model 168, Beckman Analytical). The identities of methyl esters and free acids were assigned based on retention time and on-line UV absorbance spectra.

MK-886 (1–30 μM, DMSO solution) or RP 64966 [20 μM ethanol/1 M HCl (2:1, v/v)] was added to cell or cell-lysate preparations 5 min before the LTA_4 or 14,15-dehydro-LTA_4.

For studies on the irreversible inactivation of LTA_4 hydrolase, human PMNL were preincubated for 5 min at 37 °C with 14,15-dehydro-LTA_4 (0.1–10 μM) or with solvent alone, centrifuged twice at RT and 200 ***g***, resuspended in 1 ml of PBS, and finally added with LTA_4 (1 μM).

Incubations were terminated after 1 min by addition of 2 vol. of ice-cold methanol containing the HPLC internal standard prostaglandin B_2 (LTA_4 experiments) or prostaglandin B_1 (14,15-dehydro-LTA_4 experiments).

For glutathione S-transferase activity evaluation, washed human platelets were resuspended in lysis buffer [0.05 M phosphate buffer (pH 7.4)/0.1 M NaCl/2 mM EDTA/aprotinin (0.1 units/ml)/pepstatin (1 μg/ml)/leupeptin (1 μg/ml)] at a concentration of (3–5) × 10^9 ml^{-1}, and sonicated for 4 × 15 s on ice, using a sonifier (power setting 5; model XL; Heat Systems, Farmingdale, NY, U.S.A.), equipped with a microtip. Disrupted platelets were centrifuged for 20 min at 4 °C and 12000 ***g*** and supernatant, representing cytosol and microsomal membranes, was tested for enzymic activity.

Proteins were quantified spectrophotometrically using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.), with BSA as standard.

Glutathione S-transferase (EC 2.5.1.18) in platelet lysates (100 μg of protein) was assayed spectrophotometrically at 340 nm in a reaction system containing 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (Sigma), 5% (v/v) ethanol and 0.1 M sodium phosphate, pH 6.5, at 25 °C [17]. The increase in absorbance at 340 nm was monitored for 3 min using a Jasco spectrophotometer (V-530, Tokyo, Japan), in the presence or absence of MK-886 (30 μM). Results were expressed as nmol of 1-chloro-2,4-dinitrobenzene–glutathione conjugate formed/min per mg of protein, using an absorption coefficient of 9600 $mol \cdot l^{-1} \cdot cm^{-1}$ at 340 nm.

LTA_4-derived metabolites were analysed by RP-HPLC coupled to diode-array UV detection, as described previously [18]. IC_{50} was evaluated by non-linear regression using the computer program Allfit QB [19].

Eicosanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). 14,15-Dehydro-LTA_4 methyl ester was synthesized as described previously [20]. HPLC-grade solvents were obtained from Merck. Type I 'plus' water was obtained using a MilliQ Plus water purifier (Millipore, Molsheim, France), fed with double-distilled water.

RESULTS

In agreement with a preliminary report [20], RP-HPLC analysis of incubates of human platelets exposed to 14,15-dehydro-LTA_4 (1 μM) revealed a chromatographic profile similar to that observed using authentic LTA_4, except all the chromatographic peaks moved to shorter retention times. The peak corresponding to 14,15-dehydro-LTC_4 showed a unique UV absorption spectrum, with λ_{max} at 279 nm and shoulders at 269 and 290 nm, and was structurally characterized by controlled metabolism and electrospray tandem mass spectrometry, collision induced dissociation [20].

In order to rule out the possibility that the conversion of 14,15-dehydro-LTA_4 into 14,15-dehydro-LTC_4 was the result of glutathione S-transferase activity, rather than the unique LTC_4 synthase, we tested the effect of compound MK-886, which we recently reported was an inhibitor of the human platelet LTC_4 synthase [21]. MK-886 dose-dependently inhibited the formation of 14,15-dehydro-LTC_4 with an IC_{50} of 4.7 μM (Table 1), which is similar to that observed using authentic LTA_4 as substrate. A glutathione S-transferase activity of 26.6 ± 9.0 nmol/min per mg of protein (mean ± S.E.M., $n = 3$) was evaluated in lysed human platelets, and was only slightly affected (22 ± 9% inhibition) by the presence of MK-886 at a concentration (30 μM) resulting in complete inhibition of 14,15-dehydro-LTC_4 formation in intact platelets.

The affinity of 14,15-dehydro-LTA_4 for LTC_4 synthase was very similar to that observed with authentic LTA_4 (Figure 1): apparent K_m and V_{max} of 1.6 μM and 200 pmol/min per 10^8 platelets were measured, to be compared with 2 μM and 200 pmol/min per 10^8 platelets respectively for LTA_4. Addition of LTA_4 to lysed erythrocytes resulted in the formation of LTB_4; this was completely prevented by preincubation with 20 μM RP 64966, a specific inhibitor of LTA_4 hydrolase [22]. No difference was observed between the chromatographic profiles obtained in the presence or in the absence of the inhibitor when 14,15-dehydro-LTA_4 was used, indicating lack of metabolism by the red-blood-cell LTA_4 hydrolase (Figure 2).

Table 1 Effect of MK-886 on LTC_4 synthase metabolism of 14,15-dehydro-LTA_4 in intact human platelets

Washed human platelets (10^8 cells) were incubated for 1 min with 14,15-dehydro-LTA_4 (1 μM) in the absence or the presence of MK-886 (1–30 μM). Samples were analysed by RP-HPLC. Control incubations showed production of 75 ± 9 pmol of 14,15-dehydro-LTC_4. Results are expressed as the percentage of control, as means ± S.E.M. ($n = 3$).

MK-886 (μM)	Production of 14,15-dehydro-LTC_4 (%)
1	92.3 ± 1.9
3	81.4 ± 3
10	23.1 ± 2.4
30	7.3 ± 3

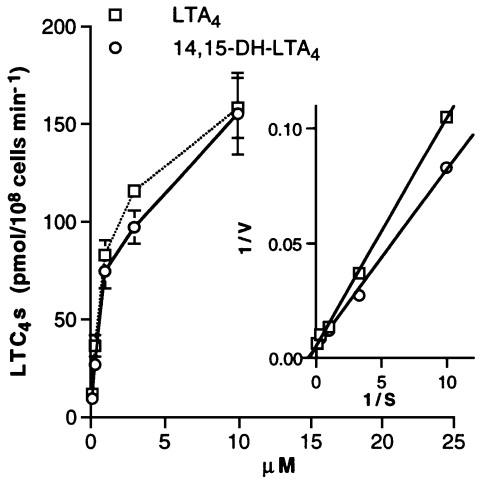

Figure 1 Substrate-dependent transformation of LTA_4 and 14,15-dehydro-LTA_4 by washed human platelets

Washed human platelets (10^8 cells) were incubated for 1 min with 14,15-dehydro (DH)-LTA_4 or LTA_4 (0.1–10 μM). Samples were analysed by RP-HPLC. Data are means ± S.E.M. ($n = 3$). The inset shows Lineweaver–Burk transformations of the data.

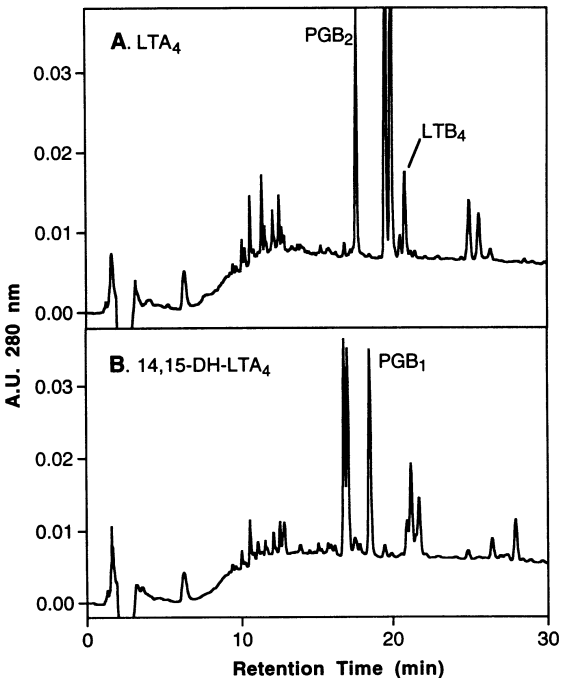

Figure 2 RP-HPLC separation of LTA_4 and 14,15-dehydro-LTA_4 metabolites after incubation with red blood cell lysates

Red-blood-cell lysates (5 mg of protein) were incubated for 1 min with (**A**) 1 μM LTA_4 and (**B**) 1 μM 14,15-dehydro-LTA_4. LTB_4 was identified by on-line UV-spectrum analysis and retention times of synthetic standards. The chromatographic profile obtained with 14,15-dehydro-LTA_4 was not modified by the presence of 20 μM RP-64966, a specific LTA_4 hydrolase inhibitor, and was identical with that obtained in the absence of red-blood-cell lysate. Significant peaks eluting before and after the internal standard, prostaglandin B_1 (PGB_1), represent the products of non-enzymic hydrolysis of 14,15-dehydro-LTA_4. A.U., absorbance units.

Addition of authentic LTA_4 to human PMNL resulted in the formation of significant amounts of LTB_4 and of its ω-oxidized metabolites, namely 20-hydroxy- and 20-carboxy-LTB_4 (Figure 3A), in agreement with previously published data [18,23]. No significant metabolism was observed on addition of up to 10 μM 14,15-dehydro-LTA_4, except for minor amounts of 14,15-dehydro-LTC_4 (Figure 3B). In order to test if eosinophil contamination could account for the 14,15-dehydro-LTC_4 production by PMNL, highly purified neutrophils and eosinophil-enriched preparations were checked. The results obtained showed that neutrophils were not able to enzymically process 14,15-dehydro-LTA_4 to any extent, whereas significant amounts of 14,15-dehydro-LTC_4 were synthesized by eosinophil-enriched preparations (Figure 4).

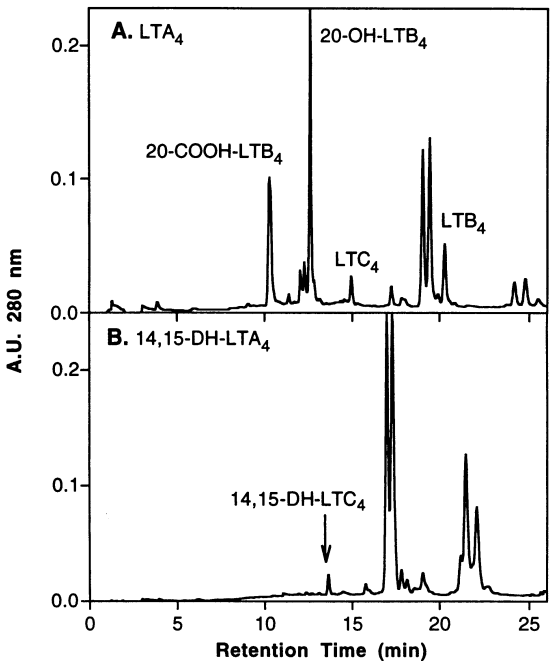

Figure 3 RP-HPLC separation of LTA_4 and 14,15-dehydro-LTA_4 metabolites after incubation with human PMNL

Human PMNL (10^7 cells) were incubated for 10 min with (**A**) 10 μM LTA_4 and (**B**) 10 μM 14,15-dehydro-LTA_4. Incubation was terminated by addition of 2 vol. of ice-cold methanol. Sample was extracted on a solid-phase cartridge (Supelclean C18) and analysed by RP-HPLC. LTA_4 metabolites [LTB_4, 20-hydroxy-LTB_4 (20-OH-LTB_4), 20-carboxy-LTB_4(20-COOH-LTB_4)] were identified by on-line UV-spectrum analysis and retention times of synthetic standards. 14,15-Dehydro-LTC_4 was identified by on-line UV-spectrum analysis and retention times of mass spectrometry-characterized platelet metabolites. A.U., absorbance units.

Preincubation of neutrophils with 14,15-dehydro-LTA_4 (0.1–10 μM) caused irreversible inactivation of LTA_4 hydrolase, with an IC_{50} of 0.73 μM (Table 2).

DISCUSSION

The results obtained using a unique LTA_4 analogue show that substitution of a double with a triple bond at C-14,15 leaves a substrate suitable for metabolism by LTC_4 synthase. The resulting 14,15-dehydro-LTC_4 analogue has been previously characterized by RP-HPLC with on-line UV spectroscopy, enzymic conversion and mass spectrometry.

LTC_4 synthase, as shown by recent cloning and expression [5,6,24], does not belong to the family of glutathione S-transferases, but presents significant similarity to another protein involved in leukotriene metabolism, namely the 'five lipoxygenase activating protein'. Further supporting this observation, a known 5-lipoxygenase-activating-protein inhibitor, compound MK-886,

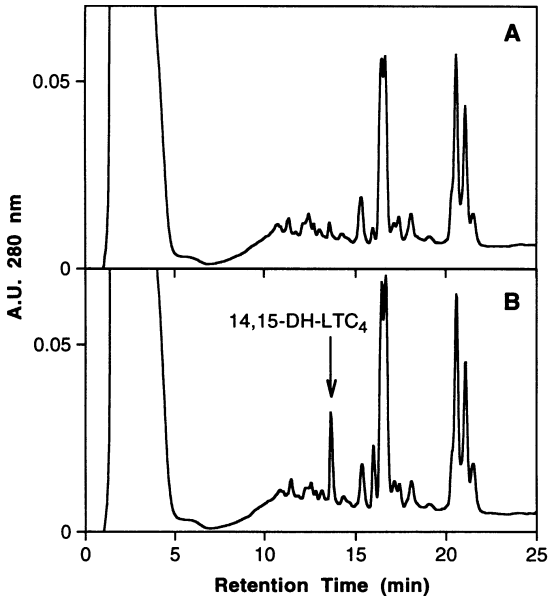

Figure 4 RP-HPLC separation of 14,15-dehydro-LTA_4 metabolites after incubation with purified human neutrophils and enriched eosinophil preparations

(**A**) Purified human neutrophils (10^7 cells) and (**B**) enriched eosinophils (5×10^6 cells) were incubated for 10 min with 10 μM 14,15-dehydro-LTA_4 (14,15-DH-LTA_4). Incubation was terminated by addition of 2 vol. of ice-cold methanol and, after centrifugation, an aliquot of the sample was directly analysed by RP-HPLC. A.U., absorbance units.

Table 2 Effect of preincubation with 14,15-dehydro-LTA_4 on metabolism of synthetic LTA_4 by human PMNL

Human PMNL (10^7 cells) were incubated for 10 min in the absence or the presence of 14,15-dehydro-LTA_4 (0.1–10 μM). Cells were washed twice with PBS and incubated with LTA_4 (1 μM) for 10 min. After addition of 2 vol. ice-cold methanol and centrifugation, an aliquot of the sample was directly analysed by RP-HPLC. Control incubations showed production of 308 ± 16 pmol of LTA_4 hydrolase-derived metabolites, namely LTB_4, 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (LTB_4s). Results are expressed as the percentage of control $\pm$ S.E.M. ($n = 3$).

14,15-Dehydro-LTA_4 (μM)	Production of LTB_4s (%)
0.1	96.0 ± 13.5
0.3	72.2 ± 11.8
1	38.7 ± 6.3
3	27.6 ± 8.4
10	3.7 ± 1.5

was reported to dose-dependently inhibit cloned, expressed LTC_4 synthase. In agreement with these data, we showed recently that MK-886, as well as a different, structurally unrelated 5-lipoxygenase-activating-protein inhibitor, was able to modulate human platelet LTC_4 synthase [21].

Inhibition of 14,15-dehydro-LTA_4 metabolism by human platelets on preincubation with compound MK-886, provides pharmacological identification of LTC_4 synthase as the enzymic activity responsible for the synthesis of the observed 14,15-dehydro-LTC_4 analogue.

A previous report had shown that saturation of the 14,15 double bond of LTA_4 did not affect metabolism by purified guinea-pig LTC_4 synthase [9]; in contrast, later evidence pointed to significant differences in substrate specificity between human platelet and guinea-pig LTC_4 synthase. Using the guinea-pig enzyme, LTA_4 methyl ester showed a V_{max} that was 4-fold higher than that for LTA_4, whereas the same substrate presented a V_{max} that was 14-fold lower than for LTA_4 using human platelet LTC_4 synthase [10]. Another LTA_4 analogue, 14,15-LTA_4, was found not to be processed by the human platelet LTC_4 synthase, but still provided a good substrate for the guinea-pig enzyme, with a V_{max} of twice that for LTA_4. In the present paper we provide evidence that 14,15-dehydro-LTA_4 represents a substrate that is indistinguishable from authentic LTA_4 by human LTC_4 synthase. Therefore modifications of the lipophilic chain of LTA_4 do not affect either binding or catalytic conversion by LTC_4 synthase, thus providing additional information on the structural requirements for interactions with this biologically relevant enzyme.

14,15-Dehydro-LTA_4 was not enzymically converted by human PMNL or red-blood-cell LTA_4 hydrolase. Furthermore, preincubation with 14,15-dehydro-LTA_4 before addition of LTA_4 resulted in potent, irreversible inhibition of LTA_4 hydrolase activity. These data support results obtained with leukotriene A_3 and rat neutrophils, showing that the double bond at C-14,15 represents a critical feature for the enzymic conversion carried out by the specific LTA_4 hydrolase. Interaction with the binding site of the enzyme does not appear to be affected by the modification of the Δ^{14} double bond. In fact leukotriene A_3 and 14,15-dehydro-LTA_4, as well as authentic LTA_4, irreversibly bind to human LTA_4 hydrolase preventing further enzymic activity. On LTA_4, the cis geometry of the double bond at C-14,15 might impose a specific orientation of the lipophilic chain, resulting in correct interaction of the conjugated system with the enzyme.

Reviewing data obtained with different LTA_4 analogues shows that the overall structure of the lipophilic chain seems to be less important than the presence of the 14,15 double bond in connection with the possibility of metabolism by the LTA_4 hydrolase: leukotriene A_5, possessing an additional double bond at C-17,18 is still efficiently metabolized to leukotriene B_5, although the apparent V_{max} is severalfold lower than that for authentic LTA_4 [12]. Interestingly, leukotriene A_5 is also a suitable substrate for LTC_4 synthase, but enzymic conversion appears to be 20-fold less efficient than with the specific substrate LTA_4.

In conclusion, we found that the substitution with a triple bond of the double bond at C-14,15 of LTA_4 does not affect the metabolism by human platelet LTC_4 synthase, but completely prevents metabolism by erythrocyte and PMNL LTA_4 hydrolase. These data provide additional information on the structural requirements for binding and metabolism by these two important enzymes. Furthermore, the observation of a preserved biological activity by 14,15-dehydro-LTC_4 [20] suggests that 14,15-dehydro-LTA_4 may represent a valuable tool for the study of the pathophysiological role of transcellular synthesis of cysteinyl leukotrienes in complex organ systems [25].

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