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LEUKOTRIENE C_4: THE MAJOR LIPOXYGENASE METABOLITE OF ARACHIDONIC ACID IN DOG SPLEEN

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SUMMARY: Slices of dog spleen converted [^{14}C]-arachidonic acid (AA) to a polar material which conjugated with [^{3}H]-glutatione. Nordihydroguaiaretic acid (NDGA) and 5,8,11,14, Eicosatetraynoic acid (ETYA) but not indomethacin, inhibited the conversion of [^{14}C]-arachidonic acid by the spleen slices into the polar material indicating that it is derived through the lipoxygenase pathway. Physicochemical analysis of the polar metabolite of arachidonic acid after thin-layer chromatography and high pressure liquid chromatography revealed that it has chemical properties identical to authentic leukotriene C_4 standard (LTC_4). The biological activity of the purified material was found to be similar to the slow reacting substance of anaphylaxis (SRS-A), viz, it caused contraction of the guinea-pig ileum which was abolished by FPL-55172, a specific SRS-A receptor antagonist. These data suggest that dog spleen slices convert arachidonic acid through lipoxygenase pathway into a polar material that appears to be identical to LTC_4.

INTRODUCTION: Hamberg reported that the major metabolites of arachidonic acid formed in the guinea-pig spleen were thromboxane and 12L-hydroxy-5,8,10,14 eicosatetraynoic acid (12 ho-17:3) (1). The amount of 12 ho-17:3 formed in the spleen was greater than thromboxane, and was also much higher when compared to that formed in the lung, indicating an active lipoxygenase pathway for the metabolism of arachidonic acid in the spleen. Among those arachidonic acid metabolites formed by lipoxygenase, leukotriene-C_4 (LTC_4), a conjugate of hydroxy arachidonic acid with glutathione, was recently found to be generated by mastocytoma cells stimulated by the calcium ionophore A-23817 (2). Leukotriene C_4, which appears

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Abbreviations: AA = arachidonic acid; NDGA = Nordihydroguaiaretic acid; ETYA = 5,8,11,14, eicostetraynoic acid; LT = Leukotriene

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to be identical to slow reacting substance of anaphylaxis (SRS-A), is also generated by other cell types (3,4). In the present study, a precursor role of arachidonic acid in LTC_4 synthesis by the dog spleen slices has been demonstrated. The material formed from arachidonic acid by the spleen appears to be identical to SRS generated non-immunologically in RBL-1 cells (5).

MATERIALS AND METHODS: Dogs weighing 20-25 kg were anesthetized with sodium pentobarbital i.v., and the abdomen opened by a mid-line incision. The vascular connections of the spleen with stomach, pancreas, liver and intestine were ligated and the splenic artery was cannulated. The spleen was immediately removed from the animal and flushed with oxygenated Tyrode solution (37°C) until the venous effluent became clear and free of blood. The spleen was cut into thin slices and separated from vascular segments. Slices (1g) were then incubated with [1-^{14}C]-arachidonic acid (1 µCi/g of tissue, New England Nuclear, specific activity 55 mCi/m mole) in the absence and in the presence of indomethacin, 5 µg/ml, and/or 5,8,11,14-eicosatetraynoic acid (ETYA), 10 µg/ml, in 10 ml of Tyrode solution at 37°C for 1 hr with constant shaking and exposure to a mixture of 95% O_2, 5% CO_2. In some experiments, the spleen slices were homogenized in 5 vol. ice cold Tris-HCl buffer, (pH 7.4, 50 mM) with a Polytron homogenizer before incubation with [1-^{14}C]-arachidonic acid (AA) as will be described. The incubation medium was then separated from the slices by low speed centrifugation (1,500 g, 10 min), acidified to pH 3 with formic acid and extracted three times with an equal volume of ethyl acetate. When spleen homogenate was used, it was acidified and extracted directly with ethyl acetate. The combined ethyl acetate phase was evaporated to dryness under vacuum and the residue dissolved in 300 µl mixture of chloroform-methanol (4:1, v/v). An aliquot (20 - 40 µl) of this extract, and authentic prostaglandins (PG) E_2, $F_{2\alpha}$, 6-keto-$F_{1\alpha}$, D_2, A_2, B_2 as well as thromboxane (Tx) B_2, arachidonic acid (AA) and phosphatidylcholine were applied to thin layer chromatographic (TLC) silica-gel G plates, 0.25 mm thick (Brinkman Instruments). These were developed twice using the solvent system iso-octane-ethyl acetate-acetic acid-water (25:55:10:50, v/v). Phospholipid and PG spots were identified by spraying with 10% phosphomolybdic acid in ethanol. The Rf values of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, PGA_2, PGB_2 and AA were approximately 0.19, 0.29, 0.43, 0.47, 0.64, 0.76 and 0.94, respectively. Radiolabeled products on the TLC plates were localized by scanning on a radiochromatograph scanner (Packard, Model 7320). After detection of the radiolabeled products, the area in the origin of the plates corresponding to phospholipid area (the major radioactive peak), was scraped and eluted with methanol. The methanol extract was evaporated to dryness and redissolved in 300 µl of chloroform-methanol (4:1, v/v) for each g of spleen slices. It was analyzed as follows:

Bioassay of the polar material: 100 µl of the sample was concentrated to a volume of 10-20 µl, reconstituted in 500 µl of saline,

and assayed by superfusion over isolated strips of guinea-pig ileum. The isolated strips of ileum were suspended in glass chambers and superfused with Krebs' solution (3 ml/min, 37°C), their contractions being measured with a Harvard muscle transducer and recorded on a physiograph (Rikadenki).

Purification of the polar metabolite of arachidonic acid by octadecylsilyl silica column: The polar metabolite extracted after incubation of 25g of spleen slices with 250 µg of arachidonic acid, and separated by thin-layer chromatography, was resuspended in distilled water and applied onto a column of octadecysilyl silica (ODS, silica, 10 x 10 cm, Water Associates), then washed with 20 ml of 15% ethanol, petroleum ether, methyl formate, and 80% ethanol, respectively (6). The fractions were dried under vacuum and resuspended in 500 µl of saline, then tested for their biological activity on the isolated guinea-pig ileum as described above. The fractions which contained biological activity were further purified by high pressure liquid chromatography (HPLC).

Purification of the polar metabolite of arachidonic acid by HPLC: Reverse phase HPLC (RP-HPLC) of the polar metabolite was performed on a C_{18}-µBondapak Column (4.6 mm x 30 cm, Water Associates). The column was prepared with methanol and the samples were suspended in 200 µl of methanol and injected to the HPLC by the use of an automatic injector (WISP-700B). The sample was eluted from the column isocratically with a solvent containing methanol:water:acetic acid (63:35:0.01, v/v) as described by Morris et al. (3). Fractions of 1 ml were collected with an on-line fraction collector (Gilson 800A) and simultaneously monitored for absorbance at 280 nm, using a Schoeffel variable wave length U.V. spectrometer.

Bioassay of the polar material purified by HPLC: Fractions separated by HPLC and having the same retention time as authentic LTC_4 standard were pooled and dried with nitrogen and suspended in saline for the assay of SRS activity on the isolated guinea-pig ileum superfused with Krebs' solution containing atropine (1 µM) and pyrilamine maleate (1 µM). One unit (U) of SRS activity was defined as that amount producing a contraction equal in amplitude to that elicited by 5 ng of histamine (7). The effect of LTC-like material was also tested on the isolated guinea-pig ileum exposed to FPL 55712 (10 µg/ml) (Fisons Pharmaceuticals, Loughborough, England), a specific antagonist of SRS (8).

Incorporation of [^{3}H]-glutathione into the polar material formed from [1-^{14}C]-arachidonic acid by the spleen slices. Glutathione, L [glycine-2-^{3}H] 2 µCi (specific activity 1.5 mCi/mmole, New England Nuclear) was added to the incubation medium containing 1g of spleen slices and 1 µCi of [^{14}C]-AA in 10 ml of Tyrode solution. The mixture was incubated for 1 hr at 37°C by exposure to 95% O_2 + 5% CO_2. The incubation medium was separated from the slices and extracted with ethanol as described earlier.

RESULTS AND DISCUSSION: When [1-^{14}C]-AA was incubated with slices of dog spleen and the radiolabeled products separated by thin-layer chromatography, the major radioactive product detected was

at the origin, corresponding to the phospholipid (PL) area. The levels of radioactivity detected in the PG and TxB_2 zones were very low compared to the PL area (Fig. 1a). Generation of this polar product from [^{14}C]-AA was also observed in spleen slices obtained from other species (e.g., rat, cat, and rabbit) (Malik and Wong, unpublished observation). When spleen slices were incubated with [^{14}C]-AA for 1 hr, washed three times with Tyrode solution and then incubated with [^{14}C]-AA-free Tyrode solution for 1 hr, the polar material was also released into the incubation medium (unpublished observations). In contrast, homogenates of spleen failed to form this polar material from [^{14}C]-AA (Fig. 1b). However, incubation of slices of spleen with the spleen homogenate did not prevent conversion of [^{14}C]-AA into the polar material, nor was the polar material hydrolyzed or degraded when eluted from thin-layer plates and incubated with the spleen homogenate for 3 hrs in Tyrode solution. In the presence of indomethacin (5 μg/ml), conversion of [^{14}C]-AA to the polar material was not affected, whereas ETYA (10 μg/ml) inhibited the formation of this material (3 experiments) (Fig. 1c). Nordihydroguaiaretic acid (10 μM), a specific lipoxygenase inhibitor, completely abolished the formation of the polar material from spleen slices (Fig. 1d).

When radioactive [^{3}H]-glutathione was added to the incubation medium together with [^{14}C]-AA, the polar meterial released from the spleen slices contained both [^{14}C]-AA and [^{3}H]-glutathione, indicating the incorporation of this tripeptide to the arachidonic acid metabolite (Table 1).

The polar metabolite of arachidonic acid recovered from TLC plates, when separated further by ODS-silica column, revealed that the biologically active substance had moderate polarity and could be separated from phospholipids with 15% ethanol. When

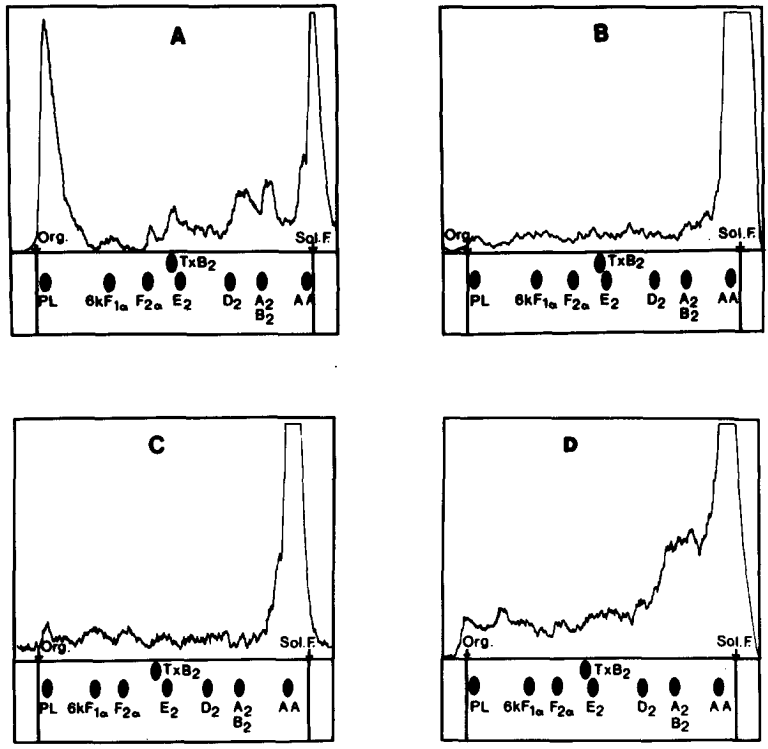

Figure 1: Chromatograms of the radioactive products formed from [^{14}C]-arachidonic acid by dog spleen slices (A) and homogenate (B) incubated in the absence and by dog spleen slices (C) incubated in the presence of 5,8,11,14 eicosatetraynoic acid (ETYA), 10 µg/ml (D) incubated in the presence of nordihydroguaiaretic acid (DNGA, 10 µM). The radioactive products were extracted from the incubation medium with ethylacetate and separated by thin-layer chromatography using isooctane-ethylacetate-acetic-water (25:55:10:50, v/v) as the solvent system. PL=Phospholipid area, prostaglandin 6-keto-$F_{1\alpha}$, $F_{2\alpha}$, F_2, D_2, A_2, B_2, thromboxane (Tx) B_2 and AA=arachidonic acid. Org.=origin, Sol. F=solvent front.

the material recovered in the fraction was further chromatographed on a C_{18}-RP-HPLC with on-line spectrophotometric monitoring at 280 nm, it yielded only one absorbance and radioactive peak with

Table I. Incorporation of [^{14}C]-arachidonic acid and [^{3}H]-glutathione into the polar material and LTC_4 before and after HPLC purification.

	Radioactivity (DPM)		
	Octadecylsilyl Column	HPLC Fraction (retention time 21 min)	Molar Ratio
[^{14}C]-arachidonic acid	264,825	66,206	0.545
[^{3}H]-glutathione	9,455,200	2,363,800	0.51

retention time of 21±1.2 min, which co-incided with authentic LTC_4 standard, but not with LTD_4 (Fig. 2a). The biological activity of the collected fractions on guinea-pig ileum corresponded directly to the absorbance peak (Fig. 2b) and remained unchanged before and after C_{18}-RP-HPLC. After HPLC, the biologically active fractions also co-incided with the radioactivity of both [^{14}C]-AA and [^{3}H]-glutathione, as measured by dual-channel liquid scintillation counting. FPL-55712, the antagonist of SRS (5,8), abolished the biological activity of the fraction corresponding to LTC_4 on guinea-pig ileum, but not that of histamine, acetylchonine or substance P (Fig. 3).

In this study we have demonstrated that [^{14}C]-AA, incubated with slices of dog spleen, releases into the medium a biologically active polar metabolite of arachidonic acid having TLC properties unlike that of any of the known prostaglandins or related substances. The polar metabolite caused contraction of guinea-pig ileum before

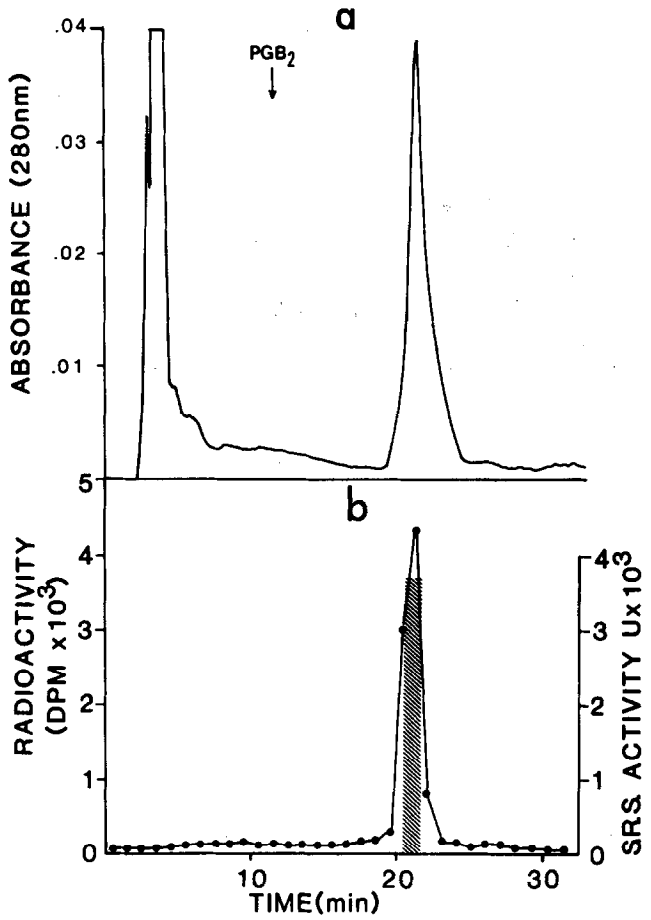

Figure 2: Reverse-phase HPLC profiles of LTC_4 derived from dog spleen previously separated from O.D.S. column. Stationary phase: μ Bondapak C_{18} (3.9 mm x 30 cm); mobile phase:methanol:water:acetic acid (65:35:0.01, v/v); Flow rate:2 ml/min. The HPLC eluate was monitored for U.V. absorbance at 280 nm (a); ^{14}C radioactivity (-0-0-) and SRS-A bioactivity (//////) (b).

and after purification using ODS-column and HPLC, suggesting that this substance is LTC_4 (2,3,4,5). Further, since incubation of homogenates from dog spleen failed to convert [^{14}C]-AA to this polar material, integrity of the cell membrane appears to be required for its formation. While it is possible that homogenization

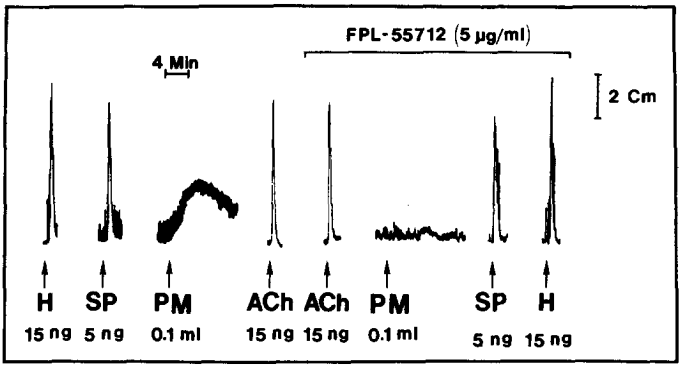

Figure 3: Effect of histamine (H), substance P (SP), polar material (PM) purified by HPLC and acetylcholine (Ach) on the isolated guinea-pig ileum superfused with Krebs' solution without and with FPL-55712, an inhibitor of SRS.

of the spleen may release an inactivator or an inhibitor of the formations of LTC_4, this is unlikely. Because incubation of the [^{14}C]-LTC_4 fraction with the homogenates of the spleen caused no appreciable breakdown of this material, nor did incubation of slices from spleen with homogenates of the spleen prevent conversion of [^{14}C]-AA to LTC_4.

Formation of this polar metabolite from [^{14}C]-AA by slices from dog spleen involve lipoxygenase rather than cyclo-oxygenase. This is inferred from our observations that indomethacin, the inhibitor of cyclo-oxygenase, failed to inhibit the conversion of [^{14}C]-AA to the polar material. However, ETYA and NDGA, both specific inhibitors of the lipoxygenase (5,12), blocked conversion of [^{14}C]-AA to LTC_4 by spleen slices. This suggests involvement of lipoxygenase pathway in the metabolism of arachidonic acid in the spleen.

The demonstration that the LTC_4 generated from AA by the dog spleen slices caused contraction of the guinea-pig ileum and that this contraction was abolished by SRS-antagonist FPL

55712, further supported our results in chromatographic analysis that the polar material is SRS-like and identical to LTC_4. The fact that the biologically active moiety was derived from [^{14}C]-AA and conjugated with [^{3}H]-glutathione, plus the evidence on C_{18}-RP-HPLC of a single peak as monitored by absorption at 280 nm, with retention time co-incided with LTC_4 standard, indicates that the polar material released from the dog spleen slices is identical to that of LTC_4 as described by Hammarstrom et al. (10), Murphy et al. (2), Morris et al. (3) and Hansson (11).

Although LTC_4 has been reported to be released from leukocytes (12), tumor cell lines (2) and guinea-pig lung after anaphylaxic shock (8), this is the first report that intact tissue slices can synthesize this highly biologically active substance and release it into the incubation medium. Thus, the predominant role of the lipoxygenase pathway in the metabolism of arachidonic acid in the spleen may be a unique immunoprotective function of this organ.

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