Isolation and characterization of 15-hydroxylated metabolites of leukotriene C_4

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A polar metabolite of leukotriene C_4 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 C_3). 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 C_4 and the position of the sulfur containing substituent [5,6]. The product formed by the action of soybean lipoxygenase on leukotriene C_4 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 C_4 and its congeners leukotriene D_4 [12] and leukotriene E_4 [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-$^3H_8$]Leukotriene C_4 was prepared using 5,8,11,14-

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eicosatetraenoic acid as starting material according to [1]. Leukotrienes D_4 and E_4 were obtained by enzymatic degradation from leukotriene C_4 [12,13]. Synthetic leukotrienes C_4, D_4 and E_4 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 C_4 (20 nmol) plus 2 μCi [^{3}H]leukotriene C_4 (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 C_{18} Polygosil (500 × 10 mm) using methanol/water/acetic acid (65:35:0.1, by vol.; adjusted to pH 5.4 with NH_4OH) as mobile phase. The products were detected by their UV-absorption at 307 nm.

Preparations of hydroxylated metabolites of leukotrienes D_4 and E_4 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 C_4, D_4 and E_4. 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 D_4 and E_4, respectively, compared with leukotriene C_4.

Fig.1 shows an HPLC-chromatogram for the product obtained from leukotriene C_4. The retention time was 0.33 relative to leukotriene C_4. The metabolite from leukotriene D_4 and that from leukotriene E_4 had the same retention time (0.46 relative to leukotriene C_4).

The product from leukotriene C_4 was treated with γ-glutamyl transpeptidase [12]. Analyses by HPLC showed traces of the product formed from leukotriene C_4 and a less polar metabolite. The less polar product was found to be identical to the pro-

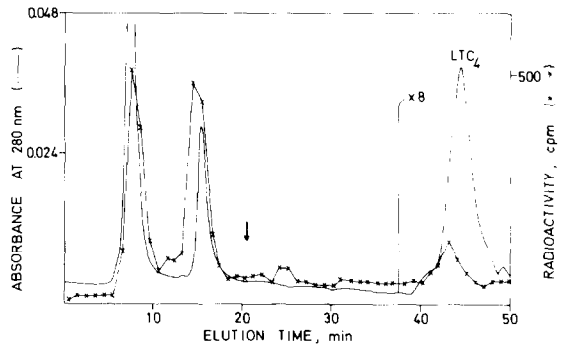

Fig.1. HPLC chromatogram of a metabolite formed from leukotriene C_4 by the action of soybean lipoxygenase and liver peroxidase (elution time = 15 min). The arrow indicates the elution time of corresponding products formed from leukotrienes D_4 and E_4 in separate experiments (21 min). For further details on formation of leukotriene metabolites and HPLC, see section 2.

duct obtained by treating leukotriene D_4 with soybean lipoxygenase and high speed supernatant from mouse liver homogenates.

Fig.2 shows the UV-spectrum of the product from leukotriene C_4. 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 C_4 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 NH_4OH).

UV-spectra of the products from leukotrienes D_4 and E_4 were similar to that of the leukotriene C_4 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 C_4 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(Me_3SiO^- $-CH-(CH_2)_3COOCH_3$) and 173(Me_3SiO^- $-CH-(CH_2)_4CH_3$). The spectrum was identical to that of 5,15-dihydroxyeicosaenoic acid (methyl ester, bis trimethylsilylether derivative [14]).

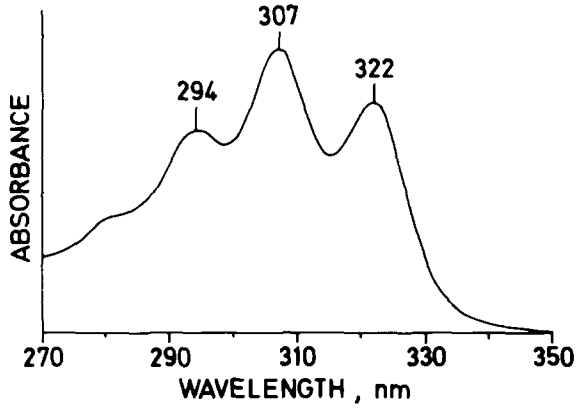

Fig.2. Ultraviolet spectrum of the metabolite of leukotriene C_4 isolated by HPLC in fig.1. The spectrum was recorded in methanol:water:acetic acid 65:35:0.1 (by vol.) adjusted to pH 5.4 by NH_4OH.

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 C_4 during the incubations with γ-glutamyl transpeptidase the structure of the leukotriene C_4 metabolite is 5,15-dihydroxy-6-*S*-glutathionyl-7,9,11,13-eicosatetraenoic 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 C_3). By the same nomenclature the products obtained from leukotrienes D_4 and E_4 are 15(*S*)-hydroxy-Δ^{13}-*trans*-leukotrienes D_3 and E_3, respectively. The formation and structure of the leukotriene C_4 metabolite are shown in fig.3.

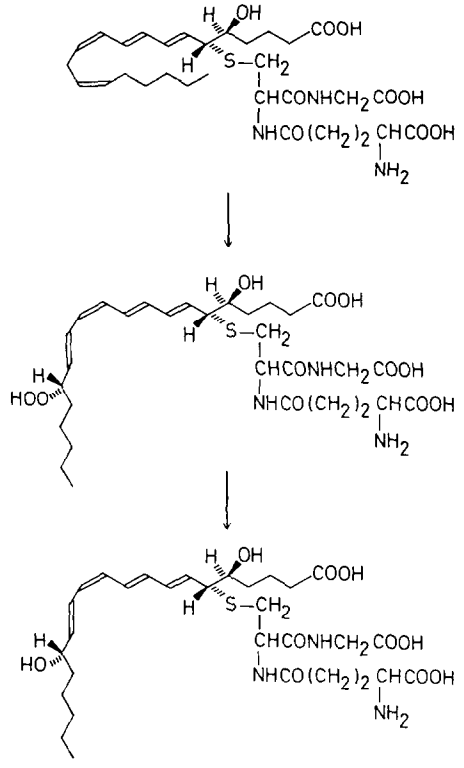

Fig.3. Structure and formation of 15(*S*)-hydroxy-Δ^{13}-*trans*-leukotriene C_3 from leukotriene C_4 by sequential conversions with soybean lipoxygenase and liver peroxidase.

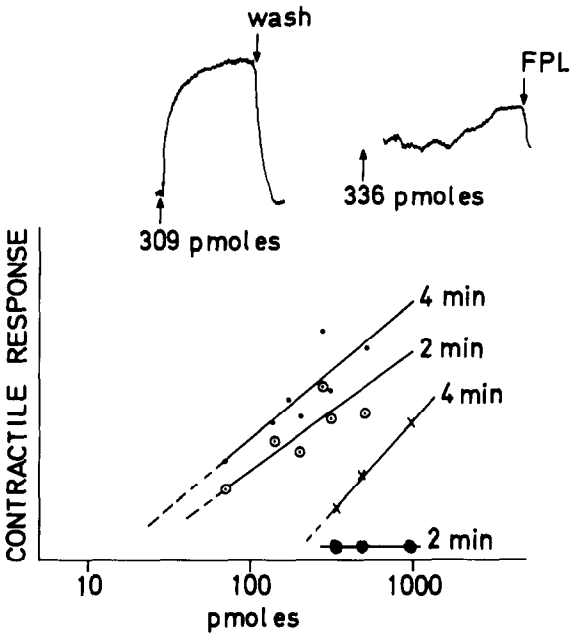

Fig.4. Contractile effects of leukotriene C_4 and 15-hydroxy-Δ^{13}-*trans*-leukotriene C_3 on isolated guinea pig ileum. The upper part shows recordings of contractions of guinea pig ileum after addition of leukotriene C_4 (309 pmol) and 15-hydroxy-Δ^{13}-leukotriene C_3 (336 pmol) to the organ bath. The lower part shows dose response curves for leukotriene C_4 (·——· at 4 min and ○—○ at 2 min) and 15-hydroxy-Δ^{13}-leukotriene C_3 (×—× at 4 min and ●—● at 2 min after injection to the organ bath).

Fig.4 shows dose–response curves for 15-hydroxy-Δ^{13}-leukotriene C_3 and leukotriene C_4 on isolated guinea pig ileum. 15-Hydroxy-Δ^{13}-leukotriene C_3 gave a contraction that was reversed by the addition of FPL 55712. On a molar basis 15-hydroxy-Δ^{13}-leukotriene C_3 was 7–11-times less potent than leukotriene C_4. A lag phase of about 3 min occurred between the injection of 15-hydroxy-Δ^{13}-leukotriene C_3 and the onset of the contraction. After 2 min when the contraction due to leukotriene C_4 was almost maximal, no contraction was observed with 15-hydroxy-Δ^{13}-leukotriene C_3. The lag phase may suggest that 15-hydroxy-Δ^{13}-leukotriene C_3 was metabolized further prior to eliciting the contraction.

To summarize, leukotriene C_4 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 C_4 metabolism is presently being investigated.

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