DETECTION OF LEUKOTRIENE A_4 AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF LEUKOTRIENES C_4 and D_4

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1. Introduction

Leukotrienes are a new group of compounds derived from arachidonic acid [1,2], eicosapentaenoic acid [3] and eicosatrienoic acid [4]. Each of these acids is the precursor of a series of compounds, which have been designated alphabetically and using subscripts to indicate the number of double bonds [5]. Leukotrienes C_4 and D_4 are the biologically active components of 'slow-reacting substance of anaphylaxis' (SRS-A [6]), a smooth muscle contracting factor first described in 1938 [7] and subsequently suggested to be an important mediator of bronchoconstriction in allergic asthma [8]. These leukotrienes are exceedingly active on guinea pig and human respiratory smooth muscle in vitro and in vivo [9–11]. Leukotrienes C_4 and/or D_4 formation has been demonstrated in mouse mastocytoma cells [1,2] rat basophilic leukemia cells [12–15], rat mononuclear cells [16,17] and in human [17,18] and guinea pig lung [19].

A pathway for leukotriene C_4 biosynthesis involving glutathione conjugation of an epoxide derived from arachidonic acid [20], has been proposed [1,2]. The epoxide, designated leukotriene A_4 [5], is unstable [20] and has been isolated from leukocytes by extraction after esterification, using synthetic leukotriene A_4 [21] as a carrier [22]. A more convenient way of determination is based on the chemical conversion of leukotriene A_4 to diastereoisomeric 5(*S*)-hydroxy-12(*S*,*R*)-methoxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acids in acidic methanol [20]. The 12-methoxy compounds can be quantitatively analyzed by high performance liquid chromatography (HPLC) using prostaglandin B_2 as internal reference [20]. By this method leukotriene A_4 has now been demonstrated to be transiently detectable during the biosynthesis of leukotrienes C_4 and D_4.

2. Materials and methods

Ionophore A23187 was purchased from Calbiochem-Behring. Rats with tumors of basophilic leukemia were generously supplied by B. H. Leonard, ICI, England. RBL cells were grown in Spinner cultures [12] and mastocytoma cells were propagated as ascites tumors [1]. Cells (10^7/ml) were suspended in a phosphate-buffered salt solution containing D-glucose [1].

2.1. *Material for structural analyses*

Mastocytoma or basophilic leukemia cells (2.4×10^9) were incubated for 30 s at 37°C with 20 μM ionophore A23187 and 166 μM arachidonic acid. The reaction was stopped with acidic methanol [20]. After adjusting the pH to 7 with NH_4OH, the solution was filtered and concentrated under reduced pressure. The aqueous solution was acidified to pH 3, extracted with diethyl ether and the extract was purified on a column of SilicAR CC7 (Mallinkrodt) using diethyl ether/hexane (1/4, v/v) and ethyl acetate as eluents. The latter fraction was subjected to reverse-phase HPLC using a column (500 × 10 mm) containing C_{18} Polygosil and methanol/water (4/1, v/v) plus 0.01% acetic acid as mobile phase (flow rate 4.5 ml/min). The absorbance at 280 nm was monitored at the column effluent.

2.2. *Time course experiments*

Mastocytoma or basophilic leukemia cells (4×10^8) were incubated (2 min, 37°C) with L-cysteine (10 mM) prior to addition of ionophore A23187 (20 μM) and arachidonic acid (90 μM). Incubations were terminated with acidic methanol after varying lengths of time. The pH was adjusted to 6–6.5 with NH_4OH and prostaglandin B_2 (6 nmol) was added as an internal reference [20].

2.3. *Isolation of leukotrienes*

After filtration and evaporation of the solvent, the remaining material was chromatographed on 10 ml columns of Amberlite XAD-8 [1]. The aqueous eluates were acidified and extracted with diethyl ether. These extracts were combined with the 80% ethanol eluates from XAD-8 [1] prior to silicic acid chromatography [1]. Ethyl acetate, methanol/ethyl acetate (1/1, v/v) and methanol eluates contained dihydroxyacids plus 12-methoxy compounds, leukotriene D_4 and leukotriene C_4, respectively.

The ethyl acetate eluates were analyzed by HPLC on C_{18} Nucleosil (250 × 4.6 mm) using methanol/water (75/25, v/v) plus 0.01% acetic acid as mobile phase at 1 ml/min [20]. The methanol/ethyl acetate (1/1, v/v) and the methanol eluates were fractionated by HPLC as above except that the mobile phase was methanol/water (7/3, v/v) plus 0.1% acetic acid, adjusted to pH 5.4 with NH_4OH.

2.4. *Analytical methods*

Ultraviolet spectra were recorded with a Cary 219 spectrophotometer. Gas-chromatography–mass spectrometry (GLC–MS) was performed using an LKB 9000 S instrument with a column containing 3% OV-210. Prior to analyses, samples were converted to methyl ester, trimethylsilyl ether derivatives [20].

3. Results

Mastocytoma and basophilic leukemia cells were incubated under conditions leading to the generation of leukotrienes C_4 [1] and D_4 [12]. The reactions were performed for varying periods of time and terminated by addition of acidic methanol [20]. Fig.1 shows a chromatogram from a 30 s incubation with basophilic leukemia cells. In addition to prostaglandin B_2, four major components were observed. Compounds I and II had the same elution times on HPLC as the diastereoisomeric 5(*S*),12(*S*,*R*)-dihydroxy-6,8,10-*trans*-14-*cis* eicosatetraenoic acids [23]. Similarly, compounds III and IV were eluted at the same positions as the 12-*O*-methyl derivatives of these compounds [20]. The ultraviolet spectra of compounds I–IV were identical to those of the dihydroxy acids and the 12-*O*-methyl derivatives mentioned above (λ_{max} at 268; shoulders at 280 and 258 nm, cf. [20,23]) indicating the presence of all-*trans* conjugated trienes [23]. Similar results were obtained with

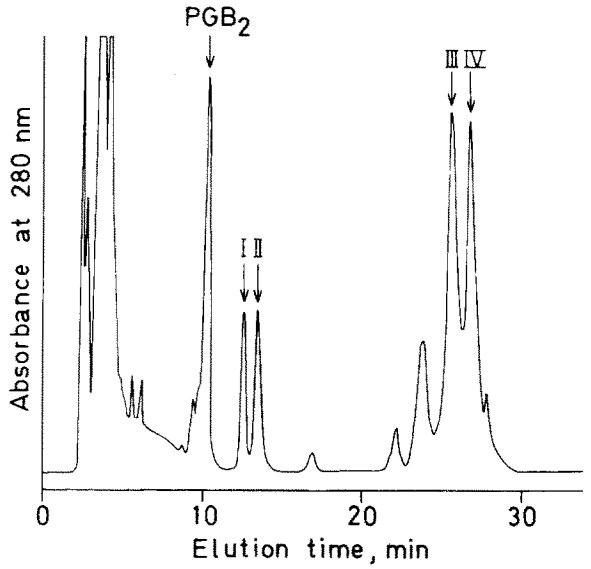

Fig.1. Reverse phase HPLC of 5,12-dihydroxyeicosatetraenoic acids (I,II) and 5-hydroxy-12-methoxy-eicosatetraenoic acids (III,IV) formed by rat basophilic leukemia cells (4×10^8) during an incubation (30 s, 37°C) with ionophore A23187 (20 μM) plus arachidonic acid (90 μM). The incubation was terminated by addition of 10 vol. acidic methanol to convert leukotriene A_4 to 5-hydroxy-12-methoxy-6,8,10,14-eicosatetraenoic acids. Prostaglandin (PG) B_2 was added as an internal reference, prior to purification [20].

mastocytoma cells. For analyses by GLC–MS, larger amounts of compounds I and II (~35 nmol each) and III and IV (~80 nmol each) were prepared as in section 2 and converted to methyl ester, trimethylsilyl ether derivatives. These derivatives of compound I and II had the same C-value (25.2) as 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acids from leukocytes [23] and the mass spectra of both compounds were indistinguishable from those reported [23,24] for the 5,12-dihydroxy acids

(Ions at *m/e* 494 (M): 479 (M-15, $^{\cdot}CH_3$), 463 (M-31, $^{\cdot}OCH_3$), 404 (M-90, $(CH_3)_3SiOH$), 383 (M-111, $^{\cdot}CH_2{-}CH{=}CH{-}(CH_2)_2{-}CH_3$), 327, 317, 293 (M-111-90), 282, 267, 261, 229, 217 ($(CH_3)_3Si{-}O{-}CH{=}CH{-}CH{=}\overset{+}{O}{-}Si(CH_3)_3$), 203 ($(CH_3)_3Si{-}\overset{+}{O}{=}CH{-}(CH_2)_3{-}COOCH_3$) and (M-111-2 × 90)), 191, 189, 171, 167, 161, 159 and 129).

Similarly, compounds III and IV had the same C-value (25.1) as methyl 5-trimethylsilyloxy-12-methoxy-6,8,10,14-eicosatetraenoates [20] and their mass spectra were identical

(Ions at *m/e* 463 (M): 421 (M-15, $^{\cdot}CH_3$), 404 (M-32, CH_3OH), 389, 373, 325 (M-111, $^{\cdot}CH_2{-}CH{=}CH{-}$

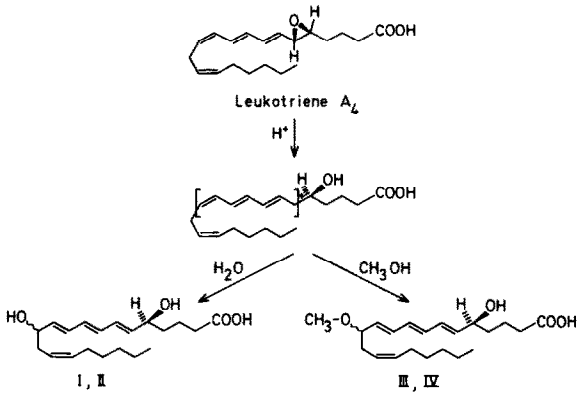

Fig.2. Chemical transformations of leukotriene A_4 to compounds I–IV.

$(CH_2)_4$–CH_3), 293 (M-111-32), 267, 241, 235, 203 ($(CH_3)_3Si$–$\dot{O}$=CH–$(CH_2)_3COOCH_3$ and M-111-90-32), 189, 171, 161, 159, 143, 133 and 129) to those reported before [20]. The structures of compounds I–IV and their formation from leukotriene A_4 are shown in fig.2.

The amounts of compounds III and IV formed, varied depending on the incubation time. The highest concentrations were observed after 15–30 s (results for basophilic leukemia cells are shown in fig.3; similar results were obtained with mastocytoma cells). The formation of leukotriene D_4 and its 11-*trans* isomer by basophilic leukemia cells ([12,13], fig.3) and leukotriene C_4 [1,2] and 11-*trans* leukotriene C_4 [25] by mastocytoma cells was determined by HPLC and ultraviolet spectroscopy. The results showed that the formation of compounds III and IV largely preceded leukotriene C_4 and leukotriene D_4 biosynthesis.

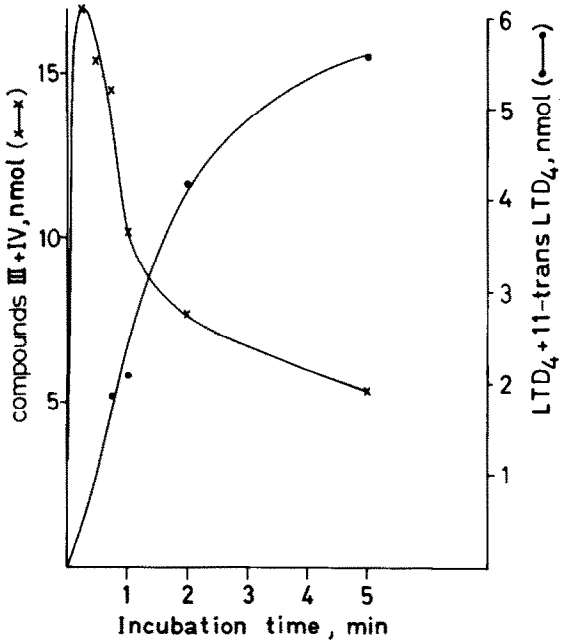

Fig.3. Temporal relationship between the formation of 5-hydroxy-12-methoxyeicosatetraenoic acids (compounds III, IV) and the biosynthesis of leukotriene D_4 plus 11-*trans* leukotriene D_4 (•——•) in basophilic leukemia cells. The experiment was performed as described in the legend to fig.1 but the incubation time was varied.

4. Discussion

Mastocytoma cells [1,2] and rat basophilic leukemia cells [12,13] were used for the structural determination of the slow-reacting substances, leukotrienes C_4 and D_4. Based on the structures deduced a biosynthetic pathway was postulated involving an allylic epoxide formed from arachidonic acid (leukotriene A_4 [5,20]) as an intermediate. The present results show that the proposed intermediate can be detected during the biosynthesis of leukotrienes C_4 and D_4 in mastocytoma and basophilic leukemia cells. This was shown by conversion in methanol of the intermediate to stable derivatives [20] which were unambiguously identified from their retention times on HPLC and GLC and from their ultraviolet and mass spectra. Compounds III and IV (fig.1) were thus the 12(*R*) and 12(*S*) diastereoisomers of 5(*S*)-hydroxy-12-methoxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (fig.2). In addition corresponding hydrolysis products of leukotriene A_4, 5(*S*),12(*R*)- and 5(*S*),12(*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acids (compounds I and II, respectively, fig.1) were formed (fig.2). The results support the proposal that leukotriene A_4 is an intermediate in the biosynthesis of leukotrienes C_4 and D_4 (fig.4). Evidence suggesting that leukotriene C_4 is an intermediate in leukotriene D_4 biosynthesis as well as evidence for enzymatic conversion of leukotriene A_4 to C_4 has been reported [12,26].

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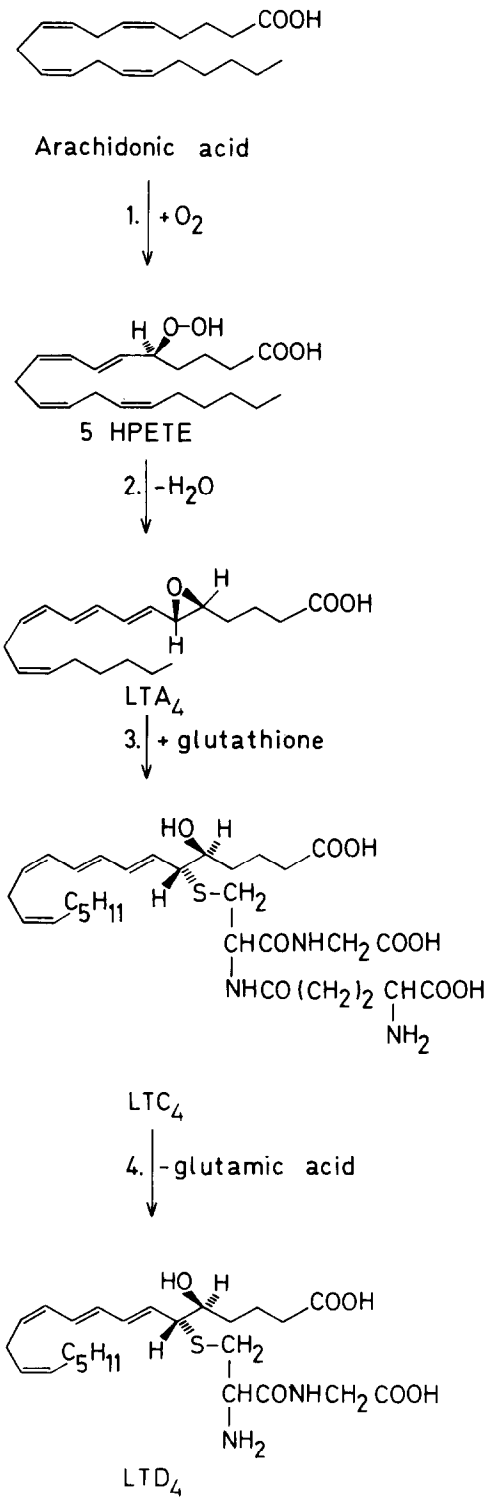

Fig.4. Pathway for the biosynthesis of leukotrienes C_4 and D_4: (1) lipoxygenase; (2) dehydrase; (3) glutathione-*S*-transferase; (4) γ-glutamyl transpeptidase.

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