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Selective inhibitors of platelet arachidonic acid metabolism: aggregation is independent of lipoxygenase

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SELECTIVE INHIBITORS OF PLATELET ARACHIDONIC ACID METABOLISM: AGGREGATION IS INDEPENDENT OF LIPOXYGENASE

A THESIS
SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

BY
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IN CONJUNCTION WITH
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Selective Inhibitors of Platelet Arachidonic Acid Metabolism: Aggregation Is Independent of Lipoxygenase

Advisor: Dr. Cyril L. Moore/Dr. Philip Needleman

Master of Science degree conferred July, 1981.
Thesis dated July 8, 1981

The use of acetylenic acid analogues differing in chain length or position of the triple bond permitted the systematic study of structural activity relationships for both the arachidonate metabolizing enzymes (i.e., cyclooxygenase and lipoxygenase) in platelets and the relationship of the enzyme to aggregation. Analogues were found that preferentially inhibited: a) cyclooxygenase only; b) 12-lipoxygenase only (HETE); c) both the cyclooxygenase and lipoxygenase; d) neither enzyme in platelets. There was a direct correlation between the rank order of potency of the acetylenic analogues to inhibit platelet cyclooxygenase and to suppress aggregation. Certain structural features of the triynoic acetylenic analogues were critical in influencing platelet function; thus, the presence of a triple bond at position 14 as well as the lack of the triple bond at position 5 resulted in a compound that inhibited both cyclooxygenase and platelet aggregation. Analogues that inhibited the platelet 12-lipoxygenase but not the C.O. were very weak inhibitors of platelet aggregation. These acetylenic acid analogues provide potentially powerful tools for dissociating the two arachidonate metabolic pathways and if other tissues are as readily manipulated as platelets,
the analogues could be especially useful to gain insight into the contribution of lipoxygenase products to other biological functions.
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<td>Arachidonic Acid</td>
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<tr>
<td>cAMP</td>
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<td>ETYA</td>
<td>5,8,11,14-eicosatetraynoic acid</td>
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<tr>
<td>SRS-A</td>
<td>Slow Reacting Substance of Anaphylaxis</td>
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<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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CHAPTER I

INTRODUCTION

Platelets contain two arachidonic acid metabolizing enzymes. Cyclooxygenase converts arachidonate via prostaglandin (PG) endoperoxide which is further metabolized to predominately thromboxane A\textsubscript{2} (TxA\textsubscript{2}) and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT). Small amounts of PGE\textsubscript{2}, PGD\textsubscript{2} and PGF\textsubscript{2α} are also formed as a result of the catabolism of arachidonic acid in platelets. Lipoxygenase, the second arachidonic metabolizing enzyme, yields 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE; Hambert, M. et al., 1974). The aqueous decay products of these arachidonate metabolic pathways are thromboxane B\textsubscript{2} (TxB\textsubscript{2}) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). The critical participation of thromboxane A\textsubscript{2} and PG-endoperoxides in platelet aggregation has frequently been demonstrated by Nugteren (1975), Hamberg and Samuelsson (1974), and Hamberg (1975). Indeed, exogenous addition of thromboxane A\textsubscript{2} will aggregate aspirin-treated platelets (Hamberg et al., 1975). What has remained elusive is the role of the lipoxygenase pathway in platelet function. Inhibitors of the lipoxygenase pathway, such as the acetylenic acid 5,8,11,14-eicosatetraynoic acid (ETYA; Hamberg, M. et al., 1974) or BW755C (Higgs, G.A. et al., 1979), also inhibited cyclooxygenase. Recently several compounds have been described which preferentially inhibit platelet lipoxygenase over the cyclooxygenase. Hammarström et al. (1977) showed that 5,8,11-eicosatriynoic acid inhibited platelet lipoxygenase preferentially over the platelet cyclooxygenase. Vanderhoek et al. (1980) reported that 15-hydroxy-5,8,11,14-eicosatetraenoic acid selectively inhibited platelet lipoxygenase. Wilhelm et al. (1981) and Sun et al. (1981) observed that the acetylenic analogues 4,7,10,13-ETYA
and 5,8,11,14-heneicosatetraynoic acid inhibit platelet lipoxygenase at concentrations that did not block TxA₂ synthesis. However, the effects of these compounds on platelet aggregation were not extensively analyzed. Such experiments seem particularly pertinent since Dutilh et al. (1978, 1981) reported that low concentrations of ETYA block platelet lipoxygenase and not cyclooxygenase and, simultaneously, reverse arachidonate induced aggregation. They concluded that the lipoxygenase is essential for blood platelet aggregation.

At present there are no agents that inhibit lipoxygenase without simultaneously inhibiting cyclooxygenase. In this investigation a comparative study of the structural requirements of numerous acetylenic analogues to preferentially inhibit either platelet cyclooxygenase or lipoxygenase and to determine the effectiveness of these compounds to inhibit human platelet aggregation was undertaken. From these studies emerged a series of selective enzyme inhibitors that will permit the study of the biochemical and pharmacological effects of the different arachidonic acid metabolites.
Prostaglandins and Arachidonic Acid Metabolism

In 1930, Kurzrok and Lieb observed that strips of human uterus relax or contracted when exposed to human semen (Kurzrok and Lieb, 1930). Subsequently, initial characteristics of prostaglandins were discovered by Glodblatt (1933, 1935) and Von Euler (1934, 1936) who independently reported smooth muscle contraction and vaso-depressor activity in seminal fluid and reproductive glands. Von Euler identified this active material as a lipid-soluble acid which he named "Prostaglandin." The structure of prostaglandins was elucidated by Bergstrom and Samuelsson in 1963, which then paved the way for many biological studies (Bergstrom, 1968).

Prostaglandins have a broad spectrum of effects. They have been studied in many biological systems, most notably renal, cardiovascular and respiratory. Prostaglandins (PGs) consist of 20 carbons having a cyclopentane ring and two aliphatic side chains that may contain one, two or three double bonds. PGs are formed by enzymatic oxygenation of certain essential polyunsaturated fatty acids consisting of either three, four or five double bonds. These precursor fatty acids are:

- series one, containing three double bonds, 8,11,14-eicosatrienoic acid (dihomo-γ-linolenic acid);
- series two, consisting of three double bonds, 5,8,11,14-eicosatetraenoic acid (arachidonic acid);
- series three, having five double bonds, 5,8,11,14,17-eicosapentaenoic acid.

There is little evidence that prostaglandins of the one or three series are important in humans; however, significant amounts of series three are present in fish and marine animals.
Arachidonic acid, in man, is the most abundant precursor of prostaglandins. It is released from membrane phospholipids by the activation of a lipase. Once released, it is rapidly metabolized to oxygenated products by two distinct enzymatic mechanisms, cyclooxygenase and lipoxygenase, or reacetylated for insertion back into the phospholipid.

In the cyclooxygenase pathway, the free unesterified precursor acid (arachidonic) is oxygenated and cyclized to form the cyclic endoperoxide derivatives prostaglandin G (PGG₂), a hydroperoxide, and prostaglandin H₂ (PGH₂), a hydroxy acid. These two chemically unstable compounds may be metabolized by a isomerase or reductase to PGE₂, PGF₂α, and PGD₂. PGH₂ is metabolized into two other compounds, thromboxane A₂ and prostacyclin (PGI₂), which are unstable and highly biologically active. Thromboxane A₂ (TxA₂), produced by the enzyme thromboxane synthetase, later breaks down nonenzymatically into the stable metabolite thromboxane B₂ (TxB₂; Hamberg et al., 1975; Piper and Vane, 1969). Also, a 17-carbon hydroxy acid (HHT) can be formed nonenzymatically from thromboxane synthetase (McMillian et al., 1978; Anderson et al., 1978). These two metabolites, thromboxane B₂ and HHT are produced exclusively in human platelets. The other unstable compound, prostacyclin (PGI₂), is formed by the enzyme, prostacyclin synthetase, and is later hydrolyzed nonenzymatically to a stable compound 6-keto-PGF₁α.

Other metabolic products of arachidonic acid originate from the lipoxygenase pathway. This enzyme was first identified in plants and the discovery of the mammalian enzyme was initiated by Hamberg and Samuelsson in 1974. In contrast to cyclooxygenase, which is widely distributed, lipoxygenase has been found in lung, platelets and leukocytes. The compounds produced by the lipoxygenase enzyme are a family of mono and dihydroxy fatty acids. Little is known about the pharmaco-
logical activity of these compounds, although 12-HETE (12-hydroxy 5,8,10,14-eicosatetraenoic acid), which is found in increasing amounts in human platelets, is believed to be chemotactic for polymorphonuclear leukocytes and alveolar macrophages (Sun et al., 1977; Fig. 1).

It has been shown that the arachidonic acid is a precursor for a family of compounds called leukotrienes. Included in this group is an inflammatory mediator termed SRS-A (slow-reacting substance of anaphylaxis), which is a product of a separate lipoxygenase pathway not yet studied in platelets (Jakschik et al., 1978).

**Aspirin-Like Drugs**

Aspirin irreversibly acetylates the enzyme (Roth et al., 1975) inhibiting the cyclooxygenase but not the peroxidase activity; other nonsteroid antiinflammatory drugs demonstrate the same inhibitory pattern, but reversibly. Among such drugs are meclofenamic acid, indomethacin, mefenamic acid, naproxen, phenylbutazone and ibuprofen (Flower, 1974). Eicosatetraynoic acid (ETYA) inhibits both cyclooxygenase and lipoxygenase activity; related acetylenic fatty acids display differential specificities (Wilhelm et al., 1981; Sun et al., 1981; Chapter III, Fig. 3). Other fatty acids, including oleic, linoleic, and linolenic acid also inhibit PG synthesis (Pace-Asciak and Wolfe, 1968). Cyclooxygenase inhibition by many fatty acids has both concentration and time dependent components (Flower, 1974). Several endogenous cyclooxygenase inhibitors have been reported but are not well-characterized (Saeed et al., 1977; Terragno et al., 1978; Fig. 2).

There are some experiments which suggest that endoperoxide synthetase is coupled in some fashion to enzymes acting on its metabolites. For example, little or no endoperoxide escapes the platelet enzyme complex to form PGD or E; it is all directly converted to thromboxane
Fig. 1. Metabolism of arachidonic acid.
Fig. 2. Enzymes and inhibitors of prostaglandin biosynthesis.
and HHT (Needleman et al., 1979). Only in the presence of a thromboxane synthetase inhibitor, such as imidazole, are alternate products observed.

Platelets

A. Production

Platelets are derived from the megakaryocytes of bone marrow by a process that leaves each platelet without a nucleus, with little endoplasmic reticulum or RNA of any type (Becker et al., 1963). Platelets are unable to respond to environmental influences via RNA transcription and subsequent translation to form new proteins. Under normal conditions, platelets circulate in the blood for 10 days as smooth disc-shaped cells that are non-adherent to each other and to normal vascular endothelium (Marcus et al., 1978). Platelets have unusual biological properties which will cause them to adhere to collagen, fragments of basement membrane and microfibrils afterward forming a haemostatic plug (aggregation; Wright and Minot, 1917), through a process in which the platelet undergoes a secretory phase during which substances found in the granules in the platelets are secreted (release reaction; Grette, 1962). This may occur when the endothelium lining in the blood vessel is broken or when disruption of the vessel allows the blood to come into contact with elements of the vessel wall on subendothelial tissue.

B. Aggregation

During the development of the haemostatic plug, two secreting types of granules have been identified within the cytoplasm of platelets. The majority, which are known as alpha granules, contain enzymes such as cathepsin and acid hydrolases that are characteristically associated with lysosomes ranging from 20-200 granules per platelet (White, J.C., 1972). The second type of granule, called a dense body, not nearly as numerous but ranging between 2-10 per platelet, contains the storage
sites for ADP, adenosine triphosphate (ATP), serotonin and calcium through studies by David and White (1968). The serotonin storage is similar to the catecholamine storage in the dense core granules of the adrenal medulla. In vitro evidence indicates that serotonin can associate with ATP and calcium or magnesium as micelles, whereas in vivo is still uncertain.

The ADP stored in the dense bodies accounts for over 50% of the total ADP in human platelets. This ADP is nonmetabolic, or storage pool ADP, which is to be distinguished from the metabolic pool of ADP (Holmsen et al., 1969). The ADP actively participates in the metabolism of the blood platelet and provides energy for maintenance of the membrane transport systems, and responses to stimuli as aggregation.

Holmsen and Day correlated the release reaction with the alpha granules and dense bodies (Day and Holmsen, 1971). They have called the selective process that releases the contents of the dense bodies, Release I, and the process that releases the contents of the alpha granules, Release II. Recent evidence confirming the separation of these two processes has been the finding that aspirin can inhibit Release I but not Release II, suggesting that these two processes may be governed by different mechanisms (Holmsen et al., 1975).

Thus, the events following vascular injury occur in the following sequence: adhesion, release reaction, platelet aggregation, primary haemostatic plug and arrest of bleeding.

C. Arachidonic acid response to aggregation

Arachidonic acid, PGG₂ and PGH₂ cause platelet aggregation and release of dense body granules, including ADP (Hamberg et al., 1975; Vargafting and Zirinis, 1973). Thromboxane A₂ was deduced to be an aggregative agent more potent than either PGG₂ or PGH₂ (Hamberg et al.,
1975). Imidazole blocks thromboxane \( A_2 \) synthesis but does not affect arachidonic acid-induced aggregation or release in wash platelet suspension (Needleman et al., 1977). Consequently, PGH\(_2\) is sufficient and thromboxane \( A_2 \) is not necessary to mediate arachidonic acid-induced platelet aggregation. The conclusion has been challenged by Gorman et al. (1977), who found that a synthetic thromboxane \( A_2 \) analogue inhibited the effects of PGH\(_2\) upon human platelets without increasing cAMP. However, the selectivity of the analogue for competition with \( A_2 \) against the prostaglandin endoperoxides is not established.

The role of HETE and HHT in platelet aggregation and release is unclear. Platelets incubated with exogenous arachidonic acid also synthesize very small amounts of PGE\(_2\), PGF\(_{2\alpha}\) and PGD\(_2\). PGD\(_2\) is an inhibitor of human platelet aggregation and release; also, PGD\(_2\) has been postulated to act as a feedback inhibitor of arachidonic acid-induced platelet aggregation and release (Oelz et al., 1977). PGE\(_2\) facilitates platelet aggregation (Weiss et al., 1976). Prostacyclin (PGI\(_2\)) has been shown to be a very potent inhibitor of arachidonic acid-induced platelet aggregation and release (Moncada et al., 1976), but this molecule has not been demonstrated to be synthesized by platelets. Finally, PGE\(_1\) is also a potent inhibitor of platelet aggregation and release in vitro (Sinha and Colman, 1978). PGD\(_2\), PGI\(_2\) and PGE\(_1\) probably exert their inhibitory action by increasing platelet cAMP concentration (Tateson et al., 1977; Fig. 3).
Fig. 3. Arachidonic acid responses to aggregation.
CHAPTER III

MATERIALS AND METHODS

Materials

[\(^{14}\text{C}\)]-Arachidonic acid (AA; sp. act., 55 mCi/mmol) was obtained from New England Nuclear, Boston, MA. Prostaglandin (PG) standards were supplied by Dr. John Pike of The Upjohn Company, Kalamazoo, MI. Thin layer chromatography plates were from Brinkman Instruments, Westbury, NY. Inophore A32187 was from Calbiochem, La Jolla, CA. Thrombin was obtained from Drs. Craig Jackson and Phillip Majerus, Washington University, St. Louis, MO. Acetylenic compounds were synthesized and kindly supplied by Dr. Howard Sprecher, Department of Physiological Chemistry, Ohio State University, Columbus, OH.

Methods

Preparation of Washed Platelets

It is important to take a medical history from the patient at the time of testing and a complete list of all medication should be noted to the aggregation results. Aspirin-like drugs have simultaneously shown to inhibit prostaglandin release from human platelets and must absolutely be avoided. It is also important that in preparation of platelets, be sure to use well siliconized glassware or plastic ware. Contact with glass will cause aggregation of platelets.

Blood from a healthy donor was collected in 2.6 ml of 0.077 M EDTA for every 35 ml of whole blood. The blood was then centrifuged for 10 minutes at 1000 rpm at room temperature. After spinning, the supernatant was removed with a plastic pipet and transferred into a 15 ml conical tube and plasma volume noted. The plasma was then centrifuged 6 minutes at 4000 rpm at room temperature. Following centrifugation,
the supernatant was discarded and the platelets pellet was resuspended in wash fluid to a volume of 2 ml less than original plasma volume. The suspension was gently mixed and centrifuged 5 minutes at 2000 rpm at room temperature. After centrifugation, the supernatant was discarded and the pellet immediately resuspended with zero calcium Krebs solution to a volume of 1/2 ml less than the original volume. The platelets were then in the final form and were ready to use. The platelets are good up to 3 to 4 hours and testing should be started within 30 minutes of collection.

Solution for Preparation of Platelets Suspension

0.077 M EDTA (Ethylenediamine Tetraacetic Acid Trisodium salt)
12.9 gms in 500 ml of distilled water

Wash Fluid

90 parts 0.15 M NaCl
8 parts 0.15 M Tris-HCl
2 parts 0.077 M EDTA

0.15 M NaCl
8.7 gms in 1 liter distilled water

Zero Calcium Krebs 10x Stock

70 gms NaCl
3.5 gms KCl
3 gms MgSO₄·7H₂O
1.6 gms KH₂PO₄

Note: Add above ingredients to approximately 1/2 the amount of distilled water needed; then fill to 1 liter. Stir for approximately 1 hour. Solution is stable indefinitely. Store at 4°C.
**Zero Calcium Krebs Solution**

890 ml Distilled Water

100 ml Zero Calcium Krebs 10x Stock

10 ml Dextrose 100 Stock

2.1 gms Sodium Bicarbonate

**Dextrose 100x Stock (3.5 liters)**

630 gms of dextrose powder was added to 2 liters of stirring distilled water; then distilled water added to 3.5 liters.

Store at 4°C.

**Platelet Aggregation Method**

Aggregation was assessed photoelectrically in a temperature controlled, continually stirred siliconized system (Payton Dual Channel Aggregometer) that was connected to a strip recording chart (Beckman R511 Recorder). Human washed platelets (WP), which were used in all experiments, are turbid and stirred continually in a glass cuvette. The transmittance of light through the sample relative to the WP blank is recorded. When the aggregating agent was added, the formation of increasingly large platelet aggregates is accompanied by a clearing in the wash platelets and, therefore, light transmittance through the sample is increased. The light received through the sample is converted into electronic signals, amplified and recorded on chart paper.

In this series of experiments ETYA was used as the control, and the acetylenic acid analogs were first incubated in 0.3 ml of wash platelets at 37°C in the aggregometer for 3 minutes before the addition of the aggregating agents.
Aggregating Agents - Stock Solutions

Arachidonic Acid (AA) 100 μg/ml
Thrombin 90 m/U store at 20°C
Ionophore (A23187) 100 μg/ml
ETYA 100 μg/ml to 1 mg/ml
Acetylenic Acids Analogs 100 μg/ml to 1 mg/ml

All of the above agents were resuspended in 0.9% saline except A23187 which was resuspended in 50 mM PO4 buffer. Thrombin and A23187 should be kept on ice while the ETYA and acetylenic analogs should be wrapped in foil and kept at room temperature while in use.

Preparation of Glassware and Stir Bars

Glassware, cuvettes and stir bars were siliconized with Prosil-28 siliconizing solution.

Radiochemistry

The human washed platelets (WP) were prepared as described earlier. Washed platelets (0.4 ml) were preincubated along with acetylenic acid ranging from 0.8 μM to 25.0 μM for 5 minutes at 37°C using an immersible magnetic stir bar. After preincubation, the mixture was incubated an additional 20 minutes with 100 μl of [14C]-arachidonic acid (300,000 cpm).

Extraction

After incubating add 100 μl of 2 N formic acid to the incubation mixture (spot check pH which should range between 3-3.5, mix well). The extraction was done twice with equal or larger volume of ethylacetate and mixed well. After mixing completely the organic layers were then combined. (If the layers do not separate, add a few crystals of NaCl, mix and centrifuge 1000 rpm for 2 minutes).
Thin Layer Chromatography

The extract was then totally dried under N2 and 50 μl of 2:1 chloroform:methanol was added, rinsing the walls of the tubes well, followed by the addition of 1 drop of cold prostaglandin (PG) standard. The solution was then streaked in lanes on silica gel plates. Each sample was rinsed with an additional 20 ml of 2:1 chloroform:methanol and allowed to dry at room temperature.

System C

Solvent system C (CHCl₃:MeOH:Acetic Acid:Distilled H₂O; 90:80:1:0.8) was made up fresh daily and allowed to equilibrate for 1 hour. The platelet was allowed to run from 1-1/2 to 2 hours.

Development of X-Ray

After chromatography, the plates were dried and positions of the PG standards were determined by iodine staining. After identifying the standards on plates, the plates were left to stand until the iodine stains had dissolved. The thin layer chromatography plate was then placed on Kodak X-Omat R X-ray film in a cassette at room temperature for 48 hours.

Qualitative and Quantitative Analysis

After developing, the positions of the zones were compared to that of the standards as visualized by iodine vapor, the appropriate lanes were cut and were counted (qualitative) in scintillation fluor (4a20-RPI Corp) in a Beckman LS-230 scintillation counter.

For the quantitative approach, the actual X-ray plate was scanned by a Joyce-Loel Densitometer and the area under the curve formula was used for each identifiable band that was detected to determine its quantity.
A complete dose-response curve was constructed. The term IC\textsubscript{50} designated the concentration of acetylenic compound required to cause a 50% reduction in response compared to the control. The IC\textsubscript{50} for cyclooxygenase was calculated as the concentration of ETYA analogue needed to cause a 50% reduction in the sum of the TxB\textsubscript{2} and HHT zones from the thin layer plates. The IC\textsubscript{50} for lipoxygenase was calculated from the HETE peak. Each experiment was repeated 3 to 4 times and mean values were determined. The standard errors were 10-15% of the mean. In each aggregation or radiochemistry experiment ETYA was run as a control antagonist.

Radiochemistry Solutions

**Indomethacin** 10 mg/ml: To 100 mg of powder add 10 ml of 0.1 M Tris buffer pH 8.2 and 4-6 drops of 1 N NaOH. Mix for 10 minutes before adding more NaOH. Store at 40°C, stable for several days.

**1 M Tris Buffer**

\begin{align*}
1 \text{ M Tris base} & \quad 40.3 \text{ ml} \\
4 \text{ N HCl} & \quad 3.2 \text{ ml}
\end{align*}

pH 8.2

Add sufficient distilled water to make 400 ml

**1 M Tris Base**

Tris base (Sigma) 60.5 g

Distilled water 500 ml

**ETYA Analogs**

Same as ETYA
Fig. 1. The above data represents the effectiveness of the Joyce Lobel Densitometer. This instrument was used to scan actual X-ray plates to obtain the area under the curve.
<table>
<thead>
<tr>
<th>Acetylenic Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 18:4 Δ5 ω4</td>
</tr>
<tr>
<td>2. 19:4 Δ5 ω5</td>
</tr>
<tr>
<td>3. 18:3 Δ5 ω7</td>
</tr>
<tr>
<td>4. 21:4 Δ5 ω7</td>
</tr>
<tr>
<td>5. 20:3 Δ5 ω9</td>
</tr>
<tr>
<td>6. 19:3 Δ5 ω8</td>
</tr>
<tr>
<td>7. 19:4 Δ6 ω4</td>
</tr>
<tr>
<td>8. 20:4 Δ6 ω5</td>
</tr>
<tr>
<td>9. 18:3 Δ6 ω6</td>
</tr>
<tr>
<td>10. 22:4 Δ4 ω7</td>
</tr>
<tr>
<td>11. 21:3 Δ6 ω9</td>
</tr>
<tr>
<td>12. 18:4 Δ6 ω3</td>
</tr>
<tr>
<td>13. 20:3 Δ6 ω8</td>
</tr>
<tr>
<td>14. 20:4 Δ7 ω4</td>
</tr>
<tr>
<td>15. 22:4 Δ7 ω6</td>
</tr>
<tr>
<td>16. 20:3 Δ7 ω7</td>
</tr>
<tr>
<td>17. 21:3 Δ7 ω8</td>
</tr>
<tr>
<td>18. 18:3 Δ8 ω4</td>
</tr>
<tr>
<td>19. 21:4 Δ8 ω4</td>
</tr>
<tr>
<td>20. 19:3 Δ8 ω5</td>
</tr>
<tr>
<td>21. 20:3 Δ8 ω6</td>
</tr>
<tr>
<td>22. 20:2 Δ8 ω9</td>
</tr>
<tr>
<td>23. 21:3 Δ8 ω7</td>
</tr>
<tr>
<td>24. 20:4 Δ8 ω3</td>
</tr>
</tbody>
</table>
25. 22:3 Δ 8 ω6  8,11,14  docosatriynoic acid
26. 17:3 Δ 8 ω3  8,11,14  heptadecatriynoic acid
27. 22:3 Δ10 ω6  10,13,16  docosatriynoic acid
28. 20:2 Δ10 ω7  10,13  eicosadiynoic acid
29. 19:4 Δ 4 ω6  4,7,10,13  nonadecatetraynoic acid
30. 20:4 Δ 4 ω7  4,7,10,13  eicosatetraynoic acid
31. 21:4 Δ 4 ω8  4,7,10,13  heneicosatetraynoic acid
32. 16:3 Δ 4 ω6  4,7,10  hexadecatriynoic acid

20:4 Δ 5 ω6  5,8,11,14  eicosatetraynoic acid (ETYA)
20:4 Δ 5 ω6  5,8,11,14  eicosatetraenoic acid (AA)
When identifying long chain fatty acid structures, it is customary to name the structure either by its omega (ω) or delta (Δ). Omega designates the position of the last double bond counted from the terminal (ω) carbon on the fatty acid. Delta designates the position of the first double bond counted from the carboxyl end of the fatty acid. Thus, 20:4 (ω5, Δ6) is 5,8,11,14-eicosatetraenoic acid (AA).

The following abbreviations are sometimes used when describing the nature and geometry of carbon-carbon bonds in fatty acids:

\[ \text{C = cis} \quad -\text{C}≡\text{C}- (\text{ethylenic double bonds}) \]
\[ \text{t = trans} \quad -\text{C}≡\text{C}- (\text{acetylenic triple bonds}) \]
CHAPTER IV

RESULTS

The Effect of Acetylenic Acids on Platelet Arachidonate Metabolism

As a primary control of this metabolic study, 5,8,11,14-eicosatetraynoic acid (ETYA) was used. This particular acetylenic fatty acid will simultaneously inhibit both the platelet lipooxygenase and cyclooxygenase activity in a concentration dependent fashion with an IC$_{50}$ of 1.7 µM (Fig. 1). The autoradiograph data from the radiochemical experiment are presented in Fig. 2. The control (far right lane) illustrates the separation of the metabolites following the incubation of intact washed human platelets with $[^{14}\text{C}]$-arachidonic acid. The primary cyclooxygenase products were thromboxane B$_2$ (TXB$_2$) and a 17-carbon hydroxy acid (HHT) and the primary lipooxygenase product was 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). Also, there were trace amounts of other metabolites that were visualized (i.e., PGE, PGD, PGF$_{2\alpha}$).

After careful study of the acetylenic acids tested, they were grouped in order of selectivity (i.e., inhibitors of cyclooxygenase, lipooxygenase, cyclooxygenase and lipooxygenase, and neither; Table 2). Preincubation of washed platelets with the acetylenic acids that simultaneously inhibited both enzymes (22:3 Δ10 w6) results showed the disappearance of TXB$_2$ and HHT with a consistent recovery of the lipooxygenase product, HETE, at concentrations up to 30 µM. Quantitation of the individual zones by scraping from the thin layer plate and counting demonstrated, in fact, that the cyclooxygenase was inhibited and that more of the arachidonic acid is shuttled into the lipooxygenase product, HETE (Fig. 3A,B). Alternatively, a number of acetylenic analogues proved to be selective lipooxygenase inhibitors. The autoradiogram presented in
Fig. 4 demonstrates that 21:4 (Δ5, ω7) abolished the HETE band without altering the cyclooxygenase products. In fact, careful quantitation shows that more arachidonic acid is shuttled into the TxB₂ and HHT bands with enhanced lipoxygenase inhibition by 21:4 (Δ5, ω7) (Fig. 5A,B) (20:4 Δ4, ω7).

With the validation that the acetylenic acid analogue possessed a high degree of selectivity for inhibition of the metabolic enzymes of cyclooxygenase and lipoxygenase, the next step was to evaluate the effects of the compounds on the aggregation of platelets.

The data, in respect to platelet aggregation, rather strikingly indicated a biomodal distribution of acetylenic acids based on their rank order of potency to inhibit platelet aggregation (Table 1). The acetylenic acid analogues that either selectively inhibited cyclooxygenase alone or inhibited both the cyclooxygenase and the lipoxygenase at concentrations of <2.5 μM of arachidonate induced aggregation of washed human platelet suspensions (Table 1). In contrast, the acetylenic acid analogues that were potent lipoxygenase inhibitors, or that inhibited neither the cyclooxygenase nor the lipoxygenase, were much less potent in suppressing aggregation requiring <5 μM to produce a 50% inhibition.

There was no detectable difference in potency in suppressing arachidonate induced platelet aggregation between the inactive acetylenic acids (i.e., inhibited neither or both enzyme) and those that were potent inhibitors of lipoxygenase alone. A comparison of the effectiveness as enzyme and aggregation inhibitors of all the acetylenic acids tested are listed in Table 2. The fatty acid analogues are grouped according to their enzyme specificity. Clearly, there is a direct correlation between the potency of the analogues to inhibit cyclooxygenase and to suppress arachidonate induced platelet aggregation.
Table 1. Comparative potency of acetylenic acid analogues as inhibitors of platelet arachidonate enzymatic metabolism and aggregation.

<table>
<thead>
<tr>
<th>Enzyme inhibition</th>
<th>Enzyme IC$_{50}$ ($\mu$moles/l)</th>
<th>Aggregation IC$_{50}$ ($\mu$moles/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclooxygenase only</td>
<td>2.5 - 8.3</td>
<td>0.6 - 2.3</td>
</tr>
<tr>
<td>Both cyclooxygenase and lipoxygenase</td>
<td>4.2 - 7.5</td>
<td>1.4 - 2.3</td>
</tr>
<tr>
<td>Lipoxygenase only</td>
<td>1.7 - 8.3</td>
<td>5.1 - 16.0</td>
</tr>
<tr>
<td>Neither cyclooxygenase nor lipoxygenase</td>
<td>$&gt;83$</td>
<td>4.8 - 21.0</td>
</tr>
</tbody>
</table>
Table 2. The rank order potency of acetylenic acid analogues as inhibitors of arachidonic acid metabolism and aggregation in washed human platelets. The enzymatic IC\textsubscript{50} is the concentration of acetylenic acid needed to cause a 50% reduction in the sum of TxB\textsubscript{2} and HHT for cyclooxygenase inhibition, or 50% fall in HETE levels of lipooxygenase activity.

<table>
<thead>
<tr>
<th>ACETYLENIC ACID</th>
<th>ω</th>
<th>Δ</th>
<th>ENZYMATIC IC\textsubscript{50} μmoles/l</th>
<th>AGGREGATION IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>I. INHIBITS CYCLOOXYGENASE ONLY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triynoics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19:3</td>
<td>5</td>
<td>8</td>
<td>2.5</td>
<td>0.6</td>
</tr>
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<td>18:3</td>
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<td>8</td>
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<td>Tetraynoics</td>
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<td></td>
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<td>5</td>
<td>2.5</td>
<td>0.9</td>
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<td>4</td>
<td>7</td>
<td>5.0</td>
<td>1.4</td>
</tr>
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<td>3</td>
<td>8</td>
<td>8.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Diynoic</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>7</td>
<td>10</td>
<td>6.7</td>
<td>1.8</td>
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<td>II. INHIBITS BOTH CYCLOOXYGENASE AND LIPOXYGENASE</td>
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</tr>
<tr>
<td>20:4</td>
<td>6</td>
<td>5</td>
<td>4.2</td>
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<td>6</td>
<td>7.5</td>
<td>2.3</td>
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<td>III. INHIBITS LIPOXYGENASE ONLY</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>21:4</td>
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<td>5</td>
<td>1.7</td>
<td>5.1</td>
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<td>4</td>
<td>2.5</td>
<td>16.0</td>
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</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>ACETYLENIC ACID</th>
<th>ω</th>
<th>Δ</th>
<th>ENZYMATIC IC₅₀ (μmole/l)</th>
<th>AGGREGATION IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:2</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>6.3</td>
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<tr>
<td>21:3</td>
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<td>7</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>19:3</td>
<td>8</td>
<td>5</td>
<td>0</td>
<td>5.4</td>
</tr>
<tr>
<td>18:3</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>16:3</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>21.0</td>
</tr>
</tbody>
</table>

IV. INHIBITS NEITHER CYCLOOXYGENASE NOR LIP oxyGENASE
Table 3. Comparison of Δ5 acetylenic acids.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>ENZYME INHIBITION</th>
<th>AGGREGATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.O.</td>
<td>L.O.</td>
</tr>
<tr>
<td>18:3 (ω7)</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>19:3 (ω8)</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>20:3 (ω9)</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>18:4 (ω4)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>19:4 (ω5)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>20:4 (ω6)</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>21:4 (ω7)</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>
Table 4. Comparison of 20-carbon acetylenics on platelet arachidonate metabolizing enzymes and aggregation.

<table>
<thead>
<tr>
<th>EICOSATETRAYNOICS</th>
<th>ENZYME INHIBITION</th>
<th>AGGREGATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.O.</td>
<td>L.O.</td>
</tr>
<tr>
<td>20:4 (ω7)</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>20:4 (ω6)</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>20:4 (ω5)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>20:4 (ω4)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>20:4 (ω3)</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>
Fig. 1. Concentration dependent inhibition of arachidonic acid metabolism in human washed platelet suspensions by 5,8,11,14-eicosatetraynoic acid (ETYA).
Fig. 2. Autoradiograph of the effects of the acetylenic fatty acids 22:3 (ω6) and 20:2 (ω7) on platelet arachidonate metabolism. The diynoic fatty acid 20:2 (ω7) inhibited only the TxB₂ and HHT and enhanced the HETE peak control representing 20:4 (ω6) (AA). Authentic standards were applied on both sides of the thin layer chromatogram and were visualized by iodine staining.
Fig. 3A. Concentration dependent inhibitors of platelet cyclooxygenase and simultaneous enhancement of the lipoxygenase by 18:4 (ω4).
Fig. 3B. Concentration dependent inhibitors of platelet cyclooxygenase and simultaneous enhancement of the lipoxygenase by 19:4 (ω4).
Fig. 4. Autoradiograph of the effectiveness of the 21:4 (ω7) acetylenic analogue as a lipoxygenase inhibitor.
Fig. 5A. Concentration dependent inhibition of platelet lipoxygenase by the 21:4 (ω7) analogue.
Fig. 5B. Concentration dependent inhibition of platelet lipoxygenase by the 20:4 (ω7) analogue.
Fig. 6. Concentration dependent inhibition of platelet 22:4 (ω7) inhibition of both.
Fig. 7. Concentration dependent inhibition of platelet 16:3 (ω6) inhibition of neither lipoxygenase nor cyclooxygenase.
CHAPTER V

DISCUSSION AND SUMMARY

The data clearly differ from that of Dutilh et al. (1978, 1981) who demonstrated that ETYA was a more potent inhibitor of lipoxygenase than cyclooxygenase and that the former enzyme influences platelet aggregation. In this particular system ETYA had an equipotential as a lipoxygenase and as a cyclooxygenase inhibitor. Similarly, Hammarström et al. (1977) and Sun et al. (1981) have found no apparent selectivity of ETYA in suppressing platelet arachidonic metabolism. Our data demonstrating the direct relationship between cyclooxygenase and aggregation seem to correlate for a large number of analogues.

The number of analogues studied permits a partial analysis of structure activity relationships. If acetylenic fatty acids that possess their initial triple bond at the 5 position (i.e., \( \Delta 5 \)) are compared, some conclusions can be drawn (Table 3). The triynoic analogues (18-20 carbons) that possess three triple bonds and lack \( \Delta 14 \) are ineffective as cyclooxygenase inhibitors and are impotent as inhibitors of platelet aggregation. Goetz et al. (1976) have previously studied some 20 carbon triynoic acid analogues and observed that the rank order of potency for the inhibition of bovine seminal vesicle cyclooxygenase and the inhibition of platelet aggregation was as follows: 20:3 (\( \omega 6 \))>20:3 (\( \omega 7 \))>20:3 (\( \omega 9 \)). We found that both 20:3 (\( \omega 8 \)) and 20:3 (\( \omega 6 \)) were potent cyclooxygenase inhibitors and potent inhibitors of platelet aggregation, while 20:3 (\( \omega 9 \)) inhibited platelet lipoxygenase only and was a weak antithrombotic agent (Table 2). Comparison of the triynoic acetylenic derivatives with triple bonds at positions 8,11,14 (i.e., \( \Delta 8 \)) clearly demonstrates that unsaturation at \( \Delta 14 \) is critical for inhibition of cyclooxygenase.
activity, whereas the lack of the triple bond at A5 is not essential to suppress TxA₂ biosynthesis (Table 3). This relationship seems fairly independent of chain length, i.e., from 17-20 carbon acetylenics; however, when the chain length was extended to 20 carbons, the analogue no longer inhibited the cyclooxygenase but was preferentially effective against the 12-lipoxygenase and, therefore, a poor antithrombotic agent. The tetraynoic analogues that possess triple bonds at the 5,8,11,14 positions demonstrate the impact of the length of the ω-carbon chain. Thus, with 18-20 carbons they are potent inhibitors of cyclooxygenase and platelet aggregation (Table 3). At 20 carbons the ω5 acetylenic fatty acid inhibits both cyclooxygenase, while further extension of the ω-chain to 21 carbons results in a compound which inhibits only the lipoxygenase. Variation of the position of the triple bonds also profoundly influences the inhibitory effects of these analogues. With the chain length fixed at 20 carbons and comparing only tetraynoic acid compounds, it appears that the ω3, ω4, and ω5 compounds inhibit cyclooxygenase only and are inhibitors of aggregation (Table 4). The ω6 compound (ETYA) inhibits both, but the ω7 inhibits lipoxygenase only.

The apparent lack of participation of the 12-lipoxygenase in platelet aggregation leaves the function of this enzyme unresolved in this tissue. An exciting possibility was recently demonstrated by Borgeat at the 1981 Winter Prostaglandin Meeting (April, 1981). His work indicated that leukocytes metabolized arachidonic acid to 5-HETE. However, when he mixed leukocytes and platelets, 5,12-diHETE was formed, suggesting the transfer of the 5-HETE to platelets where the 12-lipoxygenase catalyzed the formation of 5,12-diHETE. Thus, two cell types were required to participate in the synthesis of a potent biological compound. Such cell-cell communication and interaction would have profound implications.
In summary, the availability of agents with such a wide spectrum of activity provides a family of tools that will permit discrimination of metabolic pathways. What remains to be determined is the utility of these analogues in other cell types (see Appendix).
LITERATURE CITFD


Vargaftig, B.B. and Zirinis, P. (1973) Platelet aggregation induced by arachidonic acid is accompanied by release of potential inflammatory mediators distinct from PGE\(_2\) and PGF\(_{2\alpha}\). Nature New Biol. 244:114-116.


APPENDICES
MODULATION OF LEUKOTRIENE FORMATION

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ABSTRACT

Utilizing a cell-free enzyme system for the synthesis of the different leukotrienes we studied various factors influencing leukotriene synthesis. We found that calcium stimulates the first step in the pathway (arachidonic acid → 5 hydroperoxy arachidonate) and that glutathione is essential for the formation of slow reacting substance (SRS, leukotriene C and D). An investigation of the unsaturation requirements of polyenoic fatty acids as substrates showed that double bonds located at positions 5,8,11 were important. Thus such fatty acids as 20:4 (5,8,11,14), 20:5 (5,8,11,14,17), 20:3 (5,8,11,), 19:4 (5,8,11,14) and 18:4 (5,8,11,14) were readily converted to compounds that comigrated with 5-HETE and 5,12-DiHETE (leukotriene B) and to biologically active SRS. Chain length did not have an influence on the formation of these hydroxyacids. Fatty acids with the initial unsaturation at Δ⁴, Δ⁶, Δ⁷ or Δ⁸ were poor substrates for the leukotriene enzyme system. The double bond requirement at positions 5,8,11 and the stimulation by calcium distinguishes this lipoxygenase enzyme system from the lipoxygenase in platelets. Studying the effect of acetylenic acid analogs on leukotriene formation, we found that 8,11,14-icosatriynoic acid seems to block the synthesis of leukotriene A, as well as SRS, but not the initial lipoxygenase which converts arachidonic acid into 5-HPETE.
The availability of a simple cell free enzymatic generating system for leukotrienes allows the study of the formation of these compounds in detail. With such a system substrate and cofactors can be readily controlled and one need not be concerned about uptake into tissue or cells. We obtained a cell-free leukotriene enzyme system by homogenizing rat basophilic leukemia (RBL-1) cells under gentle conditions in the presence of EDTA and indomethacin (Steinhoff et al., 1980, and Jakschik et al. 1980a).

Stimulation of leukotriene formation by calcium. The formation of 5,12-DiHETE (leukotriene B) was studied by incubating the 10,000 xg supernatant with $^{14}$C-arachidonic acid. The products formed were separated by thin layer chromatography and quantitated by scraping the radioactive bands and liquid scintillation spectrometry. When the 10,000 xg supernatant was incubated with $^{14}$C-arachidonate alone, only insignificant amounts of lipoxygenase products were synthesized. However, the addition of calcium markedly stimulated the formation of hydroxyacids (Jakschik, et al., 1980). Two major products were observed 5-HETE and 5,12-DiHETE. Upon addition of 1 mM calcium 20-40% of the added arachidonic acid was converted to 5,12-DiHETE and 10-25% to 5-HETE. This enhancement by calcium was dose dependent. A similar enhancement by calcium was observed when SRS synthesis was studied (B.A. Jakschik and L.H. Lee, 1980).

The stimulation of this pathway by calcium was suggested earlier when we observed enhanced formation of leukotrienes when RBL-1 cells were incubated with arachidonic acid in the presence of the calcium ionophore A23187 (Jakschik et al. 1977 and 1978). Similar results were reported by Borgeat and Samuelsson (1979) for polymorphonuclear leukocytes. Calcium probably acts at the first step (arachidonic acid $\rightarrow$ 5-hydroperoxyarachidonate) since in the absence of exogenous calcium neither 5,12-DiHETE nor 5-HETE were formed in significant amounts. The calcium dependence of this pathway in basophils is of great interest. It suggests a close relationship between leukotriene formation and the release reaction of these cells which is well known to be calcium dependent.
Modulation of leukotriene formation

Effect of glutathione on leukotriene formation. The cell-free enzyme system was also utilized to study SRS formation. The synthesis of SRS was monitored on the isolated, perfused guinea pig ileum (Jakschik et al., 1978). It is very likely that a mixture of leukotriene C and D (Parker et al., 1979; Orning et al., 1980) is formed by the 10,000 xg supernatant of RBL-1 cells. These studies of SRS formation with the cell-free enzyme system showed very clearly that glutathione is essential for the synthesis of these leukotrienes. Addition of cysteine to the incubation mixture instead of glutathione did not produce SRS activity. The SRS activity generated by this cell-free enzyme system was readily blocked by the specific SRS receptor antagonist FPL55712 (Augstein et al., 1973).

The addition of glutathione to the incubation mixture did not only produce SRS but also reduced the formation of 5,12-DiHETE (Jakschik and Lee, 1980). This observation suggests the preferential formation of SRS over 5,12-DiHETE when relatively low concentrations of substrate are available.

Various polyenoic acids as substrates for leukotriene enzymes. Utilizing a variety of fatty acids, differing in chain length, degree and position of unsaturation, we investigated the substrate specificity for the enzymatic production of biologically active SRS and of the other leukotrienes (Jakschik et al., 1980b). The various fatty acids, except arachidonic acid and dihomo-γ-linolenic were prepared by total organic synthesis (Sprecher, 1971). The primary structural requirement observed for the conversion by this lipoxygenase enzyme system was an unsaturation in a polyenoic fatty acid at 5,8,11. Such fatty acids as 20:4 (5,8,11,14) 20:5 (5,8,11,14,17), 20:3 (5,8,11), 19:4 (5,8,11,14) and 18:4 (5,8,11,14) were excellent substrates for these enzymes. These fatty acids were readily converted to compounds that comigrated with 5-HETE and 5,12-DiHETE and to biologically active SRS. Chain length appeared to be less of an influence on the formation of these hydroxyacids. Fatty acids with the initial unsaturation at Δ⁴, Δ⁶, Δ⁷ or Δ⁸ were a poor substrate for the leukotriene enzyme system.
The finding that 20:5 (5,8,11,14,17) and 20:3 (5,8,11) are readily converted and form a potent SRS has pertinent biological implications. 20:3 (5,8,11) accumulates in essential fatty acid deficiency and is not converted by the cyclooxygenase (Ziboh et al., 1972). 20:5 (5,8,11,14,17) is a poor substrate for platelet cyclooxygenase and in fact is a good antagonist (Needleman et al., 1979 and Whitaker et al., 1979). It is thought that this mechanism may explain the suppressed platelet aggregation observed in Greenland Eskimos who possess a high 20:5 to 20:4 ratio in their tissue lipids because of their cold water fish diets (Dyerberg et al., 1978). These discoveries have stimulated an interest in dietary manipulation to increase 20:5 tissue levels. The observation that 20:5 is readily converted into the potent bronchoconstrictor, SRS, suggests a re-evaluation of such a dietary manipulation of fatty acid levels, especially in asthmatics. The formation of leukotrienes from 20:5 (5,8,11,14,17) and 20:3 (5,8,11) has also recently been reported by Samuelsson and Hammarstrom (1980).

The requirement of a double bond in the 5,8,11 position for leukotriene formation clearly differentiates this enzyme system from the platelet lipoxygenase which does not have such a double bond requirement for substrate. The platelet lipoxygenase is also not stimulated by calcium (Jakschik et al., 1980a).

Inhibition by Δ⁸ acetylenic acids. In recent investigations with the cell-free enzyme system we tested the effect of different acetylenic analogs of arachidonic acid. Studies with Δ⁸ acetylenic acids indicate that these compounds might block different steps in the leukotriene pathway. As can be seen from Figure 1 and 3 8,11,14-icosatriynoic acid somewhat inhibited 5,12-DiHETE formation and had no effect on 5-HETE synthesis. However the inhibition of SRS was significantly greater than 5,12-DiHETE. At an acetylenic acid concentration of 3 μg/ml 5,12,-DiHETE synthesis is not significantly different from control, but SRS formation is inhibited by 50% (Fig. 3). From this data we conclude that 8,11,14-icosatriynoic acid inhibits the pathway at two points:
Modulation of leukotriene formation

it partially blocks leukotriene A formation and also acts at a later step inhibiting SRS (leukotriene C and D) synthesis (see Fig. 4). This acetylenic acid, therefore, inhibits a certain specificity for different steps in the leukotriene pathway. Other acetylenic acid analogs tested so far block the lipoxygenase which converts arachidonic acid to hydroperoxy-arachidonate.

Work with Δ⁸ acetylenic acid with different carbon chain length indicates that as the carbon number is increased the tendency to block at the first step (arachidonate → 5HPETE) increases (Sams et al, 1980). The results suggest that by manipulating carbon chain length and position of acetylenic bonds it might be possible to obtain fatty acid analogs which will inhibit the formation of one leukotriene and not another.

ACKNOWLEDGEMENTS

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Figure 1: Effect of 5,8,11-icosatriynoic acid (18:3 (8,11,14)) on 5,12-DiHETE formation. 0.5 ml of 10,000 xg supernatant of RBL-1 cell homogenate was preincubated for 15 min, 4°C, with Ca++, 1.5 mM, and 18:3 (8,11,14), 20 μg/ml. Then 14C-arachidonic acid (AA), 200,000 cpm, was added and the mixture incubated for 15 min at 37°C. In the control 8,11,14-icosatriynoic acid was omitted. The protein was precipitated with 3 volumes of acetone and the supernatant acidified (pH 3.4) and extracted twice with 2 ml of chloroform. Thin layer chromatography was performed in the organic phase of ethyl acetate: 2,2,4-trimethyl pentane:acetic acid:water (110:50:20:100). The bands were visualized by fluorography and quantitated by scraping and liquid scintillation counting.
Modulation of leukotriene formation

18:3 (8,11,14)

Figure 2: Effect of the 8,11,14-icosatriynoic acid (18:3 (8,11, 14)) on SRS formation. 10,000 xg supernatant of RBL-1 cell homogenates was preincubated at 4°C for 15 min with Ca++, 1.5 mM and 18:3 (8,11,14) as indicated. Then arachidonic acid, 1 μg/ml, and GSH, 1 mM, were added and the mixture incubated for 20 min at 37°C. The acetylenic acid was omitted in the control. The incubation mixture (0.2 ml) was applied directly to the superfused guinea pig ileum. The size of the contractions were compared utilizing the area under the curve.
Dose-dependent effect of 8,11,14-icosatriynoic acid (18:3 (8,11,14)) on leukotriene synthesis. For experimental details see legend of figures 1 and 2. The data are presented as mean ± SEM (n=8, 5-HETE and 5,12-DiHETE; n=5 SRS).
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**Figure 4:** Schematic of the site of action of 8,11,14-icosatriynoic acid (18:3 (8,11,14)).


Modulation of leukotriene formation


FATTY ACID STRUCTURAL REQUIREMENTS FOR LEUKOTRIENE BIOSYNTHESIS*

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Abstract

Utilizing a variety of fatty acids, differing in chain length, degree and position of unsaturation, we investigated the substrate specificity for the enzymatic production of biologically active slow reacting substances (SRS) and of the other leukotrienes. A cell-free enzyme system obtained from RBL-1 cells was used in this study. The primary structural requirement observed for the conversion by this lipoxygenase enzyme system was a Δ9,11 unsaturation in a polyenoic fatty acid. Such fatty acids as 20:4 (5,8,11,14), 20:5 (5,8,11,14,17), 20:3 (5,8,11), 19:4 (5,8,11,14) and 18:4 (5,8,11,14) were readily converted to compounds that comigrated with 5-HETE and 5,12-DiHETE and to biologically active SRS. Chain length did not have an influence on the formation of these hydroxyacids. Fatty acids with the initial unsaturation at Δ9, Δ9, Δ9 or Δ9 were a poor substrate for the leukotriene enzyme system. Therefore, this lipoxygenase pathway in leukocytes is quite different from the lipoxygenase in platelets which does not exhibit this specificity.

Introduction

We have reported earlier that rat basophilic leukemia (RBL-1) cells produce the potent bronchoconstrictor SRS when stimulated with the calcium ionophore A23187 (1) and that labeled arachidonic acid (20:4, 5,8,11,14) is incorporated into slow reacting substance (SRS) (2). SRS from guinea pig lung (3), a murine mastocytoma (4) and RBL-1 cells (5,6) has been shown to have a UV absorption spectrum characteristic of conjugated trienes with a maximum at 280 nm. It has therefore been proposed that SRS is formed by the same pathway as other conjugated triene hydroxy acids synthesized by leukocytes (4). The reaction sequence involves an initial lipoxygenase conversion of arachidonic acid to the 5 position mono-hydroperoxy acid which is converted to 5-hydroxycotetraenoic acid (5-HETE) or to a

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labile intermediate, a 5,6-epoxide termed leukotriene (LT)A. The labile intermediate is then converted either to a 5,12-DIHETE (LTB) or through the enzymatic incorporation of glutathione into LTC which possesses the SRS activity (4,7). While it is believed that a glutathione S-transferase incorporates glutathione into SRS (4,7), Morris et al. (6) reported that the amino acid portion of SRS formed by RBL-T cells consists of cysteinylglycine. We have found that a cell-free preparation from RBL-1 cells readily formed 5-HETE and 5,12-DIHETE only when incubated in the presence of calcium, whereas the platelet lipoxygenase was calcium independent (8). The calcium dependent cell free RBL-1 preparation also assembled the biologically active SRS when incubated in the presence of glutathione (9).

The elucidation of the reaction sequence in the synthesis of the SRS as well as the availability of a simple cell free enzymatic SRS generating system permits an investigation of the structural requirements necessary for the production of biologically active SRS and of the other leukotrienes. We previously studied a variety of polyenoic fatty acids and demonstrated a high degree of substrate specificity for the enzymatic conversion by platelet cyclooxygenase into prostaglandins and thromboxanes (10). However, no apparent specificity was detected for the platelet lipoxygenase conversion of the various fatty acids (11). In this investigation we evaluated the suitability of a variety of fatty acids, differing in chain length, degree and position of unsaturation, as substrates for the RBL-1 leukotriene enzymes, as well as the extent of SRS receptor recognition and response activation of the various enzymatically generated SRSs.

Methods

RBL-1 cells were grown and harvested as described previously (12). The cells were washed with 50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% gelatin and 14 μM indomethacin, resuspended at 5 x 10⁶ cells/ml and homogenized as described (13). The homogenate was centrifuged at 10,000 x g and the supernatant used for the experiments.

For the study of hydroxy acid formation, [14C] fatty acids (150,000 cpm) were incubated with 0.5 ml of the 10,000 x g supernatant in the presence of 2 mM calcium chloride for 30 min. The reaction was stopped by precipitating the proteins with acetone. The pH of the supernatant was adjusted to 3.2 and extraction performed twice with two volumes of chloroform. Thin layer chromatography was performed on silica gel plates in solvent system A9, the organic phase of ethyl acetate:2,2,4-trimethyl pentane:acetic acid:water (110:50:20:100) (14). Standards were visualized by iodine staining. Radioactive peaks were located by fluorography (15) and quantitated by scraping and liquid scintillation counting. SRS was synthesized by incubating the various fatty acids (5 and 15 μg/ml) with 10,000 x g supernatant in the presence of 2 mM calcium and 1 mM reduced glutathione for 15 min at 37°C. SRS forma-
tion was monitored on the isolated, superfused guinea pig ileum (12).

The various fatty acids, except arachidonic acid and dihomo-γ-linolenic, were prepared by total organic synthesis (13-25 Ci/mol) (16). [14C]-Arachidonic acid (55 Ci/mol) was obtained from New England Nuclear (Boston, MA). [14C]-dihomo-γ-linolenic acid (55 Ci/mol) from Amersham (Arlington Heights, IL), arachidonic acid and dihomo-γ-linolenic acid from Nu Check Prep (Elyson, IL) and reduced glutathione from Sigma (St. Louis, MO). 5-HETE was kindly supplied by R.C. Kelly of the Upjohn Co. (Kalamazoo, MI), icosatetraynoic acid by Roche Laboratory and indomethacin by Merck, Sharp and Dohme (West Point, PA).

Results

The formation of mono- and, DiHETE from the various fatty acids was investigated by incubating 14C-labeled fatty acids with the 10,000 x g supernatant fraction of indomethacin-treated RBL-1 cells in the presence of calcium. An illustrative autoradiograph of a thin layer chromatographic separation is presented in Figure 1. The primary detectable products produced from arachidonic acid were 5-HETE and 5,12-DiHETE. The structure of these products was previously confirmed by their high performance liquid chromatography separation profile and gas chromatographic - mass spectrometric analysis (8). The migration of standards of these hydroxy fatty acids are indicated in the center of the plate. The fatty acids which possessed a 5, 8, 11 unsaturation were all readily converted to products that comigrate with 5-HETE and 5,12-DiHETE. The structure of these products was previously confirmed by their high performance liquid chromatography separation profile and gas chromatographic - mass spectrometric analysis (8). The migration of standards of these hydroxy fatty acids are indicated in the center of the plate. The fatty acids which possessed a 5, 8, 11 unsaturation were all readily converted to products that comigrate with 5-HETE and 5,12-DiHETE regardless of the degree of unsaturation. Thus, the 20:3 (5,8,11), 20:4 (5,8,11,-14), and 20:5 (5,8,11,14,17) fatty acids all were excellent substrates for these enzymes. Chain length appeared to be less of an influence than the necessity for double bonds at 5, 8, 11, as evidenced by the striking differences in conversion of the Δ5 versus the Δ19 carbon tetraenoates (Fig. 1 and Table I). Fatty acids with a Δ19 unsaturation, such as 19:4 (4,7,10,13) and 19:5 (4,7,10,13,16), were converted, though poorly, to a hydroxy acid which comigrated with 5-HETE and is presumably 4-HETE. However, conversion to DhETE was negligible. Fatty acids with their first double bond at Δ5, Δ6, or Δ19 were hydroxylated to compounds which migrated further than the 5-HETE standard.

The formation of SRS activity was monitored on superfused guinea pig ileum assay strips. An aliquot of the reaction mixture following incubation of the various fatty acids in the presence of the enzyme, calcium and glutathione, was injected over a bank of tissues. A profound contractile response was produced by the incubations containing the 20:3 (5,8,11), 20:4 (5,8,11,14), and 20:5 (5,8,11,14,17) fatty acids (Fig. 2). All of these fatty acids have a double bond at 5, 8, 11. The response generated when arachidonate was used as the substrate remained similar throughout the course of the experiment. No contractile response was produced in the absence
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Figure 1: Radioautogram of thin layer chromatograms of fatty acid products. 10,000 x g supernatant (0.5 ml) of RBL-1 homogenates was incubated with [14C] fatty acid (150,000 cpm) in the presence of 2 mM calcium for 30 min, 37°C. The incubation mixture was extracted and chromatography performed as indicated in Methods.
Figure 2: SRS formation by various fatty acids. 10,000 x g supernatant of RBL-1 homogenates was incubated with the fatty acid indicated in the presence of 2 mM calcium and 1 mM glutathione for 15 min, 37°C. An aliquot of the incubation mixture was applied to the superfused guinea pig ileum. FPL 55712 was applied to the tissue in a 0.2 µg/ml concentration.
TABLE I
CONVERSION OF THE VARIOUS FATTY ACIDS INTO LEUKOTRIENES

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>DHETE</th>
<th>HETE</th>
<th>Relative SRS Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:5 (5,8,11,14,17)</td>
<td>31.6 ± 2.5</td>
<td>34.2 ± 1.8</td>
<td>high</td>
</tr>
<tr>
<td>20:4 (5,8,11,14)</td>
<td>21.4 ± 3.4</td>
<td>25.8 ± 2.7</td>
<td>high</td>
</tr>
<tr>
<td>20:3 (5,8,11)</td>
<td>11.7 ± 0.9</td>
<td>31.2 ± 4.0</td>
<td>high</td>
</tr>
<tr>
<td>19:4 (5,8,11,14)</td>
<td>21.1 ± 2.4</td>
<td>42.7 ± 2.1</td>
<td>moderate</td>
</tr>
<tr>
<td>18:4 (5,8,11,14)</td>
<td>11.9 ± 2.1</td>
<td>36.6 ± 9.0</td>
<td>moderate</td>
</tr>
<tr>
<td>19:5 (4,7,10,13,16)</td>
<td>2.0 ± 0.3</td>
<td>10.0 ± 1.7</td>
<td>very low</td>
</tr>
<tr>
<td>19:4 (4,7,10,13)</td>
<td>1.7 ± 0.2</td>
<td>8.9 ± 2.1</td>
<td>low</td>
</tr>
<tr>
<td>21:5 (6,9,12,15,18)</td>
<td>2.9 ± 0.8</td>
<td>7.0 ± 1.3</td>
<td>very low</td>
</tr>
<tr>
<td>21:4 (6,9,12,15)</td>
<td>2.7 ± 0.4</td>
<td>6.6 ± 2.5</td>
<td>v. low-inactive</td>
</tr>
<tr>
<td>22:4 (7,10,13,16)</td>
<td>1.7 ± 0.8</td>
<td>7.5 ± 3.8</td>
<td>low</td>
</tr>
<tr>
<td>20:4 (8,11,14,17)</td>
<td>1.6 ± 0.3</td>
<td>9.7 ± 2.0</td>
<td>low</td>
</tr>
<tr>
<td>20:3 (8,11,14)</td>
<td>1.3 ± 0.4</td>
<td>7.1 ± 3.0</td>
<td>low</td>
</tr>
</tbody>
</table>

*The various [14C]fatty acids were incubated with the 10,000 x g supernatant of RBL-1 cell homogenates as described in the legend of Figure 1. After chromatography, the radioactive bands were scraped and quantitated by liquid scintillation counting. The data are stated as mean ± SEM, n=6.

**The bioactivity was evaluated on the isolated perfused guinea pig ileum as indicated in the legend of Figure 2. The relative SRS activity generated from the different fatty acids was assessed by comparing the contractile response produced to that of SRS synthesized from arachidonic acid. n=4-10.
of GSH. The SRS receptor antagonist FPL 55712 efficiently abolished the contractile response of the fatty acid incubations (Fig. 2).

The guinea pig ileum assay does not lend itself to accurate quantitative comparison. Relative potency was assessed either by trying to match the contractile response of the fatty acid under study with the response produced by arachidonate or by comparing equal concentrations of the various fatty acids. An extensive comparison of the various fatty acids is shown in Table 1. The compounds appeared to subdivide into two groups based on the intensity of the biological response and the relative conversion to DiHETE. The long chain (18-22 carbons) polyenoic fatty acids were all enzymatically converted to active SRS-like compounds with the possible exception of 21:4 (6,9,12,15). This latter fatty acid is enzymatically converted by platelets or blood vessels into its appropriate endoperoxide, thromboxane, and prostacyclin all of which proved to be biologically inactive (10). The most striking result was the parallelism between DiHETE and HETE formation and detectable SRS activity and the obvious preference for a double bond at positions 5, 8, 11 of the fatty acid. When the initial unsaturation was in position 4, 6, 7, or 8 very much less hydroxy fatty acid was formed (Table 1). The low SRS activity obtained from incubations of these latter fatty acids could reflect the low conversion rate and/or low biological activity. The latter issue will require careful isolation of the leukotriene like compound for quantitation. The SRS-like activity of all the fatty acids was abolished by the absence of GSH, or by the receptor blocker FPL 55712. There was no direct relationship between the suitability of the various fatty acids as lipoxygenase substrates with the RBL-1 enzyme source (leukotriene pathway) versus that from platelets (11).

Discussion

The primary structural requirement for conversion by the enzyme of the leukotriene pathway appears to be the presence of 5, 8, 11 unsaturation in a polyenoic fatty acid. There was a direct parallelism between the capacity of the enzyme system (in the absence of GSH) to form the mono-and dihydroxy fatty acids and SRS (in the presence of glutathione).

The finding that 20:5 (5,8,11,14,17) and 20:3 (5,8,11) are readily converted and form a potent SRS has pertinent biological implications. We previously demonstrated that 20:5 is a poor substrate for platelet cyclooxygenase even though this fatty acid is readily incorporated and released from platelet phospholipids (17). However, 20:5 is an effective antagonist of arachidonate metabolism by platelet cyclooxygenase (17,18). Thus, 20:5 inhibits platelet thromboxane A2 formation which presumably interferes with platelet aggregation. This latter mechanism may explain the suppressed platelet aggregation observed in Greenland Eskimos that possess a high 20:5 to arachidonate ratio in their tissue lipids because of their cold water fish diet (19). These discoveries have stimulated
an interest in dietary manipulation to increase tissue 20:5 levels. The finding that 20:5 can readily be converted into the potent bronchial constrictor forces a re-evaluation of the implications of dietary manipulation of fatty acid levels. Indeed it seems worthwhile to determine if the Greenland Eskimos have a predisposition to bronchial constriction. It is conceivable that they may have enhanced SRS formation with a fatty acid like 20:5 since none of the substrate is diverted to prostaglandin or thromboxane synthesis. A similar situation exists with 20:3 (5,8,11). This fatty acid accumulates in essential fatty acid deficiency and is not converted by cyclooxygenase (20). Thus, fatty acids that were thought to be biologically inactive may well be precursors of SRS.

Morris et al. (21) studied the effect of a number of polyenoic acids on SRS-A release from chopped sensitized guinea pig lung. They found that some of the fatty acids such as arachidonic, 20:5 (5,8,11,14,17), 22:6 (4,7,10,13,16,19) and 18:3 (5,9,12) enhanced SRS-A release while 20:3 (5,8,11), 20:2 (11,14), 20:3 (11,14,17), 18:3 (9,12,15) and oleic had no effect. They concluded from their results that the latter fatty acids were not a substrate for the lung lipoxygenase. These data are therefore in disagreement with our results. However, it is very difficult to interpret the study by Morris et al. where whole lung tissue which consists of a large number of different cells was utilized and fatty acid uptake may vary with the cell types. Moreover, it is not clear whether these exogenously added fatty acids were accessible to the lipoxygenase of the leukotriene pathway since no incorporation of label from [14C]-arachidonic acid into SRS-A was achieved. Recently, Hammerström and Samuelsson presented evidence (22) that 20:5 (5,8,11,14,17) and 20:3 (5,8,11) are converted by intact cells in the presence of ionophore into leukotrienes.

A comparison of the known characteristics of the lipoxygenases from platelets and RBL-1 cells, clearly shows that the two enzymes are quite different. The lipoxygenase of the leukotriene pathway (RBL-1) has a requirement for a 5, 8, 11 double bond and is stimulated by calcium (8). In contrast, the platelet lipoxygenase is calcium independent and no fatty acid specificity was apparent when exposed to the same fatty acids that were tested on the leukotriene pathway (11).

The availability of a cell-free SRS generating system and structural analogs will permit a continued elucidation of the enzymatic and receptor recognition characteristics and the potential evolution of potent enzyme or receptor antagonists for SRS and the other leukotrienes.

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