Effect of Fibrinogen Concentration on the Velocity of Platelet Aggregation

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The relationship between fibrinogen binding to its receptor and platelet aggregation has been investigated by comparing 125I-fibrinogen binding and aggregation velocities of gel-filtered platelets in the presence of adenosine diphosphate (ADP). Aggregometric responses at various fibrinogen concentrations are found to be bell-shaped and show a maximum at filtered platelets in the presence of adenosine diphosphate.

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Platelet aggregation has been investigated by comparing fibrinogen concentrations (FmJ similar to the 'Z51-fibrinogen binding and aggregation velocities of gel-filtered platelets were measured in the presence of plasma fibrinogen concentrations on platelet function. Our study aimed to relate the velocity of aggregation to the saturation of the fibrinogen receptor and to evaluate the possible influence of plasma fibrinogen concentration on platelet function.

MATERIALS AND METHODS

Platelet Preparation

Venous blood obtained from healthy donors was collected in plastic tubes containing sodium citrate (final concentration 0.38%) as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation at 120g for 20 minutes. Platelets were gel-filtered twice on 25 x 1.5 cm Sepharose 2B columns equilibrated with HEPESS-buffered modified Tyrode’s solution, pH 7.4, containing 0.2% bovine serum albumin, 0.13 mol/L NaCl, 2.6 mmol/L KCl, 0.39 mmol/L NaH2PO4, 12 mmol/L NaHCO3, 10 mmol/L HEPESS, and 5.5 mmol/L glucose. Platelet concentration was then adjusted to about 3.5 x 10^10/mL. Platelets were counted electronically by a TOA PL 100 platelet counter (Medical Electronics, Kobe, Japan).

Preparation of Fibrinogen

Human adult fibrinogen (grade L) purchased from Kabi (Stockholm, Sweden) was purified according to Lipinska et al. 9 This fibrinogen preparation had a clottability greater than 90% and was greater than 99% pure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed under both reducing and nonreducing conditions. The fibrinogen concentration was measured spectrophotometrically by using an extinction coefficient E (280 nm, 1 mg/mL) = 1.51. 10 Fibrinogen solution aliquots were stored at -80°C until use.

Aggregometric Studies

The aggregatory response of gel-filtered platelets to various ADP doses was tested in the presence of different concentrations of fibrinogen as described elsewhere, 11 with some modifications. In a typical experiment, 80 μL of platelet suspension was added to 150 μL of fibrinogen solution (at final concentrations ranging between 0.015 and 66 x 10^-4 mol/L) in a plastic cuvette. The suspension was then stirred at 1,000 rpm at 37°C in an Elvi 840 dual-channel aggregometer (Elvi, Milan, Italy) connected to a double-pen recorder. At 30-second intervals, 10 μL of CaCl2 (final concentrations 1 to 10 mmol/L) and 10 μL of ADP (final concentrations 0.5 to 10 μmol/L) were added. Transmittance signal variations were used as measures of aggregation following the assumption that the transmittance values are inversely proportional to the number of nonaggregated platelets in the suspension. 11 The velocity of aggregation was derived from the transmittance signal variation per minute of the initial part of aggregation tracings and expressed in arbitrary units (plain buffer = 100% transmittance). In all cases, aggregometric studies were performed within 2 hours of blood collection.

125I-Labeled Fibrinogen Binding Studies

Labeling procedures. Purified human fibrinogen was labeled with carrier-free Na125I using the Iodogen technique. 12 The specific activity of 125I-labeled fibrinogen ranged between 20 and 50 μCi/mg of protein. C1ottability of fibrinogen was not affected by the labeling procedure. A Packard (Warrenville, IL) 240 CGD automatic counter was used for all determinations.

125I-fibrinogen binding experiments. 125I-labeled fibrinogen binding to gel filtered platelets was evaluated as previously described, 6 with minor modifications. In brief, 150 μL of gel-filtered platelets were incubated for 20 minutes at 37°C with 10 μL 25 mmol/L CaCl2, 10 μL ADP (final concentration 10 μmol/L), and 80 μL of 125I-labeled fibrinogen (final concentrations ranging from 0.015 to...
15 × 10⁻⁶ mol/L). One hundred microliters of the suspensions was then gently stratified on 400 μL of 20% (wt/vol) sucrose in plastic cuvettes preincubated at 37°C. After centrifugation at 12,000 rpm in a Beckman Microfuge (Beckman, Brea, CA), the supernatants were aspirated and discarded while the tips of the tubes containing the platelet pellets were cut and counted. The data, taken as radioactivity in counts per minute (cpm), were analyzed as previously described.

RESULTS

Platelet Aggregation Velocities at Different Fibrinogen Concentrations

The influence of fibrinogen concentration on aggregation was studied by measuring aggregation velocities of gel-filtered platelets in the presence of fibrinogen concentrations ranging from 0.015 to 66 × 10⁻⁶ and of a saturating ADP concentration (10 μmol/L). Dose-response curves (Fig 1) obtained under these conditions demonstrated that aggregation velocity positively correlates with fibrinogen level up to concentrations of approximately 1 × 10⁻⁶ mol/L. Above this level, there is a plateau phase between 1 and 3 × 10⁻⁶ mol/L, where aggregation velocity reaches its maximum, and then a progressive decrease at higher fibrinogen concentrations. In subsequent experiments we tried to elucidate whether the shape of the dose-response curve might be affected by variables as stirring velocity, platelet number, and calcium concentration. Modifications of platelet number (between 0.5 and 4 × 10⁸/mL), of stirring velocity (between 500 and 1,500 rpm), and of calcium concentration (between 1 and 10 mmol/L) changed only the absolute value of the aggregation velocity. The inhibition at high fibrinogen concentrations was unmodified so that the relationship between fibrinogen concentration and aggregation velocity followed, in each case, a bell-shaped curve (see Fig 1).

In all other experiments we routinely used a stirring velocity of 1,000 rpm and a calcium concentration of 1 mmol/L.

![Fig 1. Aggregation velocity of gel-filtered platelets at various fibrinogen concentrations. Velocities are calculated as transmittance signal variations per minute in the initial part of aggregometric data. The data reported refer to measurements performed at final calcium concentrations of 1 (△), 2 (■), and 10 mmol/L (□). In all cases, the relationship between fibrinogen concentration and aggregation velocity follows a bell-shaped curve.](image)

![Fig 2. ¹²⁵I-fibrinogen bound and aggregation velocities as functions of fibrinogen concentration. The experiments were performed by using gel-filtered platelets from the same donor. Bound fibrinogen is reported in the upper part of the figure. The kd value was 1.55 × 10⁻⁶ mol/L, the standard error of the fitting procedure is 2.200 molecules. For the analysis of binding data see ref 8. Initial aggregation velocities, reported in the lower part of the figure, were analyzed as described in the text (see equations 1 and 2). The standard error of the fitting procedure was 7 ± 0.4 AU. F₅₀ was 1.71 × 10⁻⁶ mol/L. ¹²⁵I-fibrinogen binding data and aggregation experiments were performed at 10 μmol/L ADP.](image)

Relationship Between Platelet Aggregation Velocity and Fibrinogen Receptor Occupancy

This relationship was investigated by performing both aggregometric and ¹²⁵I-fibrinogen binding measurements using platelets from the same donor. In Fig 2, aggregation velocities and bound ¹²⁵I-fibrinogen are plotted as functions of fibrinogen concentration. As shown by Fig 2, significant aggregometric responses are observed only in the binding range of fibrinogen concentrations and become negligible toward the asymptotic regions of the binding curve. Because the curve of aggregation velocity is unimodal and symmetrical, the modal value of the curve can be taken as the fibrinogen concentration giving the maximal aggregation (F₅₀). This parameter value is found to be very close to the hemisaturating dose (kd) calculated from binding experiments.
This finding and the evidence that the velocity of platelet aggregation as a function of fibrinogen concentration does not have a “saturable” behavior, prompted us to hypothesize that the process of aggregation involves at least two subsequent steps: (1) the binding of the fibrinogen molecule to the platelet receptor, and then (2) the interaction of bound fibrinogen with the fraction of free receptor on a colliding platelet. The process (2) is assumed to be kinetically much slower than the reaction (1) and the simultaneous binding of opposite ends of fibrinogen molecule to different platelets is considered negligible. The relationship between receptor saturation and aggregation velocity observed under our experimental conditions was thus tentatively investigated using the following scheme of aggregation: \( P + F \leftrightarrow PF \) (1a); \( PF + P \rightarrow PF_P \) (1b); where \( P \) is the unbound platelet, \( F \) is fibrinogen, and \( PF_P \) is the platelet-fibrinogen aggregate. The equation describing this simplified scheme is given by: 

\[
V_t = k_{up} \times S \times (1 - S) \quad (2)
\]

where \( V_t \) is the initial aggregation velocity at a given fibrinogen concentration, \( k_{up} \) is an overall kinetic constant of the platelet aggregation reaction, and \( S \) is the fractional saturation of the receptor. In this equation the velocity is assumed to be a function of the product of the saturated \( (S) \) for the unsaturated \( (1 - S) \) receptor fraction. This value is maximum when \( S = 0.5 \) and symmetrically decreases at higher and lower S values. Figure 2 shows the good fitting of experimental data to the above reported equation.

According to this interpretation, \( F_{max} \) is an aggregometric measurement of kd. From \(^{125}\text{I}\)-fibrinogen binding experiments the receptor saturation \( (S) \) is calculated as follows:

\[
S = \frac{x}{kd + x} \quad (3)
\]

where \( x \) expresses the fibrinogen concentration.

In nine normal subjects, mean \( F_{max} \) at 10 \( \mu \text{mol/L} \) ADP was \( 1.65 \pm 0.45 \times 10^{-5} \text{ mol/L} \). In three of these subjects the kd, measured by \(^{125}\text{I}\)-fibrinogen binding experiments, had an average value \( 1.35 \times 10^{-4} \text{ mol/L} \).

**Effect of ADP Concentration on the Relationship Between Fibrinogen Concentration and Aggregation Velocity**

Aggregometric experiments using different ADP doses were performed in five different normal donors. A typical experiment is reported in Fig 3. By lowering the ADP dose there is a progressive reduction of maximal aggregation velocity and a gradual increase of \( F_{max} \) from 1.41 to 4.03 AU. This experiment was performed in five different normal donors; the data reported in this figure refer to a typical experiment. The range of fibrinogen concentration 100 to 1,000 mg% is comprised between \(-\log 5.5\) and \(-\log 4.5\).

![Fig 3. Effect of the ADP dose on the relationship between fibrinogen concentration and velocity of platelet aggregation. The ADP concentrations were 4 \( \mu \text{mol/L} \), 2 \( \mu \text{mol/L} \), 1 \( \mu \text{mol/L} \), and 0.6 \( \mu \text{mol/L} \). The aggregation velocity decreases at progressively lower ADP doses. \( F_{max} \) values at 4, 2, 1, and 0.5 \( \mu \text{mol/L} \) ADP were 1.25, 1.83, 2.50, and 3.12 \( \times 10^{-4} \text{ mol/L} \). The standard errors of the fitting procedures were 19.54, 7.3, 3.33, and 4.03 AU. This experiment was performed in five different normal donors; the data reported in this figure refer to a typical experiment. The range of fibrinogen concentration 100 to 1,000 mg% is comprised between \(-\log 5.5\) and \(-\log 4.5\).](image-url)

**DISCUSSION**

The interaction of platelets with fibrinogen has been recognized and investigated only over the last two decades. The fibrinogen binding process is negligible in resting platelets. After platelet activation the fibrinogen receptor becomes functionally active probably through an allosteric transition in which the activity of the membrane Na⁺/H⁺ exchanger seems to play a role. The fibrinogen binding process follows the law of mass action and \(^{125}\text{I}\)-fibrinogen binding curves show the typical saturation effect. Platelet aggregation velocity has a rather different type of dependence from fibrinogen concentration. In fact, the curve of aggregation velocity, due to the progressive inhibition at high fibrinogen concentrations, is bell-shaped.

This overall behavior is relatively independent from variables in regards to platelet number, stirring velocity, ADP dose (Fig 3), and calcium concentration (Fig 1). This last finding rules out the hypothesis that the inhibitory effect of fibrinogen might be referred to changes of free calcium concentration. At calcium concentration above 1 mmol/L aggregation velocity is reduced. This phenomenon can likely be attributed to the inhibition exerted by this ion on the fibrinogen binding process and might be mediated by some interaction of calcium with platelet membrane.

To explain the type of dependence of aggregation velocity from fibrinogen concentration we tried to relate the aggregometric data to the binding process and to analyze the curves of velocities in these terms. The striking evidence that at high fibrinogen concentrations the aggregation could be markedly or totally inhibited, led us to choose, for the interpretation of experimental data, a two-step model. This model assumes that aggregation derives from two
sequential events: first, the binding of fibrinogen to activated platelets and, second, the interaction of bound fibrinogen with a colliding platelet. Kinetic analysis of experimental data was based on the hypothesis that only the second event is rate limiting. Aggregation velocities were thus considered to estimate the occurrence of this process, with the contribution of simultaneous binding of opposite ends of fibrinogen molecule to different platelets considered negligible. The good fitting of experimental data to equation 2 seems to confirm the suitability of this tentative schematization of the aggregatory process.

In previous studies on the relationship between fibrinogen concentration and aggregation, the inhibitory effect of high fibrinogen levels had not been extensively investigated or evaluated. In those studies, aggregometric estimation of k_d values was derived from a simple monophasic dose-response curve in which the fibrinogen concentration giving the maximum platelet response (i.e., F_max value) was considered the saturating dose. Such an approach, in addition to underestimating the k_d values, has led to the erroneous conclusion that plasma fibrinogen concentration is saturating and does not affect platelet function. As demonstrated in the contrary in this study by both binding and aggregometric experiments, full saturation of the receptor requires fibrinogen concentrations well above the plasma level. This finding implies that any variation of plasma fibrinogen concentration, by affecting the fibrinogen receptor fractional saturation, has to influence the aggregation velocity. Experimental data are in agreement with this interpretation. Any variation of fibrinogen concentration in the physiopathologic range affects the velocity of platelet aggregation. In addition, similar changes of plasma fibrinogen can have different effects on the velocity of platelet response at the various ADP concentrations. This phenomenon had been previously observed by Meude et al. Those investigators, in fact, reported opposite effects of increasing plasma fibrinogen concentrations on platelet aggregation induced by low and high ADP doses. These apparently discrepant results can be rationalized by taking into consideration the modulatory effect of ADP on the receptor-fibrinogen interaction and the relationship between receptor saturation and aggregation velocity discussed above. The important effect of fibrinogen concentration on the velocity of platelet aggregation has to be taken into consideration in all studies in which platelet function is evaluated in vitro by the turbidimetric method. Most of these studies are commonly performed using PRPs of variable fibrinogen concentrations. More properly, platelet aggregation should be measured by using gel-filtered platelet suspensions containing fixed fibrinogen concentrations. This method would also avoid modifications of platelet function arising from possible variations of other plasma proteins, such as von Willebrand factor and fibronectin. These proteins, in fact, are known to play a role in platelet aggregation through their interaction with the fibrinogen receptor. Finally, the possible role of plasma fibrinogen in affecting platelet aggregation in vivo needs to be considered. Clinical studies seem to demonstrate that plasma fibrinogen plays a role in those vascular accidents in which platelet aggregation is maximally involved. Our findings strongly suggest that plasma fibrinogen concentration might affect platelet response in vivo and, thus, encourage further investigations in this field.

ACKNOWLEDGMENT

We are grateful to Professor Carlo Patrono for stimulating discussions.

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