THE EFFECTS OF DEXTRAN SULFATE, HEPARIN AND PGE₁ ON
ADENYLATE CYCLASE ACTIVITY AND AGGREGATION OF
HUMAN PLATELETS

Avinoam RECHES, Amiram ELDOR and Yoram SALOMON†
Departments of Neurology and Hematology, Hadassah University Hospital,
Jerusalem, Israel and Department of Hormone Research, The Weizmann
Institute of Science, Rehovot, Israel
(Received 8.3.1979; in revised form 15.5.1979.
Accepted by Editor D. Danon)

ABSTRACT
Prostaglandin E₁ (PGE₁) sensitive adenylate cyclase [EC.4.6.1.1
ATP-pyrophosphate lyase (cyclizing)] in human platelet lysates is
inhibited by dextran sulfate. Inhibition is dose-dependent at the
range of 1-10 μg/ml. Maximal inhibition obtained was 83% at 30
μg/ml. At a similar range of concentrations dextran sulfate was
found to antagonize the anti-aggregating effect of PGE₁ on adenosine
5' diphosphate (ADP) induced human platelet aggregation. Heparin,
like dextran sulfate, effectively antagonizes the anti-aggregating
effect of PGE₁ in human platelets. It is possible that the aggrega-
gating effects of both heparin and dextran sulfate on human plate-
lets may be mediated via inhibition of platelet PGE₁-sensitive
adenylate cyclase.

INTRODUCTION

The ability of sulfated glycosaminoglycans to modulate adenylate cyclase
activity derived from several tissues has recently been demonstrated in our
laboratories (1,2). Heparin, a potent anticoagulant, was found to inhibit
PGE₁ sensitive adenylate cyclase in human platelet lysates (3). Heparin was
also found to antagonize the antiaggregating effect of PGE₁ on ADP induced
platelet aggregation (4,5).

It has been suggested that activation of platelet adenylate cyclase (6)
or inhibition of platelet cyclic nucleotide phosphodiesterase (7) mediates
the antiaggregating properties of agents like PGE₁ and dipyridamole deriva-
tives, respectively. On the other hand, aggregating agents like epinephrine
were found to inhibit platelet adenylate cyclase activity (8).

† To whom reprint requests should be addressed.

Key words: Adenylate cyclase, dextran sulfate, heparin, human platelets,
prostaglandin E₁
In view of our findings we have suggested (4,5) that the paradoxical platelet aggregating activity of heparin (9,10,11,12) may stem from the ability of this anticoagulant to inhibit PGE\textsubscript{1} sensitive adenylate cyclase activity in human platelets.

The purpose of the present study was to determine if the observed effect on human platelets is unique to heparin or whether other polyanions also show this characteristic. Dextran sulphate, a high molecular weight polyanion, has been demonstrated by Wolff et al. (13) to inhibit thyroid stimulating hormone (TSH) sensitive adenylate cyclase. Similarly dopamine sensitive adenylate cyclase of rat caudate nucleus and the gonadotropin sensitive enzyme from the rat ovary have been shown by us to be inhibited by dextran sulfate (3). We determined therefore the effect of dextran sulfate on PGE\textsubscript{1} sensitive adenylate cyclase in human platelet lysates and studied the relationship between dextran sulfate and PGE\textsubscript{1} on ADP induced platelet aggregation.

**MATERIALS AND METHODS**

**Materials**

\[ \text{[}^{32}\text{P}] \text{ ATP, [8-}\text{H}]-3':5' \text{ cyclic AMP were purchased from the Radiochemical Center, Amersham, England. ATP, ADP, 3':5' cyclic AMP, phosphocreatine, creatine phosphokinase, dextran sulfate, (MW 500K) and heparin (Porcine Intestinal mucosa Grade I 162 USP J-A U/mg) were products of Sigma Chemical Co. PGE}_1 \text{ was a generous gift from the Upjohn Co., Kalamazoo, Mich.} \]

**Preparation of platelet lysates**

Preparation of human platelet lysates was performed by a modification of the method described by Jakobs et al. (8). Blood was drawn from healthy volunteers who had not taken drugs for at least one week prior to blood collection. Blood was taken with citrate-phosphate-dextrose solution as an anticoagulant. The platelet pellet was washed and resuspended with 150 mM NaCl, 10 mM Tris maleate, pH 7.5. The suspension was rapidly frozen in liquid nitrogen and thawed only once shortly before use.

**Adenylate cyclase assay**

Adenylate cyclase was determined by measuring the conversion of \([^{32}\text{P}] \text{ adenosine 5'} \text{ triphosphate (ATP)} \text{ to } [^{32}\text{P}] \text{ cyclic AMP. The standard assay mixture (final volume 50 } \mu\text{l)} \text{ contained 25 mM Tris-maleate, pH 7.5, 5 mM magnesium acetate, 0.5 mM [}^{32}\text{P}] \text{ ATP (2-4 x 10}^6 \text{ cpm), 1 mM dithiothreitol, 10 mM theophylline, 50 } \mu\text{M guanosine 5'} \text{ triphosphate (GTP), 5.0 mM creatine phosphate, 50 U/ml creatine phosphokinase and the test substances at the concentration indicated in the individual experiments. The reaction was initiated by the addition of platelet lysates } [5-10 \mu\text{g protein (14)}]. \text{ Incubation was carried out at 30}^\circ\text{C for 10 minutes. Termination of the reaction and isolation of } [^{32}\text{P}] \text{ cyclic AMP were performed according to Salomon et al. (15)}]. \]

**Platelet aggregation**

Blood collection and preparation of citrate platelet rich plasma (PRP) and platelet poor plasma (PPP) were as described by Han and Ardlie (16). PPP was used to adjust the platelet count of PRP (which was kept at room temperature) to 200,000/\mu\text{l}. PRP (0.4 ml) was stirred at 37\degree\text{C for 1 min. Normal saline, dextran sulfate or heparin followed by either PGE}_1 \text{ or normal saline were }
added as indicated to a final volume of 0.45 ml. To induce aggregation ADP was added (0.05 ml) to a final concentration of 2 μM. The time interval between successive additions was 20 sec. All experiments were performed within 1½ hours of PRP preparation during which no significant change in platelet response to ADP was observed. Platelet aggregation was measured by the turbidometric technique of Born (17), using a dual channel platelet aggregometer (Chrono-Log Corp. Model 340 with Omni-Scribe Recorder, Houston Instruments, Texas). Upon addition of ADP platelet aggregation was induced concomitantly with a gradual increase in light transmittance which was recorded for 4 min. The amplitude of the aggregation wave was directly related to the increment in light transmittance induced by ADP. Any change in aggregation was calculated relative to this basal aggregation wave.

The experiments presented were repeated with 3 platelet preparations obtained from 3 donors.

RESULTS

1. The effect of dextran sulfate on adenylate cyclase activity in platelet lysates

Basal adenylate cyclase activity in human platelet lysates 218 pmol cyclic AMP/10 min/mg protein is stimulated over 10-fold to 2236 pmol cyclic AMP/ 10 min/mg protein by PGE₁ (2.8 μM). Addition of dextran sulfate to the incubation medium resulted in a gradual inhibition of enzyme activity (Fig. 1). This inhibition was dose dependent in the range of 1-10 μg/ml of dextran sulfate. Maximal inhibition obtained was 86% at dextran sulfate concentration of 30 μg/ml. Increasing dextran sulfate concentration up to 100 μg/ml had no further effect on the extent of inhibition. Basal adenylate cyclase activity was inhibited to the same extent. Thus, the adenylate cyclase activity ratio: activity in the presence of PGE₁/activity in its absence, was unaffected by the presence of dextran sulfate.

Adenylate cyclase activity was determined at increasing PGE₁ concentrations (0.145-8.4 μM) in the absence or presence of dextran sulfate (10 μg/ml). It is shown (Fig. 2) that inhibition by dextran sulfate was of a mixed type when data was analysed according to Lineweaver and Burke (18). Dextran sulfate appears to reduce by 3-fold the apparent affinity of the enzyme system to PGE₁ and decrease maximal enzyme velocity by nearly 2-fold.

2. The effect of dextran sulfate and heparin on aggregation of human platelets

ADP induced wave of aggregation (Fig. 3a) was inhibited (85%) in the presence of 0.02 μM of PGE₁ (Fig. 3b). This antiaggregating effect of PGE₁ could be effectively antagonized by the addition of dextran sulfate. At 10 μg/ml (Fig. 3c) dextran sulfate reduced the inhibition by PGE₁ from 85 to 75%. Further increase in dextran sulfate concentration to 25, 35 and 50 μg/ml resulted in a progressive enhancement of the aggregating wave, thus decreasing the antiaggregating effect of PGE₁ to 64% (Fig. 3d) 22% (Fig. 3e) and 10% (Fig. 3f) of its original value respectively.

Closer examination of the data presented in Fig. 3 shows that dextran sulfate has only a small effect on the primary phase of aggregation and seems
Inhibition of basal and PGE₁-stimulated adenylate cyclase activity in human platelet lysates at increasing concentrations of dextran sulfate.

Adenylate cyclase activity was measured in the absence (0) or presence (0) of PGE₁ (2.8 μM) at increasing concentrations of dextran sulfate. The amount of lysate protein was 4.8 μg/assay. All other details were as described in "Methods".

to induce a secondary aggregating wave about one min after addition of ADP. The extent of augmentation increases with increasing concentration of the sulfated poly sugar.

Heparin was found to act in a manner similar to that exerted by dextran sulfate in promoting ADP induced aggregation of human platelets and in antagonizing the effects of PGE₁. The effect of several concentrations of heparin on platelet aggregation in the absence or presence of PGE₁ was studied. Presented in Fig. 4 is a summary of aggregation studies performed with heparin in the same manner as described for dextran sulfate in Fig. 3. The extent of aggregation at 4 min of 20 determinations is shown. It can be seen that ADP induced aggregation is augmented by heparin in a dose dependent manner. The half maximal effect is seen at 2 μg/ml of heparin and saturation is reached at nearly 10 μg/ml. PGE₁ 0.02 μM and 0.06 μM inhibited ADP induced aggregation by 30 and 68 percent respectively. As little as 1 μg/ml heparin abolished the effect of 0.02 μM PGE₁ and even augmented aggregation by 30%. The effect of heparin increased in a dose dependent...
*Inhibition by dextran sulfate of the antiaggregating effect of PGE1 in ADP induced aggregation of human platelets.

The experiment was carried out as described under "Methods". The concentration of ADP (2 μM) and PGE1 (0.02 μM) were kept constant. The sequence of other additions was as follows:

- (a) NS + NS + ADP;
- (b) NS + PGE1 + ADP;
- (c) DS (10 μg/ml) + PGE1 + ADP;
- (d) DS (25 μg/ml) + PGE1 + ADP;
- (e) DS (35 μg/ml) + PGE1 + ADP;
- (f) DS (50 μg/ml) + PGE1 + ADP.

The experiment was carried out as described in legend to Fig. 3, substituting heparin at the indicated concentration for dextran sulfate. Each point represents the extent of aggregation obtained 4 min. after the addition of ADP. This series of experiments was repeated in the absence (O) and in the presence of PGE1 0.02 μM (O) and 0.06 μM (V).

manner reaching 120% at 10 μg/ml in spite of the presence of 0.02 μM PGE1.

At the same concentration (10 μg/ml) heparin almost totally abolished the inhibitory effect exerted by 0.06 μM PGE1. Furthermore, in the absence of added PGE, the augmentation by heparin (10 μg/ml) of platelet aggregation exceeded ADP induced response by 300%.

* NS - normal saline; ADP - Adenosine 5' diphosphate, PGE1 - Prostaglandin E1; DS - dextran sulfate.
ADP induced aggregation (Fig. 5a) was inhibited at increasing concentrations of PGE, 0.02 μM (Fig. 5b), 0.04 μM (Fig. 5c) and 0.08 μM (Fig. 5d) by 85, 90 and 96 percent respectively. The addition of dextran sulfate (50 μg/ml) antagonized the inhibitory effect exerted by PGE, Thus attenuating the inhibitory effect to 0, 19 and 69 percent in the presence of 0.02 μM (Fig. 5f), 0.04 μM (Fig. 5g) and 0.08 μM PGE, (Fig. 5h) respectively. In line with the observations shown in Fig. 3 dextran sulfate induced a secondary phase of aggregation (Figs. 5g, f). In the absence of PGE, (Fig. 5e) dextran sulfate hastened the appearance of the secondary phase as compared to Fig. 5a in which a clear delay in the appearance of the secondary phase is observed.

In an effort to establish the relationship between adenylate cyclase activity in platelet lysates, changes in cyclic AMP levels in intact cells and platelet aggregation, we studied intracellular levels of cyclic AMP in intact platelets. These experiments were performed by prelabelling intact platelets with 3H adenine by a modification (19) of the method described by Humes (20). Intracellular accumulation of cyclic AMP was stimulated by PGE, (0.14 -2.8 μM) in a dose-dependent manner. Maximal stimulation obtained was 11-fold over unstimulated controls. The addition of either heparin or dextran sulfate (1-100 μg/ml) to the platelet suspension had no effect on cyclic AMP levels determined in the absence or presence of PGE, following 45 sec to 5 min incubation.

The inhibition by dextran sulfate of the antiaggregating effect of PGE, in ADP induced aggregation of human platelets.

The experiment was carried out as described in legend to Fig. 3. The concentrations of ADP (2 μM) and dextran sulfate (50 μg/ml) were kept constant. The sequence of other additions was as follows: (a) NS + NS + ADP; (b) NS + PGE, (0.02 μM) + ADP; (c) NS + PGE, (0.04 μM) + ADP; (d) NS + PGE, (0.08 μM) + ADP; (e) DS + NS + ADP; (f) DS + PGE, (0.02 μM) + ADP; (g) DS + PGE, (0.04 μM) + ADP; (h) DS + PGE, (0.08 μM) + ADP.

*NS - normal saline; ADP - Adenosine 5'-diphosphate; PGE, - Prostaglandin E,; DS - dextran sulfate.
DISCUSSION

In the present report we demonstrated that PGE₁-sensitive adenylate cyclase activity in human platelet lysate is inhibited by relatively low concentrations (1-30 µg/ml) of dextran sulfate (Fig. 1). In that range dextran sulfate was also shown to augment ADP induced platelet aggregation and to antagonize the antiaggregating effect exerted by PGE₁ (Figs. 3 and 5).

In a similar manner we have shown that heparin inhibits PGE₁-sensitive adenylate cyclase activity in human platelet lysates (3,4,5) and antagonizes the antiaggregating activity of PGE₁ (Fig. 4).

It has been suggested that activation of platelet adenylate cyclase may play a role in platelet aggregation. Agents like PGE₁ which stimulate this enzyme system (6) have been shown to inhibit platelet aggregation (7). Dipyridamole and its derivatives which are clinically used as antiplatelet aggregating agents have been shown to inhibit platelet cyclic AMP phosphodiesterase (7). In accordance, agents which induce platelet aggregation like norepinephrine were suggested among other things to activate platelet cyclic AMP phosphodiesterase (22) and to inhibit PGE₁ dependent increase in cyclic AMP levels (22,23,24). It has been suggested that this effect of epinephrine on platelet adenylate cyclase activity is mediated by the alpha adrenergic receptor (25,26).

Heparin which is currently used in clinical practice as a potent anticoagulant also shows a paradoxical platelet aggregating activity (27) and its administration in humans is sometimes associated with a thrombocytopenic effect (10,11,12). In view of our findings we have suggested (4,5) that heparin, like epinephrine, may exert its procoagulant effect via inhibition of platelet adenylate cyclase or by preventing the response of this enzyme system to naturally accruing stimulants like PGE₁.

Dextran sulfate, a highly charged poly-anion, was previously demonstrated to inhibit TSH-sensitive adenylate cyclase activity of bovine thyroid gland (13) dopamine sensitive enzyme activity in rat caudate nucleus and LH-sensitive adenylate cyclase of rat ovary (2). In the present experiments we have demonstrated that dextran sulfate also inhibits basal and PGE₁ sensitive adenylate cyclase in human platelet lysates. Likewise, dextran sulfate was also shown to inhibit the antiaggregating effect of PGE₁.

Presumably both heparin and dextran sulfate exert their effect on the platelet plasma membrane in a similar mechanism. It has been shown that heparin's action in the ovarian preparation is, in part, associated with interference with hormone binding to its specific receptor (2). It is postulated that by adsorbing to opposite charges on the plasma membrane, heparin and dextran sulfate might restrict the relative mobility of the hormone receptor and the enzyme and thus interfere with their normal function.

While a correlation between the effects of dextran sulfate and heparin on adenylate cyclase activity and platelet aggregation has been demonstrated in our experiments no direct evidence could be shown for an effect of these polyanions on cyclic AMP levels in intact cells. The failure to detect a parallel effect on cyclic AMP accumulation in intact platelets may stem from the size of the pools of cyclic AMP which are relevant to platelet aggregation. Furthermore, these findings may fit the suggestions (28,29) that cyclic AMP is stored within the platelets in functional compartments which might respond
separately to various stimuli. Further studies on the intracellular localization and function of cyclic AMP in platelets are therefore still required to verify the proposed mechanism.

An alternative hypothesis is that heparin and dextran sulfate act like other proaggregatory substances such as thrombin, collagen and epinephrine. As suggested by Gorman (30) these substances stimulate the liberation of arachidonic acid and thus enhance the synthesis of thromboxane A₂ which in turn promotes platelet aggregation. This may explain the delayed secondary phase of aggregation induced by these polyanions. Furthermore, according to this model and previous suggestions by others (31,32) high levels of cyclic AMP induced by PGE₁ are inhibitory for the platelet phospholipase A₂. It is suggested that heparin and dextran sulfate may overcome this inhibition and thus antagonize the antiaggregating effect of PGE₁. If this hypothesis is valid one may expect that the proaggregatory effects of heparin and dextran sulfate will be antagonized by indomethacin or imidazole - a question currently under investigation.

ACKNOWLEDGEMENT

This work was supported partly by a grant to Y.S. from the U.S.-Israel Binational Science Foundation (BSF), Jerusalem, and in part by a grant to Professor H.R. Lindner by the Ford Foundation and Population Council, Inc., New York. Y.S. is the incumbent of the Charles and Tillie Lubin Career Development Chair. We wish to thank Drs. H.R. Lindner and M. Frojmuc for helpful discussions and Mrs. M. Kopelowitz for excellent secretarial assistance.

REFERENCES


