FORMATION OF A NOVEL DIHYDROXYEICOSATETRAENOIC ACID*

Leukocytes

A new metabolite of arachidonic acid, 5-d-(S),12-d-(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid, was found upon incubation of the fatty acid with a suspension of rabbit peritoneal polymorphonuclear leukocytes collected 4 h after injection of glycogen into the peritoneal cavity. The yield of the dihydroxy acid was 0.5 to 2%. The compound possesses three conjugated double bonds and was found to be stereochemically pure at C-5 and C-12. Incubation of the cells with 8,11,14-eicosatrienoic acid did not lead to the formation of the analogous triunsaturated dihydroxy acid.

Recently, two novel monohydroxy acids, i.e. 5-d-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid and 8-d-(S)-hydroxy-9,11,14-eicosatrienoic acid were found to be the major metabolites of arachidonic acid and 8,11,14-eicosatrienoic acid, respectively, in rabbit peritoneal neutrophils (1). In view of the growing interest in the involvement of polyunsaturated fatty acids and their oxygenated metabolites (monohydroxy acids, prostaglandins, and thromboxanes) in various functions of leukocytes (2), we have extended our studies on the transformation of arachidonic acid by rabbit peritoneal polymorphonuclear leukocytes.

In this paper, we report the structure of a novel dihydroxy derivative of arachidonic acid.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid (specific activity ≥ 50 mCi/mmol) was purchased from the Radiochemical Centre, Amersham and purified by silicic acid column chromatography before use. Arachidonic acid was obtained from Nu-Chek Prep. (Elysian, Minn.). [5,6,8,9,11,12,14,15]0ctadeuteroarachidonic acid was prepared as described previously (3). 2-L-Hydroxyadipic acid was prepared by anodic coupling of ethyl 2-L-acetoxy-3-carboxypropionate and methyl hydroxycetate with chromic acid followed by reduction with sodium borohydride and hydrolysis (4).

Suspension of Rabbit Polymorphonuclear Leukocytes—Rabbit peritoneal leukocytes were obtained and prepared as previously described (1, 5) with the following minor modifications. Dulbecco's phosphate-buffered saline (100 ml) (6) containing 3 units/ml of heparin was used (instead of a 0.9% sodium chloride solution) to wash the peritoneal cavity of rabbits. After treatment of the cells with a buffered ammonium chloride solution (0.75%) to induce red cell lysis (7), the cells were centrifuged and resuspended in minimum essential medium (without calcium and glutamine, and supplemented with 25 mM Hepes' buffer) and kept in this medium at 5°C until incubation with arachidonic acid. The cell suspension was then centrifuged (250 x g for 15 min) and the pellets were resuspended in phosphate-buffered saline (instead of Krebs-Ringer bicarbonate). The viability of the cells as measured by the trypan blue exclusion test (8) was greater than 90%.

Incubation Conditions—The cells (35 x 10^9/ml) were incubated in phosphate-buffered saline (6) with [1-14C]arachidonic acid (4000 dpm/pg) at the concentration of 0.166 mM. Arachidonic acid was added to the cells in solution in ethanol (the final concentration of ethanol in the incubation buffer was 0.1%). The incubations were performed at 37°C for 4 min, under a normal atmosphere, and stopped by addition of 3 volumes of methanol.

Extraction Procedures—Extractions were performed as described before (1).

Column and Thin Layer Chromatography—Silicic acid column chromatography was performed in 5-mm diameter glass columns packed with 1 g of silicic acid (Mallinkrodt, 100 mesh) activated at 120°C and as follows. The dry residue from ether extraction was dissolved in 0.5 ml of diethyl ether/hexane, 25/75, v/v, and applied to the column. Different fractions of the sample were eluted using 30 ml of each of the following solvents in order: diethyl ether/hexane, 25/75, v/v; diethyl ether/hexane, 45/55, v/v; and ethyl acetate fraction of the silicic acid column chromatography was dissolved in 1 ml of methanol and treated with diazomethane (in diethyl ether) 5 min at room temperature to form the methyl esters. A similar procedure was used to form the ethyl esters, using diazoethane. The esterified samples were applied on the plates, which were developed three times in diethyl ether/hexane, 70/30, v/v.

Catalytic Hydrogenation—The samples (5 to 10 µg of the methyl esters) were dissolved in 0.5 ml of methanol. One milligram of platinum oxide was added and hydrogen was bubbled into the solution during 90 s at room temperature. The catalyst was rapidly removed by filtration on a column (3 x 10 mm) of silicic acid.

Gas Chromatography and Mass Spectrometry—The columns used for both radioisotopic chromatography and gas chromatography-mass spectrometry were packed with SE-30 (1%) Ultraphase on Gas-chrom Q or OV-210 (5%) on Supelcoport as indicated. Compounds (methyl esters) were eluted from thin layer plates with diethyl ether/hexane, 90/10, v/v, and transformed into trimethylsilyl (Me3Si) ethers by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine (60, 30, and 80 µl, respectively), 15 min at room temperature. The reagents were evaporated under a stream of nitrogen and the samples were dissolved in hexane for injection into the gas chromatograph. [H3]Me3Si derivatives were prepared by treatment of the

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The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me3Si, trimethylsilyl.

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samples with [3H]trimethylchlorosilane in pyridine (30 and 60 μl, respectively), 2 h at room temperature. The samples were first analyzed by radiogas chromatography (Barber-Colman Instrument, series 3000) for determination of the equivalent chain length of 14C-labeled metabolites. The samples were then studied by gas chromatography-mass spectrometry (LKB 9000 G Instrument) and mass spectra of the 14C-labeled compounds were recorded under electron impact with an ionizing energy of 22.5 eV and with a computer system (previously described (9)).

Steric Analysis of Alcohol Groups—The methyl ester of Compound I was converted into the (-)-dimethoxycarbonyl derivative as previously described (4). The dimethoxycarbonyl derivative was purified by thin layer chromatography (solvent system: diethyl ether/light petroleum ether, 15/85, v/v, three successive developments) and subjected to oxidative ozonolysis (10). Gas chromatographic-mass spectrometric analysis of the short chain menthoxycarbonyl derivatives formed was performed using a column of OV-210 (3%) and helium as the carrier gas.

Mass spectra were recorded during the elution of compounds to confirm the identity of the reaction products. The menthoxycarbonyl derivatives of dimethyl 2-hydroxyadipate and dimethyl malate were used as references. The menthoxycarbonyl derivatives of the 2-L-hydroxy esters gave single peaks on gas chromatography, whereas the menthoxycarbonyl derivatives of the 2-3,4-hydroxy esters gave two peaks of equal size (separation factor, 1.10 to 1.12). The more rapidly eluting peaks corresponded to the 2-L-hydroxy derivatives.

FIG. 1. Thin layer radiochromatogram of products (methyl esters) eluted in the ethyl acetate fraction of the silicic acid column.

RESULTS

A 100-ml suspension of rabbit neutrophils (35 X 10^9/ml) was incubated for 4 min with 1-[14C]arachidonic acid, 0.166 mM. The ether extract was subjected to silicic acid chromatography (as described above) and the compounds eluted in the ethyl acetate fraction were treated with diazomethane in ether and further purified by thin layer chromatography. Fig. 1 shows the pattern of 14C-labeled compounds (methyl esters) eluted in the ethyl acetate fraction of the silicic acid column.

Stable Carbon-13 Labeling—The methyl ester of Compound I is the major metabolite of this fraction. In order to obtain a sample suitable for structural analysis (free from contaminating material), Compound I was eluted from the thin layer plate and further purified by thin layer chromatography, using the same solvent system (three successive developments). The yield of the purified compound varied from 0.5 to 2% of added arachidonic acid in several experiments performed as described above. The second fraction eluted from the silicic acid column (in diethyl ether/hexane, 45/55, v/v) contained the 5-(2-hydroxy-4,8,11,14-eicosatetraenoic acid. The yield of this compound (purified as described previously, see Ref. 1) was 5 to 10 times higher than the yield of Compound I isolated from the same incubations (see “Discussion”).

The structure of Compound I is described here. The ultraviolet spectrum of the methyl ester of Compound I showed three main bands λ_max = 270 nm (ε = 51,000), 281 nm (ε = 39,500), and 260 nm (ε = 38,000), indicating three conjugated double bonds. This is in agreement with the reported ultraviolet spectrum of α-elaeeostearic acid CH_h-C(=CH-CH=CH-CH=CH-COOH) which has a λ_max = 270 nm (ε = 47,000) and two other absorption bands at 281 nm (ε = 36,500) and 261 nm (ε = 35,000) (11). The infrared spectrum (in CS2) showed bands inter alia at 992 cm\(^{-1}\) and 967 cm\(^{-1}\), the former being much stronger, in agreement with a conjugated triene consisting of two trans and one cis double bonds (reported for α-elaeeostearic acid in CS2, 992 cm\(^{-1}\) (strong) and 964 cm\(^{-1}\) (weak) (11)). Gas chromatographic analysis of the trimethylsilyl (Me_3Si ether derivative of the methyl ester of Compound I showed a peak with equivalent chain length C-23.6 (1% SE-30) and C-23.8 (3% OV-210). The mass spectrum is given in Fig. 2. Ions of high intensity were present at m/e 494 (M), 479 (M - 15), 404 (M - 90, loss of trimethylsilanol), 383 (M - 111, loss of CH_2=CH=CH=CH), 393 (M - (111 + 90)), 287, 297, 295 (M - 181), 191, 189, 187 (203 - 32), 167, and 129 supporting a tetraene with a conjugated triene consisting of two trans and one cis double bond (reported for α-elaeeostearic acid in CS2, 992 cm\(^{-1}\) (strong) and 964 cm\(^{-1}\) (weak) (11)). Gas chromatographic analysis of the trimethylsilyl (Me_3Si ether derivative of the hydrogenated compound which showed ions of high intensity at m/e 487 (M - 15), 471 (M - 31), 401 (M - 101, loss of CH_2=CH(CH_2)=COOCH_3), 389 (M - 113, loss of CH_2=CH(CH_2)=CH), 311 (M - (101 + 90)), 299 (M - (111 + 90)), 215 (base peak, Me_3SiO+), and 203 (Me_3SiO+), respectively, 2 h at room temperature. The samples were first analyzed by radiogas chromatography (Barber-Colman Instrument, series 3000) for determination of the equivalent chain length of 14C-labeled metabolites. The samples were then studied by gas chromatography-mass spectrometry (LKB 9000 G Instrument) and mass spectra of the 14C-labeled compounds were recorded under electron impact with an ionizing energy of 22.5 eV and with a computer system (previously described (9)).

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FIG. 1. Thin layer radiochromatogram of products (methyl esters) obtained from the ethyl acetate fraction of the silicic acid column chromatography of the ether extract (free acids). Rabbit polymorphonuclear leukocytes were incubated 4 min with arachidonic acid and the reaction was stopped with 3 volumes of methanol. Solvent system: the plate was developed three times in diethyl ether/hexane (70/30, v/v).

FIG. 2. Mass spectrum of the Me_3Si ether derivative of the methyl ester of Compound I.
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FIG. 3. Mass spectrum of the MeZi derivative of the methyl ester of hydrogenated Compound I.

FIG. 4. Steric analysis of alcohols of Compound I. A shows the total ionization recording of the fragments obtained after oxidative ozonolysis of the (-)-menthoxycarbonyl derivative of the methyl ester of Compound I. B shows the total ionization recording of a standard of (-)-menthoxycarbonyl dimethyl malate and (-)-menthoxycarbonyl dimethyl 2,6,8,10,14-eicosatetraenoate.

FIG. 5. Scheme of the transformation of arachidonic acid by rabbit polymorphonuclear leukocytes.

was reported to be formed from the double dioxygenation of arachidonic acid by soybean lipoxygenase (12, 13).

A sample of the new metabolite was isolated from the ether extract of an incubation mixture of rabbit peritoneal polymorphonuclear leukocytes and arachidonic acid. The extract was purified by silicic acid column chromatography of the free acids and thin layer chromatography of the methyl esters (Fig. 1). The presence and position of two hydroxyl groups as well as of four double bonds was unambiguously established by gas chromatographic-mass spectrometric analysis of several derivatives of the compound, including the hydrogenated compound (Figs. 2 and 3). The analysis of the fragments generated by oxidative ozonolysis of the (-)-menthoxycarbonyl derivative of the methyl ester of Compound I (Fig. 4) fully supported the positions of the two hydroxyl groups and of the four double bonds assigned by mass spectrometric analysis and allowed determination of the absolute configuration of the alcohols at C-5 and C-12. However, the geometry of the three conjugated double bonds at \( \Delta^5, \Delta^8, \) and \( \Delta^{10} \) remains partially unresolved. Infrared and ultraviolet data indicate that the conjugated triene consists of two trans and one cis double bond (see Ref. 11 for ultraviolet and infrared absorption data on synthetic geometrical isomers of 9,11,13-octadecatrienoic acid). These data however, do not allow the assignment of the geometry of individual ethylenic bonds. It is nevertheless likely that the double bond at \( \Delta^{14} \) has retained the cis geometry from arachidonic acid since it is not involved in the transformation of the fatty acid into the dihydroxy acid.

Compound I is the second most abundant metabolite of arachidonic acid in rabbit polymorphonuclear leukocytes. The major metabolite being the already described (1) 5-D-(S)-hydroxy 6,8,11,14-eicosatetraenoic acid. In view of structural

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Compound I is the second most abundant metabolite of arachidonic acid in rabbit polymorphonuclear leukocytes. The major metabolite being the already described (1) 5-D-(S)-hydroxy 6,8,11,14-eicosatetraenoic acid. In view of structural
similarities between the two metabolites, it seems possible that the formation of these two compounds is related. The percentage conversions of arachidonic acid into the monohydroxy acid and the dihydroxy acid are, respectively, 2 to 10% and 0.5 to 2% as measured by counting of the radioactivity of the two compounds purified by silicic acid chromatography and thin layer chromatography (data not shown). There was usually a 5- to 10-fold difference in the yield of the two metabolites obtained from the same experiments. Preliminary data (not shown) indicated that the formation of the 5,12-dihydroxy acid was maximal at substrate concentration around 0.08 mM. Addition of 8,11,14-eicosatrienoic acid to a suspension of rabbit polymorphonuclear leukocytes (data not shown) did not lead to the formation of detectable amounts of the corresponding dihydroxy trunsaturated C-20 acid but only to the formation of 8-[(S)]-hydroxy-9,11,14-eicosatrienoic acid as described before (1), pointing out the substrate specificity of the transformation. In order to rule out the possibility that the formation of Compound I could be attributed to platelets contaminating the leukocyte preparation, a control experiment with rabbit washed platelets was performed (data not shown). There was no detectable formation of Compound I or 5-hydroxy-6,8,11,14-eicosatetraenoic acid.

Studies are in progress to elucidate the pathway of formation of this novel metabolite of arachidonic acid in rabbit polymorphonuclear leukocytes and to examine its possible biological significance.

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