Prostaglandin E_1 Inhibits Platelet Aggregation by a Pathway Independent of Adenosine 3′,5′-Monophosphate

Abstract. *Aggregation of human blood platelets induced by adenosine diphosphate or l-epinephrine was inhibited when the platelets were suspended in plasma which had been previously exposed to an insolubilized ω-aminohexylagarose derivative of prostaglandin E_1. This decrease of platelet aggregation was not accompanied by a change in the concentration of adenosine 3′,5′-monophosphate (cyclic AMP) in platelets. The results demonstrate the existence of an alternative pathway independent of cyclic AMP for the inhibition of platelet aggregation by plasma.*

A crucial step in hemostasis, the aggregation of human blood platelets by adenosine diphosphate (ADP) and *l*-epinephrine, is believed to be achieved by stimulatory and inhibitory humoral messages. Stimulation results in part in the initiation of the intracellular synthesis of prostaglandin endoperoxides and thromboxane A2 (*1*). In contrast, inhibition is mediated primarily by intracellular formation of adenosine 3′,5′-monophosphate (cyclic AMP), the synthesis of which is stimulated by exogenous substances such as prostaglandin E_1 (PGE_1) and prostacyclin (*2*). However, the effect of PGE_1 on the inhibition of platelet aggregation may not be due to the increase in cyclic AMP alone. Inhibition of platelet aggregation in the presence of PGE_1 persists despite decreasing cyclic AMP concentrations (*3*), and the removal of plasma containing PGE_1 from platelets reverses the inhibition of platelet aggregation (*4*). Furthermore, the inhibition by PGE_1 at 10°C is identical to that at 25°C, making it unlikely that stimulation of platelet adenylate cyclase is the only action of PGE_1. Here we report that plasma exposed to insolubilized PGE_1 inhibits ADP- and epinephrine-induced platelet aggregation in the absence of changes in the concentration of platelet cyclic AMP.

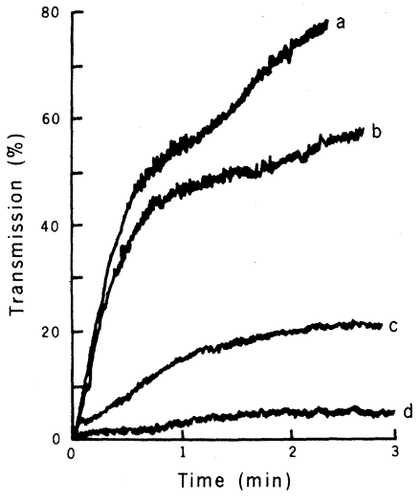

Fig. 1. Effect of plasma exposed to PGE_1-hexylagarose on platelet aggregation by ADP. Platelet-free plasma was prepared by centrifuging platelet-rich plasma at 27,000*g* at 10°C for 30 minutes. No platelets were detected by phase microscopy and the platelet count was less than 1000 per milliliter. Platelet-free plasma was passed through 0.2 ml of packed PGE_1-hexylagarose contained in the tip of a plastic tube (0.3 by 10 cm) fitted with a small nylon plug. A similar volume of the same plasma was passed through an identical column containing ω-NH_2-hexylagarose and served as a control. The *p*H of the plasma was checked before and after it was passed through the columns. No change in *p*H (7.21 in both cases) was noted. Platelets separated from 1 ml of platelet-rich plasma by centrifugation at 700*g* for 10 minutes at 23°C were resuspended in each plasma sample to a final cell count of 2×10^8 platelets per milliliter, and within 15 minutes were tested for aggregation with ADP (2 and 4 μM). (Curve a) Plasma exposed to ω-NH_2-hexylagarose and 2 μM ADP. (Curve b) Plasma exposed to PGE_1-butylagarose and 2 μM ADP. (Curve c) Plasma exposed to PGE_1-hexylagarose and 4 μM ADP. (Curve d) Plasma exposed to PGE_1-hexylagarose and 2 μM ADP. The PGE_1-butylagarose was prepared in the same way as PGE_1-hexylagarose except that ω-NH_2-butylagarose was used. The results shown are representative of more than ten individual experiments.

Prostaglandin E_1 covalently coupled to ω-NH_2-hexylagarose (hereafter referred to as PGE_1-hexylagarose) caused a fourfold increase in the concentration of platelet cyclic AMP, an effect similar to that of soluble PGE_1 (Table 1). Under the same conditions, both forms of PGE_1 completely inhibited platelet aggregation (data not shown) induced by ADP (2 μM), or by *l*-epinephrine (5 μM), as determined by the turbidometric method of Born (*5*). Thus, the effect of PGE_1-hexylagarose on the platelet cyclic AMP concentration and platelet aggregation is similar to that of the soluble hormone.

Insoluble PGE_1 can be separated from platelets by differential centrifugation at 170*g* for 10 minutes at 25°C as documented by results obtained with ^{14}C-labeled PGE_1-hexylagarose. However, the ability of these platelets to aggregate after exposure to ADP and epinephrine was not restored (data not shown). This effect could not be attributed to a persistent increase in platelet cyclic AMP because exposure of the platelet-free plasma to PGE_1-hexylagarose induced in the plasma the ability to inhibit ADP-induced platelet aggregation (Fig. 1, curve d). This result is not due to an effect of the hexylagarose since platelet-free plasma exposed to that gel produces full platelet aggregation (Fig. 1, curve a) identical to that obtained with unexposed plasma (not shown). The inhibition was less marked when the concentration of ADP was increased (Fig. 1, curve c) but even with 20 μM ADP the inhibitory effect of the plasma could not be completely reversed. The effect of the insolubilized PGE_1 appeared to be dependent on the chain length of the spacer arms. We found that PGE_1-butylagarose, in which the spacer arm is two carbons shorter than that in PGE_1-hexylagarose, failed to induce significant inhibitory activity in platelet-free plasma (Fig. 1, curve b). A similar lack of inhibition was noted when PGE_1 was coupled to ω-NH_2-propylagarose. The specificity of PGE_1-hexylagarose was tested by using insolubilized preparations of two prostaglandins which do not inhibit platelet function. Thus PGE_2-hexylagarose and $PGF_{2\alpha}$-hexylagarose were prepared by the identical methods used for the preparation of the PGE_1 derivative. Plasma exposed to either PGE_2-hexylagarose or $PGF_{2\alpha}$-hexylagarose failed to inhibit platelet aggregation induced by ADP or epinephrine (data not shown).

The inhibitory effect of PGE_1-hexylagarose might have resulted from the

Table 1. Effect of PGE_1 and PGE_1-hexylagarose on platelet cyclic AMP. The PGE_1-hexylagarose was prepared by incubating 5 ml (packed volume) of ω-NH_2-hexylagarose (*12*), 14.2 μmole of PGE_1, and 600 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (*13*) in 10 ml of 50 percent *N*,*N*-dimethyl formamide at *p*H 6.0 for 48 hours at 23°C. The derivative was subsequently washed with 250 ml of 50 percent dimethyl formamide, 150 ml of 50 percent CH_3OH, 4 liters of H_2O, 2 liters of 1.2*M* NaCl, and 1 liter of 0.15*M* NaCl solution. The resultant PGE_1-hexylagarose contained 3.6 nmole of PGE_1 per milliliter of packed gel as determined by means of radioactive PGE_1. Plasma (0.5 ml) containing 3.8×10^8 platelets per milliliter prepared from human blood as described (*6*) was incubated at 37°C for 1 minute with constant stirring at 1200 rev/min with either PGE_1 or PGE_1-hexylagarose, and cyclic AMP concentrations were determined (*14*). The results of three separate experiments, each run in triplicate, are expressed as means ± standard error.

Addition	Concentration (μM)	Cyclic AMP (pmole/10^8 platelets)
None		1.25 ± 0.17
PGE_1	0.26	4.50 ± 0.12
PGE_1-hexylagarose	0.36	4.80 ± 0.24

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elution of PGE_1 from the agarose into the plasma. Therefore, we measured the PGE_1 eluted by 1.5 ml of plasma from 0.2 ml of PGE_1-hexylagarose. The concentration of PGE_1 in the plasma was 1.2 n*M* as determined by means of a radioimmunoassay system developed by Miles Laboratories. This value compares with a minimum inhibitory concentration of PGE_1 of 20 n*M* (*6*). In another experiment, ^{14}C-labeled PGE_1-hexylagarose was prepared by including in the incubation mixture previously described (Table 1) 20 μCi of [^{14}C]PGE_1 (New England Nuclear; specific activity, 40 mCi/mmole). The [^{14}C]PGE_1-hexylagarose prepared in this way contained 8500 counts per minute per millilter. Radioactivity eluted out from the [^{14}C]PGE_1-hexylagarose column (1 ml) was 16 ± 6.5 and 18 ± 2.4 counts per minute per milliliter of plasma and Tyrode's buffer, respectively (*7*). These values are nearly equal to the background counts (15.4 ± 5.5) of the counting machine (Packard Tri-Carb liquid scintillation spectrometer). Elution of the column with albumin (1 mg/ml), fibrinogen (0.3 mg/ml), Tyrode's buffer, or water, and the addition of these eluates to unexposed plasma failed to produce inhibition of platelet aggregation. Thus, it appears that alteration of some plasma components rather than elution of PGE_1 is responsible for the inhibitory effect. Further support for a plasma-mediated mechanism comes from the observation that platelets resuspended in plasma previously exposed to PGE_1-hexylagarose failed to show any significant increase in cyclic AMP concentration despite the inability of the platelets to respond to ADP (Table 2).

One possible explanation of these results might be the removal by PGE_1-hexylagarose of some plasma cofactor that is necessary for epinephrine- and ADP-induced platelet aggregation. Attempts to elute such a corrective factor from the column with up to 4*M* NaCl were unsuccessful. Since fibrinogen is a known cofactor for ADP-induced platelet aggregation (*8*) we measured fibrinogen (*9*) in the plasma exposed to insolubilized PGE_1 but found no change. Addition of 0.3 mg/ml of highly purified human fibrinogen (AB Kabi, Stockholm) also failed to correct the aggregation abnormality. Further evidence against the removal of an essential factor from plasma is the observation that the inhibitory activity of plasma that has been exposed to PGE_1-hexylagarose (Fig. 2, curve b) is lost after incubation at 8°C for 16 hours (Fig. 2, curve a). Reexposure of this plasma to PGE_1-hexylagarose produces the same inhibitory effect in plasma, suggesting that a reversible change occurred during the 16 hours of incubation. In experiments to identify the factor altered by exposure to insoluble PGE_1, extensive dialysis of the plasma prior to exposure to PGE_1-hexylagarose did not prevent the induction of inhibitory activity in the plasma. The inhibitory activity in plasma was present in the void volume of a Sephadex G-25 column. Thus, the factor responsible appears to have a molecular weight in excess of 5000. These findings are in agreement with other investigators who have found that a plasma protein is required in addition to fibrinogen to modulate platelet aggregation induced by ADP (*10*).

Table 2. Effect of plasma exposed to PGE_1-hexylagarose on platelet cyclic AMP. After resuspension of the platelets in the respective plasmas for 1 minute at 37°C, cyclic AMP was determined (*14*). The results of three experiments run in triplicate are expressed as means ± standard error. The difference of cyclic AMP concentration in platelets resuspended in normal plasma or in platelets resuspended in plasma exposed to PGE_1-hexylagarose is not significant.

Treatment of platelets	Cyclic AMP (pmole/10^8 platelets)
Resuspended in normal plasma	1.18 ± 0.10
Resuspended in plasma exposed to PGE_1-hexylagarose	1.38 ± 0.14
Resuspended in plasma exposed to ω-NH_2-hexylagarose	1.30 ± 0.16

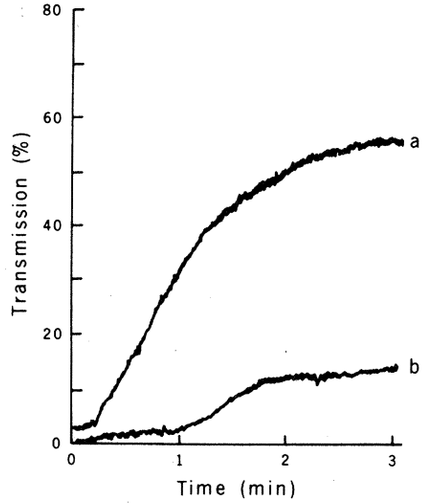

Fig. 2. Reversal of the inhibitory effect on ADP-induced platelet aggregation of plasma exposed to PGE_1-hexylagarose. Plasma exposed to PGE_1-hexylagarose was tested for its ability to inhibit platelet aggregation as in Fig. 1 after 30 minutes of incubation at 8°C (curve b) and after 16 hours of incubation at 8°C (curve a). The *p*H of the plasma exposed to PGE_1-hexylagarose was 7.21 at 30 minutes and changed to 7.30 after 16 hours of incubation at 8°C. Normal plasma which was stored under identical conditions at 8°C for 16 hours showed a similar change in *p*H. Such changes of *p*H did not influence the inhibition of platelet aggregation induced by ADP or epinephrine.

Although cyclic AMP does not appear to be involved in the inhibition of platelet aggregation by plasma exposed to PGE_1-hexylagarose, the involvement of other platelet constituents such as protein kinases or protein kinase modulator (*11*) can not be excluded. Platelet-free plasma prepared by high-speed centrifugation might contain constituents released from platelets which might not sediment and which might be modified by PGE_1.

Our data indicate that PGE_1 not only inhibits platelet aggregation by increasing cyclic AMP concentrations but also induces an alteration or formation of an inhibitory macromolecule in plasma which can regulate platelet functions independently of platelet cyclic AMP. The presence of such components in plasma should have an important role in the regulation of thrombogenesis.

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