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Research Article

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Abnormal Platelet Response to Thromboxane A₂

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ABSTRACT To determine the pathogenetic mechanism of a hereditary primary platelet release disorder, arachidonic acid metabolism via the cyclooxygenase pathway was investigated. The propositus' platelets exhibited defective release reaction and second-wave aggregation when stimulated by sodium arachidonate or U46619, a thromboxane A₂ (TXA₂) agonist. The lack of platelet response to U46619 suggested that the defect was beyond the thromboxane synthetase level. Furthermore, thromboxane B₂ (TXB₂) formation in the propositus' platelets (558.52 ng/10⁸ platelets) was within the normal range (574.29 ± SD 27.39 ng/10⁸ platelets) and TXA₂ formation appeared to be adequate for aggregating normal platelets. The results were indicative of an abnormal platelet response to TXA₂. Failure of the propositus' platelets to aggregate in response to TXA₂ formed in normal platelet-rich plasma induced by arachidonate confirmed this notion. To gain further insight, platelet cyclic (c)AMP content was determined. Prostacyclin induced a significant elevation of the propositus' platelet cAMP level comparable to normal values. U46619 suppressed prostaglandin I₂-induced cAMP elevation in normal subjects but had no such effect in the patient. We conclude that the primary release disorder observed in this kindred is due to an abnormal platelet response to TXA₂ possibly because of TXA₂/PGH₂ receptor abnormalities.

INTRODUCTION

Thromboxane A₂ (TXA₂)¹ plays an important role in mediating the platelet release reaction (1). When platelets are stimulated by aggregating agents, arachidonic

acid is liberated from the membrane phospholipids and converted by cyclooxygenase to the cyclic endoperoxides (prostaglandin G₂ and H₂) that are further converted to TXA₂ by thromboxane synthetase (2-5). Although the mechanism of TXA₂ action in platelet aggregation is unknown, it appears to be involved in the regulation of intracellular cyclic (c)AMP levels. In this regard, TXA₂ does not lower the basal level of cAMP in platelets, yet it does inhibit PGI₂-stimulated cAMP accumulation (6). The physiological significance of TXA₂ may be demonstrated in a heterogenous group of human bleeding disorders characterized by a cyclooxygenase deficiency and a primary defect in the platelet release reaction (7-9). We have previously reported a kindred with a similar release disorder (10) in that platelet aggregation in response to arachidonic acid was subnormal. Further investigations of this patient demonstrated that the release defect was not due to altered production of TXA₂ but rather due to a defect in platelet responsiveness to TXA₂.

METHODS

Patient. Clinical and functional abnormalities of this kindred were described (10). Only the propositus was available for this study. In brief, she was a 26-yr-old woman with a life-long history of mild bleeding. She exhibited a prolonged bleeding time and reduced platelet release reaction and second-wave aggregation in response to ADP, collagen, and epinephrine. Shape change and primary aggregation in response to ADP were normal. Furthermore, her platelets aggregated normally to thrombin, ionophore A23187, and ristocetin.

Materials. Sodium arachidonate was prepared by dissolving arachidonic acid (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M sodium carbonate, pH 10.0. U46619 [(15S)-hydroxy-11 α ,9 α -(epoxymethanol)-prosta-5Z, and 13E dienoic acid] and prostacyclin (PGI₂), kindly supplied by Upjohn Co., Kalamazoo, Mich., were dissolved in ethanol and in 0.05 M Tris buffer, pH 9.40, respectively.

Platelet thromboxane B₂ (TXB₂) formation. Blood was drawn from an antecubital vein into polypropylene tubes containing one-tenth vol of 3.8% sodium citrate, mixed and centrifuged at 200 g for 10 min. Platelet-rich plasma (PRP) was collected and the remaining sample was further centrifuged at 1,000 g for 20 min to prepare platelet-poor plasma.

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¹Abbreviations used in this paper: PGH₂, prostaglandin H₂; PGI, prostacyclin; PRP, platelet-rich plasma; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂.

Platelet concentration of the PRP was adjusted to $3 \times 10^8/\text{ml}$ with autologous platelet-poor plasma. PRP (0.5 ml) was preincubated in a cuvette of the Payton dual channel aggregometer (Payton Associates, Buffalo, N. Y.) for 1 min and 1 mM sodium arachidonate was added. The mixture was stirred at 37°C for 3 min and then acidified to pH 3.0 with 1 N HCl. TXB_2 was extracted with 3 ml ethylacetate twice and dried under nitrogen gas at 50°C . The dried material was reconstituted and applied to a silicic acid column (0.5 $\mu\text{g}/\text{ml}$) and the TXB_2 fraction was collected and dried under nitrogen (11). The dried extract was reconstituted in 1 ml of radioimmunoassay buffer and assayed according to a previously described radioimmunoassay method (12). TXB_2 recovery was determined by adding a known quantity of [^3H] TXB_2 (New England Nuclear, Boston, Mass.) in a control sample and the recovery at the end of extraction was calculated. The average yield in our laboratory was 80%. The TXB_2 radioimmunoassay displayed minimal cross-reactivity with other prostaglandin-type compounds and proved to be sensitive to 5 pg per assay tube (12).

Platelet aggregation study. Platelet aggregation was performed as previously described (13) using a Payton dual channel aggregometer. To demonstrate TXA_2 formation, transfer experiments were carried out in the aggregometer according to the procedure described by Hamberg et al. (4). In brief, 1 mM arachidonate was added to 0.5 ml of PRP in a Payton aggregometer. After stirring at 37°C for 30 s, 0.1 ml was rapidly transferred to the second tube that contained 0.45 ml normal PRP pretreated with aspirin, and the aggregation was determined.

Platelet cAMP study. The functional responsiveness of platelets to TXA_2 was investigated by examining their response to U46619, a relatively stable TXA_2 agonist. PGI_2 stimulates adenylate cyclase, leading to an increase in basal cAMP levels (14). Since TXA_2 or U46619 inhibits PGI_2 -stimulated cAMP accumulation in normal platelets, we investigated the ability of U46619 to inhibit PGI_2 -stimulated cAMP in the propositus' platelets. PRP was divided into four 4-ml aliquots. In aliquot 1, PRP was incubated with U46619 (final concentration 3 μM) for 90 s at room temperature. 3 nM freshly prepared PGI_2 was added and incubated for an additional 90 s. In aliquot 2, PRP was incubated with ethanol for 90 s followed by PGI_2 for an additional 90 s. In aliquot 3, PRP was incubated with U46619 for 90 s followed by Tris buffer for an additional 90 s, and in aliquot 4, PRP was incubated with ethanol for 90 s followed by Tris buffer for an additional 90 s. The mixtures were quickly frozen in liquid nitrogen and stored at -70°C until assay. The samples were kept at -70°C <48 h. The cAMP content in these samples was determined in quadruplicate by the protein binding method of Gilman (15).

RESULTS

Platelet aggregation in response to sodium arachidonate was completely absent in the propositus (Fig. 1A, B). These results were suggestive of either cyclooxygenase or thromboxane synthetase deficiency. However, platelet TXA_2 formation in the patient (558.52 ng $\text{TXB}_2/10^8$ platelets) was within the normal range ($n = 5$, $574.29 \pm \text{SD } 27.39$ ng $\text{TXB}_2/10^8$ platelets). Furthermore, U46619, a TXA_2 agonist that normally induces maximal aggregation at 4 μM , had no apparent effect on the patient's platelets (Fig. 1C, D). These findings led us to postulate that the release de-

fect was probably due to an abnormal platelet response to TXA_2 . To test this hypothesis, transfer experiments were performed. No aggregation was observed when arachidonate-treated normal PRP was transferred to the patient's platelets. By contrast, transfer of arachidonate-treated patient PRP to normal PRP resulted in platelet aggregation (Fig. 1E, F). These findings indicate that the patient's platelets were in fact capable of synthesizing TXA_2 in response to arachidonate, but that they were unresponsive to the TXA_2 that was produced.

To further substantiate the platelet unresponsiveness to TXA_2 , the effects of PGI_2 and U46619 on platelet cAMP levels were investigated. While PGI_2 induced a significant elevation of platelet cAMP in the patient, U46619 failed to suppress this increase (Fig. 2). In contrast, U46619 was capable of suppressing cAMP elevation induced by PGI_2 in five normal controls. Although the PGI_2 -induced cAMP elevation in the patient appears lower than control, it is within two standard deviations of the normal value.

DISCUSSION

It is generally believed that the primary release disorder of platelets is related to abnormality of one or more of the enzymes involved in arachidonic acid metabolism. However, the disorder observed in this patient appears to be due to a different mechanism since platelet TXA_2 formation in response to arachidonic acid is completely normal. We believe therefore, that the disorder is due to a defect in platelet responsiveness to TXA_2 . This notion is supported by three lines of evidence. Firstly, the transfer of TXA_2 formed in normal PRP failed to elicit platelet aggregation in this patient. Secondly, platelet aggregation in response to U46619, which presumably acts directly on $\text{TXA}_2/\text{PGH}_2$ receptors (16), was absent in this patient. Thirdly, U46619 failed to suppress the cAMP elevation induced by PGI_2 in this patient, while it was capable of antagonizing the PGI_2 effect in normal platelets.

The abnormal platelet responsiveness may be due to a number of mechanisms, notably membrane abnormalities and/or a generalized disturbance in intracellular Ca^{2+} mobilization. The latter seems unlikely, however, because the patient's platelets respond normally to ionophore A23187 and thrombin, both of which are thought to act through the redistribution of intraplatelet Ca^{2+} . Moreover, the platelets exhibit normal shape change and primary aggregation when stimulated by ADP. Based on these considerations it would appear that the basic defect is most likely related to membrane abnormalities. Since, however, the patient's platelets respond normally to a myriad of aggregating agents with distinctive receptor sites on the platelet membrane, we do not think that there is a

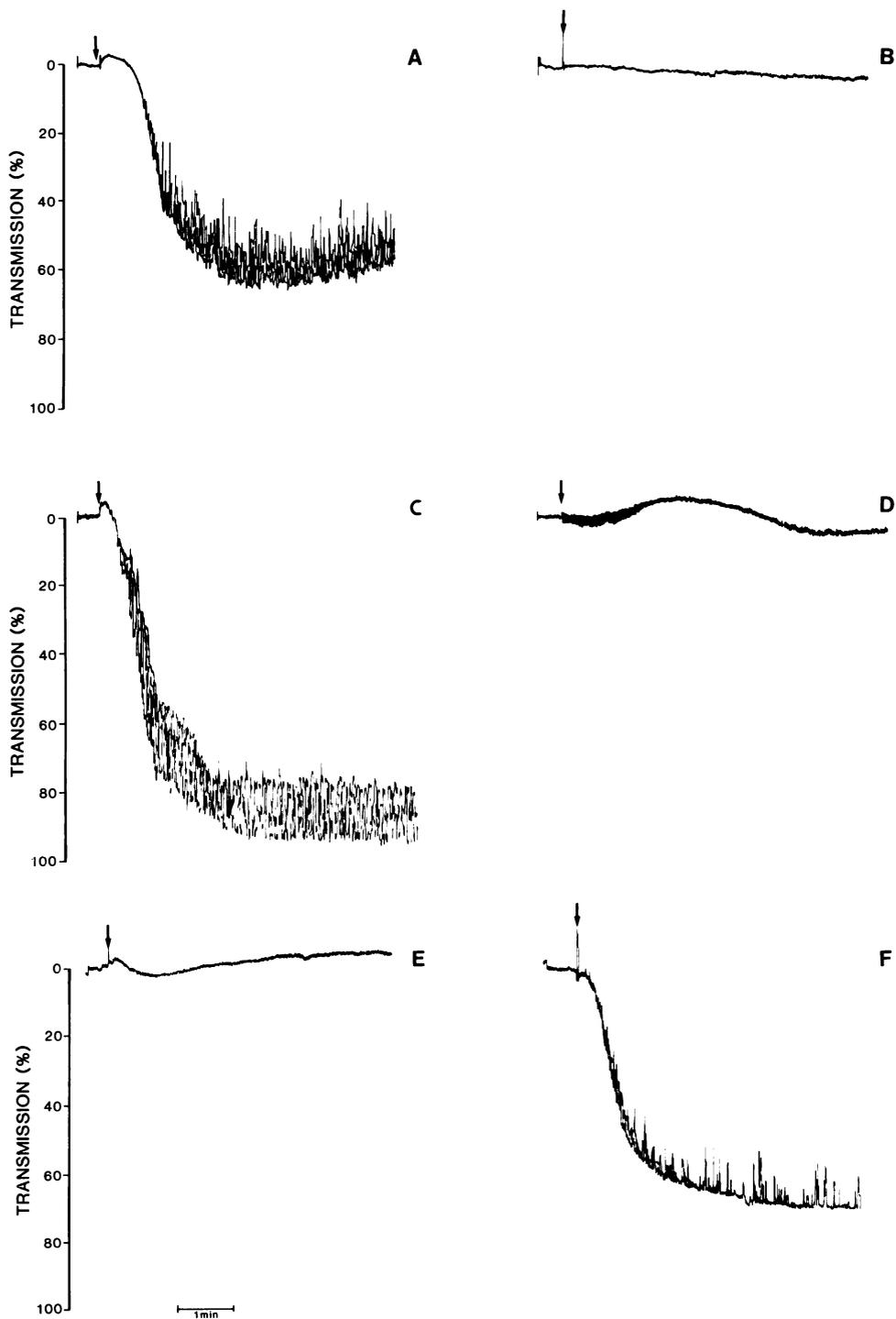


FIGURE 1 Representative platelet aggregation tracings: (A) Platelet aggregation in response to sodium arachidonate (1 mM) in a normal subject; (B) Arachidonate-induced aggregation in the propositus; (C) Aggregation in response to U46619 (4 μ M) in normal subjects; (D) U46619-induced aggregation in the propositus. E and F are aggregation tracings from the transfer experiments. Aggregation did not occur when 0.1 ml of normal PRP, preincubated with sodium arachidonate for 30 s was rapidly transferred to patient PRP (E). In contrast, transfer of patient PRP pretreated with sodium arachidonate resulted in aggregation in normal platelets (F). Arrows refer to addition of aggregating agents.

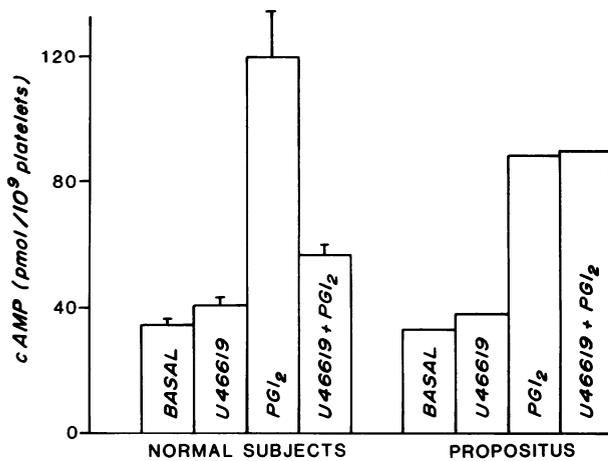


FIGURE 2 Changes in platelet cAMP content in response to PGI₂ and U46619. cAMP was determined in quadruplicate. Five normal subjects were included and each bar represents mean ± SD. Basal and U46619-induced platelet cAMP levels of the propositus were comparable to those of normal subjects. The PGI₂-induced cAMP level in the patient was slightly below 1 SD but within 2 SD of the normal value. Pretreatment of patient's platelets with U46619 failed to suppress PGI₂-induced cAMP elevation. *n* = 5.

global membrane defect. In view of the fact that the patient's platelets have a subnormal response to TXA₂ and its agonist in terms of cAMP suppression and platelet aggregation, we suggest that the defect is due to a specific TXA₂/PGH₂ receptor abnormality.

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