Synthesis of Hydroxy Fatty Acids from 4,7,10,13,16,19-[1-14C] Docosahexaenoic Acid by Human Platelets*

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Human platelets incubated in the presence of 54 μM [1-14C]22:6 produced hydroxydocosahexaenoic acid (HDHE) at about half the rate with which 12-hydroxy-5,8,10,14-eicosatetraenoic acid is produced from [1-14C]arachidonic acid. More than 90% of the radioactivity in HDHE was distributed among two major isomers, 14-HDHE and 11-HDHE. The production of HDHEs was unaffected by indomethacin but completely inhibited by 5,8,11,14-heneicosatetraenoic acid, which suggests that the hydroxy fatty acids are produced by lipoxigenase. The proportions of HDHE isomers varied with the concentration of 22:6. The ratio 14-HDHE/11-HDHE was higher at 6.8 μM 22:6 than when platelets were incubated with 54 μM 22:6. It is suggested that the amounts of these isomers produced will depend both on the availability of 22:6 as well as by competition of this acid with other acids for lipoxigenase.

Recently it was shown that rabbit kidney medullary microsomes convert 7,10,13,16-docosatetraenoic acid to a new family of prostaglandins (1). In human platelets a number of polyunsaturated fatty acids differing in chain length and in the degree and position of unsaturation can be used to various extents to synthesize cyclooxygenase and lipoxigenase products (2, 3). These studies suggest that any polyunsaturated acid found in membrane lipids could potentially be converted by mammalian cells into oxygenated metabolites.

Dietary supplementation with fish oil, which is high in 5,8,11,14,17-eicosapentaenoic acid, increases the level of this acid in platelet lipids and prolongs bleeding times (4). This acid found in membrane lipids could potentially be converted extents to synthesize cyclooxygenase and lipoxygenase products. In human platelets a number of enzymes convert 7,10,13,16-docosatetraenoic acid to a new family of prostaglandins (1). In human platelets a number of polyunsaturated fatty acids differing in chain length and in the degree and position of unsaturation can be used to various extents to synthesize cyclooxygenase and lipoxigenase products (2, 3). These studies suggest that any polyunsaturated acid found in membrane lipids could potentially be converted by mammalian cells into oxygenated metabolites. Even though small amounts of thromboxane A2 are produced from 5,8,11,14,17-eicosapentaenoic acid, this product is only weakly pro-aggregatory (6, 7). Goodnight et al. (8) recently reviewed the evidence suggesting that diets high in 5,8,11,14,17-eicosapentaenoic acid may be antithrombotic. This hypothesis fails to recognize that 5,8,11,14,17-eicosapentaenoate, or any (n - 3) fatty acid, may well be converted to 22:6 via a series of microsomal desaturation and chain elongation reactions and incorporated into tissue lipids. Most fish oils themselves are rich sources of 22:6. (8) When adult men were fed a high mackerel diet for 7 days, the content of 22:6 in platelet lipids increased 6-fold (9). The role of 22:6 in platelet function is not known. Nugteren (10) reported that 22:6 was oxygenated by platelets but no structural studies were carried out on the products. In the present study, we show that washed human platelets efficiently transform 22:6 into two hydroxy fatty acids. These findings suggest that after release from tissue lipids all naturally occurring polyenoic acids may serve as substrates for conversion to oxygenated metabolites with the potential of modulating cell function.

MATERIALS AND METHODS

Fatty Acids—[1-14C]22:6 was synthesized by coupling the di-Griignard complex of 3,6-heptadecyn-1-ol with 1-bromo-2,5,8,11-tetradecatetraynoic acid to give 3,6,9,12,15,18-heneicosahexyn-1-ol. This acetylenic alcohol was converted to [1-14C]22:6 (40 Ci/mol) using the procedures previously described for the synthesis of [1-14C]arachidonic acid (11). [1-14C]Arachidonic acid (47 Ci/mol) was from New England Nuclear (Boston, MA), while arachidonic acid and 22:6 were purchased from Nu-Chek Preparations (Elysian, MN).

Incubation of Platelets and Extraction of Metabolites—Blood was drawn from healthy donors who were not on any medication during the 2 weeks prior to collection. The blood was collected in 7.5% (v/v) 77 mM disodium EDTA and centrifuged at 200 x g for 15 min. The platelet-rich plasma was centrifuged at 2000 x g for 20 min and the platelet pellet was suspended in 0.15 M NaCl, 0.15 M Tris (pH 7.4), 77 mM disodium EDTA (90:8:2 (v/v)) (12). The platelets were recovered by centrifugation at 2000 x g for 15 min and resuspended in the above medium at a concentration of 3 x 10^7/ml.

Platelet suspensions in plastic tubes were preincubated at 37°C for 2 min. The reactions were initiated by adding the potassium salt of the fatty acid to the magnetically stirred solution. The concentration of fatty acid was either 6.8 or 54 μM (300,000 dpm). When noted, indomethacin or 5,8,11,14-heneicosatetraenoic acid were added in 10 μl of ethanol at the beginning of the preincubation. After stopping the reactions with 0.2 volume of 2 N formic acid, the cells plus the medium were extracted three times with 3 volumes of ethyl acetate. Radioactive recovery was greater than 90%.

In order to obtain sufficient material for characterization, a large scale incubation was carried out using outdated platelets from the Red Cross. The platelets were washed and resuspended at 3.4 x 10^7 cells/ml and incubated with 400 μM fatty acid (1 Ci/mmol) for 90 min. Reactions were terminated by centrifuging at 2000 x g for 15 min at 4°C. The supernatant was acidified and extracted with ethyl acetate. The cells were extracted with chloroform/methanol 2:1 (v/v). The radioactive metabolites were separated from phospholipids by eluting a silicic acid column with 1% methanol in chloroform (10). This fraction was then dried and esterified with ethereal diazomethane containing 0.1 volume of methanol.

High Performance Liquid Chromatography—High performance liquid chromatography was carried out with a DuPont HPLC system.
Metabolism of 4,7,10,13,16,19-Docosahexaenoic Acid

equipped with an 870 pump, 8800 series gradient controller, column oven, and variable wavelength detector. Radioactivity was quantitated with a model HP radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc. Tampa, FL). All solvents were from Burdick and Jackson, Muskegon, MI.

Reverse phase HPLC was done with columns packed with 5-µm particle size ODS (0.4 x 3 cm Apex, Jones Chromatography, Columbus, OH). Free acids were separated at 35°C by eluting with 25% acetonitrile, aqueous H₃PO₄ (pH 3) at 1 ml/min. After 2.5 and 10 min, acetonitrile concentration increased to 50 and 100%, respectively, over 1 min. A 1:3 volume ratio between eluent and scintillation mixture was used. Counting efficiency was about 70% and was not significantly altered by changes in acetonitrile concentration. H₂ platelets were incubated with 3.4 nmol (300,000 cpm) of [1-¹⁴C]20:4 or 22:6 in 0.5 ml of NaCl/Tris-HCl/EDTA, for 5 min at 37°C. Ethyl acetate extracts were prepared, dried under N₂ and dissolved in methanol. 45,000 cpm were injected onto an ODS column (0.4 cm i.d. x 3 cm) and resolved using acetonitrile/aqueous (pH 3) at 1 ml/min. The runs started at 25% acetonitrile to separate thromboxane B₂. After 2.5 and 10.5 min, acetonitrile concentration increased to 50 and 100%, respectively, over 1 min. B, samples prepared as indicated in A, and were converted to methyl esters with diazomethane and injected in 1% isopropanol in hexane onto an Ultrasphere-Si column (1% SP-2100) and were converted to methyl esters with diazomethane and converted to methyl esters with ethereal diazomethane and converted to trimethylsilyl ether derivatives by reaction with 10 µl of N,O-bis(trimethylsilyl)trifluoroacetic acid (Perkin-Elmer Chemical Co., Rockford, IL) in 10 µl of pyridine for 30 min at 90°C. Methyl esters were hydrogenated by bubbling hydrogen for 30 s into a solution of the methyl esters in 1 ml of methanol which contained about 1 mg of platinum oxide. The methyl esters were recovered by transferring the entire reaction mixture to a silicic acid column followed by elution with 25% eluting methanol. The metabolites were purified as methyl esters with a model HP radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc. Tampa, FL). All solvents were from Burdick and Jackson, Muskegon, MI.

Gas Chromatography-Mass Spectrometry—Hydroxy acids were estimated by reaction with ethereal diazomethane and converted to trimethylsilyl ether derivatives by reaction with 10 µl of N,O-bis(trimethylsilyl)trifluoroacetic acid (Perkin-Elmer Chemical Co., Rockford, IL) in 10 µl of pyridine for 30 min at 90°C. Methyl esters were hydrogenated by bubbling hydrogen for 30 s into a solution of the methyl esters in 1 ml of methanol which contained about 1 mg of platinum oxide. The methyl esters were recovered by transferring the entire reaction mixture to a silicic acid column followed by elution with 25% eluting methanol. The metabolites were purified as methyl esters with a model HP radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc. Tampa, FL). All solvents were from Burdick and Jackson, Muskegon, MI.

RESULTS

Fig. 1A shows that upon analysis by reverse phase HPLC that [1-¹⁴C]22:6 is metabolized to a compound with a retention time similar to that of HETE which is the product made by lipoxygenase when arachidonate is incubated with platelets (14). Key peaks were metabolites of arachidonate, which is consistent with conjugated double bonds. The radiochromatogram in Fig. 1B shows the separation of methyl esters of metabolites produced from labeled 22:6 and arachidonate by HPLC on a silicic acid column. Arachidonate was metabolized primarily to a single 20-carbon hydroxy acid although two small radioactive components were detected with retention times between HETE and HHT. Conversely, 22:6 was metabolized primarily to two hydroxy acids as well as several minor compounds.

In order to characterize these compounds, a preparative isolation was carried out after incubating 22:6 with platelets from the Red Cross. These studies were all carried out using the metabolites isolated from the cells since the majority of hydroxy acids from both arachidonate and 22:6 was retained in the platelets following the incubation. Both reverse phase and subsequent analysis by straight phase HPLC revealed that there were no significant differences between results obtained with freshly isolated versus outdated platelets. The radioactive ratio between compounds I and II (Fig. 1B) matched the area ratio at 234 nm (3.95 and 3.90, respectively), showing that both HDHE isomers had the same specific activity.

The electron impact mass spectrum of the ME-TMS derivative of compound I ([ECL = 23.1]) had a major ion at m/z 321 (loss of: (CH₂=CH=CH₂) - CH₂=CH₂) as well as at 199 (321 - (32 + 99) loss of CH₃OH + Me₂SiOH). Other ions in the low mass region were present at m/z 155, 171, 181, and 193, and 207. It was not possible to detect ions in the high mass region; however, as shown in Fig. 2B, the ammonia chemical ionization mass spectrum had major ions at 448 (M⁺ + 18), 356 (M + 18 - 90) and 341 (M + 1 - 90). The electron impact mass spectrum of the ME-TMS derivative of compound II after reduction (ECL = 24.1) had small ions at m/z 427 (M - 15; loss of CH₂=CH₂, 1.1%), 411 (M - 31; loss of OCH₃, 1.7%), 395 (M - 47; loss of CH₃OH + CH₃, 3.1%) and major ions at 329 (M - (CH₂)₆CH₂), 86%) and the base peak at 215 (M - (CH₂)₇CO₂CH₃). The ammonia chemical ionization spectrum had characteristic ions at both m/z 443 (M + 1) and at 469 (M + 18). Compound I had λ₂₅₀ at 237 nm when analyzed in hexane or methanol which is consistent with conjugated double bonds in the cis-trans configuration (15). Compound I is thus likely to be 14-hydroxy-4,7,10,13,16,19-docosahexaenoic acid in which the double bond at position 12 probably has the trans-configuration.

The electron impact mass spectrum of compound II (Fig. 2A)
that the two HDHE isomers are made by lipoxygenase. In these conditions, the production of HHT rapidly reached a plateau while HETE synthesis continued throughout the incubation period. At time periods less than 4 min, HDHE was produced at a rate of about 40-50% of that for HETE. When the HDHE isomers from the experiment shown in Fig. 3 were separated by silicic acid HPLC, it was observed that the ratio between 14-HDHE and 11-HDHE remained constant throughout the incubation period. The distribution of radioactivity among HDHE at all times of analysis was as follows: 14-HDHE, 61.5 ± 2.9%; 11-HDHE, 35.4 ± 2.1%; other isomers, 3.9 ± 2.7%. Both isomers are produced at the same rate during the 16-min incubation.

The results in Table II show that the ratio of 14-HDHE/11-HDHE was not affected by indomethacin. However, this ratio was considerably higher at low versus high concentrations of 22:6. Thus, an 8-fold increase in substrate resulted in a 3.2-fold increase in the production of 14-HDHE (1.8 ± 0.02

The time course for the production of hydroxy fatty acids from 22:6 is compared to that of arachidonate in Fig. 3. Under these conditions, the production of HHT rapidly reached a
the synthesis of both isomers is unaffected by indomethacin, primarily to 14- and 11-
products were dissolved in methanol and separated by HPLC. Ethyl acetate extracts were prepared. After drying under \( \text{N}_2 \), the products were dissolved in methanol and separated by HPLC.

**Table II**

**Effect of precursor concentration on the proportions of HDHE isomers**

Ethyl acetate extracts from the experiment described in Table I were taken to dryness and converted to methyl esters and resolved by HPLC as described in Fig. 1B. Values represent the per cent distribution of radioactivity among HDHE isomers ± S.D. from three samples.

<table>
<thead>
<tr>
<th>Concentration of 22:6</th>
<th>Distribution of (^{14}\text{C} )</th>
<th>Ratio 14/11-HDHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu \text{M} )</td>
<td>14-HDHE</td>
<td>11-HDHE</td>
</tr>
<tr>
<td>6.8</td>
<td>76.7 ± 1.2</td>
<td>18.3 ± 1.5</td>
</tr>
<tr>
<td>+Indomethacin</td>
<td>77.5 ± 1.2</td>
<td>19.0 ± 1.3</td>
</tr>
<tr>
<td>54</td>
<td>62.9 ± 2.0</td>
<td>33.2 ± 0.9</td>
</tr>
<tr>
<td>+Indomethacin</td>
<td>63.5 ± 1.7</td>
<td>33.9 ± 1.1</td>
</tr>
</tbody>
</table>

to 5.94 ± 0.31 nmol) whereas 11-HDHE synthesis increased 7-fold (0.45 ± 0.04 to 3.14 ± 0.31 nmol). This finding suggests that the relative amounts of these two metabolites produced in vivo will depend on the availability of 22:6.

**DISCUSSION**

This study shows that human platelets metabolize 22:6 primarily to 14- and 11-hydroxydocosahexaenoic acids. Since the synthesis of both isomers is unaffected by indomethacin, but inhibited by 5,8,11,14-heneicosatetraynoic acid which is a selective lipoperoxidase inhibitor (17), it is suggested that both hydroxy fatty acids are produced by lipoxygenase.

Previous studies have shown that a wide variety of polyunsaturated fatty acids are substrates for platelet lipoxygenase while cyclooxygenase has a more stringent requirement for substrate recognition (2, 3). The characterization of hydroxy fatty acids produced by platelets has been confined to the metabolites derived from arachidonate (14) 20:2 (n = 6) (19) and 20:5 (n = 3) (20). With all three substrates, the product is the 12-hydroxy fatty acid. If the terminal part of the molecule is the recognition site for lipoxygenase, then a 22-carbon fatty acid should give a 14-hydroxy fatty acid. This is consistent with our finding that the major metabolite from 22:6 is 14-HDHE.

It is not certain whether the two HDHE isomers are produced by a single or different lipoxygenases. The finding that the ratio of 14-HDHE to 11-HDHE decreased as the substrate concentration increased suggests that platelets may have two lipoxygenases with different affinities for 22:6. If this is the case, then by analogy one would expect to detect 9-HETE when arachidonate is used as a substrate. Among other hydroxy fatty acids, 9-HETE is produced from arachidonate by polymorphonuclear leukocytes (21). With platelets, we did detect two other minor metabolites but they were produced in such small proportions that characterization was not attempted. Recently, Bryant et al. (22) reported that a highly purified reticulocyte lipoxygenase metabolized arachidonate, 20:3 (n = 6), and 20:3 (n = 3) primarily to 15-hydroxy acids although with all substrates small amounts of the 12-hydroxy isomers were also produced. The possibility thus exists that a single platelet lipooxygenase may metabolize 22:6 to both 11- and 14-HDHE.

The functions of the two hydroxy acid isomers of 22:6 remain to be elucidated. Conflicting evidence has been presented as to whether HETE does (23, 24) or does not (25) mediate platelet aggregation. A detailed study of individual platelet lipid fatty acids has not been undertaken after feeding diets high in various (n = 3) fatty acids. It remains to be established whether 22:6 is released from platelet lipids by lipases. If 22:6 is released, then presumably it could exert its effect either by modulating the amounts of products produced from arachidonate or the metabolites from 22:6 themselves may directly influence platelet aggregation.

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