Inhibitory Effect of Piracetam on Platelet-rich Thrombus Formation in an Animal Model


From the Center for Molecular and Vascular Biology and Laboratory for Thrombosis Research, IRC, K.U. Leuven, Leuven-Kortrijk, Belgium; UCB-Pharma, Braine l’Alleud, Belgium; Department of Hand Surgery and Department of Anesthesia and Critical Care, NUS, Umeå, Sweden; and Division of Plastic Surgery and Department of Anesthesia, UPMC, Pittsburgh, PA, USA

Summary

Intravenous administration of piracetam to hamsters reduced the formation of a platelet-rich venous thrombus induced by a standardised crush injury, in a dose-dependent fashion with an IC₅₀ of 68 ± 8 mg/kg. 200 mg/kg piracetam also significantly reduced in vivo thrombus formation in rats. However, in vitro aggregation of rat platelets was only inhibited with piracetam-concentrations at least 10-fold higher than plasma concentrations (6.2 ± 1.1 mM) obtained in the treated animals. No effects were seen on clotting tests.

In vitro human platelet aggregation, induced by a variety of agonists, was inhibited by piracetam, with IC₅₀’s of 25-60 mM. The broad inhibition spectrum could be explained by the capacity of piracetam to prevent fibrinogen binding to activated human platelets. Ex vivo aggregations and bleeding times were only minimally affected after administration of 400 mg/kg piracetam i.v. to healthy male volunteers, resulting in peak plasma levels of 5.8 ± 0.3 mM.

A possible antiplatelet effect of piracetam could be due to the documented beneficial effect on red blood cell deformability leading to a putative reduction of ADP release by damaged erythrocytes. However similarly high concentrations were needed to prevent stirring-induced “spontaneous” platelet aggregation in human whole blood.

It is concluded that the observed antithrombotic action of piracetam cannot satisfactorily be explained by an isolated direct effect on platelets. An additional influence of piracetam on the rheology of the circulating blood and/or on the vessel wall itself must therefore be taken into consideration.

Conclusion

Piracetam has rheological effects which to some extent have been attributed to the induction of cell membrane changes in erythrocytes. This is believed to result in increased cell deformability and decreased adherence to the vessel wall (6-8). However, piracetam also lowers blood viscosity by reducing the levels of e.g. fibrinogen, macroglobulin and von Willebrand factor (9).

Platelet-inhibitory effects have been suggested to be due to a reduced responsiveness to ADP or to inhibition of TXA₂-synthesis. The doses necessary to achieve rheological and/or antiplatelet effects are of the order of 80-160 mg/kg, which is about 2-4 times more than the doses needed to obtain nootropic effects (10). Even at these elevated, and even higher, doses piracetam is well tolerated and few adverse effects have been recorded in human subjects (11).

Since the proposed activities of piracetam could result in an antithrombotic effect, we studied the influence of piracetam in a standardized thrombosis model (12) which has previously been used to document the antithrombotic activities of several other drugs such as argatroban, a specific thrombin inhibitor (13, 14), ridogrel, a dual thromboxane synthase and receptor blocker (15), G4120, a GPIIb/IIIa-antagonist (14, 16), calin from the leech saliva (17), anticoagulants (18) and thrombolytic agents where the antithrombotic effect mainly correlated with the fibrinogenolytic action (19). In addition, we tried to better define the observed antithrombotic action of piracetam by further studies on isolated rat and human platelets.

Materials and Methods

The study was performed on animals (rats, hamsters) and on human volunteers. All animal experiments were conducted according to the guidelines of the International Society on Thrombosis and Haemostasis. The part of the study which employed human volunteers was conducted according to the declaration of Helsinki, and approvals were obtained from the institutional review board of the University of Umeå, and the National Board of Health and Welfare, Stockholm, Sweden.

In Vivo Experiments in Rats and Hamsters

Male rats with a body weight of 150-200 g were anesthetized with pentobarbital (50 mg/kg IP). Outbred hamsters (245, Pfd, Gold, University of Leuven), with body weights of 80 to 120 g, were premedicated with 1.25 mg/kg atropine intraperitoneally (IP) and anesthetized by an IP injection of 50 mg/kg of sodium pentobarbital. The femoral vessels were exposed and mounted on a transilluminator. The formation of two opposing mural thrombi was initiated by a vessel wall trauma with a clamp mounted on a micromanipulator (12). The platelet-rich thrombi were visualized by increased light transmission through the transilluminated blood vessel segment. The upstream thrombus was used for analysis.
Thrombus formation and degradation was continuously monitored over 35 min under a video-equipped Zeiss OPMI-1 (Carl Zeiss, Oberkochen, Germany) operating microscope. Both femoral veins were used in each experiment and were subjected to the same procedure: one to establish a control value in the absence of test substance and the second to quantitate any effect of the drug. Piracetam was injected as a bolus via a jugular vein cannula, 15 min before induction of trauma to the vein in each animal.

Analysis

The videotape of the experiment was analysed off line using a dedicated automated image analysis system (Mac Vision AT1; Datacube Inc., Peabody, MA) described previously (12). Frames at 10 s intervals were analysed by measuring the brightness and surface of the digitised image of the thrombus.

First the image with maximal thrombus size was visually selected. This frame was digitised and an elliptic region was delineated that overlaid the thrombus and in addition, about 20% of the surrounding area (thrombus zone TZ). Around this TZ, a second concentric reference zone (RZ) was delineated. The two ellipses were stored in an overlay screen and were used for the evaluation.

The respective areas of the TZ and RZ (expressed as number of pixels, NTZ and NRZ) and their respective mean intensity (average pixel grey value, XTZ and XRZ) were determined. To account for differences in mean intensity between the TZ and RZ in the absence of a thrombus, the XRZ values were corrected by A which is the difference between XRZ and XTZ as determined from the last 10 frames of the experiment, when the thrombus had completely disintegrated. To correct for variability due to differences in vessel size or disintegration. To correct for variability due to differences in vessel size or brightness) is a measure of to -

\[ T = \text{NTZ} \times \frac{(\text{XTZ} - (\text{XRZ} + A))}{(\text{XRZ} + A)} \]

The total thrombus light intensity (surface \times brightness) is a measure of total thrombus size and has been found to correlate with the number of platelets incorporated (12).

In Vitro Studies on Rat Platelets

Aggregation in PRP

Blood was drawn from adult rats (250-300 g) after 12 h fasting. Blood was drawn into syringes containing 1 tenth volume 4% trisodium citrate. Platelet-rich (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation for 12 min at 150 \times g or for 12 min at 1,500 \times g respectively. The PRP platelet count was adjusted to 250,000-300,000/ml by dilution with PPP. Aggregations were performed by the turbidimetric method in an Elvi 840 Dual channel aggregometer (Elvi, Milan, Italy). For in vitro experiments GABA (Sigma Chemical Co., St. Louis, MO), collagen (Hormon Chemie, München, Germany) and ristocetin (Lundbeck, Copenhagen, Denmark).

Aggregation on PRP, preincubated for 15 min with either 200 mM GABA, 5, 10, 20, 100 or 200 mM piracetam was determined using the same routines as for the rat platelet experiment. Results are expressed in percent of maximal amplitude (difference in light transmission between PRP and PPP).

Aggregation in Whole Blood

Blood was collected as above and aliquots of 1 ml were transferred to poly-styrene cuvettes and a teflon coated magnetic stirrer was added. Whole blood shear stress induced aggregation was obtained by stirring at various stirring speeds for 4 min. Aliquots of 40 \mu l were transferred into 10 ml of cooling solution. To prevent disaggregation of small platelet clumps during handling, 0.47% formaldehyde was added to the cooling solution. Blood samples were preincubated for 15 min with 200 mM GABA (controls) or 200, 100, 50 or 20 mM piracetam. The volume added was kept constant for all concentrations.

The whole blood platelet aggregation studies were done using the “residual single platelet count technique”, and the results express the percent decrease in residual single platelet count from starting value (21).

Fibrinogen Binding

Human fibrinogen (courtesy of Dr. Marguerie, Grenoble, France) was \(^{125}\text{I}\)-labeled using iodogen reagent (Pierce Chemical Company, Rockford, ILL) as described (22). Platelets were isolated from fresh human blood drawn into acid/citrate/dextrose by differential centrifugation followed by gel filtration in a modified Tyrode buffer (pH 7.2) containing 2% bovine serum albumin, as previously described (22). Binding of \(^{125}\text{I}\)-fibrinogen (25,000 cpn/mg) to washed platelets was performed with 10\(^{-6}\) cells/ml in the presence of 0.5 mM/l Ca\(^{2+}\), 25 mM/l ADP and piracetam at the indicated concentrations. After an incubation period of 30 min, the bound ligand was separated from the free ligand by centrifugation of 80 % aliquots of the reaction mixture through a 1% sucrose solution and was quantified by measuring the platelet associated radioactivity.

Human Ex Vivo Study

An open study with a single intravenous bolus infusion of 400 mg/kg piracetam was performed in 10 healthy male volunteers. The dose of piracetam given to each volunteer was calculated with the intention to achieve peak plasma concentrations around 5 mM piracetam.

All volunteers were tested before infusion of piracetam and 5, 150 and 300 min after the end of infusion.

Drug Infusion

An IV solution of 200 mg/ml piracetam was obtained from UCB Pharma. Each volunteer received a bolus IV infusion of 400 mg/kg in the antecubital vein. The appropriate dose for each volunteer was prepared in an IV solution bag. Blood sampling and bleeding time assessments were performed on the contralateral arm.

Aggregation Studies

Blood for platelet aggregation was collected on 1:10 vol 3.15% trisodium citrate, under free flow conditions and by separate vein puncture at each time point (0, 5, 150 and 300 min). PRP and PPP were prepared as described above. The PRP platelet count was adjusted to 250,000-300,000/ml by dilution with PPP. Aggregations were induced with ADP or U46619 (9,11-dideoxy-11-epoxy-methano-PGF\(_{2\alpha}\), Sigma Chemical Co.,}
Bleeding Times

Bleeding times were performed before, 5 and 300 min after the end of the IV infusion of piracetam. To this purpose, an automated template device (Simplate II, General Diagnostics, Morris Plains, NJ) was used. Transverse incisions were made in a standardised manner in the proximal ventral forearm of the non-infused arm. All bleeding times were determined by the same operator.

Coagulation Studies

PT and APTT were measured using the hospital standard techniques.

Statistical Methods

Results are given as mean ± standard error of the mean unless otherwise specified. Student’s t-test is used for comparison of unpaired or paired samples as appropriate whereas multiple variance analysis was used for the ex vivo study in human volunteers. Results were considered to be statistically different when p < 0.05.

Results

In Vivo Experiments in Rats and Hamsters

Thrombus formation in 10 rats was induced in the left femoral vein, before drug administration, and in the right femoral vein, 15 min after bolus injection of 200 mg/kg piracetam. The growth and disappearance of the thrombus was continuously followed for 30 min and analyzed every 10 s (Fig. 1).

Both the maximum size or maximum thrombus volume and the "total" thrombus volume formed during the 30 min observation period (area under the curve) expressed in arbitrary light units were significantly (p < 0.05) reduced after piracetam administration, whereas also the time from clamp release needed to reach maximal thrombus size (T_max) was shorter in the animals treated with piracetam (Table 1). The time from maximal thrombus size to 50% disappearance (T_1/2) was not altered by piracetam. The average peak plasma concentration at the moment of vessel trauma was 6.2 ± 1.1 mM as determined in a separate series of 6 rats.

The dose-dependency of the antithrombotic effect of piracetam was studied in hamsters (Fig. 2) and confirmed the antithrombotic action of piracetam, which already could be observed at a dose of 50 mg/kg, and which was significant at doses exceeding 100 mg/kg. Further handling of the data allowed to calculate an IC_{50} of 68 ± 8 mg/kg.

In Vitro Studies on Rat Platelets

Aggregation in PRP

Piracetam dose-dependently inhibited the activation-dependent aggregation (ADP), with an IC_{50}-value of 120 mM and nearly complete inhibition at 200 mM, whereas no such effects were seen with 200 mM GABA (Fig. 3).

In Vitro Studies on Human Platelets

Aggregation in PRP

Piracetam dose-dependently inhibited activation-dependent human platelet aggregation induced by threshold concentrations of ADP, U46619, collagen, with respective IC_{50}-values of 59 mM, 26 mM and 30.5 mM respectively. Ristocetin-induced vWF-dependent platelet agglutination was only marginally affected by piracetam.

Aggregation in Whole Blood

Piracetam inhibited stirring-induced platelet aggregation in whole blood with an IC_{50} of 44 mM (Fig.5).

Fibrinogen Binding

In view of the effect of piracetam on platelet activation-dependent aggregation induced by a range of inducers, it seems likely that piracetam acts on a central step in platelet activation, such as cAMP-forma-
tion or fibrinogen-binding to GPIIb/IIIa. Whereas no effects of piracetam on platelet cAMP-levels could be detected (results not shown), binding studies using $^{125}$I-fibrinogen on the other hand revealed that piracetam dose-dependently inhibited the binding of fibrinogen to ADP-activated platelets with an IC$_{50}$ of 44.5 mM (Fig. 6).

**Human Ex Vivo Study**

**Aggregation Studies**

ADP-induced activation was not affected after IV administration of 400 mg/kg piracetam. U46619-induced aggregations were inhibited at threshold concentrations of U46619, but not at double threshold concentration (Table 2).

**Bleeding Times**

Five min after the end of the infusion the bleeding time was significantly prolonged from 349 ± 23 s to 419 ± 16 s ($p = 0.007$). The bleeding times were again within normal limits 5 h after the end of the infusion (Table 2).

**Table 2** Effects of piracetam on hemostatic parameters in human volunteers

<table>
<thead>
<tr>
<th>time</th>
<th>Before</th>
<th>5 min</th>
<th>150 min</th>
<th>300 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP 1x threshold</td>
<td>56.6±4.7</td>
<td>49.1±6.2</td>
<td>60.9±6.8</td>
<td>54.5±8.9</td>
</tr>
<tr>
<td>2x threshold</td>
<td>56.5±2.9</td>
<td>60.9±5.3</td>
<td>61.6±5.6</td>
<td></td>
</tr>
<tr>
<td>U46619 1x threshold</td>
<td>82.2±4.7</td>
<td>21.9±0.4</td>
<td>29.3±8.8</td>
<td>31.0±12.2 *</td>
</tr>
<tr>
<td>2x threshold</td>
<td>78.5±4.6</td>
<td>83.7±2.9</td>
<td>83.0±2.9</td>
<td></td>
</tr>
<tr>
<td>Bleeding time (s)</td>
<td>349±23</td>
<td>419±16 *</td>
<td>388±20</td>
<td></td>
</tr>
<tr>
<td>APTT (s)</td>
<td>29.8±0.9</td>
<td>29.8±0.9</td>
<td>28.1±0.4</td>
<td></td>
</tr>
<tr>
<td>PT (s)</td>
<td>95.9±3.2</td>
<td>89.5±2.8</td>
<td>97.9±4.4</td>
<td></td>
</tr>
</tbody>
</table>

Effect of piracetam on ADP and U46619-induced aggregation (maximal amplitude), bleeding time, coagulation tests and plasma levels of piracetam (mM) in healthy volunteers. Measurements were made before and 5, 150 and 300 min after IV infusion of 400 mg/kg piracetam ($n = 10$, mean ± SEM), *$p < 0.05$.
Coagulation Studies

The tested coagulation parameters were not affected by the infusion of piracetam.

Discussion

In this study, various effects of the GABA-derivative piracetam on hemostasis and thrombus formation were studied in rats, hamsters and human volunteers.

The animal experiments indicated that piracetam exerts an inhibitory effect on the size of platelet-rich thrombus formation after vessel wall trauma.

Since in this model the thrombus that is formed mainly consists of platelets (12), our first hypothesis to explain the observed antithrombotic effect was to look for an anti-platelet effect. Piracetam indeed was able to inhibit in vitro rat platelet aggregation dose-dependently; however, concentrations at least tenfold higher than the ones obtained in vivo, were required. Furthermore, no ex vivo effect of piracetam on aggregation of rat platelets could be observed.

Also for human platelets 25-44 mM was needed to cause a 50% inhibition of aggregations induced by a series of activators such as ADP, U46619 or collagen. Ristocetin-induced platelet agglutination, which occurs independently of platelet activation or GPIIb/IIIa involvement, was much less affected. Since this may indicate that the drug acts on a central mechanism of platelet aggregation, e.g. by increasing the cAMP-levels or inhibiting the fibrinogen receptor, we specifically studied these functions. Although we found no evidence that piracetam affects platelet cAMP-levels, the drug dose-dependently prevents the binding of fibrinogen to activated platelets. Further investigation will be needed in order to clarify if piracetam directly or indirectly competes with fibrinogen for activated GPIIb/IIIa or if the drug prevents activation of GPIIb/IIIa by e.g. an action on the platelet membrane fluidity.

Platelet aggregation occurs when whole blood is stirred, presumably because of release of ADP from damaged erythrocytes (21). Since piracetam has been shown to influence blood rheology by increasing red cell compliance (6-8), we hypothetized that the more deformable erythrocytes would also be less vulnerable and less likely to release ADP. The antiaggregatory effect of piracetam would hence to some extent be erythrocyte-mediated. However, since the stirring-induced aggregation in whole blood is only inhibited at relatively high plasma concentrations, the hypothesis appears not to be valid.

Based upon our findings, we conclude that the antithrombotic capacity of piracetam is mediated through factors which do not operate in vitro. This could occur with a direct effect on the vessel wall or plasma contents, e.g. by reducing blood viscosity as a consequence of lowering fibrinogen and/or von Willebrand factor levels (9). The reported long-lasting (up to 8 h) reduction of any of these factors could explain the weak ex vivo inhibition of U46619-induced human platelet aggregation for up to 5 h after infusion, and also the slight increase in bleeding time that piracetam caused in the human volunteers.

In conclusion, we have observed that piracetam has an antithrombotic effect in the model studied. However, at present we have not been able to define the exact mechanism by which piracetam is exerting its action. The apparent central inhibition of platelets with high doses of piracetam merits further study, since a better definition of its mode of action may allow for a focused screening for more active derivatives.

On the other hand, since piracetam itself, at levels in the pharmacological range, is antithrombotic in our model, it can be extrapolated that also in certain clinical thrombotic events a similar beneficial action may be expected.

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References


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