

LEUKOTRIENE C₄: ISOLATION FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Received 13 October 1980

1. Introduction

A new group of arachidonic acid metabolites, the leukotrienes [1–3] has been found to include the slow-reacting substance of anaphylaxis (SRS-A). SRS-A is a mediator in various hypersensitivity reactions, among these asthma [4]. The first slow reacting substance identified was leukotriene C₄, which was isolated from incubations of murine mastocytoma cells with ionophore A23187 and L-cysteine [5–8]. The 11-*trans*-isomer of LTC₄ was also formed as shown later by synthetic studies [9]. A metabolite of LTC₄, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene D₄, LTD₄) was originally detected in RBL-cells [10]. LTC₄ and LTD₄ were also isolated from rat peritoneal cells [11–13], and human lung [12,13]. Partial support for the formation of LTD₄ in RBL-cells and in sensitized guinea-pig lung has also been reported from other laboratories [14–16].

LTC₄ was early proposed to be biosynthesized from arachidonic acid via 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE) and the unstable allylic epoxide (5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene A₄, LTA₄)) (fig.1) [2,5]. The intermediate LTA₄ [17] has been isolated from human PMNL [18] and shown to be converted into LTC₄ in these cells [19]. LTA₄ was also shown to be the precursor of 5(*S*),12(*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid (leukotriene B₄, LTB₄) [20] which had been isolated together with compounds I, II, IV and V (fig.1) from rabbit PMNL [21,22] and from human PMNL where the ionophore A23187 enhanced the biosynthesis [23]. The ionophore also stimulates the formation of SRS in human leukocytes [24,25].

This paper concerns the identification of the SRS produced in human PMNL, when incubated with

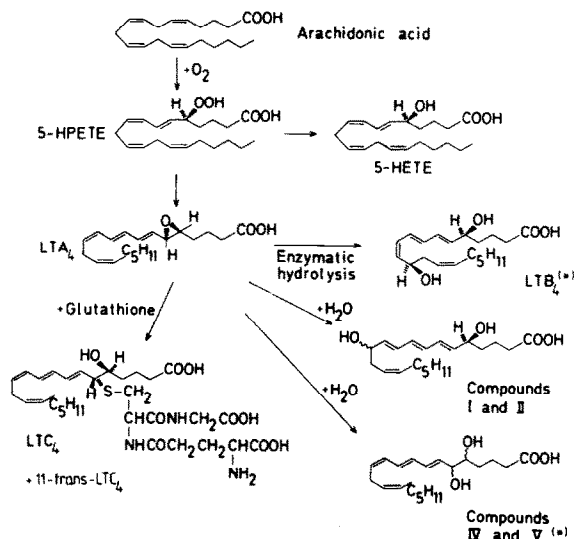


Fig.1. Leukotriene biosynthesis in human polymorphonuclear leukocytes. Asterisk indicates that the exact stereochemistry of the double bonds has not been determined.

ionophore A23187, arachidonic acid and L-cysteine or glutathione. This SRS is shown to be composed of leukotriene C₄ and 11-*trans*-leukotriene C₄.

2. Materials and methods

Arachidonic acid was purchased from Nu-Chek, MN, and ionophore A23187 from Calbiochem-Behring, La Jolla, CA. Dextran T-500 was obtained from Pharmacia, Uppsala and Lymphoprep from Nyegaard, Oslo. FPL 55712 was a kind gift from Fisons, Pharmaceut. Div., Loughborough and BW 755C (3-amino-1-(*m*-(trifluoromethyl)-phenyl)-2-pyrazoline) from Dr F. Kuehl, Merck, Rahway, NJ. Soybean lipoxygenase type I (EC 1.13.11.12) was

purchased from Sigma, St Louis, MO. Synthetic LTC₄ and 11-*trans*-LTC₄ were generously provided by Dr E. J. Corey, Harvard University, Cambridge, MA.

2.1. Cell preparation

Suspensions of human PMNL were prepared from concentrations of human leukocytes obtained upon blood component preparation at the Karolinska Hospital. The procedure outlined in [23] was followed, which implies centrifugation to remove platelet rich plasma, dextran sedimentation to separate white cells from red cells, ammonium-chloride lysis of remaining red cells and finally gradient centrifugation in Lymphoprep to separate PMNL from other white cells. For large scale incubations the Lymphoprep centrifugation was omitted and the whole leukocyte fraction was used.

2.2. Incubations

Around 30×10^8 cells suspended in 100 ml Dulbecco's PBS buffer (pH 7.4) were warmed to 37°C. L-Cysteine or reduced glutathione was added to a final concentration of 0.01 M. After 2 min, ethanol solutions of arachidonic acid and ionophore A23187 were added to the cell-suspension to final concentrations of 15 μ M and 5 μ M, respectively. The amount of ethanol never exceeded 0.1%. After 10 min the incubations were stopped by addition of 400 ml ethanol.

2.3. Isolation and purification

The ethanol solution from the incubation was filtered, evaporated to dryness and dissolved in dilute NaOH. The pH was then adjusted to 12. After 30 min at 37°C the sample was neutralized to pH 6 and extracted by Amberlite XAD-8 (Rohm and Haas, Philadelphia, PA) utilizing a batch-column procedure. The sample was eluted with 80% ethanol from the XAD-8 column. To the ethanol extract was added 1 g SilicAR CC-7 (Mallinckrodt, St Louis, MO) and the solvent was evaporated to dryness. The sample, with silicic acid particles, was applied to a column packed with 4 g SilicAR CC-7 in methanol-ethyl acetate (10/90, v/v) and eluted first with 200 ml of the same solvent followed by 200 ml methanol. The methanol eluate was subjected to reverse-phase HPLC.

2.4. High performance liquid chromatography (HPLC)

Reverse phase HPLC was performed on Polygosil

60-10 C₁₈ (10 \times 500 mm) and Nucleosil 5 C₁₈ (4.6 \times 250 mm, Macherey-Nagel, Düren) using a Constametric III pump with a Rheodyne 7125 injector and a Spectromonitor III variable UV-detector (LDC, Riviera Beach, FL). The solvent systems were methanol-water-acetic acid (70/30/0.1, by vol.) for the Polygosil column (flow rate 4.5 ml/min) and methanol-water-acetic acid (60/40/0.1, by vol.) for the Nucleosil column (flow rate 1.0 ml/min). The solvents were buffered to apparent pH 5.1 with ammonium hydroxide. The methanol eluate from the silicic acid column was chromatographed on the Polygosil column. The biologically active fractions were subsequently rechromatographed on the Nucleosil column.

2.5. Analytical methods

Bioassay was performed on the isolated guinea-pig ileum in a 7 ml cuvette with Tyrode's buffer containing atropine sulfate (10^{-6} M) and mepyramine maleate (10^{-6} M) as in [5]. Ultraviolet spectrum in the wavelength range 350–220 nm was obtained on a Cary 219 spectrophotometer (Varian, Palo Alto, CA). For incubation with soybean lipoxygenase, 2 nmol substrate were incubated with 50 μ g enzyme in 1 ml, 0.1 M phosphate buffer (pH 7.4 at 20°C) for 30–45 min. Amino acid analyses were performed on a Beckman 121 M instrument after hydrolysis in 6 M HCl with 0.5% phenol at 110°C for 20 h. Norleucine (5 nmol) was added as internal reference.

3. Results and discussion

Human PMNL incubated with ionophore A23187, arachidonic acid and L-cysteine produced two slow reacting substances. The presence of arachidonic acid and cysteine increased the amounts of ionophore-induced SRS several-fold, in accordance with earlier reports [26–28]. In our experiments cysteine could be replaced by glutathione.

The SRS produced in the incubations was isolated and purified by a procedure involving alkaline hydrolysis, extraction by Amberlite XAD-8, silicic acid chromatography and two steps of reverse-phase HPLC. All SRS-activity was eluted in one single fraction, both in the XAD-8 and in the silicic acid chromatography. On the first HPLC column (Polygosil C₁₈) two components with SRS-activity separated (fig.2). The major component (peak I) was eluted after 27 min and the minor product (peak II) appeared

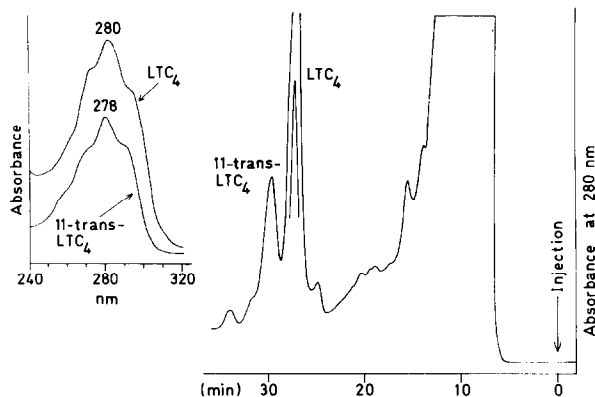


Fig. 2. Reverse-phase HPLC chromatogram of the products obtained from incubation of human PMNL with ionophore A23187, arachidonic acid and L-cysteine (ultraviolet 280 nm). The incubation was stopped with ethanol and the lipids were purified by chromatography on Amberlite XAD-8 resin followed by CC-7 silicic acid, before HPLC. The Polygosil C₁₈-column was eluted with methanol/water/acetic acid, (70/30/0.1, by vol.) pH 5.1, 4.5 ml/min. The insert shows the ultraviolet spectra of the two main products.

after 31 min. The material in the two peaks were homogenous when rechromatographed on the Nucleosil C₁₈ column. They now appeared after 35 and 41 min, respectively.

The elution times of peak I on both HPLC columns were identical with that of synthetic LTC₄. This was also shown by cochromatography on Nucleosil C₁₈ of the material in peak I mixed with an equal amount of synthetic LTC₄. The ultraviolet spectrum of compound I was identical with that of LTC₄, with a maximum at 280 nm and shoulders at 270 and 292 nm (fig. 2). Both compound I and LTC₄ gave identical results upon incubation with soybean lipoxygenase. A spectral change to a new maximum at 308 nm was seen, which indicated the formation of a conjugated tetraene. [5]. The amino acid composition of compound I and synthetic LTC₄, determined after acid hydrolysis, was identical. In the guinea-pig ileum bioassay, compound I and synthetic LTC₄ showed essentially the same properties, judged by the contractile response, the relaxation after addition of FPL 55712 (10⁻⁷ M), and the dose-response relationship (fig. 3).

Cochromatography of the material in peak II mixed with an equal amount of synthetic 11-*trans*-LTC₄ showed one homogenous peak. The ultraviolet spectrum of compound II had a maximum at 278 nm, with shoulders at 268 and 290 nm, identical with that

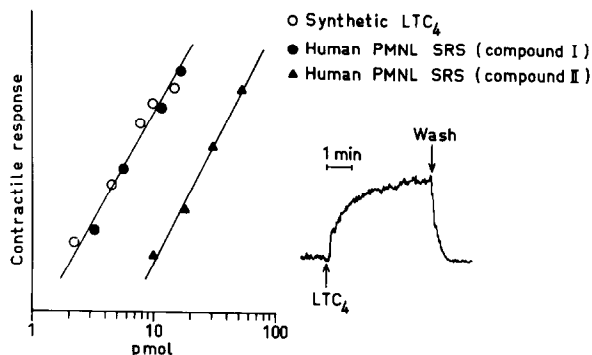


Fig. 3. Guinea-pig ileum bioassay on the two compounds obtained from incubation of human PMNL with ionophore A23187, arachidonic acid and L-cysteine. The amounts administered to the gut was estimated by ultraviolet absorption, assuming $\epsilon = 40\,000$ for these compounds.

of 11-*trans*-LTC₄ (fig. 2). Neither compound II nor 11-*trans*-LTC₄ was converted by soybean lipoxygenase, which indicated lack of the necessary 11,14-*cis* diene structure [29].

Amino acid analysis of compound II gave the same results as for compound I, LTC₄ and 11-*trans*-LTC₄. Compound II was less active than LTC₄ in the guinea-pig ileum assay (fig. 3), which is in accordance with observations in [9].

Thus, compound I and compound II in these incubations were identical with LTC₄ and 11-*trans*-LTC₄, respectively. When the lipoxygenase inhibitor BW 755C was added to the incubations (10 μ g/ml), none of these compounds could be detected [30].

Acknowledgements

The authors are indebted to Ms Siv Andell, Ms Ulla Andersson and Ms Inger Tollman-Hansson for excellent technical assistance and to Dr Hans Jörnvall for amino acid analysis. This work was supported by a grant from the Swedish Medical Research Council (project no. 03X-217).

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