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RELATIONSHIP OF THROMBOXANE GENERATION TO THE AGGREGATION OF PLATELETS FROM HUMANS: EFFECTS OF EICOSAPENTAENOIC ACID

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SUMMARY

A non-linear relationship between the percent aggregation of human platelets and the amount of TXB₂ generated requires investigators to use caution when using the data to assess antiplatelet regimens. The relationship approximates a hyperbola with a roughly linear relationship from 0 to 70% aggregation and 0 to 50 ng TXB₂ per ml of platelet-rich plasma. Above these values, the amount of TXB₂ produced may increase up to 500 ng per ml of platelet-rich plasma with no clear relationship to the observed platelet function of aggregation. Also, appreciable inhibition of TXB₂ formation can occur at high TXB₂ levels with no detectable decrease in aggregation. Thus, assessment of antiplatelet regimens using TXB₂ formation alone are unlikely to be interpretable without reference to this non-linear property of platelet function. We applied this concept when evaluating a study of forty subjects with dietary supplements of 1.8 g or 2.7 g of ethyl eicosapentaenoate (20:5n-3) for four weeks. There was a moderate, but statistically significant decrease in average values for the percent aggregation (60±15 to 45±30) and thromboxane production (51±30 to 33±31 ng/ml). Although the differences in mean values were slight relative to the overall standard deviations, reductions of platelet function were clearly evident in 31 of 40 subjects when paired results were examined relative to the recognized hyperbolic relationship.

INTRODUCTION

A report at the 1983 Winter Prostaglandin Conference described a mean difference in platelet function between control and experimental subjects receiving fish oil that was of small magnitude, but statistically significant. In evaluating these results, we realized that a large standard deviation in the results was in part due to an experimental phenomenon that obscured the changes occurring. Our three laboratories had independently encountered difficulty in obtaining a simple relationship between the amount of thromboxane generated and the expected or observed amount of aggregation. Each had results that were non-linear and seemed to be either uninterpretable or unreliable. In reviewing the problem collectively, we jointly realized that we had a common discovery that had not been previously communicated concerning the overall relationship between these two variables. This report provides a summary of our insight into this relationship to help investigators recognize the limits of linearity that can be expected for platelet aggregation and thromboxane formation. Our collective experience is provided to demonstrate the region of submaximal platelet response that is most useful for interpretive studies.

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MATERIALS AND METHODS

Platelet Aggregation (University of Michigan)

Human whole blood was obtained from healthy volunteers who had not consumed any medications for 10 days prior to pheresis at the University of Michigan Medical Center Blood Bank or hematology laboratory. Platelet rich plasma (PRP) was prepared by differential centrifugation. The PRP was stored at 22°C for the platelet storage study.

Platelet aggregation was studied by modifications of commonly-used spectrophotometric methods (1) utilizing a Sienco Dual Sample Aggregometer DP-247E (Sienco, Inc. Morrison, Colorado). Platelets were counted on a Haema-Count TM MK-4/HC (J.T. Baker Instruments, Milford, Conn.) and platelet-rich plasma was adjusted to 2×10^8 /ml platelets before testing. Baseline for 0 and 100 light transmission were set with PRP and PPP, respectively, silicone-coated stir bars 1 x 5 mm were added to siliconized glass curvettes at 37°C and stir speed adjusted to 1000 rpm. Aggregation of platelets with "soluble" bovine deep flexortendon (2.2, 10, 22, and 100 µg/ml; Ethicon, Sommerville, N.J.) was seen as an increase in percent light transmission.

Platelet Aggregation (Chiba University)

Platelet aggregation was measured according to the method of Born (1), with a "Sienco" dual channel aggregometer DP247E (Morrison, CO). Citrated venous blood was obtained and immediately centrifuged at 150 x g for 10 min at room temperature to obtain platelet-rich plasma (PRP). The residual blood was further centrifuged at 1800 x g for 15 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to 4×10^8 /ml with autologous PPP. The aggregation was carried out with stirring at 1000 rpm at 37.5°C within 120 min of venipuncture. The change in light transmission after the addition of collagen (1.0 and 1.5 µg/ml) was recorded as a percentage, and aggregation was expressed as the maximum increase in light transmission in 5 min.

Thromboxane B₂ Radioimmunoassay (University of Michigan)

Samples of plasma and platelet-rich plasma (PRP) were treated with acetonitrile (1:1). The supernatant was removed and acidified to pH 3.5 with formic acid followed by two 3 ml extractions with petroleum ether. The aqueous fraction was then extracted twice with 4 ml of ethyl acetate. The combined ethyl acetate layers were evaporated to dryness under nitrogen and dissolved in the assay buffer (0.1 M phosphate-buffered saline, pH 7.4 containing 0.1% gelatin). Tritiated thromboxane B₂ was added before extraction to monitor recoveries. Each sample was assayed in duplicate in at least two dilutions. Tritiated thromboxane B₂ was added to samples and standards, followed by antiserum. The final dilution of antibody was 1:14,000 and assay volume was 0.3 ml. Tubes were vortexed and incubated at 25° C (room temperature) for 1 hour and then at 4° C for 16 to 24 hours. The separation of the antibody-antigen complex from free antigen was achieved

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by adsorption of the free tritiated thromboxane B₂ on to cold activated dextran-coated charcoal. (Norit A, 250 mg; Dextran T-70, 25 mg/100 ml buffer.)

Following centrifugation, the supernatant containing the antigen-antibody complex was decanted from the hard charcoal pellet containing the free antigen. The supernatant was transferred to a scintillation vial containing 10 ml of Scintisol (Amersham Searle, Des Plaines, IL) counting fluid.

Radioactivity was determined to a 1% error in a Beckman Beta Spectrometer (Beckman Instrument Co., Irvine, CA) with an overall efficiency of 40%. The binding data (B/B₀) was calculated for each sample and the concentration of each sample was determined from standard curves.

Thromboxane B₂ Formation (Chiba University)

Thromboxane B₂ (TXB₂) formation by platelets was determined by radioimmunoassay in connection with the platelet aggregation study as previously reported (2). After 5 min of aggregation induced by collagen or epinephrine, 100 μ l of aggregated PRP was transferred to a plastic tube containing 200 μ l of 50 mM Tris-HCl buffer, pH 7.5 with 1% gelatin. This tube was immediately dipped into liquid nitrogen for 10 sec and stored at -80°C until the assay of TXB₂ by radioimmunoassay (TXB₂ [³H]RIA Kit, New England Nuclear, Boston). The extraction of TXB₂ from the reaction mixture was performed by the method of Green et al. (3) with minor modifications. The recovery of TXB₂ was around 75%. The cross reactivity of the RIA Kit antibody was 0.2% for Prostaglandin (PG) E₂ and less than 0.2% for PGA₂, PGF_{2 α} and 6-Keto PGF_{1 α} .

Subjects and Study Design (Chiba University)

Forty patients (20 male and 20 female, average age of 59 years) diagnosed as having diabetes mellitus, cerebrovascular disorders, ischemic heart diseases, hyperlipidemia Type IIa, IIb and IV and hypertension were used in the present study. Any medication affecting platelet aggregation was discontinued at least 2 weeks prior to the start of the study.

The encapsulated ethyleicosapentaenoate (EPA-E) was provided by Mochida Pharmaceutical Company. The EPA-E used in this study was purified from sardine oil as previously reported (4). Each gelatin-coated soft capsule weighed 400 mg and comprised ethylesters containing 75% eicosapentaenoic acid, 3.8% octadecatetraenoic acid, 6.3% eicosatetraenoic acid (ω -3), 6.2% docosahexaenoic acid, and 0.2% α -tocopherol. EPA-E in such a form is stable for at least 6 months at 25°C or 40°C, with no change in its peroxide value.

During the experimental period of 16 weeks, the patients ingested 6 capsules (EPA 1.8 g/day) or 9 capsules (EPA 2.7 g/day) with meals (divided in 3 times a day). They consumed an ordinary Japanese diet

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(1800-2200 Kcalorie) with average intake of 100 g fish meat, equivalent to 0.9 g EPA. Venous blood samples were obtained after 12 hours of fasting at the start of the experiment and 4 weeks after the beginning of the study.

RESULTS AND DISCUSSION

Aggregation of platelets generally involves the release of arachidonate from the membrane of the platelet and its oxidation to TXA_2 (via PGH_2) that signals aggregation to occur. Figure 1 illustrates the release of thromboxane A_2 , measured as TXB_2 , from platelets stimulated to aggregate with various concentrations of collagen (2.2, 10, 22, and 100 $\mu\text{g/ml}$). Although the percent aggregation tended to be linearly related to TXB_2 formation below 50% aggregation, aggregations of 50 to 95% produced thromboxane concentrations between 50 and 500 so the relationship between aggregation and thromboxane generation was not a simple dose-response association. The results in Figure 1 illustrate that platelets can be stimulated to produce much more thromboxane than is needed for aggregation. Although the amounts of thromboxane formed increased with increasing concentrations of collagen, the percent aggregation reached its limiting value with intermediate amounts of collagen. The curve also indicates that 50% aggregation can occur with relatively little thromboxane release. When both aggregation and thromboxane generation was low, there was a more linear relationship between these two measurements.

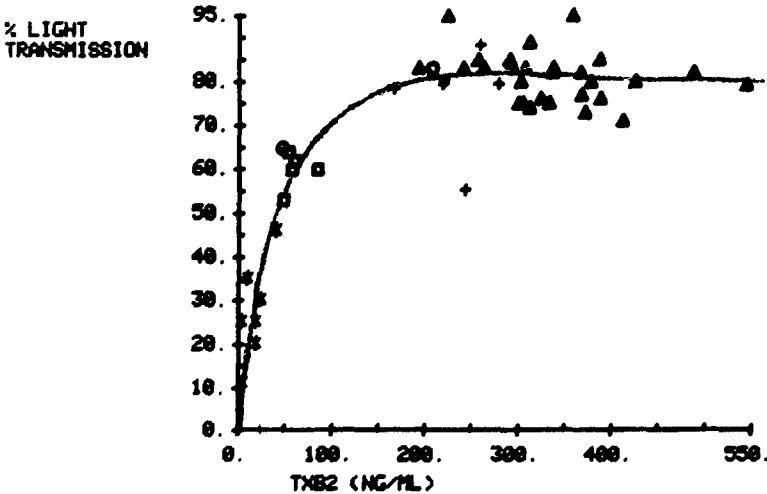


Fig. 1 Relationship between thromboxane generation and percent aggregation for normal subjects. Platelet-rich plasma was challenged with different amounts of collagen: ($-\square-$), 2.2 $\mu\text{g/ml}$; ($-0-$) 22 $\mu\text{g/ml}$; ($-+-$), 10 $\mu\text{g/ml}$; ($-\Delta-$), 100 $\mu\text{g/ml}$; ($-*-$), 2.2 $\mu\text{g/ml}$, after storage for 2 days.

It is our collective experience that increasingly stronger stimuli for aggregation give more TXB₂ formation even though no further aggregation can be detected. Essentially maximal aggregation can be achieved in fresh platelet-rich plasma (PRP) with 30 to 40 ng/ml TXB₂. This conclusion can be derived from two different results. When PRP was incubated with PGH₂, there was a burst of TXB₂ synthesis which then declined (5). When the peak burst of TXB₂ synthesis reached 30 to 40 ng/ml, maximal aggregation occurred. When results from 10 separate experiments were pooled, the average concentration of thromboxane that was associated with maximal aggregation was 36.2 ± 4.7 . Essentially the same value was found using the stable analog of PGH₂/TXA₂, U-51093 (9,11-azoprosta-5,13-dien-1-oic acid). This synthetic molecule mimics thromboxane A₂ in being a potent stimulator of human platelet aggregation (6), and it appears to be equipotent to TXA₂ in this regard (7). When dose response curves are generated with U-51093, essentially maximal aggregation was achieved with 30 to 40 ng/ml (Figure 2), and the aggregation response was linear with respect to the agonist only at values below 30 ng/ml. The average value of the experiments for the amount of U-51093 that gave maximal aggregation was 32 ± 6 ng/ml.

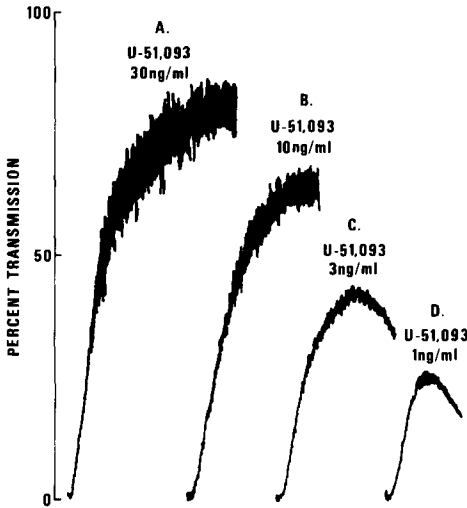


Fig. 2 Dose-response curves with U-51093. The amount of the thromboxane-mimetic agent is noted for each curve.

The above results were useful in evaluating an epidemiological study of platelet function in the residents of farming and fishing villages (8) that had a small, but statistically significant reduction in the maximum aggregation and TXB₂ formation from platelets of subjects who had eaten supplemental EPA for 4 weeks. Individuals exhibited a wide range of responsiveness to 1 μ g collagen per ml (Figure 3). A linear correlation ($r = 0.71$, $y = 1.5x + 8.8$) was observed between collagen-induced platelet

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Table 1. Changes in platelet aggregation and TXB₂ formation after ingestion of ethyl eicosapentaenoate.

		0 week (before)	4 week (after)
Platelet aggregation (%)			
Collagen	(1.0 µg/ml)	60 ± 15	45 ± 30***
	(1.5 µg/ml)	67 ± 12	53 ± 23**
TXB ₂ formation (ng/ml)			
Collagen	(1.0 µg/ml)	51 ± 30	33 ± 31**
	(1.5 µg/ml)	61 ± 29	43 ± 25***

** p < 0.01 *** p < 0.001 Mean ± S.D.

aggregation and TXB₂ formation only when the amount of TXB₂ produced was less than around 40 ng/ml PRP. No such correlation was noted when the production of TXB₂ was over 40 ng/ml PRP (Figure 3). The arrows in the figure indicate that in 31 of the 40 paired cases studied, the platelet function decreased. High levels of TXB₂ were frequently decreased with little change in aggregation, and low levels of TXB₂ decreased slightly with marked changes in aggregation.

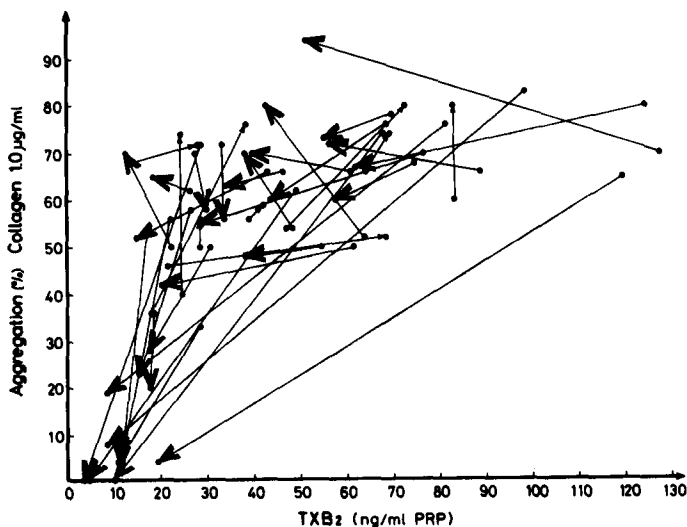


Fig. 3. Changes in platelet aggregation and thromboxane formation after 4 weeks ingestion of EPA-E. Collagen 1.0 µg/ml)-induced platelet aggregation and thromboxane B₂ formation was determined in the patients with thrombotic cardiovascular disorders before and after 4 weeks ingestion of EPA-E (1.8 or 2.7 g/day) as described in Materials and Methods. The arrows indicate the changes observed for each patient.

Results in Figure 1 approached 70 to 80% aggregation with 100 to 150 ng TXB₂ per ml (and 60% with 70 ng/ml), whereas in Figure 2 they were 70 to 80% with 30 ng/ml of the TXA₂ analog added and 60% at 10 ng/ml. In Figure 3, 70 to 80% occurred with 30 to 60 ng TXB₂/ml and 60% at about 25 ng/ml. Our collective experience leads us to emphasize the desirability of designing platelet function studies with levels of stimulating aggregant that are below the maximal value. Recently, the complexities of "strong" and "weak" aggregants at threshold and supramaximal concentrations were adumbrated extensively (9). By avoiding overstimulation of thromboxane formation, we believe that investigators can find better correlations between aggregation and TXB₂ formation to interpret the effects of antithrombotic regimens.

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