Effects of Aspirin and Prostaglandin E1 on In Vitro Thrombolysis With Urokinase
Evidence for a Possible Role of Inhibiting Platelet Activity in Thrombolysis

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The formation of thrombosis in vivo includes the activation of both platelets and the coagulation cascade. Conventional thrombolytic therapy is primarily directed toward the dissolution of fibrin. To evaluate the possibility that platelet activity impairs the lysis of thrombi, we studied the effects of aspirin and platelet-deaggregating prostaglandin E1 on thrombolysis with urokinase. Combined platelet and fibrin thrombi were produced in vitro by adding CaCl2 and collagen (1 μg/ml) to citrated platelet-rich plasma (250,000 platelets per μl). Urokinase (500–10,000 units/ml) caused a dose-dependent weight loss of the thrombi that was maximal at 2,000 units/ml. The addition of aspirin (10–200 μg/ml) to platelet-rich plasma before thrombus formation markedly enhanced thrombolysis with urokinase. This effect was most pronounced at 20 μg/ml aspirin. However, when aspirin was added after completion of thrombus formation, no significant effect on thrombolysis was noted. Prostaglandin E1 (1–100 μmol/l) improved the lysis with urokinase of the combined platelet and fibrin thrombi. This effect was maximal at 20 μmol/l prostaglandin E1. When pure fibrin thrombi were produced in platelet-free plasma, prostaglandin E1 was without effect on lysis. Thus, in vitro lysis with urokinase of combined platelet and fibrin thrombi was enhanced by the addition of platelet-deaggregating prostaglandin E1 and by pretreatment with aspirin. (Circulation 1989;79:1309–1314)

Thrombus formation in vivo is the result of a complex interaction between blood components and the vessel wall. It includes the activation of both platelets and the coagulation system. Conventional thrombolytic therapy is primarily directed toward the dissolution of fibrin, the final product of the coagulation cascade. In an in vitro model of combined platelet and fibrin thrombus formation, we studied the effects of aspirin and prostaglandin E1 on thrombolysis with urokinase. Aspirin inhibits platelets by irreversible acetylation of platelet cyclooxygenase. Prostaglandin E1 is a platelet inhibitory compound and has the additional capacity of deaggregating aggregated platelets. These actions of prostaglandin E1 are generally thought to be mediated by an elevation of platelet cyclic AMP levels.

Methods
The experiments were conducted on blood from 20 apparently healthy and nonsmoking volunteers, 12 men and eight women, with a mean age of 25.2 years (range, 22–29 years). All claimed not to have taken any drugs during 14 days before blood collection. For each experimental condition, 10–13 series of experiments were performed on blood from 10–13 donors. The blood was collected from a large antecubital vein through a needle of 1.1-mm internal diameter into a plastic syringe. Nine parts of blood were mixed in the syringe with one part of 3.13% Na-citrate.

The blood was centrifuged at 300g for 10 minutes. After removal of the platelet-rich plasma, the remaining blood was centrifuged at 2,500g for 10 minutes. Platelet-free plasma was produced by centrifugation of the supernatant plasma at 8,000g for another 5 minutes. Platelets in platelet-rich plasma were counted under a light microscope, and platelet-rich and platelet-free plasma were mixed to achieve a final count of 250,000 platelets/μl.

Aliquots of 500 μl of this standardized platelet-rich plasma were incubated for 5 minutes at 37°C in
an aggregometer (APACT, Labor, Hamburg, FRG). In the experiments on the influence of aspirin added before thrombus formation, 50 μl aspirin in isotonic NaCl (final concentrations, 10–200 μg/ml) or vehicle alone was added. Then, a stirrer was started at 1,200 rpm, and 50 μl CaCl₂ solution (final concentration, 20 mmol/l) and 50 μl collagen in isotonic glucose buffer (final concentration, 1 μg/ml; Hormon Chemie, Munich, FRG) were added. After 4–9 minutes, thrombus formation occurred around the microstirrer.

After 10 minutes of aging, the thrombus was weighed for the first time. Weighing was easily done with the help of a magnetic microstick by which the microstirrer with the thrombus could be removed from the cuvette. At the same time, the baseline light transmission of the plasma was registered. The light transmission of platelet-free plasma (plus appropriate quantities of the solvents of the added substances) was defined as 100%, and the light transmission of platelet-rich plasma (250,000 platelets/μl) was defined as 0%. Thrombolysis was started by adding 50 μl urokinase solution in double-distilled water (final concentrations, 500–10,000 units/ml; Medac, Hamburg, FRG) plus 50 μl prostaglandin E₃ (final concentrations, 1–100 μmol/l; Sigma Chemical, St. Louis, Missouri) or aspirin (final concentrations, 10–200 μg/ml) in isotonic NaCl or vehicle, where appropriate. Thrombus weight and plasma light transmission were recorded again after 30 minutes of thrombolysis.

For histologic examination, 10-minute old thrombi were fixed in 3.7% formaldehyde, embedded in paraffin, sectioned, and stained. Light microscopy revealed that the thrombi had one or more smaller cores of platelet aggregates with small amounts of fibrin between. These cores were surrounded by a somewhat larger portion consisting of fibrin only. Examination of the plasma in which the thrombi had been produced revealed that, by thrombus formation, more than 99% of the platelets and 98–99% of fibrinogen (fibrinogen kinetic test; Boehringer Mannheim, Mannheim, FRG) had been removed. No differences either in histologic composition or in the changes in platelet count or plasma fibrinogen levels were noted when the thrombi were formed in the presence or absence of aspirin.

In the experiments on thrombolysis in platelet-free plasma, thrombi were produced in 500 μl platelet-free plasma by adding 50 μl CaCl₂ and 50 μl suspension of washed homogenized human platelets and kaolin (PTT reagent; Behringwerke, Marburg, FRG). Thrombolysis was carried out as outlined above. Because lysis of the thrombi produced in platelet-free plasma was almost complete after 30 minutes, an intermediate measurement of thrombus weight and plasma light transmission was performed after 10 minutes of lysis.

Histologic examination revealed that the thrombi produced in platelet-free plasma were pure fibrin thrombi. After thrombus formation, the levels of fibrinogen in the plasma surrounding the thrombus had decreased by 98–99%.

Statistics

Statistical comparisons were performed with Student’s t tests for paired or for unpaired data. A p value of 0.05 or less was considered to be significant. All values indicated are mean±SEM.

Results

The thrombi (n=308) formed in platelet-rich plasma (250,000 platelets/μl) had a mean weight of 20.6±0.2 mg. In the control experiments (n=35 in the three series displayed in Figures 1, 3, and 4) in which no active compounds were added to the thrombi, thrombus weight during 30 minutes decreased to 70.4±1.3% of baseline. This decrease in thrombus weight was associated with almost no change in the light transmission of the plasma surrounding the thrombi (–0.6±0.2%)

The in vitro application of urokinase (500–10,000 units/ml) caused dose-dependent thrombolysis (Figure 1). The weight loss of the thrombi reached a maximum at 2,000 units/ml urokinase. Thrombolysis with
urokinase was associated with a decrease in the light transmission of the plasma surrounding the thrombi. However, although the effect of urokinase on thrombus weight was maximal at 2,000 units/ml, plasma light transmission further declined when the concentrations of urokinase exceeded that value.

When aspirin (10–200 μg/ml) was added to platelet-rich plasma before thrombus formation, the mean thrombus weights at baseline for the different concentrations of aspirin ranged between 19.6±0.6 and 20.6±0.8 mg and were, thus, identical to the value of 20.4±1.0 mg in the control experiments without aspirin in the same persons at the same blood collections. However, pretreatment of the plasma with aspirin markedly enhanced thrombolysis with urokinase (2,000 units/ml, Figure 2). The effect of aspirin pretreatment on thrombus weight was significant at concentrations of 10–100 μg/ml, and plasma light transmission decreased significantly more after aspirin at 10–50 μg/ml than in the respective control experiments without aspirin. The effect of pretreating the plasma with aspirin both on thrombus weight and plasma light transmission was maximal at a concentration of aspirin of 20 μg/ml. After 20 μg/ml aspirin, thrombus weight after 30 minutes of thrombolysis with urokinase was by 47.3±5.0% lower than in the control experiments without aspirin (p<0.0001). Plasma light transmission decreased by a mean of 25.6±1.7% with aspirin compared with 20.1±1.5% in the control experiments (p<0.01).

When aspirin was added to the plasma after completion of thrombus formation, thrombolysis with urokinase was not significantly affected (Figure 3). This was true for all concentrations of aspirin tested (10–200 μg/ml) and for its effect on both thrombus weight and light transmission of the plasma (Figure 3).

Prostaglandin E₁ added to urokinase (2,000 units/ml) improved thrombolysis (Figure 4). Compared with the control experiments with urokinase alone, thrombus weight after 30 minutes of lysis was significantly reduced by concentrations of prostaglandin E₁ of 5–20 μmol/l. The light transmission of the plasma surrounding the thrombus undergoing lysis decreased significantly more at all concentrations of prostaglandin E₁ tested (1–100 μmol/l). The maximal effect on both thrombus weight and plasma light transmission occurred at a concentration of 20 μmol/l prostaglandin E₁. With 20 μmol/l prostaglandin E₁ and after 30 minutes of thrombolysis, throm-

**FIGURE 2.** Plot of effects of aspirin (10–200 μg/ml) added to platelet-rich plasma before thrombus formation on thrombus weight and plasma light transmission after 30 minutes of thrombolysis with urokinase. Values are mean±SEM of 12 experiments. *p<0.05, **p<0.01, §p<0.001, §§p<0.0001 vs. thrombolysis with urokinase alone.

**FIGURE 3.** Plot of effects of aspirin (10–200 μg/ml) added to the plasma after completion of thrombus formation on thrombus weight and plasma light transmission after 30 minutes of thrombolysis with urokinase. Values are mean±SEM of 12 experiments. No significant differences occurred between the experiments with urokinase plus aspirin and those with urokinase alone.
bus weight was by 27.2±7.0% lower than in the control experiments with urokinase alone (p<0.01), and plasma light transmission decreased by 31.5±3.0% compared with 17.4±1.5% (p<0.001).

The mean weight of thrombi produced in platelet-free plasma was 31.5±1.5 mg (n=30) and, thus, significantly higher than that of the thrombi produced in platelet-rich plasma (p<0.0001). In the control experiments (n=10) with no active compound added to the thrombi, thrombus weight measured after 30 minutes had declined to 52.5±2.1% of baseline and, thus, in absolute values had become similar to the weight after 30 minutes of the control thrombi produced in platelet-rich plasma. For the thrombi produced in platelet-free plasma, prostaglandin E₁ (20 µmol/l) did not enhance lysis with urokinase (2,000 units/ml, Figure 5).

**Discussion**

In this study we have shown that, in an in vitro model of combined platelet and fibrin thrombus formation, inhibition of platelet activity and hence the platelet part of the thrombus enhanced thrombolysis with a fibrinolytic agent. Histologically, the thrombi generated in vitro consisted of one or more smaller cores of platelet aggregates with only small amounts of fibrin between, surrounded by a somewhat larger portion of pure fibrin without platelets. They were, thus, similar to the thrombi found on autopsy in the large epicardial arteries of patients who had died from acute myocardial infarction or unstable angina.11,12 In our experiments, both the pretreatment of the plasma with platelet inhibitory aspirin and the additional application of platelet deaggregating prostaglandin E₁ improved the lysis of these thrombi with urokinase.

Interestingly, for both aspirin and prostaglandin E₁, maximal enhancement of lysis occurred at concentrations that were not the highest tested. In the literature, aspirin-induced suppression of platelet thromboxane formation and the accumulation of cAMP in platelets after prostaglandin E₁, as well as the resulting inhibition of platelet function, have been reported to be dose dependent and maximal above certain concentrations of the two agents.4-7,9,13-15 To our knowledge, there are no data to explain the apparent decrease in the supportive effect on thrombolysis seen with concentrations of both drugs that exceed the most effective concentrations in our experiments.

In our study, the most effective concentrations in vitro were 20 µg/ml for aspirin and 20 µmol/l for prostaglandin E₁. For aspirin, plasma levels of 10–30


µg/ml were reported to be attained in vivo after ingestion of 600–1,200 mg of a rapid release formulation.16–18 However, the effect of aspirin on platelets was observed not only to depend on its peak plasma concentration but also on the duration of interaction between the drug and platelets.19,20 Thus, cumulative platelet inhibition occurs in vivo with sustained presence of aspirin in the plasma leading to maximal platelet inhibition after repeated ingestion of daily doses of 100 mg or less.13,21,22 A "best dose" for aspirin to facilitate thrombolysis in vivo, thus, cannot be predicted from our in vitro studies.

For prostaglandin E₁, which is inactivated to about 70% by a single passage through the lungs,23 only very low concentrations in the nanomolar range can be attained with systemic application.24 Thus, only by direct infusion into the occluded artery do the concentrations effective in enhancing thrombolysis in vitro seem to be attainable in vivo. When infused intra-arterially, the local concentration of prostaglandin E₁ mainly depends on blood flow and on uptake by endothelial and blood cells. As a result, the "best dose" to be applied into an occluded artery also cannot be derived from our in vitro studies but must be evaluated clinically.

In our experiments when aspirin was added to the plasma after completion of thrombus formation, no significant effect on lysis was observed. Thus, the positive effect of aspirin on the mortality of patients with myocardial infarction and undergoing thrombolytic therapy25 may be explained by an inhibition of reocclusion of the vessel opened by thrombolysis26 rather than by an increase in the primary efficacy of lysis. Although no morphologic difference was noted between thrombi formed in vitro in the presence or absence of aspirin, a less stable architecture of the platelet part of the thrombi formed under the influence of aspirin may have been the cause of the increased extent of thrombus lysis with urokinase. When prostaglandin E₁ was used in lysing thrombi produced in platelet-free plasma, no effect in addition to that of urokinase was observed. Thus, the positive effect of prostaglandin E₁ was linked to the presence of platelets in the thrombus.

The different thrombolytic regimens evaluated in our study differently affected the light transmission of the plasma surrounding the thrombi undergoing lysis. A decrease in the light transmission of plasma may either be produced by an increase in the number of particles present in the plasma or by a decrease in the size of the particles with constant total particle mass. The decrease in thrombus weight seen in the experiments where no active compounds were added to the thrombi was associated with almost no change in the light transmission of the surrounding plasma. This was true for thrombi formed in both platelet-rich and platelet-free plasma. Because it did not coincide with a change in light transmission of the plasma, the decrease in thrombus weight in the control experiments during 30 minutes of storage of plasma must, thus, reflect a loss of plasma from the thrombus.

When thrombi were lysed with increasing concentrations of urokinase, plasma light transmission continuously decreased, while the reduction in thrombus weight was greatest at a concentration of 2,000 units/ml urokinase. Thus, above that concentration, urokinase, despite a decrease in its thrombolytic potential, must have produced a decrease in the size of the particles dissolved from the thrombi. In addition, by comparing the effect on thrombolysis of aspirin pretreatment to that of adding prostaglandin E₁ to urokinase, aspirin was found to be more effective than prostaglandin E₁ in reducing thrombus weight. In contrast, the effect of prostaglandin E₁ on plasma light transmission was considerably larger than that of aspirin. Thus, prostaglandin E₁ must have led to a larger decrease in the size of the particles lysed from the thrombus.

What could be the clinical implications of these results? In patients with acute myocardial infarction undergoing thrombolytic therapy, aspirin pretreatment may significantly increase the rate and extent of coronary artery reopening. In addition, thrombi generated under the influence of aspirin may more frequently be dissolved by the body’s own thrombolytic mechanisms. The effect of aspirin described here may thereby contribute to the favorable effect of aspirin on the incidence of acute myocardial infarction observed in secondary27–36 and primary prevention trials.37 The observed effects of prostaglandin E₁ in enhancing thrombolysis with urokinase should stimulate present attempts to evaluate this substance under clinical conditions.38,39 However, with both adjunctive antiplatelet approaches, an increased bleeding tendency under in vivo conditions may limit the benefit for patients.

In conclusion, focussing on the platelet part of the thrombus by either inhibiting platelets before thrombus formation or disaggregating platelets after completion of thrombus formation may be useful to further enhance the efficacy of thrombolytic therapy in vivo. In the clinical setting, however, an increased bleeding tendency could be a limitation.

References


**KEY WORDS** • deaggregation • synergism • aspirin • thrombolysis • urokinase • prostaglandin E1
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