

Isolation and characterization of 15-hydroxylated metabolites of leukotriene C₄

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Received 24 January 1983

A polar metabolite of leukotriene C₄ was formed by sequential conversions with soybean lipoxygenase I and liver peroxidase. The structure of this product was found to be 5(*S*),15(*S*)-dihydroxy-6(*R*)-*S*-glutathionyl-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (15-hydroxy- Δ^{13} -*trans*-leukotriene C₃). The HPLC behaviour, the molar extinction coefficient and the biological activity of the metabolite are reported.

Preliminary evidence suggests that this product is formed by mammalian tissues.

15-Hydroxy leukotriene	SRS-A	Lipoxygenase product	HPLC
Guinea pig ileum bioassay		UV spectroscopy	

1. INTRODUCTION

In experiments with homogenates and isolated organs from different animal species [1,2] and also in *in vivo* experiments [3,4] a rapid and extensive metabolism of leukotrienes has been observed. Increasing amounts of polar metabolites are formed with time. Presumably, different kinds of oxygenations are involved in the formation of such products. Lipoxygenases are enzymes that stereospecifically introduce a molecule of oxygen into polyunsaturated fatty acids. They play an important role in the metabolism of arachidonic acid and initiate the pathways leading to hydroxy acids, prostanoids* and leukotrienes.

Soybean lipoxygenase-1 inserts oxygen at the C-15 position of arachidonic acid. It requires a

cis,cis-1,4-pentadiene structure in the substrate with a methylene carbon at ω -8. This structural specificity was used to determine the positions of the three conjugated double bonds in leukotriene C₄ and the position of the sulfur containing substituent [5,6]. The product formed by the action of soybean lipoxygenase on leukotriene C₄ was however, not previously characterized. Since several animal tissues and cells contain enzyme(s) which converts arachidonic acid to 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid [7-11] it seemed of interest to isolate the products formed by the action of soybean lipoxygenase on leukotriene C₄ and its congeners leukotriene D₄ [12] and leukotriene E₄ [13]. The metabolites were enzymatically reduced and characterized chemically. The biological activity on isolated guinea pig ileum was also determined.

* The initial step catalyzed by prostaglandin endoperoxide synthase (fatty acid cyclooxygenase) involves stereospecific addition of molecular oxygen at C-11 of precursor acids and can be regarded as a lipoxygenase-type of reaction

Abbreviations: HPLC, high performance liquid chromatography; 15-HETE, 15-hydroxy eicosatetraenoic acid; UV, ultraviolet

2. MATERIALS AND METHODS

2.1. Materials

Soybean lipoxygenase type 1 (EC 1.13.11.12) was purchased from Sigma Chemical Co. FPL 55712 was provided by P. Sheard of Fisons Pharmaceuticals (Loughborough). [5,6,8,9,11,12,14,15-³H]Leukotriene C₄ was prepared using 5,8,11,14-

eicosatetraenoic acid as starting material according to [1]. Leukotrienes D₄ and E₄ were obtained by enzymatic degradation from leukotriene C₄ [12,13]. Synthetic leukotrienes C₄, D₄ and E₄ were kindly provided by J. Rokach, Merck-Frosst of Canada Inc. All leukotrienes were purified by reverse phase HPLC before use.

2.2. Preparation of high-speed supernatant from mouse liver

Livers from two mice were minced and homogenized in Tyrode's buffer (2 ml/g wet wt) at 0°C for 90 s using a Potter-Elvehjem homogenizer. The mixture was centrifuged at 600 × g and 0°C for 15 min. The supernatant was decanted and recentrifuged at 60000 × g and 0°C for 20 min. This high speed supernatant was frozen in 2 ml fractions or used immediately.

2.3. Incubations

Leukotriene C₄ (20 nmol) plus 2 μCi [³H]leukotriene C₄ (10 Ci/mmol) were evaporated to dryness under a stream of argon and dissolved in 5 ml of Tyrode's buffer. Soybean lipoxygenase (50 μl; 1 mg/ml) was added and the mixture was kept at room temperature. The reaction was followed by UV spectroscopy every 5 min with scanning between 220–350 nm in a Cary 219 spectrophotometer. After 35 min no further increase in absorption at 307 nm was observed. High speed supernatant from mouse liver (5 ml) was then added and the mixture was incubated at 37°C for 15 min.

After addition of 5 vol. ethanol the mixture was left on ice for 30 min and then centrifuged at 1200 rev./min and 4°C for 5 min. The supernatant was decanted and the pellet was resuspended in 5 ml ethanol and recentrifuged. The two supernatants were pooled, evaporated to dryness and dissolved in 2 ml HPLC-phase. The preparations were then purified by HPLC with a column of C₁₈ Polygosil (500 × 10 mm) using methanol/water/acetic acid (65:35:0.1, by vol.; adjusted to pH 5.4 with NH₄OH) as mobile phase. The products were detected by their UV-absorption at 307 nm.

Preparations of hydroxylated metabolites of leukotrienes D₄ and E₄ were analogous except that 10 nmol (100 Ci/mol) of each compound were used.

2.4. Bioassay

Bioassay was performed on isolated guinea pig ileum in Tyrode's buffer containing atropine sulfate (1 μM) and mepyramine maleate (1 μM).

3. RESULTS AND DISCUSSION

Products having conjugated tetraenes as judged by UV spectroscopy were formed by the action of soybean lipoxygenase on leukotrienes C₄, D₄ and E₄. These products, which probably contained a hydroperoxy group, were reduced to the corresponding hydroxy metabolites by incubations with high speed supernatant from mouse liver homogenates. The reaction velocity in the lipoxygenase reaction was about 1.7- and 3.5-times higher for leukotrienes D₄ and E₄, respectively, compared with leukotriene C₄.

Fig.1 shows an HPLC-chromatogram for the product obtained from leukotriene C₄. The retention time was 0.33 relative to leukotriene C₄. The metabolite from leukotriene D₄ and that from leukotriene E₄ had the same retention time (0.46 relative to leukotriene C₄).

The product from leukotriene C₄ was treated with γ-glutamyl transpeptidase [12]. Analyses by HPLC showed traces of the product formed from leukotriene C₄ and a less polar metabolite. The less polar product was found to be identical to the pro-

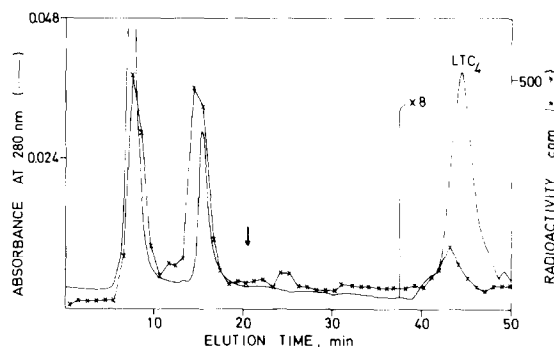


Fig.1. HPLC chromatogram of a metabolite formed from leukotriene C₄ by the action of soybean lipoxygenase and liver peroxidase (elution time = 15 min). The arrow indicates the elution time of corresponding products formed from leukotrienes D₄ and E₄ in separate experiments (21 min). For further details on formation of leukotriene metabolites and HPLC, see section 2.

duct obtained by treating leukotriene D₄ with soybean lipoxygenase and high speed supernatant from mouse liver homogenates.

Fig.2 shows the UV-spectrum of the product from leukotriene C₄. The spectrum has an absorbance maximum at 307 nm and shoulders at 294 nm and 322 nm. Since the specific activity of the tritium labeled leukotriene C₄ used as substrate was known, the molar extinction coefficient (ϵ) for the product could be calculated from UV absorbance and radioactivity measurements (73000 at 307 nm, 59000 at 322 nm and 52000 at 294 nm; solvent: methanol/water/acetic acid, 65:35:0.1 (by vol.) adjusted to pH 5.4 with NH₄OH).

UV-spectra of the products from leukotrienes D₄ and E₄ were similar to that of the leukotriene C₄ metabolite (not shown). The UV-spectra and the extinction coefficients are compatible with a conjugated tetraene structure having an allylic thioether substituent.

Desulfurization of the leukotriene C₄ product with Raney nickel and subsequent analyses by gas-liquid chromatography/mass spectrometry of the trimethylsilylether, methylester derivative showed a component at C-24.2 (OV-1) with ions at m/z 487(M-15), 471(M-31), 431(M-71), 365, 341(M-(90+71)), 311(M-(101+90)), 203(Me₃SiO⁻-CH-(CH₂)₃COOCH₃) and 173(Me₃SiO⁻-CH-(CH₂)₄CH₃). The spectrum was identical to that of 5,15-dihydroxyeicosaenoic acid (methyl ester, bis trimethylsilylether derivative [14]).

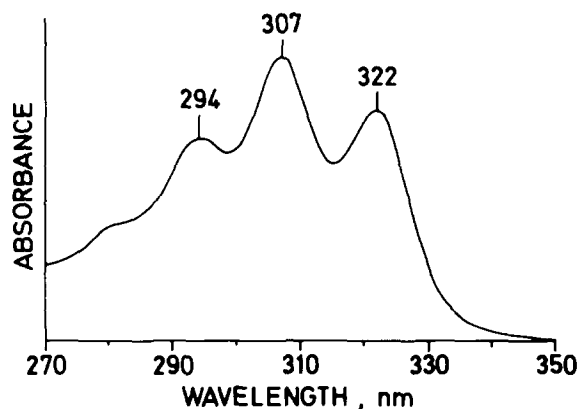


Fig.2. Ultraviolet spectrum of the metabolite of leukotriene C₄ isolated by HPLC in fig.1. The spectrum was recorded in methanol:water:acetic acid 65:35:0.1 (by vol.) adjusted to pH 5.4 by NH₄OH.

Based on the UV spectrum, the formation of 5,15-dihydroxyeicosaenoic acid by catalytic desulfurization and the conversion of the reduced lipoxygenase product of leukotriene C₄ during the incubations with γ -glutamyl transpeptidase the structure of the leukotriene C₄ metabolite is 5,15-dihydroxy-6-S-glutathionyl-7,9,11,13-eicosa-tetraenoic acid. The configuration of the 15-hydroxyl group is probably (S) and geometry of the Δ^{13} double bond is probably *trans* by analogy with the soybean lipoxygenase product formed from arachidonic acid [15]. Thus, the complete structure of the metabolite is 5(S),15(S)-dihydroxy-6(R)-S-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (15(S)-hydroxy- Δ^{13} -*trans*-leukotriene C₃). By the same nomenclature the products obtained from leukotrienes D₄ and E₄ are 15(S)-hydroxy- Δ^{13} -*trans*-leukotrienes D₃ and E₃, respectively. The formation and structure of the leukotriene C₄ metabolite are shown in fig.3.

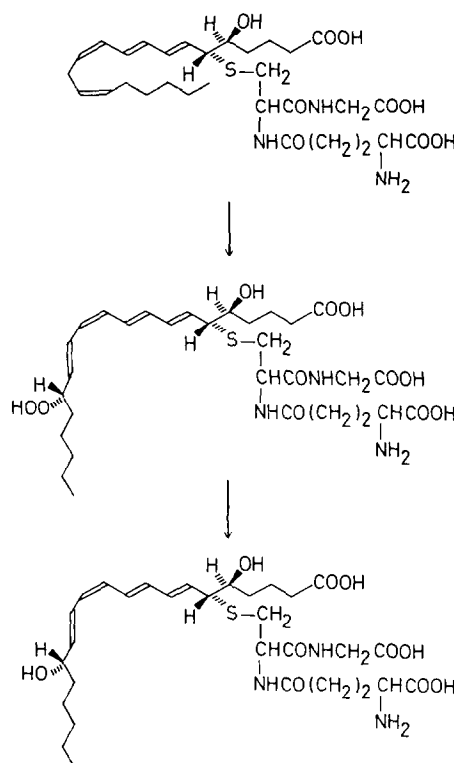


Fig.3. Structure and formation of 15(S)-hydroxy- Δ^{13} -*trans*-leukotriene C₃ from leukotriene C₄ by sequential conversions with soybean lipoxygenase and liver peroxidase.

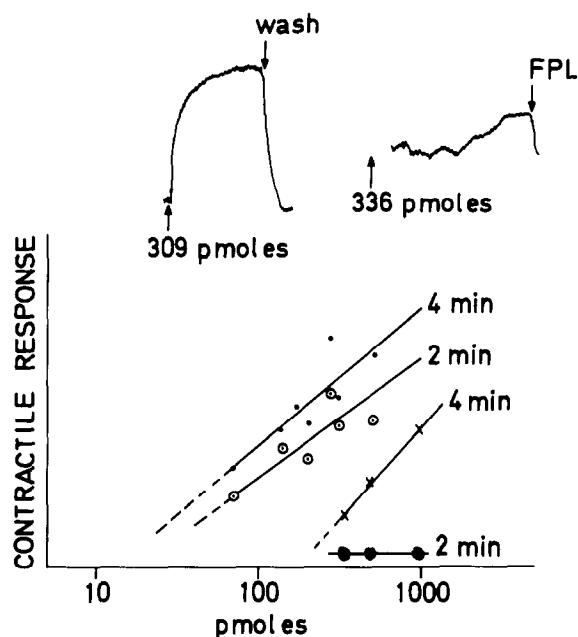


Fig.4. Contractile effects of leukotriene C₄ and 15-hydroxy-Δ¹³-trans-leukotriene C₃ on isolated guinea pig ileum. The upper part shows recordings of contractions of guinea pig ileum after addition of leukotriene C₄ (309 pmol) and 15-hydroxy-Δ¹³-leukotriene C₃ (336 pmol) to the organ bath. The lower part shows dose-response curves for leukotriene C₄ (—•— at 4 min and —○— at 2 min) and 15-hydroxy-Δ¹³-leukotriene C₃ (×—× at 4 min and —●— at 2 min after injection to the organ bath).

Fig.4 shows dose-response curves for 15-hydroxy-Δ¹³-leukotriene C₃ and leukotriene C₄ on isolated guinea pig ileum. 15-Hydroxy-Δ¹³-leukotriene C₃ gave a contraction that was reversed by the addition of FPL 55712. On a molar basis 15-hydroxy-Δ¹³-leukotriene C₃ was 7–11-times less potent than leukotriene C₄. A lag phase of about 3 min occurred between the injection of 15-hydroxy-Δ¹³-leukotriene C₃ and the onset of the contraction. After 2 min when the contraction due to leukotriene C₄ was almost maximal, no contraction was observed with 15-hydroxy-Δ¹³-leukotriene C₃. The lag phase may suggest that 15-hydroxy-Δ¹³-leukotriene C₃ was metabolized further prior to eliciting the contraction.

To summarize, leukotriene C₄ is metabolized by introduction of oxygen at C-15. This transformation diminishes the contractile activity on ileum

with about 90%. The contribution of mammalian 15-lipoxygenases to leukotriene C₄ metabolism is presently being investigated.

ACKNOWLEDGEMENTS

We wish to thank Ms Saga Elwe for excellent technical assistance, and Drs P. Sheard and J. Rokach for generous gifts of FPL 55712 and synthetic leukotrienes respectively. This work was supported by grants from the Swedish Medical Research Council (projects 03X-5914 and 03P-6396).

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