Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1038-1046

FORMATION OF THE CYSTEINYL FORM OF SLOW REACTING SUBSTANCE (LEUKOTRIENE E_4) IN HUMAN PLASMA

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Received October 27,1980

SUMMARY: The two major species of slow reacting substance (SRS) contain either a glutathionyl or cysteinyl-glycyl side chain. Incubation of these SRS's with undiluted or diluted (usually 1:10 or 1:50) human plasma at 37°C resulted in marked losses of smooth muscle contracting activity due primarily to conversion of their oligopeptide side chains to cysteine.

Our laboratory reported in 1977 that slow reacting substance (SRS)[2] is an arachidonic acid (AA) metabolite produced through the lipoxygenase pathway (1,2). Subsequently the two major naturally occurring species of SRS (3) have been shown to be 5-hydroxy- 6-S-glutathionyl, 7(trans), 9(trans), 11(cis), 14(cis) eicosatetraenoic acid (leukotriene C-4, LTC_4) (4) and 5-hydroxy-6-S(cysteinyl-glycyl) 7(trans), 9(trans), 11(cis), 14(cis) eicosatetraenoic acid (leukotriene D_4, LTD_4) (5-13). Early time course studies indicate that the glutathionyl form of SRS, LTC_4, is produced first and that the γ-glutamyl moiety is then removed to produce the cysteinyl-glycyl form, LTD_4 (14. 15). In addition, we have recently reported that when LTC_4 and LTD_4 are incubated for 60 min with RBL-1 cells, the rat basophilic leukemia cell

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[2]Abbreviations used in this paper are: slow reacting substance, SRS; arachidonic acid, AA; leukotriene C-4, LTC_4; leukotriene D-4, LTD_4; leukotriene E-4, LTE_4; phosphate buffered saline (0.15M NaCl, 0.01 M phosphate, pH 7.4), PBS; ethylenediaminetetraacetic acid, EDTA; rat basophilic leukemia cells, RBL-1 cells; high pressure liquid chromatography, HPLC.

0006-291X/80/231038-09$01.00/0

line used in early studies of SRS structure, their sulfur containing side chains are almost completely degraded to cysteine. (14,15) While precise assignments of double bond configuration have not been made, this product is presumably 5-hydroxy-6-S-cysteinyl 7(trans), 9(trans), 11(cis), 14 (cis) eicosatetraenoic acid (leukotriene E_4, LTE_4), a previously undescribed naturally occurring form of SRS with about 10% of the spasmogenic activity of LTD_4. We have also shown that commercial preparations of limpet Type B aryl sulfatase convert LTD_4 to LTE_4 presumably due to a contaminating protease. (14,15). These conversions to LTE_4 are of interest since LTE_4 differs in its biologic activity from the other leukotrienes and is relatively stable to further degradation. However, it remains to be demonstrated that the mammalian species which generate SRS have enzymatic systems which would produce this conversion in vivo. In the present study, human plasma is shown to readily degrade LTC_4 and LTD_4 to LTE_4, indicating that LTE_4 is likely to be a significant product of SRS metabolism in vivo.

METHODS

Plasma was obtained from the peripheral blood of normal healthy young adult human volunteers. The blood was anticoagulated by diluting it with 0.5 volumes of 0.15 M NaCl, 0.01 M phosphate pH 7.4 (PBS) containing 0.015 M ethylenediaminetetraacetic acid (EDTA) and the cells were removed by centrifugation at 1700 XG for 30 minutes. The plasma was stored frozen at -20°C. There was no apparent loss of activity over an 8 week period at this temperature. Plasma dilutions were made in PBS.
The SRSs used in these studies were produced biosynthetically in RBL-1 cells, and purified and characterized with regard to their spasmogenic activities on guinea pig ileal smooth muscle strips and other properties as described previously (3,5,15). The LTC_4 and LTD_4 comigrated on C18 reverse phase high pressure liquid chromatography (HPLC) columns (3,7,17) with naturally occurring and synthetic LTC_4 and LTD_4 from other laboratories (generously provided by S. Hammarstrom and J. Rokash, respectively). LTC_4 is the major component of our original Type I SRS (3,5) whereas our original Type II SRS is apparently a mixture of LTD_4 and 11-trans LTD_4 (15). LTE_4 was produced by incubating of RBL-1 cells for 20 or 60 minutes with A232187 and was characterized by HPLC, spasmogenic activity, ultraviolet absorbancy before and after treatment with soy bean lipoxygenase, cleavage with Na metal in liquid ammonia and amino acid analysis (14,15). Radiolabel was incorporated into the carboxy group of the fatty acid portion of SRS by stimulating RBL-1 cells with A23187 in the presence of [1-^{14}C] AA (1,3,5). SRS doubly labeled with ^{14}C in the glycine portion of the oligopeptide side chain (from [2-^{14}C] glycine) and with ^{3}H in the fatty acid moiety (from

[5,6,8,9,11,12,14,15-^{3}H] AA) (14,15) also was prepared. In some experiments the labeled SRS was diluted with unlabeled SRS of the same type.

At the completion of the incubation of the SRS with plasma four volumes of cold 95% ethanol were added. After thirty minutes at 4°C, insoluble material was removed by centrifugation at 35,000 X G for 30 min and the supernatent was chromatographed successively on Amberlite XAD-7 and high pressure liquid chromatography (HPLC) columns. Control studies indicated that even undiluted plasma did not affect the subsequent chromatographic behavior of SRS provided the SRS was exposed to the plasma only briefly at 4°C and the samples were handled in this manner. At plasma dilutions of 1:5 or greater the Amberlite XAD-7 column step did not affect the subsequent HPLC behavior of the SRS and was omitted. The overall recovery of radioactivity was 75% - 90%.

Two analytical HPLC systems were used in these studies. The first utilizes a Varian Micropak Monomeric C-18 column and a linear gradient from 6% methanol-0.6% t-pentanol-93.4% water buffered with 0.01 M phosphate pH 7.4, to 99.4% methanol - 0.6% pentanol. The column was operated at room temperature over a 100 ml total volume at a flow rate of 1 ml/min increasing the gradient by 1% methanol per min and collecting 1 ml fractions (3,17). The other is the isocratic μ Bondapak Waters system described by Murphy et al, which utilizes methanol: water: 0.1% acetic acid adjusted to pH 5.4 with NH_4OH as the solvent (7). The column was operated at room temperature at a flow rate of 1 ml/min, collecting 0.5 ml fractions.

RESULTS

When LTC_4 or LTD_4 doubly labeled with ^{3}H in the eicosatetraenoic acid moiety and with ^{14}C in the glycine moiety was incubated with diluted human plasma for 4 hours at 37°C a marked change in chromatographic behavior was observed (Fig. 1). With LTD_4 the bulk of the ^{3}H counts now migrated in the LTE_4 area and essentially all of the ^{14}C counts now eluted almost immediately from the column which is where free glycine is obtained. Small amounts of ^{3}H radioactivity also eluted rapidly from the column, probably due to oxidation of the lipid portion of the SRS molecule. LTC_4 was also altered by incubation with plasma although in this case the conversion was only about 75% completed. Incubation with plasma also produced a partial inactivation of SRS spasmogenic activity on guinea ileal strips. Under the conditions given in the legend to Fig. 1 there was 8-10 fold fall in spasmogenic activity of LTD_4 and most of the activity was now located in the column fractions where LTE_4 migrates as expected for a conversion of LTD_4 to LTE_4. A similar change occurred with LTC_4 although as expected from

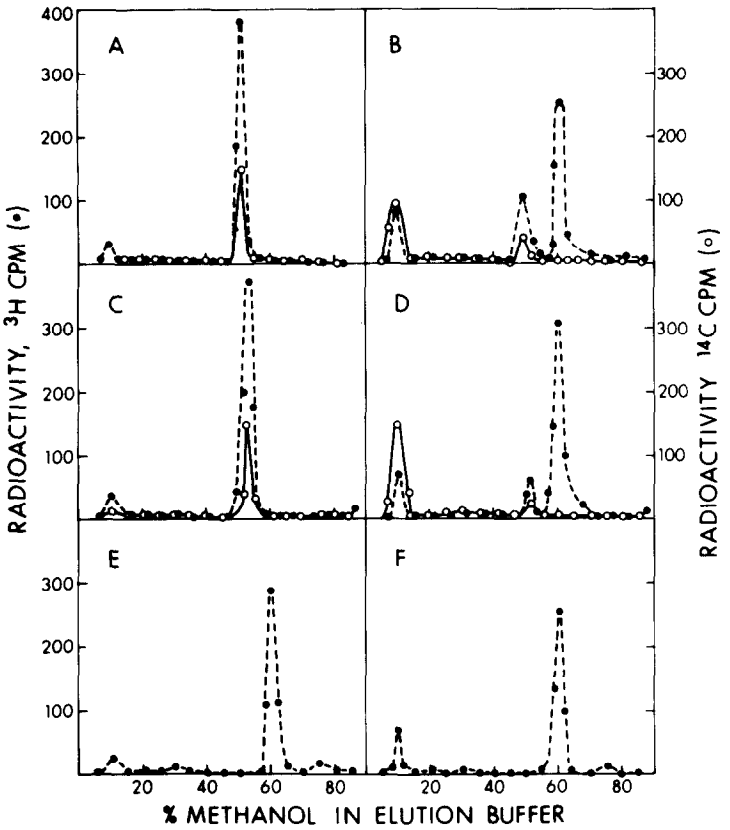

Figure 1: Samples of 300 picomoles of LTE_4, LTD_4 and LTC_4 containing about 600 ^{3}H CPM (from ^{3}H AA) were incubated for 4 hrs at 37°C in the absence and presence of plasma, 1:50, in a final volume of 0.05 ml. They were then extracted with ethanol and chromatographed on a Varian HPLC column. A, LTC_4 without plasma; B, LTC_4 with plasma; C, LTD_4 without plasma, D, LTD_4 with plasma; E, LTE_4 without plasma; F, LTE_4 with plasma. The LTC_4 and LTD_4 also contained about 240 ^{14}C CPM (from ^{14}C glycine).

the results in Figure 1 the conversion was only about 75% of the theoretical maximum.

Production of LTE_4 by plasma was confirmed with SRS labeled with ^{14}C at the 1-COOH position both on the Varian HPLC column system (not shown) and in the Bondapak HPLC system, which gives better resolution of LTC_4 from LTD_4 (Fig. 2). Again the LTC_4 and LTD_4 plasma digestion products comigrated with LTE_4. The incubation time with plasma was shorter in Fig. 2 than Fig. 1 (2 and 4 hrs, respectively) and the slower rate of conversion of LTC_4 than LTD_4 to LTE_4 is again

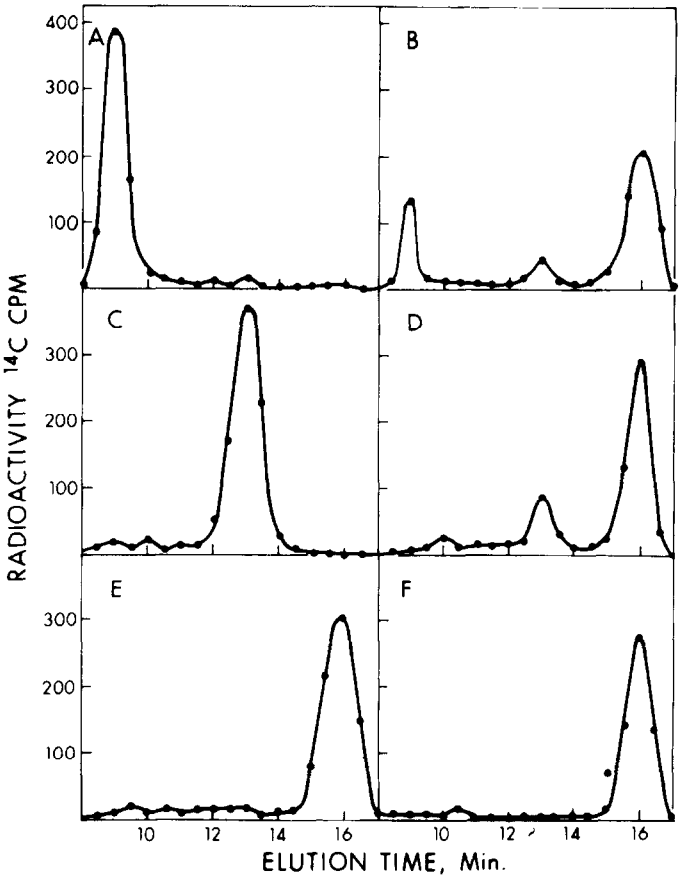

Figure 2: Samples of 300 picomoles of LTC_4, LTD_4 and LTE_4 containing about 500 ^{14}C CPM (from ^{14}C AA) were incubated in the absence and presence of plasma as described in the legend to Fig. 1, except that an 120 minute incubation time was used. The panel designations A-F are the same as those in Fig. 1.

evident. Serial studies with ^{3}H and ^{14}C labeled LTC_4 indicated that the initial product formed from LTC_4 is primarily LTD_4, which is then converted to LTE_4 (not shown but small amounts of LTD_4 can be seen in the plasma digest of LTC_4 in Fig. 2).

Further studies revealed that the conversion of LTC_4 and LTD_4 to LTE_4 is dependent both on the time and temperature of the incubation with plasma as well as the dilution of plasma used (shown for LTD_4, Table I). Degradation was actually less effective with undiluted plasma than with plasma diluted 1:10 or 1:50, presumably because binding of the

TABLE I

EFFECT OF INCUBATION WITH PLASMA ON THE CHROMATOGRAPHIC BEHAVIOR OF LTD_4

Experimental Condition		Time (Min)	Temp (°C)	Distribution of counts in bioreactive area[a]	
				49-53% Methonal	58-62% Methanol
Control	buffer	120	37	84(±6)	4(±3)
Unheated plasma	undiluted	120	37	24(±2)	47(±8)
	1:10	120	37	7(±1)	62(±12)
	1:50	120	37	6(±0)	66(±9)
	1:250	120	37	49(±14)	31(±10)
	1:1000	120	37	50(±7)	26(±2)
	1:2500	120	37	65(±4)	16(±2)
	1:5000	120	37	81(±11)	7(±4)
	undiluted	120	4	68(±6)	16(±5)
	1:50	120	4	68(±5)	13(±2)
	1:50	60	37	31(±7)	55(±11)
	1:50	30	37	41(±8)	36(±6)
	1:50	10	37	53(±14)	21(±5)
	1:50	0	—	85(±6)	6(±3)
Heated plasma[b]	1:10	120	37	78(±9)	8(±6)

Samples containing 520 SRS units and 560 ^{14}C LTD_4 CPM (from [1-^{14}C]AA) were incubated in the presence and absence of plasma as indicated. The SRS was extracted with ethanol and chromatographed on Amberlite XAD-7 and C-18 (Varian) HPLC columns or only the Varian HPLC column. See text. The data are given as the mean ± range for 2 experiments.

[a] LTC_4 and LTD_4 elute at 49-53% methanol and LTE_4 at 58-62% methanol on this column (see Fig. 1).

SRS by albumin limits its accessibility to the enzyme(s). Plasma dilutions of as much as 1:2500 resulted in some LTE_4 formation and at 1:50 dilutions considerable conversion was demonstrable within 10 min. When the plasma was heated at 90°C for 1 min before addition to the SRS,

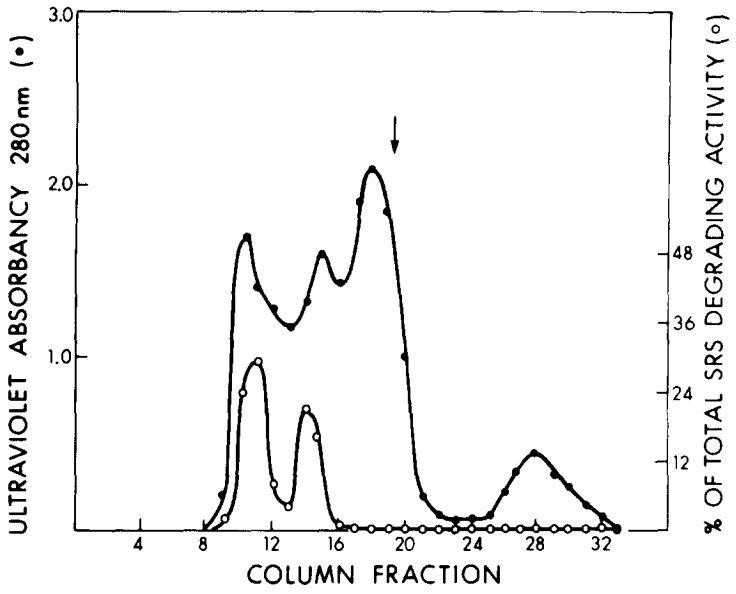

Figure 3: Fractionation of the glycine removing activity in plasma on Sephadex G-200-120. 0.25 ml of plasma was loaded onto a 31.5 X 1cm (25 ml) Sephadex column packed in 0.15 M NaCl, 0.01 M phosphate, pH 7.4, at 4°C and eluted with the same buffer collecting approximately 0.9ml fractions. Relative glycine removing activity was determined by incubating various dilutions of the fractions with 0.3 nanomoles of ^{14}C labeled LTD_4 for 2 hrs at 37°C and chromatographing on the Varian HPLC column. The calculation is based on the dilution producing approximately 25% cleavage. The last two ultraviolet absorbancy peaks correspond to human IgG and albumin. The arrow indicates the elution position of bovine serum albumin (MW 52,000).

conversion to LTE_4 was no longer observed, consistent with a role for plasma enzymes in the reaction. When plasma was fractionated by gel filtration on Sephadex G-200 columns, and the diluted column fractions were incubated with LTD_4, all of the glycine removing activity was in the large molecular weight fractions (Fig. 3). Two peaks of activity eluting before or together with IgG which has a molecular weight of about 150,000 were observed.

DISCUSSION

An understanding of how SRS is metabolized is one of the first steps in elucidating its role and likely duration of action in allergic reactions. The present study indicates that human plasma has activities which convert the two common forms of SRS, LTC_4 and LTD_4, to LTE_4.

The LTD_4 to LTE_4 conversion presumably occurs through an enzymatically mediated cleavage of the cysteinyl-glycyl peptide bond whereas the LTC_4 to LTE_4 conversion has the additional requirement that the γ-glutamyl-cysteinyl peptide bond be broken. It is known that human plasma contains at least several γ-glutamyl transpeptidases (18) carboxypeptidases (19,20) but the precise enzymes responsible for the SRS degradation in plasma will require further study. Since SRS is degraded by plasma that has been diluted as much as 1:2500, a similar conversion is likely to occur in vivo, not only in the blood but also in the interstitual fluid, where the level of large molecular weight proteins from plasma is normally relatively low. Evidence that LTC_4 and LTD_4 are metabolized to LTE_4 in allergic human beings is not yet available. However, studies in progess in our laboratory in mice injected intravenously with radiolabeled LTD_4 indicate that LTE_4 is one of several products that can be demonstrated in the urine, providing the first direct evidence that this pathway is involved in SRS degradation is vivo.

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