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IDENTIFICATION OF THE C(6)-S-CONJUGATE OF LEUKOTRIENE A WITH CYSTEINE AS A NATURALLY OCCURRING SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A). IMPORTANCE OF THE 11-CIS-GEOMETRY FOR BIOLOGICAL ACTIVITY.

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Summary: A third major chemical constituent of slow reacting substance (SRS-A) has been shown to possess the chemical structure 5(S)-hydroxy-6(R)S-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid (leukotriene E). Comparison of the biological activities of leukotriene E and the 11-trans stereoisomer on guinea pig airways, ileum, and cutaneous microvasculature has revealed a noteworthy dependence of activity on stereochemistry with leukotriene E being much more potent in each system.

Slow reacting substance of anaphylaxis (SRS-A) (1) describes a biological activity generated during immediate hypersensitivity reactions in mammalian lung (2-6) and rat peritoneal cavity (7,8). Recently, this activity from rat peritoneum (SRS-A^{rat}) has been resolved into three major chemical components by high performance liquid chromatography (HPLC) (9). The first two activity peaks eluting from C-18 reversed phase (RP) HPLC were identified in both SRS-A^{rat} and SRS-A^{human} as leukotrienes C and D (LTC and LTD) (9,10), and their biological activities were quantified on guinea pig ileum, central and peripheral airway strips, and cutaneous vasculature (9,11). The third activity peak of SRS-A^{rat} eluting from HPLC has now been identified as 5(S)-hydroxy-6(R)S-cysteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid,for which the name leukotriene E (LTE) has recently been proposed (10). Further, the effects of the specific stereochemistry of the 11,12 ethylenic bond of this molecule on its biological activities has been assessed by comparison of LTE and 11-trans-LTE and related to the LTC and LTD series.

MATERIALS AND METHODS

LTC and LTD were prepared as described (9,12), sealed in ampoules in 4:1 0.1 M phosphate buffer (pH 6.8)-ethanol under argon, and stored frozen until the day of use. Histamine diphosphate and atropine sulfate (Sigma Chemical Co., St. Louis, MO), Amberlite XAD-8 (Mallinckrodt Chemical Works, St. Louis, MO),

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DE-52 cellulose (Whatman Ltd., Springfield Mill, UK), HPLC grade chloroform and methanol (Burdick and Jackson Laboratories, Inc., Muskegon, MI), and mepyramine maleate (Merck, Sharp, and Dohme, West Point, PA) were purchased from the manufacturers.

SRS-A was produced by IgGa-dependent anaphylaxis in the rat peritoneal cavity (13) and isolated by elution from XAD-9 and DE-52 cellulose (14). After SRS-A was dried by flash evaporation and resuspended in 1 ml of the HPLC running buffer (65% methanol, 34.9% water, 0.1% acetic acid, pH 5.6), it was chromatographed by isocratic elution from a C-18 RP-HPLC column (Altex Scientific, Inc., Berlekey, CA) in that buffer as described (9). Samples (1 ml) collected with on-line monitoring of absorbance at 280 nm were assessed for biological activity on the guinea pig ileum bioassay (2) and peaks of activity were separately rechromatographed on the same HPLC system. These purified peaks were then quantitatively assessed on the guinea pig ileum bioassay, standardized by defining a unit of SRS-A activity as that produced by a 5 ng/ml solution of histamine (2).

Synthetic leukotriene E (LTE) and the 11-trans isomer: N-Trifluoroacetyl-L-cysteine methyl ester, mp 30-31°C, was prepared from L-cystyne methyl ester (15) via N-trifluoroacetylcystine methyl ester (16), mp 152-154°C, by reduction with 1.1 equiv of triphenylphosphine in 2:1 dimethoxyethane-water at 23°C for 2 hr. Reaction of leukotriene A methyl ester (12,17-19) with N-trifluoroacetyl-L-cysteine methyl ester (2 equiv), hydroquinone (1 equiv) and triethylamine (4 equiv) in a minimum of methanol at 23°C for 1.5 hr under argon afforded N-trifluoroacetyl-leukotriene E dimethyl ester purified by RP-HPLC using a Waters Associates μ-Porasil-C_{18} column, with eluent 75:25 methanol-water containing ca. 0.1% acetic acid buffered to pH 5.6 by means of ammonium hydroxide; UV_{max} (methanol) 280 nm (ε, 40,000), 270 nm (ε, 31,000) and 290 nm (ε, 31,000). Deprotection of N-trifluoroacetyl LTE dimethyl ester using 0.13 M aqueous potassium carbonate and 5 equiv of hydroquinone in 3:1 water-methanol for 18 hr at 23°C under argon afforded in quantitative yield LTE UV_{max} (methanol-water) 270, 280 (ε, 40,000) 290 nm. When the deprotection of N-trifluoroacetyl LTE dimethyl ester was performed as above but in the absence of hydroquinone a mixture of LTE and 11-trans-LTE was produced (20). The isomers were separated by RP-HPLC in the above indicated system; retention volumes: LTE 15.3 and 11-trans-LTE 13.5. The UV_{max} (methanol-water) for 11-trans LTE were at 268 (ε, 31,000) 278 (ε, 40,000) and 288 (ε, 31,000) nm.

Biological Assays: The contractile effects of the synthetic leukotrienes were additionally evaluated on guinea pig pulmonary parenchymal strips. Concentration-effect relationships for the constriction of each tissue by histamine were determined as previously described (9,14). Each synthetic leukotriene was evaluated by its serial addition in increasing quantities to the organ bath over a concentation range of 4×10^{-13} to 4×10^{-6} M, allowing 2 to 3 minutes for the response to each added compound to develop fully. The response of each tissue was normalized to that achieved by exposure to 100 μM histamine, which was assigned a value of 100.

The effects of each leukotriene on cutaneous microvasculature was assessed after intradermal injection of the compound into the shaved dorsal skin of Hartley strain guinea pigs (11). When augmented cutaneous permeability was being assessed, 0.2 ml of Coomassie Brilliant Blue R-250 dye (BioRad Laboratories, Richmond, CA), 75 mg per ml of phosphate buffered saline (PBS), was administered intraveneously 15 min after the intracutaneous injections and when vasoconstriction was being evaluated, 1.0 ml of the dye was given 5 min after the intradermal injection of the agonist. The animals were killed under anesthesia, the skins were reflected and the lesional areas were assessed for diameter and intensity of bluing in the permeability-enhancing studies and for diameter and absence of bluing in the vasoconstriction protocol.

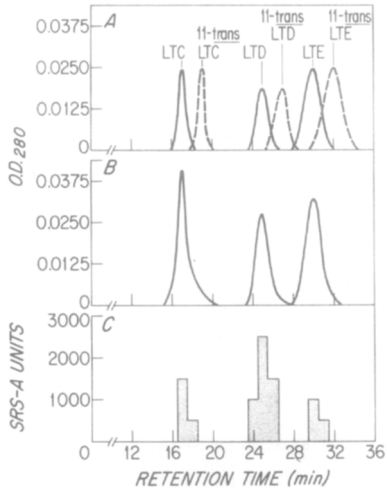

Fig. 1 RP-HPLC of three major peaks of SRS-A^{rat}: Retention times of (A) synthetic leukotrienes, (B) SRS-A^{rat} by 280 nm detection and (C) SRS-A^{rat} by measurement of biological activity with guinea pig ileum.

RESULTS

When SRS-A^{rat}, partially purified by deproteination, and elution from XAD-8 and DE-52 cellulose (14) was chromatographed on C-18 RP-HPLC, biological activity of SRS-A eluted in three areas, with the first and second corresponding to previously defined peaks of LTC and LTD at 16-18 and 25-27 min, respectively (9) and the third corresponding to LTE at 29-30 min. In four experiments, Peak I (LTC) represented 12-28% of the biological activity, Peak II (LTD), 50-64%, and Peak III, 18-36%. Recoveries compared to deproteinated material were not reduced by a second HPLC step which produced 280 nm absorbance corresponding to each region of ileum-contracting activity.

Chromatography of synthetic LTC, LTD, and LTE as well as of their 11-trans isomers on C-18 RP-HPLC with monitoring at 280 nm yielded absorbance peaks with average retention times of 17, 26, and 30 min, respectively, for the LTC, LTD and LTE and 19, 28, and 32 min for the corresponding 11-trans isomers (Fig. 1A).

The chromatogram shown in Fig. 1B is a composite of the second HPLC purification step for each peak of SRS-A^{rat} in a representative experiment. The elution pattern

of the three purified peaks of SRS-A^{rat} is displayed for both their 280 nm absorptions (Fig 1B) and their biological activities (Fig 1C). Chromatography of the six synthetic leukotrienes (Fig 1A) shows elution of LTC, LTD, and LTE at retention times corresponding to those of the three peaks of SRS-A^{rat} activity.

The specific activities of the biologically active peaks of SRS-A^{rat} were calculated utilizing the integrated areas from the HPLC chromatograms (280 nm detection), biological activities measured on the guinea pig ileum, and an assumed molar extinction coefficient at 280 nm (ε) of 40,000, as calculated for each of the leukotrienes (21). The specific activities of SRS-A^{rat} Peak I (LTC) and Peak II (LTD) were found to average 1.55 ± .13 and 4.75 ± 1.41 units/pmol (mean ± SEM), respectively. Specific activities of Peak III averaged 1.17 ± 0.05 units/pmol.

The specific activities of the six synthetic leukotrienes were calculated by directly assaying each on the guinea pig ileum on a dose-dependent basis (Fig 2). Two separate experiments were carried out to compare each pair of 11,12-cis-trans isomers. The activities for the respective leukotrienes averaged 1.4 (LTC), 1.0 (11-trans LTC), 5.0 (LTD), 1.4 (11-trans LTD), 1.0 (LTE),

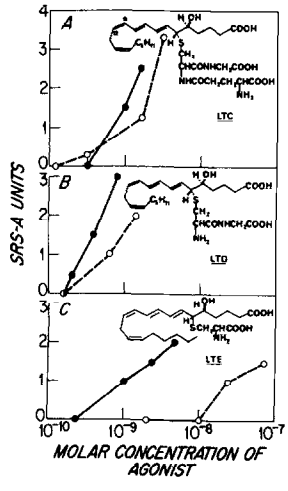

Fig. 2 Comparative dose-response contractile activities of (A) LTC, (B) LTD, and (C) LTE (solid lines), with the 11-trans isomer of each (dotted lines) on guinea pig ileum.

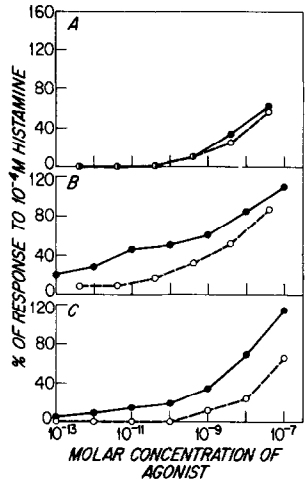

Fig. 3 Comparative dose-response contractile activities of (A) LTC, (B) LTD, and (C) LTE (solid lines), with the 11-trans isomer of each (dotted lines) on guinea pig pulmonary parenchymal strips.

and 0.04 (11-trans LTE) in units/pmol. The most noteworthy difference was the loss of activity of 11-trans LTE relative to both its 11-cis isomer and to the other assayed leukotrienes.

These observations were extended by comparison of the paired 11,12-cis-trans isomers on 4-7 guinea pig parenchymal strips (Fig 3). A contractile response representing 50% of that produced by the 100 μM histamine of the parenchymal strip was achieved with bath concentrations of 1×10^{-8} M LTC, 2×10^{-8} M 11-trans LTC (Fig 3A), 6×10^{-11} M LTD, 4×10^{-9} M 11-trans LTD (Fig 3B), 3×10^{-9} M LTE, and 5×10^{-8} M 11-trans LTE (Fig 3C). To achieve 2/3 of the maximal contractile response, bath concentations of 4×10^{-8} M (LTC), 6×10^{-8} M (11-trans LTC, extrapolated), 3×10^{-9} M (LTD), 10^{-8} M (11-trans LTD), 8×10^{-9} M (LTE), and 10^{-7} M (11-trans LTE) were required. Thus, at the higher active tensions, the pulmonary parenchymal strip assay resembled the ileum bioassay with regard to the disparity between LTE and 11-trans LTE.

As previously shown (11), intradermal injection of LTC over a range from 5-50 nanograms produced incremental vasoconstriction manifested by blanching of

increasing intensity in otherwise completely blued guinea pig skin. In contrast, both LTD and LTE elicited no vasoconstriction but each evoked a dose related lesional blueing within the same dose range in the assay for increased permeability. When LTE and 11-trans LTE were compared using assays for vasoconstriction an increased vascular permeability on three guinea pigs, the 11-trans isomer had no vasoconstrictive effects at doses up to 25 nanograms per skin site and only slightly increased vasopermeability at 50 nanograms - representing at least a 10-fold difference relative to the minimal effective dose of LTE.

The ultraviolet spectrum of purified SRS-A^{rat} Peak III was identical to that of synthetic LTE.

DISCUSSION

SRS-A^{rat}, when previously separated into its components by RP-HPLC, has been shown to be comprised of LTC, LTD, and, in 2 of 5 experiments, to possess a third uncharacterized major peak (9). SRS-A^{rat} Peak III is identified in the present study as LTE by chromatographic (RP-HPLC) (Fig 1) and ultraviolet spectral comparison with synthetic LTE of unambiguous structure. Further, the calculated specific activity of SRS-A^{rat} Peak III of 1.17 ± 0.05 (mean ± SEM) units/pmol (Fig 1) compares favorably with 1.0 units/pmol obtained from dose response experiments with synthetic LTE on the guinea pig ileum bioassay (Fig 2C).

That SRS-A^{rat} Peak III is LTE and that both the naturally occurring and synthetic compounds possess significant biological activities comparable to LTC on ileum and airway assays is a novel finding since LTE has not been detected previously in biological systems (21).

Comparison of synthetic LTE and 11-trans LTE by three bioassays demonstrated a 10-25 fold ratio of activity for the LTE/11-trans LTE on guinea pig ileum (Fig 2C), peripheral airway strips (Fig 3C), and cutaneous microvasculature. The activity ratios of each 11-cis compound to its 11-trans isomer is 1.4 for LTC, 3.6 for LTD, but 25 for LTE on guinea pig ileum. At the highest response levels assessed on the pulmonary parenchymal strip, the ratios of LTE to its 11-trans isomer exceed the ratios for LTC and LTD with theirs (Fig 3). The

greater ratios of LTD/11-trans LTD at lower airway tensions is ascribable to the interaction of LTD at low concentrations with high-affinity receptors eliciting a submaximal response (11).

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