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Epinephrine Potentiation of Arachidonate-Induced Aggregation of Cyclooxygenase-Deficient Platelets

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Evaluation of platelet physiology, biochemistry, ultrastructure, and function in a young woman without history of hemorrhagic problems revealed that her platelets were deficient in cyclooxygenase activity. Her citrate platelet-rich plasma (C-PRP) responded monophasically when stirred with aggregating agents in the same manner as aspirin-treated normal C-PRP, but could be irreversibly aggregated by high concentrations of thrombin, collagen, and ADP. Her platelets did not aggregate when stirred with AA at concentrations as high as 2 mM. Ultrastructure and levels of serotonin and adenine nucleotides were normal. Amounts of ^{14}C-AA released after stirring with thrombin were similar to normal cells. However, evaluation of prostaglandin synthesis after stirring with ^{14}C-AA revealed no evidence of endoperoxide or thromboxane production, although products of the lipoxygenase pathway were produced in normal amounts. Aggregation in response to AA was completely corrected after mixing with 10% normal C-PRP. However, equal volumes of her C-PRP and normal aspirin-treated C-PRP did not respond to AA, whereas 10% normal platelets combined with aspirin-treated cells corrected aggregation to AA. Since epinephrine pretreatment corrects the response of dog platelets that are not aggregated by AA, we evaluated the influence of epinephrine on her platelets. Preexposure to 5 μM epinephrine, a concentration that gave only primary waves of aggregation, resulted in normalization of her response to AA, even though correction was not associated with the generation of endoperoxides or thromboxanes. The results may explain why patients with platelet cyclooxygenase deficiency have mild or absent bleeding symptoms.

Key words: cyclooxygenase deficiency, membrane modulation

INTRODUCTION

Several individuals and families with a wide range of clinical symptoms of hemorrhagic disease and laboratory findings have been reported to have platelet

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cyclooxygenase deficiency [1–5]. Cyclooxygenase is the enzyme in platelets that converts arachidonic acid into endoperoxides [6–8] that are rapidly transformed by thromboxane synthetase into thromboxane A_2 [9, 10]. This unstable product of prostaglandin synthesis is a potent vasoconstrictor and one of the most powerful platelet-aggregating agents known [9–12].

Aspirin binds irreversibly to platelet cyclooxygenase, and prevents conversion of arachidonic acid derived from membrane phospholipids into endoperoxides and thromboxanes [13–15]. Although the bleeding time in normal individuals is slightly prolonged following aspirin ingestion, the risk for serious bleeding episodes is minimal. Patients with cyclooxygenase deficiency should be essentially identical to normal individuals taking aspirin and have little or no problem with serious bleeding, unless some other component of the hemostatic mechanism is compromised.

We have recently evaluated a healthy young woman with no history of unusual bleeding, whose platelets lack cyclooxygenase activity. In addition to establishing the diagnosis, we have evaluated the influence of epinephrine on the response of her platelets to sodium arachidonate-induced aggregation and secretion. The results demonstrate that, even though her platelets are unable to convert arachidonic acid into endoperoxides, they undergo irreversible aggregation in response to arachidonic acid and other agents when pretreated with epinephrine.

Case History

The proposita is a 23-year-old unmarried female in excellent health. She has never had bleeding symptoms in her life. Onset of menses at age 12 was uneventful. Her periods are regular and not associated with excessive bleeding. Wisdom teeth were extracted at age 15 without any bleeding complications. She has not been in any severe accidents and has not required major or minor surgery. Bruising and petechiae formation have not been problems, although she is involved in strenuous athletics and her work in animal management requires violent physical activity on occasion. Her family history is negative. Grandparents on both sides of the family died of normal causes. Uncles and aunts and her mother and father are free of bleeding symptoms. She has only one sibling, a sister, who is also free of any unusual history of bleeding. On physical examination the patient had no sign of petechiae, bruising, ecchymosis, or joint involvement.

MATERIALS AND METHODS

Materials

Arachidonic acid as the sodium salt was obtained from Nu Chek Prep, Elysian, Minnesota, and made up in 0.1 M Tris buffer at pH 7.4. (I-^{14}C)arachidonic acid was obtained from New England Nuclear, Boston, and 5-hydroxy (side chain-2-^{14}C) tryptamine creatinine sulfate was purchased from Amersham, Arlington Heights, Illinois. Injectable adrenalin and topical thrombin were provided by the Parke Davis Co., Detroit. Acid-soluble collagen was purchased from Worthington Biochemical Corp., Freehold New Jersey. Unless otherwise stated all other chemicals were obtained from Sigma Chemical Co., St. Louis.

Methods

Platelets for these studies were obtained from healthy adult volunteers and the patient after informed consent. Blood drawn from an anticubital vein into plastic syringes was mixed immediately with 100 mM sodium citrate buffer containing 136 mM glucose, pH 6.4, in a ratio of 9 parts of blood to 1 part anticoagulant. Platelet-rich plasma (C-PRP) was separated by centrifugation at 200g for 20 minutes at room temperature. Platelet-poor plasma (PPP) was prepared by centrifugation of anticoagulated blood at 1,500g for 20 minutes. Platelet aggregation was monitored with a dual channel Payton aggregometer at a stirring speed of 1,000 rpm and calibrated using PRP and PPP [16]. Samples were prepared by glutaraldehyde and osmic acid fixation for study in the electron microscope according to the methods established in this laboratory [17, 18].

EXPERIMENTAL PROCEDURES

Platelet Serotonin Secretion

Platelets in C-PRP were labeled with ^{14}C-5-HT by a modification [19] of the method of Jerushalmy and Zucker [20] for the evaluation of the release reaction. Samples of prelabeled platelets were exposed to an aggregating agent on the aggregometer at 37°C until maximum aggregation was obtained. After aggregation, the reaction was stopped with the addition of EDTA to a final 1% concentration, and the pellet and supernatant were separated by rapid centrifugation at 4°C. Pellet and supernatant fractions containing the labeled serotonin were separated and counted in a scintillation counter.

Platelet Metabolism of Archidonic Acid

Thrombin stimulated release of ^{14}C-arachidonic acid from platelet phospholipids was measured using a modification of the method of Bills et al [21]. Metabolites formed during platelet oxidation of arachidonic acid by the lipoxygenase enzyme, resulting in formation of 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), and the cyclooxygenase enzyme leading primarily to the formation of C17 hydroxytrienoic acid (HHT) and thromboxane B_2 was followed using a modification [16] of the method of Hamberg and Samuelsson [7] employing ^{14}C-arachidonic acid (Applied Science) as the substrate. Labeled arachidonic acid was diluted with cold arachidonic acid (Nu Chek Prep) to a specific activity of 25–28 mCi/mM, made up as a sodium salt and diluted in 0.1 M Tris buffer, pH 7.0. One-milliliter aliquots of each reaction mixture containing 1.5×10^9 platelets were incubated for 3 minutes at 37°C with 1 μg labeled arachidonic acid. At the end of the incubation period, 10 ml of ethanol was added to stop the reaction. The conversion of labeled fatty acid to hydroxy derivatives and to thromboxane B_2 was followed using thin-layer chromatography of the methyl esters on silica gel G with the organic layer of isoctane:water:ethyl acetate (100:100:50 v/v) as the eluting solvent (system A). Conversion of labeled arachidonic acid to thromboxane B_2 was also evaluated using thin-layer chromatography of the free acids on silica gel G with diethyl ether:methanol:acetic acid (135:3:3 v/v) as the eluting solvent (system B).

Thin-layer platelets developed with various systems were incubated with x-ray films (x-Omat R XR-S Kodak) for 2 days for localization of radioactive spots. For obtaining radiochromatograms, thin-layer plates were scanned with a Berthold Scanner. Radioactive spots were scraped from these plates, and the activity found in each area was determined by liquid scintillation counting. Some samples thus separated by thin-layer chromatography were further subjected to gas chromatography mass spectrometry using an LKB 9000 mass spectrometer equipped with PDP-8e data processor.

RESULTS

General

The defective function of the patient's platelets was discovered by accident. Repeated efforts to use her platelets for control purposes always revealed a release-like defect. The patient, however, consistently denied taking aspirin or aspirin-containing medications. As a result, we evaluated her in detail. History and physical findings were negative as discussed above. Routine tests of coagulation, including a partial thromboplastin time (37.2″), prothrombin time (13.6″), thrombin time (15.0″), and clot retraction were all within normal limits. Her bleeding time was 9.5 minutes on one occasion and 10.5 minutes on another. The first value is within 2 standard deviations of the normal mean in this laboratory, and the second is just outside the normal range. Her platelet function, as discussed below, was consistently abnormal. Similar studies were carried out on her sister on one occasion, and were found completely normal.

Platelet Morphology and Biochemistry

Studies in the electron microscope revealed no structural abnormalities in the patient's platelets. The number of dense bodies in her platelets was within the normal range of control platelets. Platelet peroxidase studied by a modification [22] of the method of Graham and karnovsky [23] gave a positive reaction. Serotonin and adenine nucleotide levels, measured biochemically [24, 25], were in the range of normal control platelets (Table I).

Response of COD Platelets to Aggregating Agents

Reactions of her platelets to various aggregating agents was similar to the response of aspirin-treated platelets (Fig. 1). Normal concentrations of epineph-

TABLE I. Levels of Serotonin (5 HT) and Adenine Nucleotides (AN)

	5-HT	AN μmol/10^{11} cells*		
	ngs/10^9 cells*	AMP	ADP	ATP
Normal	816 ± 52	0.63 ± 0.5	3.9 ± 0.24	5.9 ± 0.18
Patient	724 ● 43	0.69 ± 0.4	4.2 ± 0.28	5.8 ± 0.23

*Mean and the standard error (n = 3).
There were no significant differences in the levels of serotonin and adenine nucleotides in the platelets from the patient compared to the normal control platelets.

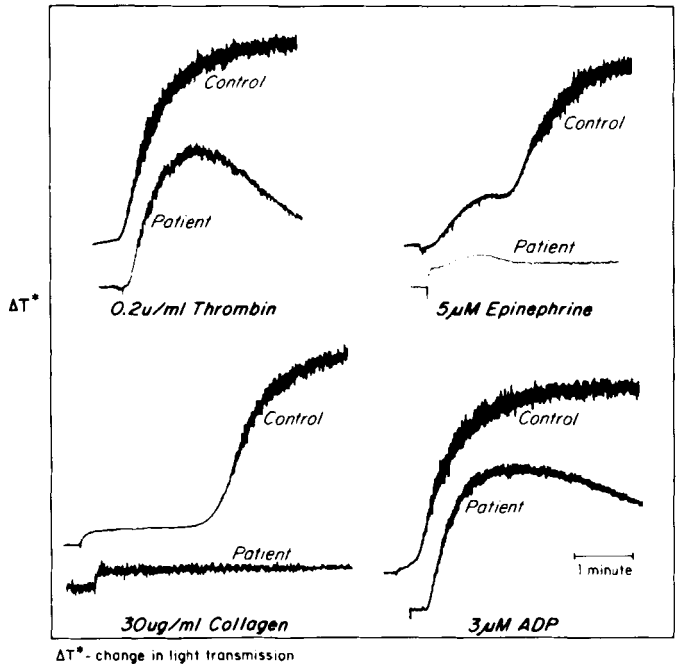

Fig. 1. Reactions of patient platelets to aggregating agents were similar to the response of aspirin-treated platelets. Our threshold concentrations of epinephrine and collagen elicited very little response. The reponse to adenosine diphosphate and thrombine resulted in reversible primary waves.

rine (5.5 μM) elicited no response, and at high concentration (10 μM) only a primary response could be observed. Similarly, serotonin exposure gave only a primary response. Platelets aggregated irreversibly to high concentrations of ADP (5 μM), collagen (100 μg/ml), and thrombin (0.3 u/ml). COD platelets did not respond to any concentration of arachidonate (0.5-2.0 mM). However, when COD platelets were pretreated with epinephrine, stimulation with 0.9 mM arachidonate gave irreversible aggregation (Fig. 2).

Mutual Correction of COD and Aspirin-Treated Platelets

Studies from this laboratory [26] have shown that when mixtures of platelets from patients with storage pool disease and aspirin-treated normal donors are stirred with aggregating agents, there exists a mutual correction response resulting in irreversible aggregation. Similar studies using 50% COD platelet and 50% of normal AT cells did not give any aggregation response. Even 90% COD with 10% AT platelets gave a negative response (Fig. 3). Although no mutual correction could be obtained with COD and AT platelet combinations, the mixture of 10% of normal platelets combined with 90% COD platelets responded with irreversible aggregation when stimulated with 0.9 mM arachidonate (Fig. 3).

Epinephrine Potentiation of the Arachidonate-Induced Release Reaction of COD Platelets

Exposure of COD platelets to epinephrine before stirring with arachidonate always induced irreversible aggregation as shown above (Fig. 2). Depending on the

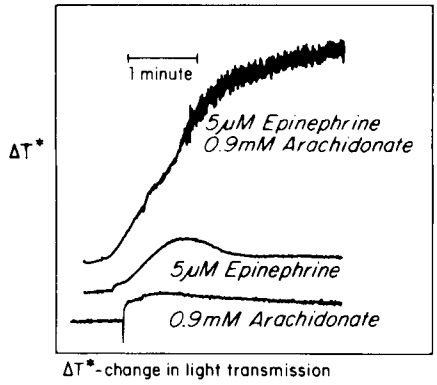

Fig. 2. Though the patient's platelets did not aggregate irreversibly in response to threshold concentrations of epinephrine or a high concentration of arachidonate, they responded with irreversible aggregation when the agents were used in combination.

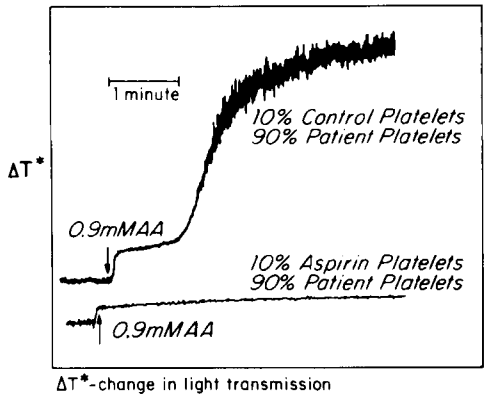

Fig. 3. Ninety percent patient platelets mixed with only 10% of aspirin platelets failed to correct the defective aggregation response when stirred with arachidonate. Only 10% of untreated normal platelets mixed with 90% of patient platelets gave an irreversible aggregation response to the action of arachidonate.

sensitivity of these platelets, the concentrations of epinephrine and arachidonate had to be adjusted each time the patient platelets were studied. Studies with ^{14}C-serotonin showed that a combination of epinephrine and arachidonate caused significant release of serotonin from COD platelets (Table II). Release induced by arachidonate in the presence of epinephrine was equal to that obtained from normal platelets stimulated with arachidonate alone.

Platelet Prostaglandin and Thromboxane Synthesis

Platelets from the COD patient were studied for their ability to convert ^{14}C-arachidonic acid to various products. Metabolites were monitored by thin-layer chromatography, radioautography, and gas chromatography – mass spectrometry. COD platelets did not produce significant thromboxane under any con-

ditions. Indeed, the counts recovered from thin-layer plates showed that COD platelets were essentially identical to normal platelets after aspirin treatment (Table III). As shown, control platelets converted significant amounts of substrate to thromboxane.

Since COD platelets gave a positive staining reaction for peroxidase, they were also evaluated for their ability to convert ^{14}C-arachidonic acid to 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), and thromboxane B_2. Patient platelets converted labeled arachidonic acid to HETE but did not show any peaks for HHT, one of the major products of prostaglandin pathway (Fig. 4). Similar results were obtained with radioautography and gas chromatography—mass spectrometry. No detectable levels of HHT or thromboxane B_2 was found in COD platelet samples by radioautography. Studies with mass spectrometry using single-ion monitoring

TABLE II. Release of ^{14}C-5 HT From Labeled Platelets in Response to Various Aggregating Agents

	CPM × 10^3		
	Pellet	Supernatant	% Release[a]
Control	63.1	5.2	7.0 ● 0.5
Epinephrine (5 μM)	60.3	7.2	10.6 ± 1.6
Arachidonate (0.9 mM)	48.6	15.5	24.0 ± 1.4
Epinephrine and arachidonate (5 μM and 0.9 mM)	26.8	36.3	59.0 ± 1.2

[a]Mean and standard error (n = 6).
Platelets stirred on the aggregometer for 5 minutes released 7% of their serotonin content. Epinephrine used as an agonist induced 10% release of serotonin, whereas arachidonate enhanced it to 24%. A combination of epinephrine and arachidonate caused as much release as one would obtain from normal platelets stimulated with arachidonate alone.

TABLE III. Conversion of ^{14}C-Arachidonic Acid to Prostaglandin Metabolites by Platelets

	CPM × 10^3[a]		
	Total counts recovered	Counts as TX B_2	% Conversion
Control platelets	94.1 ± 6.7	34.2 ± 2.7	36.3 ± 2.3
Patient platelets	129.8 ± 6.2	7.2 ● 0.8	5.6 ± 0.7
Aspirin-treated platelets	128.2 ± 7.3	6.2 ± 0.6	4.6 ± 0.9

[a]Mean and standard error (n = 6).
Platelets obtained from normal donors converted 36% of the substrate arachidonate to thromboxane. Platelets from the patient did not produce significant thromboxane. Indeed the conversion of arachidonate by these platelets was close to normal platelets after aspirin treatment.

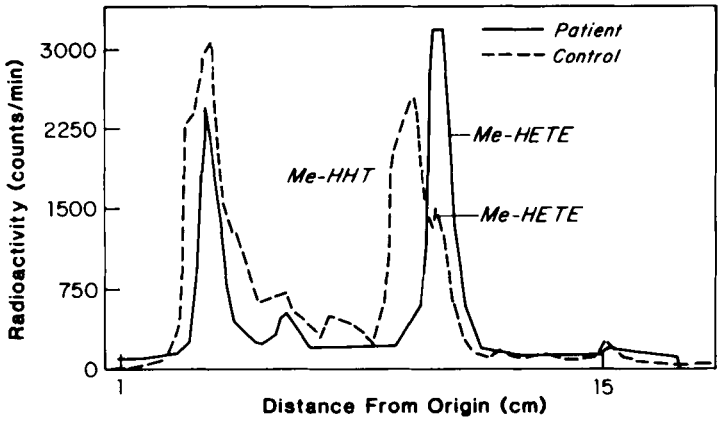

Fig. 4. Conversion of arachidonate by the lipoxygenase pathway was followed by using solvent system A described under Methods. Metabolites extracted were methylated before running on thin-layer plates. Extracts of the spots from the two main peaks of interest (identified in the figure as HHT and HETE) were subjected to GC-mass spectroscopy for characterization. Major portions of the two peaks in control platelets were contributed by HHT. In patient platelets the entire fraction consisted of HETE, a product of the lipoxygenase pathway. No cyclooxygenase product (HHT) could be detected in patient platelet samples.

techniques to detect major mass fragments for HHT and HETE showed that no detectable HHT could be found in the COD platelets and that the major product was HETE, a metabolite of the lipoxygenase pathway.

Release of ^{14}C-AA from Platelet Membranes in Response to Thrombin

There was no significant difference in the uptake or release of arachidonic acid from platelet membranes of COD platelets compared to normal control platelets. When stimulated with thrombin, COD platelets released as much arachidonic acid as control platelets (Table IV).

DISCUSSION

Results of the present investigation and repeated evaluations of the proposita indicate that her platelets are deficient in the cyclooxygenase enzyme required to convert endogenous or exogenous arachidonic acid into endoperoxides. However, it is possible that the patient, despite repeated denials, had taken aspirin or aspirin-containing drugs surreptitiously. We have tried to rule out this possibility in every conceivable way, but must point out that aspirin ingestion is virtually impossible to distinguish from cyclooxygenase deficiency by any test system available to us. If her platelets had been unable to convert the major product of the lipoxygenase pathway, HPETE, into HETE, aspirin ingestion might have been suspected. High concentrations of aspirin may influence peroxidase and conversion of HPETE to HETE [27, 28]. However, despite the fact that her platelets were unable to generate endoperoxides, we could not detect any compromise in the production of HETE. We accept, therefore, the integrity of the proposita, despite the absence of any history of easy bleeding.

TABLE IV. Release of ^{14}C-Arachidonic Acid From Platelet Membranes in Response to Thrombin

	% Release		
	Thrombin		Buffer
	0.2 u/ml	0.5 u/ml	100 μl/ml
Control	9.7 ± 1.0	16.2 ± 1.2	2.0 ± 0.3
Patient	8.6 ± 0.8	16.9 ± 1.0	1.8 ± 0.2

Mean and standard error (n = 4).
Normal platelets labeled with ^{14}C-arachidonic acid, when stirred with 0.5 u/ml thrombin, released 16% of the label compared to cells stirred with buffer, which released only 2%. When stimulated with a similar concentration of thrombin, platelets of the patient released as much labeled arachidonic acid as normal control platelets.

The defective response of the patient's platelets was not due to storage pool deficiency [29, 30]. Ultrastructural studies revealed normal numbers of dense bodies in her platelets, and levels of adenine nucleotides and serotonin were within normal limits [24, 25]. Cytochemical experiments revealed the presence of peroxidase in the dense tubular system of her platelets [22]. Since previous studies [3] have shown that peroxidase in the platelet-dense tubular system is coupled to cyclooxygenase and essential for conversion of arachidonic acid to specific endoperoxides, its presence in COD platelets could mitigate against a specific enzyme defect. However, cytochemically detectable peroxidase activity has been found in other patients with cyclooxygenase deficiency, and the findings suggest that defective function is due to an abnormal cyclooxygenase protein, rather than peroxidase.

The response of COD platelets from our patient to aggregating agents was similar to the reaction of aspirin-treated normal platelets and platelet samples from other patients with proven COD-deficient cells. Concentrations of epinephrine, ADP, and thrombin that stimulated biphasic irreversible aggregation in samples of normal C-PRP caused only reversible, single waves of response when stirred with COD platelets from our patient. The defective response of COD platelets to ADP and thrombin could be corrected by increasing the concentration of either agent, just as in the case of aspirin-treated normal platelets. The collagen response of COD platelets was also similar to that of platelets following exposure to aspirin. Sodium arachidonate at any concentration failed to aggregate the patient's platelets. These defects were regularly present in platelet samples from the patient, but were not observed in samples obtained from her sister.

The simple test system [26] developed to detect patients with platelet storage pool disease was useful for establishing the diagnosis of cyclooxygenase deficiency. Equal volumes of patients platelets and aspirin-treated normal platelets were mixed and then stirred on the platelet aggregometer with concentrations of aggregating agents, which cause biphasic responses in samples of normal platelets, but reversible, single waves of clumping in storage pool-deficient or aspirin-treated normal cells. As shown earlier, storage pool deficient and aspirin-treated normal platelets that respond monophasically, will, when mixed in equal volumes, develop irreversible aggregation when exposed to the same stimulus. Correction in this

case was shown to be due to formation of endoperoxides and thromboxanes by storage pool platelets that bypassed the influence of aspirin to cause release of the storage pool from the drug-treated normal cells. Weiss used a modification of this test system to establish the diagnosis of thromboxane synthetase deficiency in one of his patients [31]. In the present study equal volumes of patient and aspirin-treated normal cells responded to aggregating agents as if both populations had been treated with the drug. Even when the proportion of COD-deficient platelets was increased to 90%, no corrective influence on aspirin platelets or of aspirin platelets on COD cells was observed. However, as low as 10% untreated, normal platelets corrected the response of COD platelets to all aggregating agents, and gave irreversible aggregation in response to arachidonate.

Recently we have shown that aspirin-treated platelets, ordinarily unresponsive to arachidonate, react irreversibly when stirred with this agent if the cells are pretreated with epinephrine [32]. The corrective influence of epinephrine on the response of aspirin-treated platelets to arachidonate and other agents was mediated by surface membrane α-adrenergic receptor. Antogonists, such as dihydroergocryptine and yohimbine, selectively block the correction produced by epinephrine in cells exposed to aspirin [34]. The ability of epinephrine to restore sensitivity to aspirin platelets was not dependent on continuing presence of the catecholamine, since gel filtration of epinephrine-treated aspirin platelets did not result in loss of their ability to respond irreversibly to aggregation by arachidonate. A concentration of the endoperoxide analogue, U44069, too low to cause aggregation, a thromboxane synthetase inhibitor, U-51605, and an endoperoxide/thromboxane receptor antagonist, 13-azaprostanoic acid, could all block the response of platelets to arachidonic acid, and their inhibitory effects could be reversed by exposure to epinephrine prior to stirring with arachidonate [33]. These findings suggested a cooperative interaction between α-adrenergic receptors and receptors for endoperoxides, thromboxanes, and other products of prostaglandin synthesis. Furthermore, the results demonstrated that an intrinsic mechanism in the platelet membrane activated by epinephrine through α-adrenergic receptors could secure irreversible platelet aggregation in the absence of prostaglandin synthesis and the platelet release reaction.

In this investigation we examined the influence of epinephrine on the reactivity of COD platelets to arachidonate. As with aspirin platelets, COD platelets developed irreversible aggregation in response to arachidonate when the cells had been exposed earlier to epinephrine. The findings adds additional support to the concept that membrane modulation and receptor cooperativity are critical facets of platelet activation [33] and can compensate for drug-induced inhibition or inherited defects in platelet prostaglandin synthesis.

Thrombin is a powerful stimulus of platelet secretion and aggregation, and can cause release in aspirin-treated normal platelets. A previous study has shown that thrombin can also trigger the release reaction of COD platelets. In the present investigation we have evaluated the influence of epinephrine and arachidonate alone and the two agents together on the release of ^{14}C-serotonin from COD platelets. A combination of epinephrine and arachidonate caused the release of as much ^{14}C-5HT from COD platelets as from normal control cells. This result differed from our observations on aspirin-treated normal platelets, which developed irreversible aggregation in response to arachidonate after pretreatment with

epinephrine but did not secrete adenine nucleotides or serotonin. The difference in the secretory response of aspirin-treated and COD platelets to stimulation by the combination of epinephrine and arachidonate may turn out to be a diagnositic test for the inherited disorder.

Nyman et al [4] have recently reported that some patients with cyclooxygenase deficiency have no apparent platelet functional defect aisde from their failure to respond to arachidonate, and they concluded that platelets from these patients may have some enzyme activity. We have evaluated that capacity of platelets from our patient to synthesize prostaglandin products by thin-layer radiochromatography, gas chromatography, and mass spectrometry. These studies failed to detect the production of any HHT or thromboxane. The only major product of arachidonate metabolism detected was HETE. No defect in the release of substrate arachidonic acid from prelabeled membranes of COD platelets after thrombin stimulation could be demonstrated. These findings are compatible with a selective and complete, rather than partial, deficiency in platelet cyclooxygenase.

In summary, the present report has presented results of studies carried out on platelets from a patient with cyclooxygenase deficiency. Her platelets are strikingly similar in functional and biochemical behavior to aspirin-treated normal platelets. However, in contrast to aspirin platelets, her cells secrete normal amounts of ^{14}C-5/HT when stimulated by arachidonate following treatment with epinephrine. The ability of epinephrine and arachidonate together to secure irreversible aggregation and secretion in samples of COD platelets when neither agent alone can cause either response supports the concept that platelet activation and irreversible aggregation are not absolutely dependent on prostaglandin synthesis and on the release reaction. Further studies are in progress to define the nature of this newly recognized mechanism for platelet stimulus response coupling.

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