

Leukotrienes and Slow Reacting Substance of Anaphylaxis (SRS-A)

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SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A)

In 1938 Feldberg & Kellaway demonstrated that perfusates from dog and monkey lungs, treated with cobra venom, induced sustained contractions with slow onset on isolated guinea pig jejunum (19). Kellaway & Trethewie (26) named the active principle "slow reacting substance" (SRS) and showed that SRS was released by immunological challenge of sensitized guinea pig lungs.

The bioassay for SRS was subsequently refined by use of a histamine antagonist (mepyramine) in the organ bath (14). The term "slow reacting substance of anaphylaxis" (SRS-A) was introduced for immunologically released SRS and it was shown that perfused segments of human lung from asthmatic patients produced SRS-A after treatment with birch or timothy pollen. Furthermore, addition of allergens to bronchial rings from these patients resulted in a prolonged contraction of the bronchial muscle and release of SRS-A (14). This and other evidence have suggested that SRS-A is an important mediator of symptoms in asthma and other immediate hypersensitivity reactions (reviewed in 2 and 32).

The proposed pathophysiological importance of SRS-A stimulated research on the structure of this principle. With use of impure preparations and modifiers of functional groups it was suggested that SRS is a carboxylic acid with hydroxyl group(s) and one or more double bonds (47). Similar conclusions were obtained by others who also

proposed that SRS-A has amphoteric properties (iso-electric point 4.7) and a molecular weight of approximately 400 (35). Based on the observation that arylsulfatase inactivates SRS-A it was suggested that the molecule contains a sulfate ester group (34). Using cultured basophilic leukemia cells and isotopically labeled arachidonic acid it was suggested that this polyunsaturated acid is a precursor of SRS (24). These early results, however, suffered from a lack of efficient methods for the purification of SRS-A.

The use of high performance liquid chromatography (HPLC) has improved the situation and led to the resolution of multiple SRS components (5, 30). An ultraviolet spectrum of purified guinea pig lung SRS-A with an absorbance maximum around 280 nm and shoulders around 270 and 290 was interpreted as being due to aromatic (30) or a nonaromatic conjugated chromophore (29). The spectrum was furthermore used as evidence against a close relationship between SRS-A and products of arachidonic acid formed via lipoxygenase or cyclooxygenase pathways (30). Based on a recent observation that soybean lipoxygenase inactivates SRS-A it was suggested that the molecule contains a *cis,cis*-1,4-pentadiene structure (45).

ARACHIDONIC ACID METABOLISM IN POLYMORPHONUCLEAR LEUKOCYTES

Previous investigations on arachidonic acid metabolism in platelets led to the discovery of novel biologically active metabolites, the thromboxanes (formed from intermediates in

prostaglandin biosynthesis) and 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), a lipoxygenase product (reviewed in (44)). In view of these new developments it seemed of interest to investigate the metabolism of arachidonic acid in polymorphonuclear leukocytes. Initial investigations with cells obtained from the peritoneal cavity of rabbits after glycogen injection showed formation of a major product, 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), which is an isomer of 12-HETE, and is formed by a lipoxygenase (9). A more polar metabolite was identified as a dihydroxy acid containing three conjugated and one isolated double bonds (5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid (10); leukotriene B, cf. below). Stereoisomers of this compound were formed in smaller quantities (11). They were identified as the diastereo-isomeric 5(S),12-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid with 12(S) and 12(R) configurations, respectively. Two diastereoisomeric 5,6-dihydroxy-7,9,11,14-eicosatetraenoic acids were also identified (11). A characteristic feature of all these dihydroxy acids was a conjugated triene which resulted in u.v.-absorption with a maximum at about 270 nm.

It was not evident at this stage how the dihydroxy acid metabolites in the leukocytes were related to SRS-A. However, studies on their mechanism of formation had a profound effect on the development of this area of research. Isotopic oxygen experiments demonstrated that the oxygen of the alcohol group at C-5 originated in molecular oxygen, whereas the oxygen of the alcohol group at C-12 was derived from water. These findings led to the design of trapping experiments with ethanol and methanol. The ensuing studies showed the existence of an unstable intermediate, which could be converted into 12-O-alkyl derivatives. The half-life of the unstable intermediate was about 3 min. The proposed structure, 5,6-oxido-7,9,11,14-eicosatetraenoic acid (12) (leukotriene A, cf. below) of the intermediate has been confirmed by chemical synthesis and the stereochemistry

of the molecule has been determined (17, 40). The allylic epoxide intermediate, which is almost instantaneously hydrolyzed under acidic conditions, has recently been isolated from human polymorphonuclear leukocytes as the methyl ester using a novel technique for derivatization and isolation (41). The reaction mechanism described above for formation of the dihydroxy acids in leukocytes is different from that reported for dioxygenation of arachidonic acid by plant lipoxygenase (8).

A method, using HPLC and u.v.-absorption, was developed for quantitative determination of 5-HETE and the 5,12-dihydroxy acids in human polymorphonuclear leukocytes. Using this method the ionophore A23187 was found to stimulate the synthesis of 5-HETE and the 5,12-dihydroxy acids (13). This was of particular interest since the ionophore had earlier been shown to *inter alia* stimulate release of SRS from leukocytes (16, 18, 27, 46). The u.v.-absorption of the dihydroxy acids ($\lambda_{\max}$ about 270 nm) discovered in the leukocytes was similar to that ($\lambda_{\max}$ about 280 nm) reported for SRS-A (30, 35). Based on these and other considerations the hypothesis of a biogenetic link between the unstable allylic epoxide intermediate and SRS-A was developed.

ISOLATION AND STRUCTURE DETERMINATION OF SRS FROM MASTOCYTOMA CELLS

Preliminary experiments indicated that several previously described systems for generation of SRS (e.g. 3, 23, 25, 35) produced comparatively small quantities of bioactive material. However, murine mastocytoma cells, treated with ionophore A23187 and L-cysteine were found to be more suitable. A purification scheme consisting of treatment with base, chromatography on Amberlite XAD-8 and silicic acid followed by two steps of reverse phase HPLC afforded essentially pure SRS. This material gave the typical slow sustained contraction of guinea pig ileum which was reversed by the SRS antagonist FPL 55712 ((1), Fig. 1a). A characteristic property of the

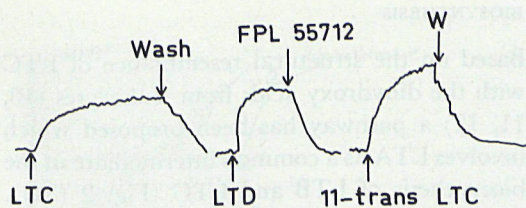


Fig. 1. Contractile responses on the isolated guinea pig ileum: a) LTC; b) LTD; c) 11-*trans*-LTC.

purified SRS was its ultraviolet spectrum with absorbance maximum at 280 nm and shoulders at 270 and 292 nm. The spectrum resembles those of the dihydroxy acids mentioned above (8, 10, 11) but was shifted 10 nm to higher wavelength. This is consistent with a sulfur substituent α to a conjugated triene (28). It has been previously reported that arachidonic acid (24) and cysteine (33) stimulated SRS synthesis. Experiments with [$^3\text{H}_8$]-arachidonic acid and [^{35}S]-cysteine showed that both tritium and sulfur-35 were converted to SRS. It was important to establish if cysteine was incorporated intact in view of the earlier proposal that SRS contains a sulfate ester (34). Experiments with [$3\text{-}^3\text{H}$] and [$\text{U-}^{14}\text{C}$] cysteine showed that the three carbon atoms of the amino acid were retained in SRS.

The ensuing structural work was performed by chemical degradations and an enzymatic transformation. Desulfurization with Raney nickel yielded 5-hydroxy-arachidic acid (plus mono-, di- and triunsaturated species) suggesting that the amino acid and arachidonyl moieties were connected by a thioether bond. The experiment also showed that all carbon atoms of the fatty acid remained in the SRS. The hydroxyl group at C-5 strongly suggested a metabolic relationship with the previously described arachidonic acid metabolites in leukocytes (see above).

Reductive ozonolysis of SRS prepared from [$^3\text{H}_8$]-arachidonic acid gave [^3H]-1-hexanol which demonstrated the presence of a Δ^{14} -double bond. The position of the conjugated triene was established in an experiment with soybean lipoxygenase. This enzyme converts

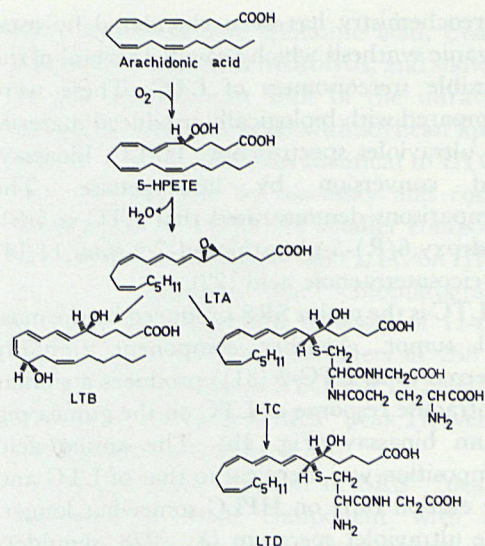


Fig. 2. Structures of leukotrienes (LT) and proposed scheme of formation.

unsaturated acids containing two methylene interrupted *cis* double bonds at the ω -6 and ω -9 positions. Oxygen is introduced at ω -6 and the ω -6 double bond is isomerized to ω -7 (20). The reaction of SRS with soybean lipoxygenase was followed by ultraviolet spectroscopy. A 30 nm shift of the spectrum to higher wavelength was observed, demonstrating the presence of a Δ^{11} -*cis* double bond and additional double bonds at Δ^7 and Δ^9 . The experiment also showed that the sulfur substituent was at C-6 since the alternative position (C-13) would give a greater bathochromic shift if the lipoxygenase reaction would indeed occur with a substituent at C-13.

Amino acid analyses of acid hydrolyzed SRS demonstrated that in addition to cysteine, 1 mol of glycine and 1 mol of glutamic acid were present per mol of SRS. The structure of the peptide as determined by end group (dansyl method and hydrazinolysis) and sequence analyses (dansyl-Edman procedure) was γ -glutamylcysteinylglycine (glutathione).

The experiments described above (21, 31, 42) showed that the structure of the SRS from murine mastocytoma cells is 5-hydroxy-6-*S*-glutathionyl-7,9,11,14-eicosatetraenoic acid (Fig. 2, leukotriene (LT)C, cf. below). The

stereochemistry has been elucidated by total organic synthesis which provided several of the possible stereoisomers of LTC. These were compared with biologically produced material by ultraviolet spectroscopy, HPLC, bioassay, and conversion by lipoxygenase. The comparisons demonstrated that LTC is 5(S)-hydroxy,6(R)-*S*-glutathionyl-7,9-*trans*,11,14-*cis*-eicosatetraenoic acid (22).

LTC is the major SRS produced by the mast cell tumor. Another component (initially referred to as LTC-2 (31)) produces a similar contractile response as LTC on the guinea pig ileum bioassay (Fig. 1b). The amino acid composition was identical to that of LTC and the elution time on HPLC somewhat longer. The ultraviolet spectrum ($\lambda_{\max}$ 278, shoulders 268 and 290 nm) and the inability to react with lipoxygenase suggested that the triene had all *trans* geometry. The structure (5(S)-hydroxy,6(R)-*S*-glutathionyl-7,9,11-*trans*,14-*cis*-eicosatetraenoic acid (Fig. 2) 11-*trans* LTC, cf. below) has been confirmed by total organic synthesis (15). The synthetic work described above was carried out by Dr E. J. Corey et al., Harvard University, Cambridge, Mass.

NOMENCLATURE

As several SRS molecules had been observed and in view of the cumbersome systematic names of the compounds described above, a nomenclature for the new group of biologically active compounds was needed. Because of their origin and the common structural feature of a conjugated triene, the trivial name "leukotriene" has been introduced (43). Various members of the group have been designated alphabetically (Fig. 2): leukotriene A is the unstable epoxide intermediate; leukotriene B, the enzymatically formed 5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid; leukotriene C, the glutathione containing SRS described above and leukotriene D, a cysteinylglycine containing SRS (see below).

BIOSYNTHESIS

Based on the structural resemblance of LTC with the dihydroxy acids from leukocytes (10, 11, 12) a pathway has been proposed which involves LTA as a common intermediate in the biosynthesis of LTB and LTC (Fig. 2 (31)). LTA has in turn been proposed to be formed from the lipoxygenase product of arachidonic acid in leukocytes, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE, Fig. 2 (12)). Recently, LTA has been isolated from leukocytes (41) and demonstrated to be a precursor of LTB (40). Furthermore, 5-HPETE has been enzymatically converted to LTB by disrupted porcine leukocytes (38). In agreement with the proposed biosynthetic pathway, lipoxygenase inhibitors have been found to inhibit formation of 5-HETE, leukotriene B and SRS in human polymorphonuclear leukocytes (39) and formation of LTC in mastocytoma cells (37).

STRUCTURE OF SRS FROM RAT BASOPHILIC LEUKEMIA (RBL-1) CELLS (36)

Treatment of RBL-1 cells with ionophore A23187, L-cysteine and arachidonic acid yielded a slow reacting substance which was less polar than LTC. The ultraviolet spectrum of the RBL SRS, the product obtained by Raney nickel desulfurization and the spectral change observed after treatment with soybean lipoxygenase were indistinguishable from those previously observed for LTC. This suggested that the fatty acid part of the molecules was identical. Amino acid analyses indicated that the RBL SRS contained cysteine and glycine but not glutamic acid. Edman degradation showed that glycine was C-terminal. Further proof for the structure was obtained by treating LTC with γ -glutamyl transpeptidase. The product was identical to the RBL SRS as judged by ultraviolet spectroscopy, conversion by lipoxygenase (the product and the RBL SRS reacted faster than LTC), bioassay on guinea pig ileum and behavior on reverse phase HPLC. The structure, 5(S)-hydroxy, 6(R) - *S* - cysteinylglycyl - 7, 9 - *trans*, 11, 14 - *cis*-eicosatetraenoic acid, LTD is shown in Fig.

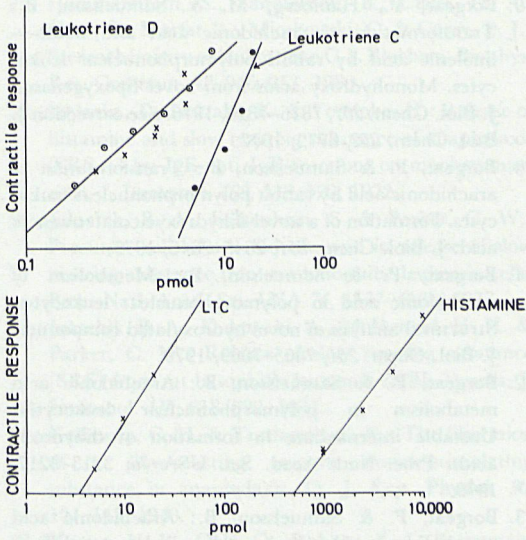


Fig. 3. Dose response relationships in the guinea pig ileum bioassay for leukotriene (LT)C, histamine and leukotriene D.

2. The contractile response on guinea pig ileum is similar to that of LTC (Fig. 1c) but the onset of the contraction is faster and LTD is more potent than LTC.

IDENTITY OF RAT MONONUCLEAR CELL SRS WITH LTC AND LTD

Rat peritoneal mononuclear cells produce a slow reacting substance (SRS^{ri}) when stimulated with ionophore A23187 and L-cysteine (3, 4). The use of straight phase HPLC resolved SRS^{ri} into two components (peaks I and II) (5).

Peak I corresponded chromatographically and by its contractile response on the guinea pig ileum bioassay to immunologically released SRS-A from human lung (5).

In collaboration with Dr M. K. Bach et al. SRS^{ri} has recently been characterized further: the more polar fraction (peak II) consisted of two components with SRS activity. The major one had an ultraviolet spectrum with absorbance maximum at 280 nm and shoulders at 270 and 292 nm which was identical to the spectra of LTC and LTD. This component gave 5-hydroxy arachidic acid

after desulfurization, glutamic acid, cysteine and glycine after acid hydrolysis, and showed a 28 nm bathochromic shift of the ultraviolet spectrum upon treatment with soybean lipoxygenase. The rat SRS was identical to LTC by these criteria and by bio-assay and cochromatography on HPLC. A smaller component of rat SRS eluted slightly after LTC on HPLC. Its u.v. spectrum and chromatographic behavior were identical to those of 11-*trans*-LTC (λ_{max} at 278 nm, shoulders at 268 and 290 nm). The yields of LTC and 11-*trans*-LTC from 3.5×10^6 units of SRS^{ri} peak II, were 40 and 10 nmol, respectively (6).

The less polar fraction of SRS^{ri} (peak I) contained a main component with SRS activity which was eluted from reverse phase HPLC in the same region where LTD elutes. The u.v. spectrum, the conversion by lipoxygenase, and the biological effects on guinea pig ileum (type of contraction and potency) of SRS^{ri} peak I and LTD were identical. The compound also cochromatographed with LTD and gave glycine and cystine after acid hydrolysis. Based on these criteria the major component of SRS^{ri} peak I was identified as LTD. Approximately 11 nmol of LTD were obtained from 3×10^6 units of SRS^{ri} . Peak I also contained a small amount of LTC (< 2 nmol) (7). Thus, about 270 units of peak I gave 1 pmol of LTD and 70 units of peak II gave 1 pmol of LTC plus 11-*trans* LTC. This agrees well with the absolute and relative potencies of LTC and LTD on the guinea pig ileum bioassay (21, 31, 36). The dose response relationships for LTC, LTD and histamine in the bioassay are shown in Fig. 3.

RELATIONSHIP BETWEEN THE LEUKOTRIENES AND SRS-A

SRS from several sources as described above has been shown to be due to one or both of leukotrienes C and D (LTC and LTD). LTC, which is the glutathionyl derivative of 5-hydroxy-eicosatetraenoic acid, is metabolized by γ -glutamyl transpeptidase (GGTP) into LTD. LTD is thus the corresponding cysteinyl-

glycine derivative. LTD is more active than LTC in the guinea pig ileum bioassay and also gives a more rapid response. LTD corresponds to the main component of human lung SRS-A (5, 7). However, SRS-A from different sources has been shown to consist of variable amounts of two components depending on the conditions used for generation. It therefore seems likely that SRS-A is due to a mixture of LTC and LTD. The relative amounts of these two leukotrienes under *in vivo* conditions remain to be determined. A third and minor product, 11-*trans*-LTC, was also detected.

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