

THE EFFECT OF TICLOPIDINE ADMINISTRATION TO HUMANS ON THE BINDING OF ADENOSINE DIPHOSPHATE TO BLOOD PLATELETS

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ABSTRACT

Administration of Ticlopidine to human volunteers resulted in a prolonged bleeding time and decreased or absent aggregation of platelets with collagen and epinephrine. Adenosine diphosphate (ADP) induced platelet aggregation was initiated by a normal shape change, but the rate of the first wave of aggregation had decreased. The second wave of aggregation was absent. ADP-binding to platelets of volunteers, consisted of two classes of binding sites: one with high affinity and one with low affinity, giving a curvilinear and a linear part in a concentration dependency curve. After Ticlopidine, the low affinity part of the curve had disappeared. Evidence will be presented that this is a specific Ticlopidine effect.

INTRODUCTION

In recent years, much attention has been paid to the development of drugs preventing diseases of the heart and vascular system. Blood platelets play an important role in these diseases. Inhibition of platelet function may prevent cerebrovascular diseases or myocardial infarction and theoretically even atherosclerosis (1-3). Acetyl salicylic acid is a drug which has potential anti-thrombotic action (14). However it has the disadvantage of inhibiting prostaglandin I_2 activity from vascular tissues (5). Ticlopidine is a new drug inhibiting platelet function (6), but without influence on prostaglandin synthe-

Key words : Human platelets, ADP-receptors, platelet aggregation inhibitors.

sis (7,8). The mechanism of action of Ticlopidine is unknown. In this study we present data on the inhibition of platelet function by Ticlopidine and the effect of the drug on ADP-binding by platelets.

MATERIALS AND METHODS

Volunteers

Six healthy male volunteers who had used no drugs the preceeding 7 days participated. Informed consent was given. On the first day we performed a bleeding time according to Mielke (9) and we took 60 ml of blood (9 volumes of blood and 1 volume of 0.113 M disodium citrate) for aggregation and ADP-binding studies. The volunteers received 2 times daily 250 mg of Ticlopidine (in dragee form) on day 2 and 3.

The bleeding time determination and blood collection for aggregation and ADP-binding studies was repeated on the 4th day. Platelet rich plasma (PRP) was prepared by centrifugation (10 min, room temp., $175 \times g_{\max}$). Platelet numbers were determined with a Thrombocounter (Coulter Electronics, Harpenden, England).

Chemicals

Ticlopidine was a gift from Labaz, (Brussels, Belgium).

[U- ^{14}C] ADP (Spec. act. 570 mCi/mMol) was purchased from the Radiochemical Centre Amersham, England. Collagen was prepared from bovine achilles tendon according to Holmsen (10). Epinephrine, ADP and PGE₁ were obtained from Sigma (St. Louis, USA) and all other chemicals used were analytical grade.

Aggregation studies

We performed aggregation studies with ADP (1, 2 and 4 μM and sometimes 6 and 10 μM (final concentration, f.c.), collagen (7.5, 15 $\mu\text{g/ml}$ and sometimes 75 $\mu\text{g/ml}$ f.c.) and epinephrine (5.5 μM or 55 μM f.c.). Dilutions of the aggregating agents were made in saline, just before use. We used 0.45 ml of platelet rich plasma, preincubated this under stirring (1000 rpm, 37°C) for 5 min in the cuvette of a Payton Dual Channel aggregometer (Payton Ass. Inc. Scarborough, Canada) and added 0.05 ml of the aggregating agent. The aggregation curve was recorded with an Omniscribe recorder (Houston Instr., Belaire, USA). 90% of full scale was set for PRP and 10% of full scale for platelet poor plasma. For aggregation studies, the platelet numbers after Ticlopidine ingestion were adjusted to the same concentrations as before.

Binding of adenosine diphosphate

We washed platelets by centrifugation (10 min, 1000xg, 4°C) and resuspension in Tris/saline/EDTA (Tris-HCl 10 mM, NaCl 150 mM and EDTA 1 mM, pH 7.4). After a second centrifugation we resuspended the platelets in Tris-saline (Tris-HCl 10 mM, NaCl 150 mM, pH 7.4). We incubated 100 μ l of this suspension for 5 min at 37°C, with 50 μ l of Tris-saline containing 8 mM calcium chloride. We then added 50 μ l of [14 C]-ADP in Tris-saline giving final concentrations of 0.6 - 100 μ M.

We incubated this mixture for 10 minutes at 37°C. The equilibrium situation always had been reached within 10 min and this equilibrium level remained constant for the next 20 min; this was found for all ADP concentrations used. We took 3 separate samples of 50 μ l from each incubation vessel. These samples were filtered over 0.8 μ l Millipore filters with vacuum aspiration, the platelets on the filters were washed twice with 6 ml of 0.154 M NaCl and transferred to counting vials and dissolved in 2 ml of ethylene glycol monomethyl ether (Merck, Darmstadt, Germany). We then added 5 ml of emulsifier scintillator (Packard, Downers Grove Ill., USA) and counted the vials in a Searle MK III (Nuclear Chicago) liquid scintillation spectrometer. Filter blanks were obtained with incubation mixtures without platelets but otherwise identical.

RESULTS

The results of bleeding time and aggregation studies are summarized in Table 1. The bleeding time was prolonged in 5 out of 6 volunteers.

The epinephrine induced aggregation at 5.5 μ M, was normal for all volunteers, before Ticlopidine. After Ticlopidine the epinephrine aggregation was absent at this concentration for all volunteers. At a higher concentration, 11 μ M, the epinephrine aggregation partly returned in 4 out of 6 volunteers. The aggregation of the two strongly inhibited volunteers became normal at the high concentration of 55 μ M.

Before Ticlopidine, platelet aggregation with collagen (7.5 μ g/ml) was normal in 5 out of 6 volunteers. After Ticlopidine the collagen aggregation was absent with this concentration. It had been diminished at 15 μ g/ml in 5 out of 6 volunteers. The volunteer whose collagen aggregation after Ticlopidine had not been diminished at 15 μ g/ml (no. 5) had also no prolonged bleeding time. Shape change was normal in ADP-challenged platelets, but the subsequent primary wave of aggregation had decreased.

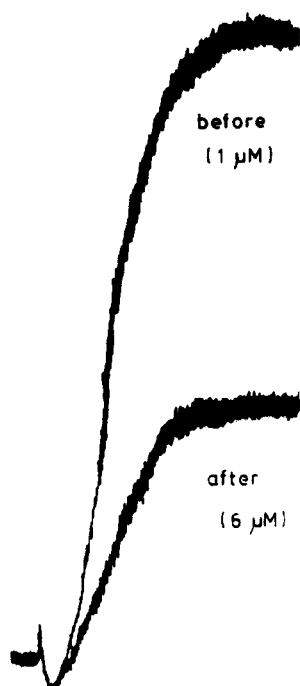
TABLE I

Effect of Ticlopidine on bleeding time and platelet aggregation.

<u>Bleeding time</u>		<u>Collagen</u>		<u>ADP</u>		<u>Epinephrine</u>	
<u>Before</u>	<u>After</u>	<u>Before</u>	<u>After</u>	<u>Before</u>	<u>After</u>	<u>Before</u>	<u>After</u>
		(7.5 μ g per ml)	(15 μ g per ml)*	(1 μ M)	(6 μ M)*	(5.5 μ M)	(11 μ M)*
1. 75"	99"	23	Absent	177	60	221	15
2. 50"	124"	193	134	185	120	189	136
3. 94"	217"	190	10	148	65	170	152
4. 41"	216"	163	5	150	70	157	24
5. 128"	128"	175	202	148	118	196	173
6. 71"	175"	186	125	186	112	195	184

The Mielke bleeding time is given as the mean value in seconds of three incisions. The epinephrine, collagen and ADP-aggregation are expressed as the maximum decrease in optical density obtained, in mm. *After ingestion of Ticlopidine, the response towards the lowest concentration of aggregating agent was absent in all volunteers. Results are given of the next higher concentration.

FIG. 1.

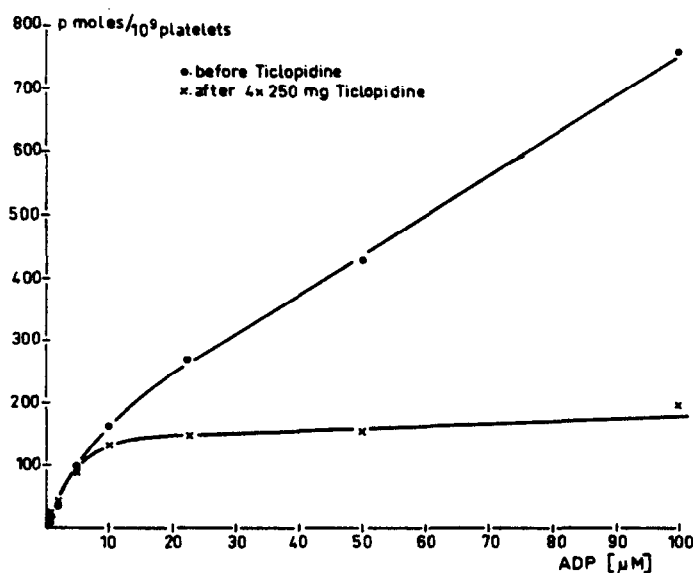


Recordings of aggregation response towards ADP, before and after 4 x 250 mg Ticlopidine. The recorder had been set 90 % of full scale for platelet rich plasma and 10 % of full scale for platelet poor plasma. In both cases the aggregating samples contained the same amount of platelets.

The second wave of ADP-aggregation had disappeared even at 6 μM (Fig. 1), in all individuals and this caused a decrease in amplitude to 55 % of the pre-Ticlopidine values. Higher concentrations of ADP (up to 100 μM) were used in one individual. Even at this concentration we could not overcome the inhibition of ADP-aggregation.

The concentration dependence of equilibrium binding of ADP before Ticlopidine consisted of a curvilinear part at low ADP-concentrations (below 20 μM), and a rectilinear part at concentrations between 20 and 100 μM . (Fig. 2).

FIG. 2.



Equilibrium binding of ADP to intact washed (centrifugation) platelets, before (●—●) and after (x—x) 4 x 250 mg Ticlopidine. Binding was determined by Millipore filtration. A representative experiment is shown.

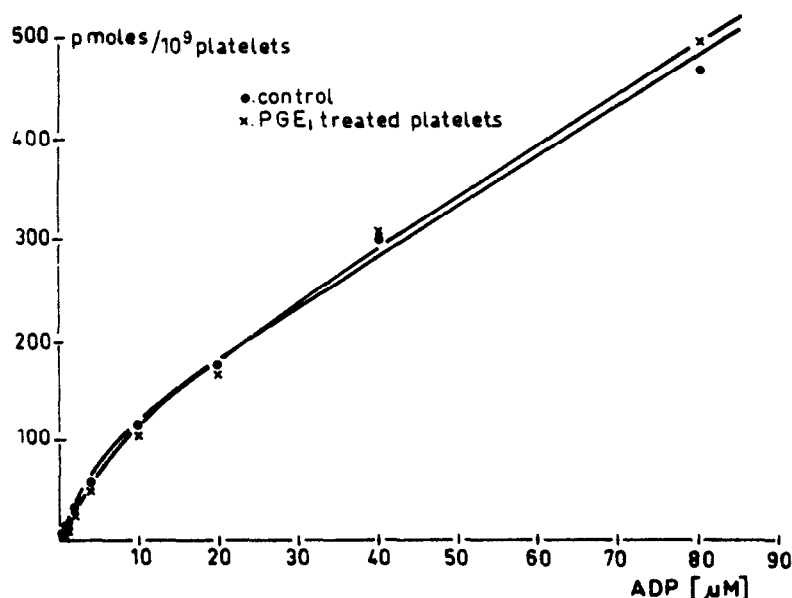
This pattern was similar to that found in previous studies (11, 12). The concentration dependence after Ticlopidine administration is shown in Fig. 2. Only the saturable high affinity system was observed; the rectilinear part of the curve had disappeared.

We tested the specificity of the response to Ticlopidine by comparing the ADP-binding before and 2 hours after the administration of 1 gram of aspirin in 2 volunteers. We observed no change in ADP-binding although collagen and epinephrine aggregation had become inhibited. Similar results were obtained with

aspirin and indomethacin in vitro (results not shown).

Ticlopidine might protect platelets against changes due to blood collection and further handling. Such mechanism was suggested by the observation that gel filtered platelets possess a much less pronounced low affinity binding system (12). We collected blood in citrate containing PGE₁ (1 μ M f.c.) and washed the platelets by means of gel filtration in a buffer according to Walsh (13) in the presence of 1 μ M PGE₁.

FIG. 3.



Equilibrium binding of ADP to intact platelets. Half of the platelet rich plasma was gel filtered in the presence of 1 μ M PGE₁, the other half gel filtered without PGE₁. Binding was determined in the presence of 10 μ M PGE₁ (x—x) or absence of PGE₁ (●—●) by means of Millipore filtration. A representative experiment is shown.

The ADP-binding studies were finally performed in the presence of 10 μ M PGE₁. Although these conditions should prevent shape change of the platelets, the low affinity ADP-binding component was still observed (Fig. 3).

DISCUSSION

In the present study we show the effect of orally administered Ticlopidine on the bleeding time, platelet aggregation and the binding of ADP to washed platelets. Four times 250 mg Ticlopidine caused a prolongation of the bleeding

time and inhibition of aggregation 18 hours after the last dragee. The aggregation inhibition after Ticlopidine differed from that with aspirin in that the ADP-binding was also inhibited. This could not be overcome by increased dosages of ADP. These results are consistent with earlier reports. (7, 14).

It seems plausible to relate the decrease of ADP-binding at higher ADP-concentrations with this inhibition of ADP-aggregation. This would imply that the low affinity system is involved in ADP-induced aggregation. We are reluctant to attach much value to the low affinity system for ADP-induced aggregation, because all our further studies (12) indicate that it is the high affinity binding system which is involved in ADP induced platelet shape change (Fig. 1). It is obvious from our study that the changes evoked by Ticlopidine are changes in the platelets, and this is in agreement with studies that have demonstrated that Ticlopidine is only active after in vivo administration and that platelet poor plasma of individuals, who received Ticlopidine, is not inhibitory (14, 15). This suggests that platelets have changed in such a way that the platelet-platelet interaction is inhibited. This may be caused by changes in the surface properties of the platelet membrane. The absent binding to the low affinity receptor may be a reflection of this membrane change. Further studies are required for elucidation of the inhibition by Ticlopidine of ADP-binding to platelets.

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