Temporal Effects of Thrombolytic Agents on Platelet Function In Vivo and Their Modulation by Prostaglandins

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To examine the temporal effects of plasmin generated in vivo on platelet function, we infused tissue-type plasminogen activator (t-PA) in rabbits over 3 hours and measured ex vivo platelet aggregation. We noted an initial increase in the aggregation response to ADP occurring 30 minutes after the start of infusion. This enhanced response was short-lived and by 180 minutes was reduced, compared with pretreatment levels. Baseline aggregation response was restored by 240 minutes. This pattern of aggregation response to t-PA infusion was also seen with thrombin as the agonist. Coinfusion of either prostaglandin I_2 or prostaglandin E, abolished the initial hyperaggregable phase induced by t-PA; the hypoaggregable phase occurred earlier (after 60 minutes) and persisted throughout the 1-hour recovery period. Similarly, streptokinase infused for 1 hour also increased platelet aggregation at early times and then reduced aggregation responses after the first hour. Plasma plasmin activity increased as expected with t-PA infusion alone, peaking at 30 minutes and returning to baseline by the first hour. Interestingly, prostaglandin E, blunted the rise in plasma plasmin activity. This same dose of prostaglandin E, or prostaglandin I_2 used alone did not appreciably alter platelet function at any time during the experiment. Our data show that therapeutic doses of t-PA or streptokinase produce a biphasic effect on platelet aggregation response in the rabbit. Coinfusion of either of the antiplatelet agents, prostaglandin E, or prostaglandin I_2, abolishes the hyperaggregable phase and prolongs the inhibitory effects on platelet aggregation produced by t-PA. These data suggest that the effects of thrombolytic agents on platelet function are complex and can be modulated by antiplatelet prostaglandins. (Circulation Research 1990; 67: 1175–1181)

Although several studies have examined the effects of plasminogen activation on platelets, effects in vivo remain controversial. There are reports that demonstrate enhancement\(^1\)\(^-\)\(^5\) and inhibition\(^1\)\(^\;\)\(^6\) of platelet aggregation, as well as disaggregation of platelets.\(^7\) Niewiarowski and colleagues\(^1\) and Schafer and coworkers\(^2\)\(^-\)\(^6\) reported in vitro evidence that explains the apparent discrepancies among other studies, namely, that the effect of plasmin on platelet function is dose and time dependent. Low doses of plasmin (<1 caseinolytic unit [CU]/ml) and long incubation times attenuate aggregation, whereas high doses (>1 CU/ml) enhance the aggregation response. Measurements of platelet function after exposure to plasmin have been reported by several groups,\(^1\)\(^3\)\(^-\)\(^5\) but none of these studies provides information on temporal changes in platelet function generated during the course of treatment with plasminogen activators. For these reasons, we examined the effects of tissue plasminogen activator (t-PA) or streptokinase infusion on platelet aggregation as a function of time and attempted to correlate these effects with generated plasma plasmin activity (PPA). Additionally, since we\(^8\)\(^,\)\(^9\) as well as others\(^10\) have previously shown that the action of t-PA on clot lysis and disaggregation of platelets can be enhanced with antiplatelet agents, we examined the effects of coinfused antiplatelet prostaglandins on platelet responses over time.

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Materials and Methods

Animal Preparation and Protocol

Female New Zealand White rabbits (2–4 kg) were initially anesthetized using ketamine (30–50 mg/kg). Pentobarbital (30–50 mg/kg) was subsequently given as a sustaining anesthetic. After induction of anesthesia, the rabbits were surgically prepared for femoral artery and venous cannulation. Approximately 1 hour later, an infusion of t-PA, prostaglandin E\(_1\) (PGE\(_1\)) or prostaglandin I\(_2\) (PGI\(_2\)) alone, t-PA plus PGE\(_1\) or PGI\(_2\), or saline was begun. t-PA was infused at a rate of 0.86 mg/kg/hour for the first hour of infusion and at 0.29 mg/kg/hr for the remaining 2 hours. This dose regimen of t-PA was chosen to mimic that given clinically. PGE\(_1\) and PGI\(_2\) were given at a dose of 1.2 \(\mu\)g/kg/hr for the entire 3-hour period. These doses of t-PA and PGE\(_1\)/PGI\(_2\) were coin infused in experiments examining the effect of combination treatment. In other experiments, streptokinase (40,000 units/kg/hr) was given in place of t-PA. Because rabbits are more resistant to streptokinase than many other mammals, we found this dose to give more reproducible changes in plasmin activity. The rabbits were monitored for an additional 1 hour after the 3-hour infusion period, at which point additional blood samples were obtained. In some rabbits, vessels were tied off, and the surgical sites were sutured; these were housed overnight for blood sampling from the contralateral femoral artery the following day. This was performed to determine whether platelet function was similar to that measured before treatment.

Blood samples (4 ml) were collected in syringes containing a final concentration of 13 mM trisodium citrate and 1 \(\mu\)M D-phenyl-L-prolyl-L-arginine chloromethylketone, a serine protease inhibitor, at 0, 1, 5, 10, 30, 60, 120, 180, and 240 minutes after the start of the infusion. This concentration of inhibitor was chosen as one that inhibits further t-PA activity while not affecting plasmin activity. An additional 2 ml blood was withdrawn into a separate tube containing a final concentration of 13 mM trisodium citrate at 0, 60, and 180 minutes.

Platelet Aggregation

Platelet-rich plasma was prepared by centrifugation of whole blood at 138g for 10 minutes at 22°C. The top two thirds of the platelet-rich plasma was removed for determination of platelet counts and aggregation responses. Platelet counts were determined with a Coulter counter (model ZM, Coulter Electronics, Inc., Hialeah, Fla.). Platelet-poor plasma was obtained by centrifuging the remaining blood after platelet-rich plasma removal at 1,240g for 10 minutes.

Platelet-rich plasma was diluted with platelet-poor plasma to give a final concentration of 1.0x10\(^2\) platelets/\(\mu\)l. Platelet aggregation\(^{12}\) was induced with 0.05 mM ADP or with thrombin (2.5 units/ml) plus \(H\)-glycyl-L-prolyl-L-arginyl-L-proline (1.5 mM final concentration) to prevent fibrin polymerization. Aggregation was monitored at 37°C while stirring (900 rpm), using a dual-channel aggregometer (Payton Associates, Inc., Buffalo). Aggregation was quantified by measuring the maximal rate of change in light transmittance. All values are normalized to those rates obtained before infusing any drug or combination of drugs.

Assays

**Plasma plasmin activity.** PPA was measured using a chromogenic substrate method.\(^{13}\) Plasma (50 \(\mu\)l) was incubated at 37°C with 0.8 mM \(H\)-d-valyl-L-leucyl-L-lysine-\(p\)-nitroanilide (S2251) (Kabi Vitrum, Stockholm) in 24-well microtiter plates. Optical density was read at 410 nm on a MiniReader II spectrophotometer (Dynatech Laboratory, Inc., Chantilly, Va.) at various time points during the incubation. Activity was calculated as change in absorbance per hour.

**Tissue-type plasminogen activator antigen.** Plasma levels of t-PA were measured using an enzyme-linked immunosorbent assay method (Diagnostica Stago, Paris).

Drugs and Reagents

t-PA was obtained from Genentech, Inc., South San Francisco. PGE\(_1\) and PGI\(_2\) were kindly provided by the Upjohn Co., Kalamazoo, Mich. All other reagents were of standard reagent grade or better.

Statistical Analysis

All data within groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s or Duncan’s multiple-comparison tests where appropriate. Data between groups were analyzed by two-way ANOVA\(^ {14}\) or by one-way ANOVA and Newman-Keuls or Duncan’s multiple-comparison test. Values are given as mean±SEM. Values of \(p<0.05\) were considered statistically significant.

Results

Platelet Aggregation

The time course of the ex vivo platelet aggregation response to ADP during t-PA infusion in rabbits is shown in Figure 1. Infusion of t-PA (Figure 1B) caused an initial increase in platelet aggregation to ADP at 30 minutes (\(p<0.05\) compared with pretreatment levels) and a later attenuation of the aggregation response at 180 minutes (\(p<0.05\) compared with pretreatment values). A biphasic response was also noted using thrombin as the agonist (\(n=3\); however, the hypoaggregable phase appeared to occur sooner (Figure 2; two-way ANOVA, \(p<0.05\)). Streptokinase infusion (\(n=3\)) produced a similar biphasic platelet aggregation response not significantly different from that noted with t-PA (Figure 3; two-way ANOVA, \(p>0.05\)).

When t-PA and PGE\(_1\) were coin infused (Figure 1D), only reduced aggregation responses were observed, beginning at 60 minutes and lasting until 240 min-
FIGURE 1. Graphs showing time course of effect of saline (panel A, n=5), tissue-type plasminogen activator (panel B, n=6), prostaglandin E₁ (panel C, n=7), and tissue-type plasminogen activator plus prostaglandin E₁ (panel D, n=6) infusion in rabbits on ex vivo platelet aggregation, using ADP (0.05 mM) as the agonist. Tissue-type plasminogen activator was infused at 0.86 mg/kg/hr for the first hour and then at 0.29 mg/kg/hr for the second and third hours; prostaglandin E₁ was infused at 1.2 μg/kg/hr. *p<0.05 vs. control period using analysis of variance and Dunnett's test.

FIGURE 2. Graph showing time course of effect of tissue-type plasminogen activator infusion in rabbits on ex vivo platelet aggregation using either ADP (0.05 mM; n=7) or thrombin (2.5 units/ml; n=6) as agonist.

utes. When coinfused with t-PA, PGE₁ completely abolished the initial hyperaggregable phase produced by t-PA infusion alone (Figure 1 and Figure 4; p<0.05). Additionally, infusion of PGE₁ and t-PA caused the hypoaggregable phase to occur earlier, reducing the aggregation response by approximately 40% at 60 minutes (Figure 4; p<0.05). Attenuated aggregation lasted only 1 hour in the t-PA group and returned to normal by 240 minutes; however, aggregation remained significantly reduced 1 hour after infusion in the rabbits treated with both t-PA and PGE₁ (Figure 4; p<0.05). PGI₂ produced responses similar to PGE₁ (Figure 4; two-way ANOVA, p>0.39).

The aggregation response pattern with thrombin was slightly different as compared with ADP when PGE₁ was coinfused with t-PA (Figure 5). PGE₁ still prevented the hyperaggregable phase, but the hypoaggregable phase occurred in the first hour and was short-lived, lasting only 30 minutes (two-way ANOVA, p<0.05).

FIGURE 3. Graph showing time course of effect of tissue-type plasminogen activator (t-PA) (n=7) or streptokinase (SK) (n=5) infusion in rabbits on ex vivo platelet aggregation using ADP (0.05 mM) as the agonist. t-PA was infused at 0.86 mg/kg/hr for the first hour and at 0.29 mg/kg/hr for the second and third hours; SK was infused at 40,000 units/kg for 1 hour.
Coinfusion of streptokinase and PGE₁ (Figure 6) similarly resulted in the abolition of the early hyper-aggregation response of streptokinase as seen within t-PA and PGE₁. As with t-PA, streptokinase plus PGE₁ (Figure 7) began to show a reduction in aggregation as early as 60 minutes, although it was not as pronounced as the reduction seen within t-PA and PGE₁. Aggregation responses in the control groups, that is, saline (Figure 1A), PGE₁ alone (Figure 1C), and PGI₂ (data not shown), remained unchanged throughout the experimental period.

Platelet aggregation at the 24-hour point was studied in the saline, PGE₁, t-PA, and t-PA plus PGE₁ groups, using ADP. The response at this point was similar to the pretreatment response in all four groups (one-way ANOVA and Dunnett’s test).

Plasmin Activity

Figure 8 shows the time course of PPA during infusion of t-PA (panel B), PGE₁ (panel C), both (panel D), and neither (panel A). There was essentially no change in PPA in the two control groups (panels A and C) throughout the experiment. As expected, t-PA infusion increased PPA, which peaked 30 minutes after the start of the infusion. PPA remained slightly elevated at 60 and 120 minutes but returned to baseline by 180 minutes. Interestingly, coinfusion of PGE₁ and t-PA (panel D) prevented the peak increase in PPA. To determine if this were the result of reduced plasma levels of t-PA,
we also measured plasma t-PA antigen in selected rabbits with the most striking comparative differences. We found that t-PA was 645 ± 148 pg/ml (n = 4) and 637 ± 238 pg/ml (n = 3) at 1 and 3 hours, respectively, in rabbits treated with t-PA. In contrast, in rabbits treated with t-PA and PGE₁ (n = 5), these values were 446 ± 104 and 370 ± 107 pg/ml at 1 hour and 3 hours, respectively (p < 0.3 for t-PA versus t-PA plus PGE₁ at both time points). These results suggest that PGE₁ may increase clearance of t-PA perhaps by increasing hepatic blood flow; however, with this small number of animals, our results did not achieve statistical significance. Importantly, no change in mean arterial pressure was detected in any of the rabbits treated with t-PA or t-PA plus PGE₁ throughout the experimental protocol.

**Platelet Count**

Platelet count did not change significantly in any t-PA groups using ADP as the agonist nor in any of the streptokinase groups.

**Discussion**

Our results show that infusion of t-PA or streptokinase in rabbits causes a biphasic platelet aggregation response. The initial hyperaggregable effect peaked at a time when PPA was greatest, approximately 30 minutes after the start of the infusion. As PPA returned toward baseline, the platelet aggregation response also returned to baseline. However, a hypoadgregable phase occurred at a time by which PPA was not essentially different from preinfusion levels.

Previous studies²,²⁶ have also shown that plasmin can cause both increased and reduced platelet aggregation responses in vitro. The fact that enhanced...
platelet aggregation occurred when PPA peaked is consistent with the data of other studies that demonstrate enhanced aggregation with high doses of plasmin. Estimates of PPA at the peak response yield approximately 0.8 CU/ml, consistent with the observation of Schafer and coworkers in in vitro studies showing that a hyperaggregative response was noted with approximately 1 CU/ml plasmin. The mechanism by which hyperaggregation occurs is thought to involve increases in cytosolic Ca++ concentration by one or more mechanisms: Plasmin may simply cause the release of Ca++ from intracellular stores or increase transport across the plasma membrane by altering membrane permeability and Ca++ channel function. Alternatively, it may alter phosphatidylinositol metabolism and hence intracellular Ca++ levels.

The second phase of attenuated aggregation occurred much later, at a time by which PPA was reduced to preinfusion levels. In support of these observations, Foltz and colleagues showed in a dog model that in vivo platelet aggregation was reduced by streptokinase infusion at pharmacological doses. In contrast, Ohlstein and coworkers failed to observe a reduction in platelet aggregation responses with t-PA infusion in rabbits, noting only increased aggregation. However, these investigators infused high doses of t-PA (up to 30 μg/kg/min) for brief periods (15 minutes) in their protocol. Schafer and Adelman reported that low doses of plasmin (<1.0 CU/ml) inhibited platelet aggregation possibly by blocking arachidonic acid release from membrane phospholipids. It has also been suggested that plasmin may inhibit aggregation by causing fibrinolysis and cleavage of the platelet glycoprotein receptor (GP Ib/IIIa) for fibrinogen binding.

Platelets have been shown by us and by others to play an important role in the efficiency of thrombolysis. Our laboratory has previously shown that PGE1 can dramatically reduce the time by which thrombolysis occurs with t-PA infusion, using a rabbit venous model, thus showing at least functional synergism between PGE1 and t-PA. In addition, our group has recently shown true pharmacological synergism between t-PA and PGE1 in vitro as effectors of platelet disaggregation in plasma. This in vivo study shows that PGE1 can eliminate the initial hyperaggregable platelet response to t-PA and cause the later hyperaggregable response to occur sooner as well as to persist for a longer time. These data suggest 1) the existence of synergism between t-PA and PGE1 in thrombolysis and 2) the importance of platelet responses in resistance to lysis or in ongoing thrombosis during lysis. Clinical data from the ISIS-2 trial support this latter point as well.

The mechanism by which PGE1 and PGI2 modulate the effect of plasmin on platelet function is not totally clear. Blockade of the early stimulatory phase of platelet aggregation by plasmin may simply be the result of the low PPA, probably brought about by increased hepatic clearance of t-PA or renal clearance of streptokinase. In addition, PGE1 and PGI2 can directly inhibit platelet aggregation by increasing cyclic AMP levels. Therefore, the earlier onset and prolonged duration of platelet inhibition with the combination of t-PA and PGE1 or PGI2 may be the result of the increasing cyclic AMP effect of both prostaglandins, which reduces aggregation responses, and of the inhibition of arachidonic acid mobilization at low doses by plasmin, which also attenuates aggregation. It is unclear why, when using thrombin as an agonist of platelet activation, PGE1 did not enhance the inhibitory phase of platelet aggregation as seen with ADP. Perhaps the greater potency of thrombin as an agonist of aggregation may be a possible explanation.

An important problem encountered during thrombolytic therapy in coronary artery disease is reocclusion. Gold and colleagues recently reported that reocclusion after coronary thrombolysis was dependent on platelet responses. Prolonged inhibition of platelet function, particularly during the critical window in time during which platelet responses are increased, may prevent or minimize reocclusion. Preliminary data in a dog arterial model in which t-PA was used with an antiplatelet prostaglandin (iloprost, a congener of PGI2) support this view. Administering PGE1 or PGI2 may be useful in this regard, since platelet activation is prevented and platelet inhibition is prolonged even when the infusion has ceased.

In summary, t-PA used in the rabbit at doses equivalent to those used in the treatment of coronary artery thrombosis in humans causes an early stimulatory and later inhibitory response in platelet aggregation corresponding to times during which PPA is high and low, respectively. Both responses last approximately 1 hour. Concomitant infusion of PGE1 abolishes the early stimulatory phase and causes the inhibitory phase to occur sooner and last longer. Thus, these data, in conjunction with our previous studies, suggest that combination treatment with t-PA and an antplatelet prostaglandin may enhance rates of thrombolysis by blunting platelet-mediated ongoing thrombosis, reduce the dose of t-PA necessary to achieve effective lysis, and minimize platelet-mediated reocclusion. Dosing and timing of antiplatelet prostaglandins must, however, be used judiciously to optimize benefits of effective thrombolysis and minimize bleeding risk.

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