Role of Platelet-Activating Factor in Hypotension and Platelet Activation Induced by Infusion of Thrombolytic Agents in Rabbits

Giuseppe Montrucchio, Giuseppe Alloatti, Filippo Mariano, Enrico Lupia, Pietro Greco Lucchina, Elma Musso, Giorgio Emanuelli, and Giovanni Camussi

Infusion of the thrombolytic agents streptokinase (SK, 666 units/kg per minute for 60 minutes) and tissue-type plasminogen activator (t-PA, 10 µg/kg per minute for 15 minutes) in rabbits induced a significant hypotension and decrease in platelet count that were completely prevented by treatment with platelet-activating factor (PAF) receptor antagonists SDZ 63-675 and WEB 2170. PAF synthesis by vascular tissue was suggested by its extraction from blood-free heart and aorta of rabbits treated in vivo with SK or t-PA but not of control rabbits. In contrast, PAF was not detected in peripheral blood. Ex vivo studies on platelet aggregation response to ADP and PAF performed on platelet-rich plasma obtained before and after SK and t-PA infusion demonstrated an early hyperaggregable phase, abrogated by PAF receptor antagonists and followed by reduced sensitivity of platelets to PAF. The ED₈₀ values for the aggregation of washed rabbit platelets induced by PAF but not thrombin were significantly increased at 60 and 120 minutes after SK and t-PA infusion, suggesting a specific desensitization of platelets to PAF. In contrast to PAF receptor antagonists, aspirin did not significantly modify the hypotension and the platelet hyperaggregability induced by SK or t-PA or the platelet hypoaggregability induced by t-PA. Thrombocytopenia induced by t-PA, but not by SK, was partially prevented by aspirin. The effect of SK, t-PA, and plasmin on the aggregation of washed platelets from untreated rabbits and from humans was also studied. Whereas SK and t-PA were inactive, plasmin induced dose-dependent platelet aggregation that was inhibited by platelet pretreatment with PAF receptor antagonists. In conclusion, the effect of PAF receptor antagonists observed in the present experimental model suggests that the hypotension and activation of platelets induced by SK and t-PA infusion are mediated by PAF. (Circulation Research 1993;72:658–670)

KEY WORDS • streptokinase • tissue-type plasminogen activators • platelet-activating factor • hypotension • platelets

Several studies support the hypothesis that platelets (PLTs) modulate the biological response to thrombolytic agents. PLT activation that occurs in patients with acute myocardial infarction treated with intravenous streptokinase (SK) and tissue-type plasminogen activator (t-PA) may limit the response to thrombolytic therapy. In experimental models of coronary thrombosis, the activation of PLTs may delay reperfusion and induce reocclusion. This biological effect of thrombolytic therapy has been related to a number of mechanisms: the local generation of plasmin, the increased biosynthesis of thromboxane A₂, and the transient release of active thrombin from the lysed clot. However, it was shown that, in the rabbit, infusion of SK or t-PA induces reduction of PLT count and promotes PLT hyperaggregation in the absence of thrombotic occlusion. Rudd et al have recently shown that a protracted infusion of SK and t-PA in the rabbit produces a biphasic effect on PLT aggregation, characterized by an early hyperaggregable phase concomitant with a rise in plasma plasmin activity and followed by a hypoaggregable phase. An additional effect of thrombolytic therapy is a significant fall in systemic blood pressure. The intravascular generation of plasmin followed by the production of bradykinin has also been proposed as a mechanism for the hypotension induced by thrombolytic agents.

The role of platelet-activating factor (PAF), a mediator that may explain both PLT aggregation and hypotension, has not been investigated as yet. PAF, which may be synthesized by a variety of cells including endothelial cells after stimulation with proteases,
TABLE 1. Experimental Protocol

<table>
<thead>
<tr>
<th>Groups</th>
<th>In vivo experiments</th>
<th>Ex vivo experiments</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hematologic assays and physiological measurements (n)</td>
<td>PAF extraction (n)</td>
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<tr>
<td>SK (N=20)</td>
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<td>Control (N=17)</td>
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PAF, platelet-activating factor; PRP, platelet-rich plasma; PLTs, platelets; SK, streptokinase; SDZ 63-675 and WEB 2170, PAF receptor antagonists; ASA, aspirin; t-PA, tissue-type plasminogen activator; N, total number of rabbits in study group; n, number of rabbits from study group in experiment.

Reagents

SK was obtained from Behringwerke AG, Marburg, FRG; t-PA (Actilyse) was provided by Boehringer Ingelheim, Ingelheim, FRG. SDZ 63-675 (Sandoz, East Hanover, N.J.) and WEB 2170 (Boehringer Ingelheim) were used as PAF receptor antagonists.28–30 Aspirin (ASA) was purchased from Synthelabo Benelux, Brussels. The chromogenic substrate S2251 (H-D-Val-Leu-Lys [pNa 2] HCl) and the human plasmin (22 nKat) used in the antiplasmin activity assays were obtained from Kabi Diagnostica, Stockholm.31,32 d-Phenyl-L-prolyl-L-arginine chloromethylketone (PPACK), a serine protease inhibitor,33 was obtained from Calbiochem, Frankfurt, FRG. Synthetic PAF (1-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was obtained from Bachem Feinchemikalien, Bubendorf, Switzerland. Porcine plasmin, human plasmin, rabbit plasminogen, ADP, and bovine thrombin were obtained from Sigma Chemical Co., St. Louis, Mo.

Animal Preparation and Experimental Design

New Zealand White rabbits of both sexes weighing 2.5–3.4 kg and fed with standard diet were used. The rabbit model is the most used for studies implicating fibrinolysis because the plasminogen activator activity in plasma of this species is similar to that of humans.14,34 The animals were initially anesthetized with ketamine (7 mg/kg i.v., Ketalar, Parke-Davis, Milan, Italy); pentobarbital (30–50 mg/kg) was subsequently given as a sustaining anesthetic. The depth of anesthesia was ascertained by the lack of the corneal reflex.

A sterile saline-filled catheter (3F, Biotrol Pharma, Paris) was inserted into the femoral artery for pressure recording and blood sampling. Thrombolytic agents and PAF receptor antagonists were diluted to the appropriate concentration in sterile saline solution and administered to the rabbits through the marginal ear veins by a constant-rate syringe infusion pump (Perfusor Secura, B. Braun, Melsungen AG, FRG). One hundred thirty-eight rabbits were randomly divided into 12 different groups (experimental protocols are schematically depicted in Table 1).

SK group. Twenty rabbits were infused with 666 units/kg per minute SK for 60 minutes. This dose of SK was chosen because it was found to produce changes in plasmin activity in rabbits comparable to those observed in humans.14

SK+SDZ 63-675 group. Five minutes before SK infusion (same schedule used in SK group), nine rabbits received a 2-ml bolus of saline containing 5 mg/kg SDZ 63-675, followed by a continuous infusion (83 μg/kg per minute) for 120 minutes.

SK+WEB 2170 group. Five minutes before SK infusion, nine rabbits received a 2-ml bolus of saline containing 0.5 mg/kg WEB 2170, followed by a continuous infusion (8.3 μg/kg per minute) for 120 minutes.

SK+ASA group. One hour before SK infusion, nine rabbits received a 2-ml bolus of saline containing ASA (15 mg/kg).

causes PLT activation and thrombocytopenia associated with hypotension when infused in experimental animals.24,25 The local generation of PAF and its association with the endothelial cell surface may favor platelet–endothelium as well as leukocyte–endothelium interaction.26,27 In the present investigation, the involvement of PAF in the biological response to thrombolytic agents was studied both in vivo and in vitro. After SK and t-PA infusion in rabbits, we evaluated the following in vivo: the generation of PAF by thrombolytic agents in blood samples and cardiac and aortic perfusates; the effect of two structurally unrelated PAF receptor antagonists, SDZ 63-67528 and WEB 2170,29,30 on hypotension, thrombocytopenia, and hyperaggregability; and the desensitization of PLTs to PAF. We evaluated the in vitro effects of SK, t-PA, and plasmin on PLT aggregation in the presence and absence of PAF receptor antagonists.

Materials and Methods
Data are presented as mean ± SEM. The number of platelets. SDZ 63-675 was injected as a bolus of 5 mg/kg, followed by continuous infusion of 83 μg/kg per minute for 120 minutes; WEB 2170 was injected as a bolus of 0.5 mg/kg, followed by a continuous infusion of 8.3 μg/kg per minute for 120 minutes. At 120 minutes, rabbits were injected with synthetic platelet-activating factor (PAF, 0.2 μg/kg). Data are expressed as percentage of the pretreatment level (mean ± SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for SDZ 63-675 vs. saline alone; †p<0.05 for WEB 2170 vs. saline alone.

t-PA group. Twenty rabbits were infused with 10 μg/kg per minute t-PA for 15 minutes. This dose of t-PA is similar to the dose used for thrombolysis in clinical practice.35

t-PA+SDZ 63-675 group. Five minutes before t-PA infusion (same schedule used in t-PA group), nine rabbits received a 2-ml bolus of saline containing 5 mg/kg SDZ 63-675, followed by a continuous infusion (83 μg/kg per minute) for 60 minutes.

t-PA+WEB 2170 group. Five minutes before t-PA infusion (same schedule used in t-PA group), nine rabbits received a 2-ml bolus of saline containing 0.5 mg/kg WEB 2170, followed by a continuous infusion (8.3 μg/kg per minute) for 60 minutes.

t-PA+ASA group. One hour before t-PA infusion, nine rabbits received a 2-ml bolus containing ASA (15 mg/kg).

SDZ 63-675 group. As a non-SK and non-t-PA control, nine rabbits were infused with 5 mg/kg SDZ 63-675, followed by a continuous infusion (83 μg/kg per minute) for 120 minutes.

WEB 2170 group. As a non-SK and non-t-PA control, nine rabbits were infused with 0.5 mg/kg WEB 2170, followed by a continuous infusion (8.3 μg/kg per minute) for 120 minutes.

ASA group. Nine rabbits were infused with ASA (15 mg/kg).

Control group. Seventeen rabbits received an equivalent volume of saline solution alone.

**Hematologic Assays and Physiological Measurements**

Blood samples (2 ml) were collected in plastic syringes containing a final concentration of 12.9 mM trisodium citrate and 1 μM PPACK before and at 30, 60, and 120 minutes after the beginning of the infusion to measure plasma levels of fibrinogen and α2-antiplasmin. One additional milliliter of blood was withdrawn into a separate tube containing EDTA at time 0 and at 15, 30, 60, 90, and 120 minutes for the PLT count and hematocrit evaluation. The plasma level of fibrinogen was determined by coagulation rate assay36; the α2-antiplasmin was assayed with the chromogenic substrate S-2251.31,32 PLT counts and hematocrit were determined with a Coulter counter (Coulter Electronics, Hertfordshire, UK). Systemic arterial pressure was measured by means of a pressure transducer (model 377, Harvard Apparatus, South Natick, Mass.), recorded on magnetic tape (model 3964 A recorder, Hewlett-Packard Co., Palo Alto, Calif.), visualized on
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FIGURE 3. Graph showing time course of effect of tissue-type plasminogen activator (t-PA, ○, n=5), t-PA+SDZ 63-675 (□, n=5), t-PA+WEB 2170 (▲, n=5), and saline (●, n=5) infusion in rabbits on mean arterial pressure. t-PA was infused at 10 μg/kg per minute for 15 minutes; SDZ 63-675 was administered 5 minutes before streptokinase infusion, at a bolus dose of 3 mg/kg, followed by a continuous infusion of 83 μg/kg per minute for 60 minutes; WEB 2170 was given 5 minutes before streptokinase infusion, at a bolus dose of 0.5 mg/kg, followed by a continuous infusion of 8.3 μg/kg per minute for 60 minutes. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for t-PA vs. saline alone; †p<0.05 for t-PA+SDZ 63-675 vs. t-PA; ‡p<0.05 for t-PA+WEB 2170 vs. t-PA.

an oscilloscope (model 5103 N, Tektronix, Beaverton, Ore.), and reproduced by a Hewlett-Packard 7015 B X-Y recorder for data analysis. Heart rate was monitored by electrocardiogram.

Extraction and Characterization of PAF

In a separate set of experiments, measurement of PAF was performed after extraction and purification of blood samples and cardiac and aortic perfusates. In rabbits infused with SK, t-PA, or saline, blood samples (8 ml) were obtained before and at 30, 60, and 90 minutes (n-4 for each group) after the beginning of infusion of thrombolytic agents or saline. Rabbits were killed at 90 minutes, and the heart and the aortic artery were rapidly excised. The heart was cannulated with a polypropylene tube via the ascending aorta and, after washing with Tyrode’s solution to remove blood, was perfused with 20 ml acidified methanol to extract PAF. A 5-cm segment of aorta was cannulated to each end with polypropylene connectors and submitted to the same procedure as the heart. In selected experiments, PAF extraction was performed after denudation of endothelium according to the technique described by Gasic et al: insertion of a glass rod into the aortic lumen, followed by gently rolling the vessel over a paper towel impregnated with perfusion buffer. The effective removal of endothelial layer was verified at the end of

FIGURE 4. Graph showing time course of effect of streptokinase (○, n=5), streptokinase+SDZ 63-675 (□, n=5), streptokinase+WEB 2170 (▲, n=5), and saline (●, n=5) infusion into rabbits on the number of platelets and plasma levels of fibrinogen and α2-antiplasmin. Streptokinase, streptokinase+SDZ 63-675, and streptokinase+WEB 2170 were used with the schedule shown in the legend of Figure 2. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for streptokinase vs. saline alone; †p<0.05 for streptokinase+SDZ 63-675 vs. streptokinase; ‡p<0.05 for streptokinase+WEB 2170 vs. streptokinase.

the experiment on histological preparations stained with 0.2% toluidine blue and examined by stereo light microscopy. The dry weights of heart and aorta were determined after lyophilization of the sliced tissue. Lipids were extracted and purified from blood samples or aortic and cardiac perfusates by methods previously described.38,39 Briefly, lipids, extracted in acidified methanol (pH 3.0–3.5) to destroy the acid-labile acetylhydrolase, after phase separation in chloroform were fractionated by thin-layer chromatography (TLC) with 65:35:6 (vol/vol/vol) chloroform: methanol:water39 as a solvent. In preliminary experiments, the overall recovery of PAF was 95–98%, as measured by addition of 10 nCi [3H]PAF to the whole blood and the aortic or cardiac perfusates before the extraction and purification procedure. PAF isolated from blood and aortic or cardiac perfusates was characterized as previously described by high-performance liquid chromatography.
Platelet Preparation and Aggregation Studies

In four rabbits of each group, 9 ml blood was withdrawn at the indicated times and mixed with 1 ml of 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 375g for 20 minutes; platelet-poor plasma (PPP) was prepared by further centrifugation at 1,400g for 10 minutes. PLTs were counted, and PRP was adjusted to $3.5 \times 10^8$ PLTs/µl with PPP. In a separate series of studies, we examined specific desensitization of PLTs to PAF after infusion of SK or t-PA. Washed rabbit PLTs were prepared according to the method of Ardlie et al., modified as previously described. The isolated washed PLTs were suspended in Ca$^{2+}$-free Tyrode-gelatin buffer, pH 6.5 (3.2$\times$10$^5$ PLTs/ml). Twenty microliters of the PLT suspension was transferred into cuvettes of the aggregometer and diluted with Tyrode-gelatin buffer, pH 7.4, containing 1.33 mM Ca$^{2+}$ to give a final concentration of (2.9$\pm$0.17)$\times$10$^5$ PLTs/µl. PLT aggregation in PRP or in washed PLTs was performed using a dual-channel aggregometer (model 840, Elvi Spa, Milan, Italy) at constant stirring of 900 rpm. In PRP studies, the percentage of light transmission was set at 10% with PRP and at 90% with PPP. Each aggregation rate was evaluated as an increase in light transmission. Aggregation response in PRP was induced with PAF (4.36 nM) and ADP (4.0 µM). Washed PLTs were stimulated with different concentrations of PAF (range, 7.5–750 pM) and thrombin (range, 0.04–2 units/ml). To compare the different responses, maximal aggregation was quantified by Weiss’s formula ($OD_0-OD_m$)$\times$100/$OD_0$, where $OD_0$ is the initial light transmission value and $OD_m$ is the minimum light transmittance. To compare the different profiles, we used $ED_{50}$ values obtained from a dose-response curve at each experimental time point. $ED_{50}$ is the concentration of each aggregating agent necessary to induce 50% of the maximal aggregation.

In Vitro Studies of Washed Rabbit PLTs

The effect of SK (range, 300–3,000 units/ml), t-PA (range, 10–100 µg/ml), and plasmin (range, 0.22–1.72 units/ml) on washed rabbit PLTs and the effect of PAF receptor antagonists SDZ 63-675 (range, 0.5–5 µM) and WEB 2170 (range, 0.5–5 µM) on PLT aggregation induced by plasmin were evaluated by aggregometry. Data are expressed as a percentage of maximal aggregation induced by the aggregating agent in the absence of any inhibition; values of $IC_{50}$ (minimal concentration of PAF receptor antagonist necessary to reduce the basal maximal aggregation to half) and $IC_{100}$ (minimal concentration required to induce a complete inhibition of PLT aggregation) were also determined.

Since plasminogen and plasmin share the same binding sites, we studied the binding of plasminogen on washed rabbit PLTs according to the method of Miles and Plow. Plasminogen was labeled by a modified chloramine T procedure with an efficiency of approximately 98%. The functional activity, evaluated on the cleavage of the tripeptide substrate S2251, was not decreased upon radiiodination. In a typical binding assay, 40 µl washed rabbit PLTs at 4.8$\times$10$^8$/ml in Tyrode’s buffer, pH 7.2, were incubated with 0.1 µM [$^{125}$I]plasminogen that had been precentrifugated for 5 minutes. [$^{125}$I]Plasminogen, unlabeled plasminogen, and

(continued...)

(Figure 5. Graph showing time course of effect of tissue-type plasminogen activator (t-PA, ○, n=5), t-PA+SDZ 63-675 (□, n=5), t-PA+WEB 2170 (▲, n=5), and saline (●, n=5) infusion into rabbits on the number of platelets and plasma levels of fibrinogen and α2-antiplasmin. t-PA, t-PA+SDZ 63-675, and t-PA+WEB 2170 were used with the schedule shown in the legend of Figure 3. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for t-PA vs. saline alone; †p<0.05 for t-PA+SDZ 63-675 vs. t-PA; ‡p<0.05 for t-PA+WEB 2170 vs. t-PA.)
PAF receptor antagonists SDZ 63-675 (3 μM) or WEB 2170 (4 μM) were added to a final volume of 200 μl. After incubation for 10 and 30 minutes at room temperature, triplicate 50-μl samples were individually layered onto 300 μl of 20% sucrose in Tyrode’s buffer in 500-μl polypropylene tubes and centrifuged for 2.5 minutes in a Beckman Microfuge. Molecules of plasminogen bound were calculated from the specific activity of the radiolabeled plasminogen.41

In Vitro Studies of Washed Human PLTs

Human PLTs were isolated according to Torr et al43 by differential centrifugation from fresh blood obtained from normal healthy volunteers who had no ingested platelet-active pharmacological agents for at least 1 month and who provided informed consent. Blood (24 ml) was collected into 3.4 ml acid-citrate-dextrose solution (85 mM trisodium citrate, 110 mM D-glucose, and 75 mM citric acid, pH 4.5) and centrifuged for 15 minutes at 200g. The resulting PLT pellet was resuspended in Tyrode’s buffer (pH 6.3). The PLT suspension was centrifuged once more at 400g, and the PLT pellet was resuspended in Tyrode’s buffer at pH 7.3. The PLT count was adjusted to 3×10⁸ by dilution with Tyrode’s buffer. PLTs were suspended in Tyrode’s buffer in an aggregometer at 37°C with a stirring rate of 1,000 rpm. PLTs were incubated at 37°C for 3 minutes before the addition of either plasmin (1.72 units/ml) or thrombin (1 unit/ml). The effect of plasmin or thrombin was studied both in the absence and presence of the

PAF receptor antagonists SDZ 63-675 (3 μM) and WEB 2170 (4 μM).

Statistical Analysis

Data are presented as the mean±SEM. Statistical analysis was performed by one-way analysis of variance with either Dunnett's or the Newman-Keuls multiple-comparison test where appropriate. In vitro experiments, IC₅₀ and IC₉₀ were determined by probit analysis.

Results

Hemodynamic Effect, Specificity, and Duration of Activity of PAF Receptor Antagonists

The infusion of SDZ 63-675 (n=5) and WEB 2170 (n=5) at the concentrations and rate described in “Materials and Methods” did not modify mean arterial pressure (Figure 1) and heart rate (not shown) over baseline values during the 120-minute observation period. The infusion of the two PAF receptor antagonists used, which were selected chemically unrelated to exclude possible aspecific pharmacological effects, completely abrogated the reduction in mean arterial pressure and the thrombocytopenia induced by infusion of synthetic PAF (0.2 μg/kg) at 120 minutes (Figure 1). Washed rabbit PLTs prepared from blood of rabbits treated with PAF receptor antagonists, obtained before the end of the experiment (120 minutes), were unresponsive to PAF (750 nM) but not to thrombin (2 units/ml) when tested by aggregometry. These data indicate that infusion of PAF receptor antagonists indeed achieved the desired pharmacological effect.
FIGURE 7. Panel A: Bar graph showing amount of platelet-activating factor (PAF, in picograms per gram dry weight cardiac tissue) obtained after perfusion (with 20 ml acidified methanol) of the heart of rabbits infused with streptokinase (SK, n=4) or tissue-type plasminogen activator (t-PA, n=4) and killed after 90 minutes. Panel B: Bar graph showing generation of PAF (in picograms per gram dry weight) by aorta with intact (open column, n=4 for each stimulus) or mechanically denuded (closed column, n=3 for each stimulus) endothelium treated with SK or t-PA. No PAF was detected in the cardiac or aortic perfusates obtained from control rabbits, which were infused with saline alone (n=4).

The PAF extraction procedure is detailed in "Materials and Methods." Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for SK vs. control; tp<0.05 for t-PA vs. control; #p<0.05 for aorta with denuded endothelium vs. intact endothelium.

In Vivo Experiments: Arterial Pressure, Heart Rate, and Hematologic Changes

As shown in Figures 2 and 3, infusion of both the thrombolytic agents SK and t-PA induced a significant hypotension in rabbits, which was evident at 30 minutes after the beginning of infusion and persisted for the duration of the experiment. In contrast, the heart rate was unaffected by the infusion of SK and t-PA (not shown). The fall in mean arterial pressure induced by SK or t-PA was completely abolished by the specific PAF receptor antagonists SDZ 63-675 and WEB 2170. The effect of SK and t-PA infusion on the number of circulating PLTs and on the plasma concentration of fibrinogen and of α2-antiplasmin is shown in Figures 4 and 5. Both SK and t-PA induced a significant decrease in PLT counts that became evident at 90 and 60 minutes, respectively, and persisted until the end of the experiments (120 minutes). The plasma levels of fibrinogen and of α2-antiplasmin were significantly reduced at 30, 60, and 120 minutes after the infusion of SK but not after the infusion of t-PA. No significant variations of hematocrit were observed after treatment with the thrombolytic agents (data not shown). The pretreatment of rabbits with SDZ 63-675 and WEB 2170 significantly reduced the fall in PLT counts induced by infusion of SK and t-PA but did not affect the SK-induced changes in plasma concentrations of fibrinogen and α2-antiplasmin (Figures 4 and 5). Infusion of saline alone, SDZ 63-675, or WEB 2170 did not modify PLT counts (Figure 1) nor plasma levels of fibrinogen and α2-antiplasmin (not shown). The pretreatment of rabbits with ASA did not modify the hypotension induced by the thrombolytic agents (Figure 6) and the fall in PLT counts induced by SK; however, ASA significantly reduced thrombocytopenia observed at 60 minutes after infusion of t-PA. The changes in plasma concentrations of fibrinogen and α2-antiplasmin induced by SK infusion were unaffected by ASA (data not shown). Infusion of ASA alone did not modify the mean arterial pressure and the hematologic parameters (Figure 6).

Extraction and Characterization of PAF

PAF was never present in blood samples of rabbits from the control group, which was infused with saline.
alone, nor in the blood samples from the rabbits infused with the thrombolytic agents. In contrast, significant amounts of PAF were detected in the cardiac (Figure 7A) and aortic (Figure 7B) perfusates obtained from rabbits treated with SK or t-PA; no PAF was detectable in the cardiac or aortic perfusates obtained from control rabbits. In addition, the removal of the endothelial layer from the aorta significantly reduced the amount of PAF detectable in the aortic perfusate (Figure 7B), suggesting that endothelium is the main source of PAF in vessels of animals subjected to thrombolitics. PAF extracted and purified from the cardiac or aortic perfusates had identical migratory properties on TLC as synthetic PAF. By HPLC, TLC-purified cardiac PAF coeluted with standard PAF in a single peak with retention time of 20 minutes. The biological activity of PAF was destroyed after base-catalyzed methanolysis (0–2% of recovered activity) or treatment with phospholipase A₂ (0–3% of recovered activity) but not by treatment with HCl (85–100% of recovered activity) or lipase A₁ (100% of recovered activity). After base-catalyzed methanolysis or inactivation with phospholipase A₂, the treatment with acetic anhydride restored 78% of the biological activity. PAF purified from the aortic or cardiac perfusates induced aggregation of washed rabbit PLTs in the presence of indomethacin, an inhibitor of cyclooxygenase, and of creatine phosphate/creatine phosphokinase as ADP scavengers. The specificity of the biological activity of aortic or cardiac PAF on washed rabbit PLTs was inferred either from specific desensitization of PLTs to a subsequent challenge with synthetic PAF or from the inhibitory effect of SDZ 63-675 or WEB 2170.

**Ex Vivo Experiments: PLT Aggregation in PRP and in Washed PLT Preparations**

The time course of PLT aggregation response to ADP and PAF was studied on PRP of rabbits infused with SK or t-PA (Figures 8 and 9). After 30 minutes, the infusion of SK and t-PA caused a significant increase in PLT aggregation to ADP that reduced thereafter and returned to pretreatment levels within 60 minutes. SDZ 63-675 and WEB 2170 completely abrogated the increased sensitivity of PLTs to ADP caused by the infusion of the thrombolytic agents (Figures 10 and 11). A similar increase in PLT aggregation response was observed to PAF at 30 minutes (Figures 8 and 9). In the experiments of t-PA infusion, this early hyperaggregability was followed by a reduced sensitivity of PLTs to PAF (Figure 9). In contrast, in the experiments of SK infusion, the reduction in PLT sensitivity was not evident in PRP (Figure 8). The infusion of the PAF receptor antagonists alone did not modify the sensitivity of PLTs to ADP (Figure 12), whereas PAF-induced aggregation was totally abrogated for the entire observation period. Pretreatment of the rabbits with ASA did not modify the alteration of PLT response to ADP or PAF observed after the infusion of SK or t-PA (Figures 13 and 14).

To better evaluate whether a specific desensitization of PLTs to PAF occurred, the sensitivity (ED₅₀) of PLTs and the changes in responsiveness of PLTs to PAF were

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**Figure 9.** Graph showing time course of effect of tissue-type plasminogen activator (t-PA, n=4) and saline (n=4) infusion into rabbits on ex vivo platelet aggregation induced by 4.0 μM ADP (Δ, t-PA; □, saline) and by 4.36 nM platelet-activating factor (○, t-PA; ●, saline) (four individual experiments performed in duplicate). t-PA, t-PA+SDZ 63-675, and t-PA+WEB 2170 were used with the schedule shown in the legend of Figure 3. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by Dunnett's multiple-comparison test. *p<0.05 for t-PA vs. saline (aggregation induced by ADP); †p<0.05 for t-PA vs. saline (aggregation induced by platelet-activating factor).

**Figure 10.** Graph showing time course of effect of streptokinase (△, n=4), streptokinase+SDZ 63-675 (○, n=4), and streptokinase+WEB 2170 (●, n=4) infusion into rabbits on ex vivo platelet aggregation induced by 4.0 μM ADP (four individual experiments performed in duplicate). Streptokinase, streptokinase+SDZ 63-675, and streptokinase+WEB 2170 were used with the schedule described in the legend of Figure 2. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by Dunnett's multiple-comparison test. *p<0.05 for streptokinase+SDZ 63-675 vs. streptokinase; †p<0.05 for streptokinase+WEB 2170 vs. streptokinase.
studied on washed rabbit PLTs prepared before, as well as 60 and 120 minutes after, the treatment with SK and t-PA (Figure 15A). The ED<sub>50</sub> values for PAF-induced PLT aggregation were significantly increased in rabbits at 60 and 120 minutes after SK and t-PA infusion but not in control rabbits. However, PLT desensitization to PAF was significantly higher after t-PA treatment than after SK treatment. In contrast, no variation in ED<sub>50</sub> was shown for thrombin-induced PLT aggregation (Figure 15B). These results, together with the reduction in responsiveness of PLTs to PAF but not to thrombin, observed after SK and t-PA infusion suggest a specific desensitization of PLTs to PAF.

**In Vitro Experiments**

Aggregation of washed rabbit PLTs challenged with SK, t-PA, and plasmin was measured by aggregometry. SK and t-PA had no direct effect on PLT aggregation. In contrast, plasmin aggregation induced in vitro dose-dependent PLT aggregation (Figure 16) with an ED<sub>50</sub> of 0.42 units/ml. Plasmin-induced aggregation was inhibited by PLT pretreatment with PAF receptor antagonists (Table 2), suggesting that plasmin aggregates PLTs by a PAF-dependent pathway. In contrast, 3 μM SDZ 63-675 and 4 μM WEB 2170, corresponding to IC<sub>50</sub> for aggregation induced by 0.86 units/ml plasmin, were totally ineffective on aggregation induced by 2 units/ml thrombin.

Washed human PLTs aggregated in response to plasmin in a dose-dependent manner (1.0–1.72 units/ml); however, they required higher doses (minimal dose giving maximal aggregation, 1.72 units/ml) of plasmin than rabbit PLTs (minimal dose giving maximal aggregation, 0.86 units/ml). SDZ 63-675 (3 μM) and WEB 2170 (4 μM) reduced the aggregation induced by plasmin (1.72 units/ml) by 88.0±2.4% and 78.8±3.1%, respectively.

SDZ 63-675 (3 μM) and WEB 2170 (4 μM) did not significantly decrease the specific binding of [125I]plasminogen to washed rabbit PLTs (variation in the number of plasminogen molecules per PLT evaluated 10 and 30 minutes after addition of [125I]plasminogen was <3% in respect to untreated PLTs).

**Discussion**

The results of the present study indicate that PAF mediates the hypotension as well as the alterations of PLT functions induced by the infusion of SK and t-PA. One limitation of the administration of the thrombolytic agents in patients with acute myocardial infarction is that they can cause a significant fall in systemic blood pressure. Experimental studies indicate that SK and, in particular, the SK–plasmin complex produce a marked fall in blood pressure that is related either to a rapid increase in serum plasmin concentration or to bradykinin generation. Indeed, the infusion of plasmin or bradykinin produces a marked hypotensive effect. Clinical studies, moreover, demonstrate that the hypotensive reaction is not a specific effect of SK but that it also occurs after high-dose intravenous infusion of tPA.
nists SDZ 63-675 or WEB 2170, which do not possess a direct pressor effect, completely abrogated SK-induced and t-PA–induced hypotension and aspirin may induce hypotension when infused in experimental animals.24,35-49 The observed alterations in PLT functions are also consistent with the involvement of PAF in the biological sequelae of thrombotic agent infusion. PLTs may play a critical role in both delaying reperfusion and mediating reocclusion after thrombolysis.1,4-6 Clinical observations indicate marked PLT activation after intravenous infusion of SK2 or t-PA2 in patients with acute myocardial infarction. Infusion of thrombotic agents in the rabbit induced in vivo thrombocytopenia7,13 and ex vivo hyperaggregability, concomitant with an elevation in plasmin activity, followed by a delayed phase of hypoaggregability.14 As suggested by in vitro studies, plasmin could mediate the effects of SK and t-PA on PLTs. Indeed, plasmin directly stimulates phosphatidylidyinositol 4,5-diphosphate–specific phospholipase C, leading to acylglycerol and phosphatidic acid formation, protein kinase C activation, and elevation of cytosolic free Ca2+.8 These intracellular events are associated with PLT secretion. However, plasmin induces the aggregation of human PLTs only at high doses.8,29 A significant reduction in PLT survival was demonstrated after injection of plasmin-treated PLTs,9 suggesting a potential role of plasmin in inducing thrombocytopenia.

In the present study, we observed a significant in vivo reduction in PLT count after infusion of both SK and t-PA and an ex vivo transient hyperaggregability to ADP and PAF. Thrombocytopenia and hyperaggregability were completely prevented by treatment with PAF receptor antagonists, suggesting that PAF mediates the activation of PLTs. In addition, the early hyperaggregability to PAF was followed by a greatly diminished responsiveness of PLTs to this mediator. This PLT desensitization that occurred 60 and 120 minutes after infusion of SK and t-PA was PAF specific, since only the ED50 for PAF- but not for thrombin-induced PLT aggregation was significantly increased. This observation is consistent with the occurrence of an in vivo PAF-mediated PLT activation with either a downregulation of PAF receptor in circulating PLTs or a decrease in coupling efficiency between PAF receptors and a second messenger–generating system.51,62 This hypoaggregability may contribute to explain the hemorrhagic complication oc-

**Figure 13.** Graphs showing time course of effect of streptokinase (●, n=4), streptokinase+aspirin (★, n=4), or aspirin (□, n=4) infusion into rabbits on ex vivo platelet aggregation induced by 4.0 μM ADP (panel A) or 4.36 nM platelet-activating factor (panel B) (four individual experiments performed in duplicate). Streptokinase, streptokinase+aspirin, and aspirin were used with the schedule described in the legend of Figure 6. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for streptokinase+aspirin vs. aspirin.

**Figure 14.** Graphs showing time course of effect of tissue-type plasminogen activator (t-PA, ●, n=4), t-PA+aspirin (★, n=4), or aspirin (□, n=4) infusion into rabbits on ex vivo platelet aggregation induced by 4.0 μM ADP (panel A) and 4.36 nM platelet-activating factor (panel B) (four individual experiments performed in duplicate). t-PA, t-PA+aspirin, and aspirin were used with the schedule described in the legend of Figure 6. Data are expressed as percentage of the pretreatment level (mean±SEM). Statistical analysis was performed with one-way analysis of variance followed by the Newman-Keuls multiple-comparison test. *p<0.05 for t-PA+aspirin vs. aspirin.
currying in vivo in patients treated with thrombolytic therapy.63

In contrast to PAF receptor antagonists, treatment of rabbits with ASA, which is a standard treatment in patients with ischemic heart disease, did not significantly modify hypotension and PLT hyperaggregability induced by SK and t-PA and hypoaggregability induced by t-PA. In addition, the reduction in PLT counts induced by SK was totally unaffected by ASA. Thrombocytopenia induced by t-PA was partially reduced at 60 minutes but not at 90 and 120 minutes, consistent with an early involvement of thromboxane generation in t-PA treatment.3

The in vitro studies excluded a direct effect of SK and t-PA on platelets. The possible role of plasmin generated by thrombolytic agents in PLT activation is supported by its ability to induce PLT aggregation in vitro.7,8,64 The inhibitory effect of PAF receptor antagonists on both rabbit and human PLTs suggests the dependence on PAF of the aggregation induced by plasmin. This observation is consistent with the involvement of serine proteases in PAF synthesis by leukocytes,23 endothelial cells,23 and PLTs.65

However, the present finding that PAF was not detected in peripheral blood of rabbits treated with SK or t-PA seems to exclude the possibility that thrombolytic agents stimulate directly or via plasmin generation the synthesis of PAF from PLTs or circulating leukocytes. In contrast, an active synthesis of PAF was detected in the heart vascular tissue and in the intact aorta of the rabbits infused with SK or t-PA but not in mechanically endothelium-denuded aorta or in unstimulated control rabbits, suggesting that vascular endothelium is the main target for PAF synthesis induced by thrombolytic agents. Indeed, we have recently found that cultured human umbilical vein–derived endothelial cells stimulated with SK and plasmin synthesize PAF (unpublished results). PAF synthesized by endothelial cells remains mainly cell associated, thus increasing PLT and leukocyte adheresiveness to endothelium.19,20,26,27 In conclusion, the hypotension and the activation of PLTs that, in the present experimental model, occur even in the absence of thrombolytic occlusion mainly depend on the synthesis of PAF. PAF synthesis could be triggered by plasmin. SK produced a significant fall in plasma fibrinogen and in α1-antiplasmin that suggests plasmin generation. However, no similar findings were observed with t-PA. This may be

![Graph showing ED50 values of platelet-activating factor (PAF, panel A, n=4) and thrombin (panel B, n=4) on ex vivo aggregation of washed platelets obtained before and 60 and 120 minutes after starting the infusion of streptokinase (△, n=4), tissue-type plasminogen activator (t-PA, ○, n=4), or saline (●, n=4) infusion. Washed platelets were stimulated with various concentrations of PAF (range, 7.5–750 μM) and thrombin (range, 0.04–2 units/ml). The extent of platelet aggregation was measured as described in “Materials and Methods.” ED50 values were calculated from dose–response curves. Data are expressed as percentage of the pretreatment levels (mean±SEM). Statistical analysis was performed using one-way analysis of variance followed by the Newman-Keuls test. *p<0.05 for streptokinase vs. saline; †p<0.05 for t-PA vs. saline; ‡p<0.05 for t-PA vs. streptokinase.

![Graph showing dose-dependent aggregation of washed rabbit platelets induced by plasmin. Results are mean±SEM for four individual experiments performed in duplicate.](http://circres.ahajournals.org/)

**Table 2. Inhibitory Effects of SDZ 63-675 and WEB 2170 on Platelet Aggregation Induced by Plasmin**

<table>
<thead>
<tr>
<th>Plasmin</th>
<th>SDZ 63-675</th>
<th>WEB 2170</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC100</td>
<td>0.43 units/ml</td>
<td>1.00±0.04</td>
</tr>
<tr>
<td></td>
<td>0.86 units/ml</td>
<td>3.00±0.04</td>
</tr>
<tr>
<td>IC50</td>
<td>0.86 units/ml</td>
<td>1.36±0.05</td>
</tr>
</tbody>
</table>

SDZ 63-675 and WEB 2170, platelet-activating factor receptor antagonists; IC100, minimal concentration of platelet activating factor receptor antagonist required to induce a complete inhibition of platelet aggregation; IC50, minimal concentration of platelet-activating factor receptor antagonist necessary to reduce the basal maximal aggregation to half.
indicative of an alternative mechanism for in vivo PAF generation after the infusion of t-PA. t-PA is a serine protease and therefore may directly stimulate leukocytes and endothelial cells to synthesize PAF. In addition, during thrombolytic therapy in patients with myocardial infarction, a transient thrombin activity was observed. Therefore, in that condition the activation of platelets may also depend on a direct effect of thrombin on platelets or on the synthesis of PAF by thrombin-stimulated endothelial cells. The recent observation that the PAF receptor antagonist prevents reocclusion in a model of thrombosis and t-PA-induced reperfusion in the femoral artery of the dog indicates that the synthesis of PAF may interfere with the beneficial effects of thrombolytic therapy.

References


Role of platelet-activating factor in hypotension and platelet activation induced by infusion of thrombolytic agents in rabbits.

G Montrucchio, G Alloatti, F Mariano, E Lupia, P G Lucchina, E Musso, G Emanuelli and G Camussi

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