15-Hydroxy-5,8,11,13-eicosatetraenoic Acid

A POTENT AND SELECTIVE INHIBITOR OF PLATELET LIPOXYGENASE*

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The functional interrelationships between the cyclooxygenase and lipoxygenase systems for arachidonic acid metabolism in platelets have not yet been clarified. Although a number of specific inhibitors of the cyclooxygenase, such as aspirin and indomethacin, have been described, few inhibitors of the lipoxygenase have been found.

Several hydroxy and hydroperoxy derivatives of arachidonic acid were prepared and purified by high performance liquid chromatography, and their structures were confirmed by gas chromatography-mass spectrometry. The effect of these compounds on oxygenation of [1-14C]arachidonic acid by platelet cyclooxygenase and lipoxygenase enzymes was monitored both with an oxygen electrode and by analysis of radioactive products formed. It was found that 15-hydroxy-5,8,11,13-eicosatetraenoic acid selectively inhibited platelet lipoxygenase activity at micromolar concentrations (I50 = 8 μM) without inhibiting the cyclooxygenase. The 15-hydroperoxy analog, although more potent, was less selective, whereas neither 12-hydroxy-5,8,10,14-eicosatetraenoic acid nor 12-hydroxy-9-octadecenoic acid (ricinoleic acid) appeared to affect either enzyme. The high degree of selectivity of the 15-hydroxy derivative of eicosatetraenoic acid makes it the most suitable inhibitor so far discovered for studying the functions of the platelet lipoxygenase system.

Arachidonic acid is metabolized in human platelets via two different pathways (1). The cyclooxygenase enzyme produces thromboxane A2 in addition to HHT, PGD2, and PGE2, whereas 12-HPETE is formed via the lipoxygenase pathway (2, 3). The importance of the cyclooxygenase pathway to platelet function is due to the formation of thromboxane A2, a potent inducer of aggregation (2). Recent reports suggest that lipoxygenase products may also be significant to the aggregation process (4, 5). A common feature of both pathways is that arachidonic acid oxygenation is the initial, rate-limiting reaction (6). The search for selective inhibitors of various enzymes of the arachidonic acid cascade is important in developing compounds with therapeutic potential to control pathological processes mediated by arachidonic acid metabolites and in providing useful biochemical tools for mechanistic investigations. A number of nonsteroidal anti-inflammatory drugs (e.g., aspirin, indomethacin, and the farnic acids) have been found to inhibit the cyclooxygenase but do not affect the lipoxygenase enzyme (1, 7). The availability of a selective lipoxygenase inhibitor would be useful for studying the functions of the platelet lipoxygenase system. 5,8,11-Eicosatrienoic acid has been reported to inhibit selectively platelet lipoxygenase (8). In the present study, we compared the inhibitory effects of various hydroxy- and hydroperoxy-fatty acid derivatives on the platelet fatty acid oxygenases with the formation of various derivatives.

MATERIALS AND METHODS

Human platelet concentrates (3 to 4 days old) obtained from the Washington, D.C. chapter of the American Red Cross, were always used within 24 h of expiration and washed platelet suspensions were prepared as described by Feinstein and Fraser (9). Antimycin A was obtained from Sigma Chemical Co. Ricinoleic acid was bought from Applied Science Laboratories. Unlabeled arachidonic acid was purchased from Nu-Chek Prep., Inc., Elysian, Minn. and [1-14C]arachidonic acid was obtained from Amersham Co. PGD2, PGE2, PGF2α, and thromboxane B2 were kindly supplied by Dr. John Pike, Upjohn Co., Kalamazoo, Mich. Precast silica gel TLC plates (Analytech) were purchased from Fisher Scientific Co. Silicic acid (100 to 200 μm) was obtained from Bio-Rad Corp.

The hydroperoxide, 15-HPETE, was prepared by soybean lipoxygenase-catalyzed oxygenation of arachidonic acid and partially purified by silicic acid column chromatography (10). Further purification was achieved by high pressure liquid chromatography (HPLC) using a Hewlett-Packard model 1084A chromatograph fitted with a µPorasil column (3.9 × 300 mm) from Waters Associates, Inc. with the solvent hexane/2-propanol/acetic acid, 387:12:1, as the mobile phase (11). The flow rate was 2 ml/min and a 254 nm detector was used to monitor the elution. Under these conditions, 15-HPETE eluted at 5.6 min. The alcohol 15-HETE, prepared by triphenylphosphine reduction of 15-HPETE (10) followed by HPLC purification, eluted at 4.2 min. The platelet lipoxygenase product 12-HETE was prepared from arachidonic acid as previously described (12) and further purified by HPLC. Under the above conditions, 12-HETE eluted at 3.8 min.

The purities of recovered 15-HPETE, 15-HETE, and 12-HETE were greater than 95% based on analysis of the HPLC elution profiles. The structures of 15-HETE and 12-HETE were confirmed by gas chromatography-mass spectrometry. The mass spectrum of the methyl ester trimethylsilyl ether derivative of 15-HETE showed prominent ions (m/e) with their relative intensities and probable mode of origin in parentheses at: 406 (0.6, M+), 391 (0.4, M+ − 15), 375 (0.2, M+ − 31), 335 (3, M+ − 71), 316 (3, M+ − 90), 225 (42), 173 (10), and 73 (100), whereas the same derivative of 12-HETE exhibited ions at 391 (0.6, M+ − 15), 375 (0.6, M+ − 31), 316 (0.9, M+ − 90), 296 (100, M+ − 111), 229 (10), and 205 (7). The data are in agreement with published spectra (1, 13).

Oxygen consumption measurements were monitored using a Yellow Springs Instrument Co., Inc. model 53 oxygen meter (Yellow Springs, Ohio) coupled to a Linear Instruments Corp. model 265 recorder (Irvin, Calif.). Platelet oxygenase determinations were carried out in a reaction vessel containing 3 to 4 mg of washed platelet suspension protein in 2 ml of medium containing 1.8 μM antimycin, 137 mM NaCl, 2.7 mM KCl, 11 mM dextrose, and 25 mM Tris-HCl (pH 7.6) at 30°C.
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RESULTS AND DISCUSSION

Fig. 1 shows the effect of exogenously added 15-HETE on platelet arachidonic acid metabolism. The formation of 12-HETE, the stable platelet lipoxygenase pathway end product, is inhibited by 90 to 95% without appreciably affecting the production of thromboxane B₂ or HHT via the cyclooxygenase route. Fig. 2 illustrates the effect of increasing concentration of 15-HETE on both 12-HETE and thromboxane B₂ formation. Fig. 2 also shows that inhibition of platelet lipoxygenase 12-HETE production by 15-HETE is accompanied by about 20% stimulation of thromboxane B₂ formation. Since platelets produce a 5- to 7-fold excess of 12-HETE over thromboxane B₂ (data not shown), the thromboxane B₂ stimulation described above may be due to increased substrate (arachidonic acid) availability to the cyclooxygenase. This observation is analogous to the observed increase in 12-HETE formation when the platelet cyclooxygenase is inhibited by indomethacin (1).

Replacing the 15-hydroxy group with the hydroperoxy functionality increased the potency of the inhibitor but decreased its selectivity (Table I). The inhibitory effects of two other hydroxy fatty acids were also tested. Neither 12-HETE nor ricinoleic acid, at the concentrations tested, appeared to affect significantly the platelet lipoxygenase or cyclooxygenase enzymes. The results are summarized in Table I. Decreased rates of oxygen uptake in the presence of 15-HETE and 15-HPETE layer was separated. Authentic thromboxane B₂, PGG₂, PGE₂, and PGF₂, standards were added to the chloroform solution and the solvent was evaporated under N₂. The residues were redissolved in a small amount of chloroform, applied to Silica Gel TLC plates and developed in Solvent System C (chloroform/methanol/acetic acid/water, 90:8:1:0.8, v/v/v). Radioactive zones were detected by autoradiography, scraped from the TLC plate, and counted by a liquid scintillation counter. The identities of thromboxane B₂, HHT, and HETE were confirmed by GC-MS as previously described (12).

Varying concentrations of hydroxy fatty acids or 15-HPETE in ethanol were added and after 1 min, 32 to 59 μM arachidonic acid (final concentration) was added to initiate the reaction. Initial velocities of oxygen consumption were determined and compared with controls in which ethanol was used in order to calculate the inhibitory effectiveness of these fatty acid derivatives.

Using [1-¹⁴C]arachidonic acid diluted with unlabeled arachidonic acid as substrate, radioactive arachidonic acid metabolites were isolated from the reaction mixture as follows. After 5-min incubation, the reaction was stopped by the addition of 0.1 ml of 9% formic acid and extracted with 10 ml of chloroform/methanol (2:1). The mixture was agitated with a Vortex mixer and centrifuged and the chloroform fraction was evaporated. The residues were redissolved in a small amount of chloroform, applied to Silica Gel TLC plates and developed in Solvent System C (chloroform/methanol/acetic acid/water, 90:8:1:0.8, v/v/v). Radioactive zones were detected by autoradiography, scraped from the TLC plate, and counted by a liquid scintillation counter. The identities of thromboxane B₂, HHT, and HETE were confirmed by GC-MS as previously described (12).

Fig. 2 illustrates the effect of increasing concentration of 15-HETE on 12-HETE production. The figure illustrates typical radioscans and autoradiographs (shown below the radioscanner traces) of arachidonic acid metabolites formed in the absence (upper panel) and presence (lower panel) of 15-HETE.

FIG. 1. Selective inhibition of platelet lipoxygenase 12-HETE formation from [1-¹⁴C]arachidonic acid by 15-HETE. A washed platelet suspension (3.3 mg) in 2 ml of Tris/physiological saline (0.9% NaCl solution) buffer (pH 7.6) containing antimycin A (1.8 μM) and dextrose (11 mM) was preincubated for 1 min with 15-HETE (12 μM). After the addition of [1-¹⁴C]arachidonic acid diluted with unlabeled material (final concentration, 53 μM), the mixture was magnetically stirred for 5 min at 30°C. The reaction was stopped, worked up, and chromatographed as described under "Materials and Methods." The figure illustrates typical radioscans and autoradiographs (shown below the radioscanner traces) of arachidonic acid metabolites formed in the absence (upper panel) and presence (lower panel) of 15-HETE.

FIG. 2. Effect of increasing concentrations of 15-HETE on platelet lipoxygenase and cyclooxygenase activities. Platelet lipoxygenase and cyclooxygenase activities were measured by the formation of 12-HETE (●——●) and thromboxane B₂ (Δ——Δ), respectively, from [1-¹⁴C]arachidonic acid as described under "Materials and Methods." Points, mean ± S.E. of three experiments.
HPETE (Table I) suggest that these fatty acid derivatives directly inhibit the platelet fatty acid oxygenases.

Platelet aggregation in platelet-rich plasma induced by ADP was not affected by 15-HETE at concentrations that inhibited the formation of the lipoxygenase product 12-HETE in washed platelets. Since platelet-rich plasma contains proteins (e.g. albumin) which could bind 15-HETE, thereby making it unavailable for its inhibitory action, these results do not prove or disprove the hypothesis that the lipoxygenase pathway is important to platelet function (4, 5).

While many drugs have been reported to inhibit the platelet cyclooxygenase, very few inhibitors of the platelet lipoxygenase have been described. 1-Phenyl-3-pyrazolidone (14) and 5,8,11,14-eicosatetraynoic acid (8) were shown to inhibit both the lipoxygenase and cyclooxygenase activities, although with little selectivity. Hammarstrom (8) reported that 5,8,11-eicosa-

<table>
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<th>Fatty acid added</th>
<th>Lipoygenase concentration for half-maximal inhibition</th>
<th>Cyclooxygenase concentration for half-maximal inhibition</th>
<th>Total oxygenase concentration for half-maximal inhibition</th>
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</thead>
<tbody>
<tr>
<td>15-HETE</td>
<td>8.2 ± 1.5</td>
<td>135 ± 10</td>
<td>23 ± 2</td>
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<tr>
<td>15-HPETE</td>
<td>2.5 ± 1.0</td>
<td>5.7 ± 0.3</td>
<td>2.8 ± 0.0</td>
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<tr>
<td>12-HETE</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>98 ± 9</td>
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<tr>
<td>Ricinoleic acid</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;350</td>
</tr>
<tr>
<td>5,8,11,14-Eicosatetraynoic acid (8)</td>
<td>4</td>
<td>8</td>
<td>ND</td>
</tr>
</tbody>
</table>
| 5,6,11-Eicosa-

As measured by 12-HETE formation from [1-14C]arachidonic acid.

As measured by thromboxane B2 formation from [1-14C]arachidonic acid.

ND, not determined.

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REFERENCES

2 J. Y. Vanderboeck, and J. M. Bailey, unpublished observations.