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ω6-OXYGENATION OF 6,9,12-OCTADECATRIENOIC ACID IN HUMAN PLATELETS

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Summary: [1-^{14}C]6,9,12-Octadecatrienoic acid was incubated with suspensions of human platelets. Three monohydroxy acids were isolated, i.e. 10L_S-hydroxy-6,8-pentadecadienoic acid, 10L_S-hydroxy-6,8,12-octadecatrienoic acid, and 13L_S-hydroxy-6,9,11-octadecatrienoic acid. Aspirin (0.5 mM) and indomethacin (10 μM) completely inhibited formation of the first mentioned compound whereas 5,8,11,14-eicosatetraynoic acid (34 μM) inhibited formation of all three compounds. Isolation of 13L_S-hydroxy-6,9,11-octadecatrienoic acid demonstrates that human platelets possess a lipoxygenase activity catalyzing ω6-oxygenation of suitable poly-unsaturated fatty acids.

Arachidonic acid incubated with human platelets is transformed into 12L_S-hydroxy-5,8,10-heptadecatrienoic acid and thromboxane A_2 (1; cyclooxygenase pathway) and into 12L_S-hydroxy-5,8,10,14-eicosatetraenoic acid (1,2; lipoxygenase pathway). The substrate specificity of platelet lipoxygenase was studied by Nugteren (2). He found that a number of C_{20} polyenoic acids as well as 6,9,12-octadecatrienoic acid and 4,7,10,13,16,19-docosahexaenoic acid served as substrates for the enzyme. In the case of 8,11,14-eicosatrienoic acid (3) and 5,8,11,14,17-eicosapentaenoic acid (4) it was later confirmed that oxygenation occurred at C-12 (ω9 position) as expected.

The present paper, which is concerned with the metabolism of 6,9,12-octadecatrienoic acid in human platelets, demonstrates the presence in platelets of a lipoxygenase activity that efficiently catalyzes oxygenation at the ω6 position, in addition to the cyclooxygenase and ω9-lipoxygenase activities previously recognized.

MATERIALS AND METHODS

[1-^{14}C]6,9,12-Octadecatrienoic acid was prepared by incubation of [1-^{14}C]stearic acid with growing cultures of Tetrahymena pyriformis, strain GL (generously provided by Dr. O. Vestergaard, Aarhus Universitet, Denmark) at room temperature for 44-

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48 h (cf. 5). The chemical and radiochemical purity of the acid obtained was 95% or better and the specific radioactivity was 1 Ci/mol. Unlabeled 6,9,12-octadecatrienoic acid was purchased from Nu-Chek-Prep., Inc., Elysian, Minn.

Suspensions of human platelets (1-1.5x10^6/µl) in 0.154 M NaCl, 25 mM tris(hydroxymethyl)aminomethane, and 0.2 mM disodium EDTA; pH 7.4 were prepared as previously described (4).

Incubations of [1-^{14}C]6,9,12-octadecatrienoic acid (7-1103 µM) with platelet suspensions (10-15 ml) were carried out at 37° for 20 min with magnetic stirring. When inhibitors were included, these were incubated with the platelet suspension for 10 min at 37° (0.5 mM aspirin) or 2 min at 37° (10 µM indomethacin; 34 µM 5,8,11,14-eicosatetraynoic acid) prior to addition of substrate. The incubations were stopped by the addition of 5 vol. of ethanol and extracted with diethyl ether. The residue obtained after evaporation of the solvent was treated with diazomethane and subjected to thin layer radiochromatography (solvent, diethyl ether - hexane (1:1; v/v)). Material recovered following thin layer chromatography was further purified by straight-phase high performance liquid chromatography using a column of Nucleosil 5 µ (250x4.6 mm) and isopropanol - hexane (1:99; v/v) as the solvent.

Gas-liquid chromatography was carried out with an F&M Biomedical gaschromatograph, model 402. The stationary phases used were 10% SP-2330 on Supelcoport (analysis of poly-unsaturated fatty acid esters), 1% SE-30 on GasChrom Q (analysis of derivatives of hydroxy acids), and 5% QF-1 on GasChrom Q (analysis of ozonolysis products). Mass spectrometry in combination with gas-liquid chromatography was performed with an LKB 9000 instrument equipped with a column of 1% OV-1 on Chromosorb W.

Catalytic hydrogenation was carried out by stirring the unsaturated compound (methyl ester); 10-50 µg) with 2 mg of platinum oxide in 2 ml of methanol for 15 min under an atmosphere of H_2.

RESULTS

Identification of Products. Four major peaks of radioactivity were observed upon thin-layer radiochromatographic analysis of the esterified product isolated following incubation of [1-^{14}C] 6,9,12-octadecatrienoic acid with human platelets (Fig. 1). The peak close to the solvent front (R_F = 0.94) corresponded to substrate remaining unconverted. Material present in the peak at the origin was rechromatographed in a more polar solvent system (methanol - diethyl ether (3:97; v/v)) using the methyl esters of thromboxane B_2 and prostaglandins E_2 and $F_{2\alpha}$ as references. No peak of radioactivity appeared in the region of the reference compounds. Instead, the labeled material remained at the origin suggesting its identity with phospholipid(s) that had incorporated the labeled 6,9,12-octadecatrienoic acid.
Material present in peak A (Fig. 1) showed strong ultraviolet absorption with λ_{max}^{EtOH} = 233 nm indicating the presence of a pair

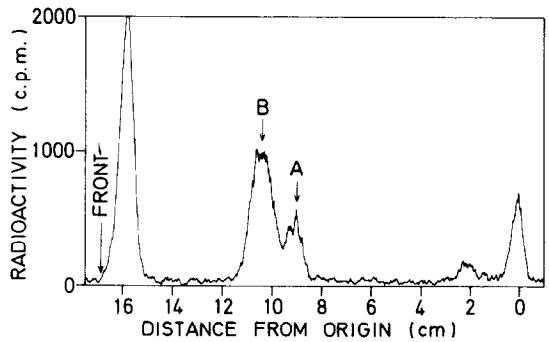

Fig. 1. Thin layer radiochromatogram of esterified product obtained after incubation of 133 µg of [1-^{14}C]6,9,12-octadecatrienoic acid with 12 ml of suspension of human platelets (1.22×10^6 platelets/ µl) at 37° for 20 min. Solvent system: diethyl ether - hexane (1:1; v/v).

of conjugated trans double bonds. Analysis of the Me_3Si [1] derivative by GC-MS showed a major peak with an equivalent chain length corresponding to C-17.5. The mass spectrum showed prominent ions at m/e 340 (M), 325 (M-15; loss of $\cdot CH_3$), 309 (M-31; loss of $\cdot OCH_3$), 269 (M-71; loss of $\cdot(CH_2)_4CH_3$), 250 (M-90; loss of Me_3SiOH), 225 (M-115; loss of $\cdot(CH_2)_4COOCH_3$), and 147 (269-(90+32)). This suggested that Compound A was the methyl ester of 10-hydroxy-6,8-pentadecadienoic acid. Mass-spectrometric analysis of the Me_3Si derivative of the hydrogenated methyl ester (C-17.0) showed ions inter alia at m/e 329 (M-15; loss of $\cdot CH_3$), 273 (M-71; loss of $\cdot(CH_2)_4CH_3$), 244 (M-100; rearrangement followed by loss of $O{=}HC{-}(CH_2)_4CH_3$), and 173 ($Me_3SiO^+{=}CH{-}(CH_2)_4CH_3$) demonstrating a methyl pentadecanoate carrying a hydroxyl group at C-10. Oxidative ozonolysis of the (-)menthoxycarbonyl derivative (cf. 6) of Compound A afforded methyl hydrogen adipate and the (-)menthoxycarbonyl derivative of 2L-hydroxyheptanoic acid. On basis of these results Compound A was assigned the structure 10L_S-hydroxy-6,8-pentadecadienoic acid (methyl ester). The abbreviation 10-HPD is proposed for this hydroxy acid.

Material present in peak B (Fig. 1) was separated into two compounds, i.e. Compounds B_I and B_{II}, by straight-phase high performance liquid chromatography (Fig. 2). Both compounds showed

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[1] The abbreviations used are: Me_3Si, trimethylsilyl; GC-MS, gas-liquid chromatography - mass spectrometry; 10-HPD, 10L_S-hydroxy-6,8-pentadecadienoic acid; 10-HOT, 10L_S-hydroxy-6,8,12-octadecatrienoic acid; 13-HOT, 13L_S-hydroxy-6,9,11-octadecatrienoic acid.

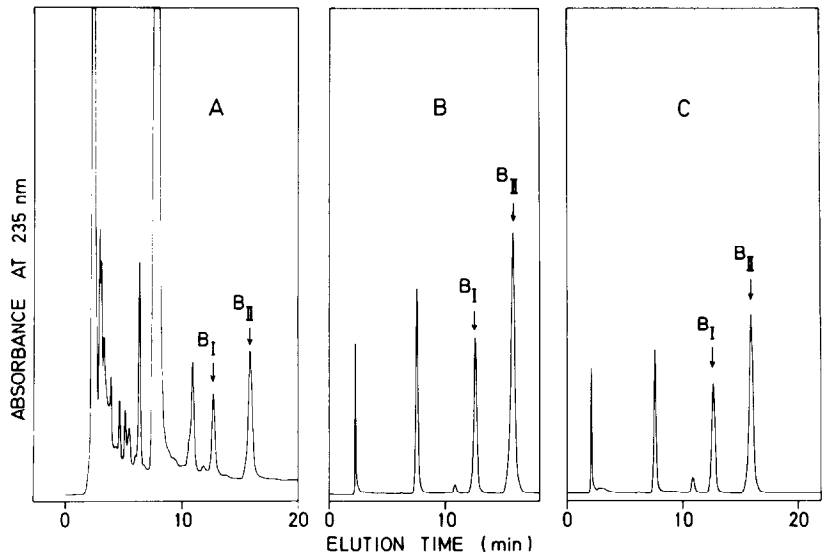

Fig. 2. Straight-phase high performance liquid chromatography of material present in peak B, Fig. 1. Column, Nucleosil 5 μ (250x4.6 mm); solvent, isopropanol - hexane (1:99; v/v); flow rate, 1.5 ml/min.
Concentrations of incubated 6,9,12-octadecatrienoic acid: in "A", 2 μg/ml; in "B", 45 μg/ml; in "C", 18 μg/ml.

strong ultraviolet absorption with λ_{max}^{EtOH} = 235 nm indicating the presence of a pair of cis/trans conjugated double bonds. The mass spectrum of the Me_3Si derivative of Compound B_I (C-19.6) showed ions of high intensity at m/e 380 (M), 309 (M-71; loss of $\cdot(CH_2)_4CH_3$), 290 (M-90; loss of Me_3SiOH), 225 ($[(CH{=}CH)_2{-}CH(OSiMe_3){-}(CH_2)_4CH_3]^+$), and 173 ($Me_3SiO^+{=}CH{-}(CH_2)_4{-}CH_3$) suggesting a methyl 13-hydroxyoctadecatrienoate. The mass spectrum of the Me_3Si derivative of hydrogenated Compound B_I (C-20.0) showed prominent ions at m/e 371 (M-15; loss of $\cdot CH_3$), 355 (M-31; loss of $\cdot OCH_3$), 339 (M-(15+32)), 315 (M-71; loss of $\cdot(CH_2)_4CH_3$), 286 (M-100; rearrangement followed by loss of $O{=}HC{-}(CH_2)_4CH_3$), and 173 ($Me_3SiO^+{=}CH{-}(CH_2)_4CH_3$) in agreement with a methyl octadecanoate carrying a hydroxyl group at C-13. Oxidative ozonolysis performed on the (-)menthoxycarbonyl derivative of Compound B_I afforded methyl hydrogen adipate and the (-)menthoxycarbonyl derivative of 2L-hydroxyheptanoic acid (Fig. 3). This result taken together with the ultraviolet data demonstrated that the double bonds of Compound B_I were located at Δ^6, Δ^9, and Δ^{11}, and that the hydroxyl group at C-13 had the L_S configuration. Compound B_I was thus methyl 13L_S-hydroxy-6,9,11-octadecatrienoate. The abbreviation 13-HOT is proposed for the parent acid. Compound B_{II} was also converted into the Me_3Si derivative and

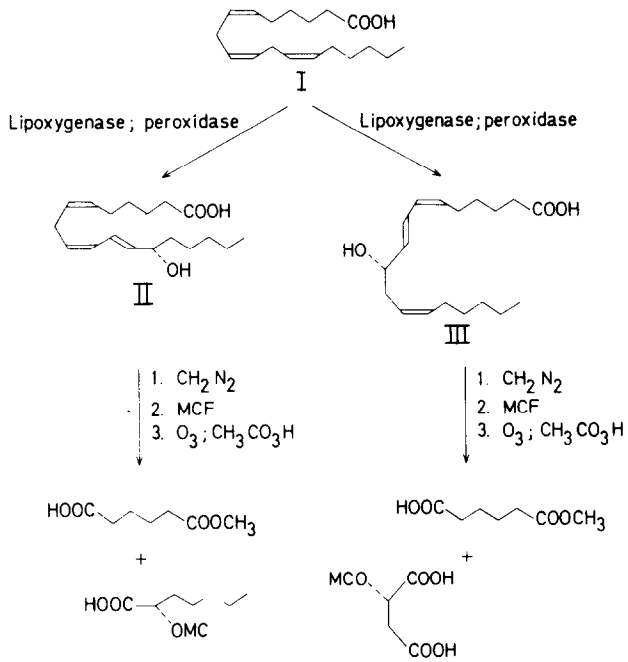

Fig. 3. Chemical degradation of 10- and 13-hydroxyoctadecatrienoates.
"I", 6,9,12-Octadecatrienoic acid; "II", $13L_S$-hydroxy-6,9,11-octadecatrienoic acid; "III", $10L_S$-hydroxy-6,8,12-octadecatrienoic acid; "MCF", (-)menthylchloroformate; "MC", (-)menthoxycarbonyl.

analyzed by GC-MS (C-19.6). The mass spectrum showed a prominent ion at m/e 269 (M-111; loss of $\cdot CH_2$-CH=CH-$(CH_2)_4CH_3$) as well as ions of lower intensity at m/e 365 (M-15; loss of $\cdot CH_3$), 349 (M-31; loss of $\cdot OCH_3$), 290 (M-90; loss of Me_3SiOH), 179 (269-90), and 147 (179-32). Catalytic hydrogenation of Compound B_{II} afforded methyl 10-hydroxyoctadecanoate as judged by mass-spectrometric analysis of the Me_3Si derivative (prominent ions at, inter alia, m/e 273 (M-113; loss of $\cdot(CH_2)_7CH_3$), 244 (M-142; rearrangement followed by loss of O=HC-$(CH_2)_7CH_3$), and 215 ($(Me_3SiO^+=CH-(CH_2)_7CH_3$)). Oxidative ozonolysis of the (-)menthoxycarbonyl derivative gave rise to methyl hydrogen adipate and the (-)menthoxycarbonyl derivative of L-malic acid (Fig. 3). The data thus showed that Compound B_{II} was identical with methyl $10L_S$-hydroxy-6,8,12-octadecatrienoate. The proposed abbreviation for the parent acid is 10-HOT.

Effect of Inhibitors. Conversion of 6,9,12-octadecatrienoic acid into 10-HPD was completely blocked in the presence of either 0.5 mM aspirin or 10 μM indomethacin. Formation of the two C_{18} hydroxy acids was not affected by these agents. This shows that

10-HPD was formed by the cyclooxygenase pathway (by elimination of malonaldehyde from the C_{18}-prostaglandin endoperoxide, 9α, 11α-epidioxy-15L_S-hydroxy-2,3-dinorprost-13-enoic acid) whereas the two latter compounds were formed by action of lipoxygenase(s). In agreement with this, the cyclooxygenase and lipoxygenase inhibitor, 5,8,11,14-eicosatetraynoic acid (34 μM), blocked formation of all three compounds.

Effect of Precursor Concentration. [1-^{14}C]6,9,12-Octadecatrienoic acid at concentrations 7-1103 μM was incubated with suspensions of human platelets. The methyl esters of 10-HOT and 13-HOT were isolated by thin layer chromatography (peak B, Fig. 1) and the ratio 10-HOT/13-HOT was determined by straight-phase high performance liquid chromatography (Fig. 2). As seen from Table I the ratio between the two isomers was essentially constant, i.e. 10-HOT/13-HOT = 1.94-2.11.

DISCUSSION

The present study shows that 6,9,12-octadecatrienoic acid is converted into cyclooxygenase as well as lipoxygenase products when incubated with human platelets. This is in agreement with previous results obtained with arachidonic acid (1,2), 8,11,14-eicosatrienoic acid (3) and 5,8,11,14,17-eicosapentaenoic acid (4). However, in the case of 6,9,12-octadecatrienoic acid the only cyclooxygenase product that could be isolated was a C_{15} monohydroxy acid, 10L_S-hydroxy-6,8 -pentadecadienoic acid. Thromboxane- or prostaglandin-like material could not be detected. Another difference concerns the lipoxygenase pathway: the C_{20} polyenoic acids were each converted into a single monohydroxy acid by oxygenation at C-12 (ω9)(1-4) whereas 6,9,12-octadecatrienoic acid gave rise to two isomeric hydroxy acids by oxygenation at ω9 and ω6. The ratio between the two isomers was about 2.0. The finding that this ratio was not significantly changed when substrate concentrations ranging from 7 to 1103 μM were used suggests action of a single platelet lipoxygenase with dual positional specificity. However, further studies are needed to determine whether one or two lipoxygenases are involved. After the present paper was completed a study appeared on hydroxy acid synthesis from 4,7,10,13,16,19-docosahexaenoic acid by human platelets (7). Two major isomers, i.e. 14(ω9)- and 11(ω12)-hydroxydocosahexaenoic acids were isolated. In that study the ratio between the two isomers was found to vary with respect to

substrate concentration (14-/11-hydroxy isomer at 6.8 μM docosahexaenoic acid, 4.2; 14-/11-hydroxy isomer at 54 μM docosahexaenoic acid, 1.9; ref. 7).

Although the present paper for the first time shows efficient (Table I) conversion of a poly-unsaturated fatty acid into an ω6-monohydroxy acid by action of ω6-lipoxygenase activity in platelets two previous studies in which small amounts of dihydroxy acids inter alia oxygenated in the ω6 position should be mentioned. These are 1) the study on the transformations of 8,11,14-eicosatrienoic acid in platelets (3) in which small amounts of 14,15(ω6)-dihydroxy-8,10,12-eicosatrienoic acid and 8,15(ω6)-dihydroxy-9,11,13-eicosatrienoic acid were tentatively identified; and 2) a recent report that showed release of small amounts of 14,15(ω6)-dihydroxy-5,8,10,12-eicosatetraenoic acid from platelets upon exposure to bradykinin (8).
The significance of the ω6-lipoxygenase pathway in platelets is unknown, however, recent studies on the biological activity of eg. 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid (9) make further investigation of the platelet ω6-lipoxygenase pathway and its products of interest.

Table I

Effect of precursor concentration on the yield and proportion of hydroxy-octadecatrienoate isomers. Each incubation was carried out with 10 ml of platelet suspension preincubated with 0.5 mM aspirin. "18:3" = 6,9,12-octadecatrienoic acid.

18:3 incubated (μg)	10-HOT+13-HOT isolated * (μg)	10-HOT/13-HOT**
Donor 1		
20	N.D.	1.97
50	24	1.94
180	82	2.04
450	220	2.11
Donor 2		
67	33	2.00
567	264	2.09
3067	313	2.07

* The amounts were determined by ultraviolet spectrometry after isolation by thin layer chromatography (see Fig. 1).

**The ratios were determined by straight-phase high performance liquid chromatography (see Fig. 2).

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