Effect of thrombolysis on myocardial injury: recombinant tissue plasminogen activator vs. alfimeprase

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Effect of thrombolysis on myocardial injury: recombinant tissue plasminogen activator vs. alfimeprase. Am J Physiol Heart Circ Physiol 290: H959–H967, 2006. First published October 21, 2005; doi:10.1152/ajpheart.00649.2005.—Plasmin-dependent thrombolytic agents are potentially prothrombotic and proinflammatory. Alfimeprase, a zinc-containing metalloproteinase, degrades fibrin directly and achieves thrombolysis independent of plasmin formation. This study examines the hypothesis that thrombolysis in the absence of plasmin generation results in improved myocardial salvage on reperfusion. The thrombolytic effects of recombinant tissue plasminogen activator (rt-PA; 0.022 mg/kg, 1/10 of which was administered as a loading dose; the rest (9/10) was infused over 60 min by intracoronary (ic) administration) or alfimeprase (0.5 mg/kg over 1 min ic) were evaluated in a canine model of arterial thrombosis involving electrolytic injury of the left circumflex (LCX) coronary artery. Both agents induced thrombolysis, with onset of reperfusion being more rapid after alfimeprase compared with rt-PA (1.5 ± 0.6 vs. 10.1 ± 2.1 min). In the absence of adjunctive therapy, time to reocclusion after alfimeprase was 3.2 ± 0.5 min compared with 77.5 ± 31.9 min with rt-PA. The glycoprotein IIb/IIIa platelet receptor antagonist CRL-42796 prolonged reperfusion time after thrombolysis with alfimeprase or rt-PA. The effect of each lytic agent on myocardial infarct size was examined in a separate group of dogs subjected to 60 min of LCX coronary artery ligation and 4 h of reperfusion. Myocardial infarct size, expressed as percentage of the risk region, was larger (32.16 ± 3.95%) after rt-PA compared with alfimeprase (19.85 ± 3.61%) or that of the saline control group (18.46 ± 3.34%). rt-PA in contrast to alfimeprase, a direct-acting fibrinolytic agent, is associated with an increase in myocyte reperfusion injury.

myocardium; reperfusion injury; thrombolytic agents

THROMBOLYTIC AGENTS in current use activate the fibrinolytic system and achieve thrombolysis through the conversion of plasminogen to plasmin. In addition to its well-characterized mechanism of action on clot lysis, plasmin directly activates other enzymatic systems that contribute to proinflammatory events that may compromise the overall benefits of lytic therapy.

Previous studies demonstrated the induction of platelet activation by plasmin and plasmin-dependent thrombolytic agents (4, 26), while others have shown both activation as well as inhibitory effects (6, 27). Therapeutic reperfusion, achieved by intravenous administration of recombinant tissue plasminogen activator (rt-PA), is effective in salvaging tissue at risk of irreversible ischemic injury. The benefit of lytic therapy, however, may be limited because of failed reperfusion in 25–40% of the patients (40, 42) and the high incidence (30%) of reocclusion (43). Furthermore, because of the activation of the plasminogen-plasmin system, the mechanism for the cardioprotective effect of thrombolytic therapy remains unresolved (20).

Myocardial ischemia of sufficient duration, followed by reperfusion, is associated with an extension of irreversible tissue injury. The ensuing myocyte cell death is attributed to both the ischemic episode as well as to undefined covariates that contribute to the extension of myocardial injury aside from those commonly recognized factors (e.g., duration of ischemia, collateral blood flow). One undefined covariate involves the activation of the complement system (36, 37), which mediates a direct cytotoxic effect and, in addition, has an indirect effect by attracting polymorphonuclear neutrophils (PMNs) and monocytes to the reperfused region. The generation of plasmin by thrombolytic agents may be associated with unrecognized secondary complications, secondary to plasmin-induced activation of the complement cascade (12, 16, 33), as well as enhanced platelet aggregation (31, 32).

We hypothesize that, like rt-PA, the use of a non-plasmin-dependent lytic agent (e.g., alfimeprase) will provide effective thrombolysis, while having the added benefit of preserving tissue viability in the reperfused myocardial area at risk.

Alfimeprase is a recombinant, truncated form of fibrolase, a known direct fibrinolytic zinc metalloproteinase isolated initially from the venom of the Southern copperhead snake (Agkistrodon contortrix contortrix) (11, 21, 39). Preliminary studies demonstrated that rt-PA and alfimeprase are effective lytic agents in the canine coronary and carotid arteries and that reocclusion could be prevented by concomitant use of a platelet glycoprotein (GP) IIb/IIIa receptor antagonist (21, 39).

The present study involved the use of two experimental protocols. Protocol I was designed to determine the efficacy of the thrombolytic agents, rt-PA and alfimeprase, in achieving clot lysis in the canine model of coronary artery thrombosis. Protocol II made use of ligature occlusion of the left circumflex (LCX) coronary artery followed by intra-coronary artery administration of rt-PA or alfimeprase immediately before the reintroduction of LCX coronary artery blood flow. The effects of rt-PA or alfimeprase on myocardial infarct size in the two groups in protocol II were compared with a control group of animals that received an intra-coronary artery infusion of 0.9% sodium chloride solution at the time of reperfusion. The use of a ligature occlusion allowed for precise control over the duration of regional myocardial ischemia and involved the administration of the lytic agents at the time of reperfusion. The respective intracoronary doses of rt-PA and alfimeprase were

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determined in protocol I, in which each lytic agent was shown to achieve successful thrombolysis without inducing a systemic lytic effect.

The results indicate that myocardial infarct size, determined after 4 h of reperfusion, was larger in the rt-PA-treated group compared with the alfimeprase- and placebo-treated groups, thereby suggesting that plasmin generation contributes to the extension of myocardial injury during reperfusion.

METHODS

Animal Investigation

The study conforms to the position of the American Heart Association on research animal use adopted November 11, 1984, by the American Heart Association. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The procedures used in this study were in accordance with the guidelines of the University of Michigan (Ann Arbor) Committee on the use and care of animals and with the Guide for Care and Use of Laboratory Animals (DHHS Publication No. NIH 78–23). All procedures were approved by the Institutional Animal Care and Use Committee (Approval No. 8779).

Protocol I: Comparative Thrombolytic Effects of rt-PA and Alfimeprase

The experimental model used in this investigation is a modification of one developed by our laboratory for the study of experimentally induced arterial thrombosis (29). Purpose-bred beagle dogs (Covance Research Products, Kalamaazoo, MI) of either sex were anesthetized with pentobarbital sodium (30 mg/kg iv), intubated, and ventilated under positive pressure on room air at a stroke volume of 30 ml/kg and a frequency of 12 breaths/min maintained with a mechanical respirator (Harvard Apparatus, South Natick, MA). The left femoral vein was isolated, and a catheter was inserted for obtaining blood samples. Arterial blood pressure was monitored from the cannulated femoral artery with a Millar Mikro-Tip pressure-sensitive transducer catheter (Millar Instruments, Houston, TX). The standard limb lead II of the electrocardiograph was recorded continuously to monitor heart rate and to detect changes in the ST-segment indicative of regional myocardial ischemia. Coronary artery blood flow was recorded with a Doppler flow probe (model 100, Triton Technology, San Diego, CA). Recordings of blood pressure, the lead II electrocardiogram, and LCX coronary artery blood flow were recorded on a Grass model 7 polygraph (Grass Instrument, Quincy, MA) interfaced to a data-acquisition system (Po-Ne-Ma Data Acquisition System, Gould Instrument Systems, Valley View, OH) and downloaded to a computer hard drive.

An external stenosis was established on the LCX coronary artery distal to the Doppler flow probe by applying a suture ligature around the vessel and an 18-gauge hypodermic needle, after which the needle was withdrawn. An infusion line was fashioned from a 25-gauge needle tip attached to polyethylene tubing and inserted into the LCX coronary artery proximal to the flow probe and was used for the local administration of rt-PA, alfimeprase, or 0.9% sodium chloride solution. An intravascular electrode (Teflon-coated, 30-gauge silver-coated copper wire, Alpha Wire, Elizabeth, NJ) was inserted into the LCX coronary artery between the flow probe and the external stenosis. The electrode was positioned so that the exposed distal end (4 mm) was in firm contact with the intimal surface of the vessel. The intravascular electrode was connected to the positive pole (anode) of a dual-channel Grass S88 stimulator and a constant-current unit, model CCU1A (Grass Instrument). The cathode was connected to a distant subcutaneous site on the thorax. Application of an anodal direct current of 150 μA to the intimal surface of the artery resulted in a deep vascular wall lesion and exposure of subendothelial components, leading to the formation of a thrombogenic surface and the spontaneous development of an occlusive arterial thrombus (29).

Thirty minutes after formation of an occlusive thrombus in the LCX coronary artery, the electrolytic injury current was discontinued, and the animals were randomized to one of the following four groups.

Group 1. Group 1 (n = 6), placebo control, received sodium chloride solution (0.9%) + rt-PA [Alteplase, Genentech, San Francisco, CA; 0.022 mg/kg (12,609 IU/kg)] diluted to 10 ml with water for injection; 1 ml (10% of total amount) of rt-PA was injected over 3–4 min as a loading dose, and the remainder was administered over 1 h as an intracoronary (ic) infusion into the LCX coronary artery, immediately proximal to the occlusive thrombus.

Group 2. In group 2 (n = 6), sodium chloride solution (0.9%) + alfimeprase (Nuvelo, Sunnyvale, CA; 0.5 mg/kg) were administered over 1 min into the LCX coronary artery immediately proximal to the occlusive thrombus.

Group 3. In group 3 (n = 6), CRL-42796, a glycoprotein (GP) IIb/IIIa platelet receptor antagonist (Center of Research Cephalon, Paris, France; 30 μg/kg iv loading dose plus 1 μg·kg⁻¹·min⁻¹ iv infusion) + rt-PA were administered.

Group 4. Group 4 (n = 9) received the GPIIb/IIa platelet receptor antagonist CRL-42796 + alfimeprase.

A second dose of alfimeprase was administered if the first dose failed to initiate reflow within 10 min. The administration of sodium chloride solution (0.9%) or CRL-42796 was initiated 30 min before administration of lytic therapy with either rt-PA or alfimeprase and continued for 2.5 h as an adjunctive agent to maintain vessel patency. After discontinuation of the CRL-42796 infusion, the animals were monitored for an additional 2 h to assess the duration of vessel patency and to record the quality of arterial blood flow. The latter was assessed by the frequency of cyclic flow variations and assignment of a patency score graded from 0 to 3. The quality of coronary artery blood flow (estimate of tissue level perfusion) was evaluated using a previously published (34) scoring system, which is described briefly as follows. 1) A rating of 0 indicates the absence of blood flow. 2) A rating of 1 is applied when flow declines to 0 and is restored spontaneously. 3) A rating of 2 indicates a decline in blood flow that is restored spontaneously before it reaches zero flow. 4) A rating of 3 is applied when the blood flow is maintained relatively constant in a nonsaccilatory manner.

Tongue bleeding time determinations. Tongue bleeding time was assessed by making a uniform incision 5 mm long and 1 mm deep on the upper surface of the tongue with a Surgicut device (International Technidyne, Edison, NJ). The tongue lesion was blotted with filter paper every 15 s until the transfer of blood to the filter paper ceased. The interval was recorded as the tongue bleeding time. Bleeding time determinations were made at baseline, after administration of the adjunctive agent, and after completing the administration of thrombolytic therapy.

Ex vivo platelet aggregation studies. Blood (10 ml) was withdrawn into a plastic syringe containing 3.7% sodium citrate as the anticoagulant [1:10 citrate/blood (vol/vol)]. The platelet count was determined with an H-10 Cell Counter (Texas International Laboratories, Houston, TX). Ex vivo platelet aggregation was assessed by established spectrophotometric methods with a four-channel aggregometer (Bio-Data-PAP-4, BioData, Hatboro, PA) by recording the increase in light transmission through a stirred suspension of platelet-rich plasma (PRP) maintained at 37°C. PRP was obtained by centrifugation at 600 rpm for 10 min and diluted with platelet-poor plasma (PPP) to achieve a platelet count of 200,000/ml before use. PPP was prepared after the PRP was removed by centrifuging the remaining blood at 3,000 rpm (2,000 g) for 10 min and discarding the bottom cellular layer. Separate determinations of ex vivo platelet aggregation were performed with ADP (20 μM) or arachidonic acid (AA; 0.65 mM). A subaggregatory concentration of epinephrine (550 nM) was added to the PRP to prime the platelets before exposure to the aggregating agents. Values are expressed as percentage of aggregation, representing the percentage of
light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission, respectively.

Protocol II: Effects of rt-PA or Alfiameprase on Myocardial Infarct Size

A left thoracotomy was performed at the fifth intercostal space. The lung was retracted, and the heart was suspended in a pericardial cradle. A 15- to 18-mm segment of the LCX coronary artery was dissected free from surrounding tissue beginning at the border of the left atrial appendage. The left atrial appendage was cannulated for the administration of radiolabeled microspheres for the determination of regional myocardial blood flow (RMBF). A ligature stenosis was placed around the LCX coronary artery and adjusted to achieve a 30% reduction in the hyperemic response to a 10-s occlusion of the artery. After obtaining baseline hemodynamic measurements, the LCX was occluded by applying traction on a Silastic tube placed under the vessel and through a polyethylene sleeve placed above the artery forming a snare occluder. All dogs in protocol II were subjected to 60 min of regional myocardial ischemia.

Radioactive microspheres, for the determination of RMBF, were administered 45 min into the occlusion period via a cannula in the right atrial appendage. At the end of the 60-min occlusion period, reperfusion of the ischemic area was initiated by slowly releasing the snare until the baseline level of LCX coronary artery blood flow was attained after which the snare was removed completely. Controlled reperfusion attenuates the hyperemic response and decreases the incidence of ventricular fibrillation (antiarrhythmic agents including lidocaine were not used at any time during the study). In addition, the controlled reperfusion better simulates the restoration of blood flow that is achieved with the use of a thrombolytic agent in the presence of an occlusive thrombus.

The animals in protocol II were randomized among three treatment groups as follows.

Group I. Group 1 (n = 7) received 0.9% sodium chloride solution and served as the control group.

Group 2. Group 2 (n = 8) received rt-PA as an intracoronary injection followed by an infusion. rt-PA [Alteplase, Genentech; 0.022 mg/kg (12.609 IU/kg)] was diluted to 10 ml with water for injection; 1 ml (10% of the total amount) of rt-PA was injected over 3–4 min as a loading dose and the remainder was administered over 1 h as an LCX intracoronary infusion, immediately proximal to the occlusive thrombus.

Group 3. Group 3 (n = 7) received alfiameprase as an intra-coronary artery injection of 0.5 mg/kg (0.7 ml) over 1 min.

On completion of the experimental protocol, the animals were euthanized by application of a 9-V direct current to the apex of the heart to induce ventricular fibrillation. The hearts were removed and processed for the determination of infarct size, area at risk, and regional myocardial blood flow. The method used for determination of infarct size, which makes use of a dual-perfusion technique using Evans blue dye and triphenyltetrazolium chloride (TTC), was described previously (7, 17). The normal zone appears blue after Evans blue dye and triphenyltetrazolium chloride (TTC), was described previously (7, 17).

Infarct Size

Determination of RMBF. RMBF was determined using radiolabeled microspheres (15 μm diameter, New England Nuclear, Newton, MA) by the reference withdrawal method. Each vial of microspheres was placed in an ultrasonic bath and vortexed before injection to ensure that adequate dispersal of the microsphere suspensions was achieved before administration. Radiolabeled microspheres (103Ru, 0.1 mCi) were injected into the left atrium 45 min after applying the occlusive ligature to the LCX coronary artery. Simultaneous reference arterial blood samples were withdrawn from the femoral and carotid arteries at a constant rate with a withdrawal pump, beginning immediately before the injection of microspheres into the left atrium and ending 2 min later. The reference sample counts were averaged for calculation of myocardial blood flow.

Tissue samples weighing 0.10–0.05 g (wet weight) were dissected from the papillary muscle, endocardial, midmyocardial, and epicardial sections of the heart in the regions of distribution of the left anterior descending (LAD) coronary artery. Four transverse ventricular sections from each heart were used so that blood flow to each region represents the average of four samples for each experiment. The mean RMBFs from the inner two-thirds of the myocardium were used to determine whether an excessive collateral blood supply (>0.16 ml·min⁻¹·g tissue⁻¹) was present 45 min after occlusion of the LCX coronary artery. Animals that failed to satisfy the preestablished criterion regarding RMBF were excluded from the final data analysis.

Statistical Analysis

Data are expressed as means ± SE. The data were analyzed by one-way ANOVA for group comparisons and for repeated measures followed by a Bonferroni’s post hoc t-test to determine the level of significance. Coronary artery flow patency scores were compared by a chi-square test. Values are considered statistically different at a level of P < 0.05.

RESULTS

Protocol I: Thrombolytic Effects of rt-PA vs. Alfiameprase

Survival. There were 29 animals allocated to protocol I in which the LCX coronary artery of each animal was subjected to electrolytic induction of vessel wall injury with subsequent formation of an occlusive thrombus. Two animals were excluded because of intractable ventricular fibrillation that occurred during the induction of vessel wall injury and thrombus formation. Three animals had excessive blood loss during the experimental procedure and did not complete the entire protocol. Twenty-four animals completed the experimental protocol and are included in the final data analysis (see Table 1).

No significant differences were observed among the groups with respect to body weight, hemodynamic parameters, and time to thrombotic occlusion in the LCX coronary artery in response to electrolytic induction of deep vessel wall injury (Table 1).

Thrombolytic effects of rt-PA vs. alfiameprase. In the absence of adjunctive therapy, successful thrombolysis was observed in each of the animals treated with either rt-PA or alfiameprase administered immediately proximal to the occlusive thrombus. The average time to thrombolysis in the alfiameprase-treated group was significantly shorter (1.5 ± 0.6 min) compared with that of the rt-PA-treated animals (10.1 ± 2.1 min). Reclosure occurred in each of the vessels in the alfiameprase-treated animals after a mean time of 3.2 ± 0.5 min and remained occluded for the duration of the experimental protocol. The
incidence of reocclusion in the rt-PA-treated group was 50% (3/6), and the total reflow time was 77.5 ± 31.9 min, indicating a longer duration of vessel patency after rt-PA-induced thrombolysis (Table 1).

**Thrombolysis in the presence of platelet GPIIb/IIIa receptor inhibition.** After pretreatment (intravenous loading dose plus infusion) with CRL-42796, a GPIIb/IIIa platelet receptor antagonist, the incidence of thrombolysis was 66.7% (4/6) in the rt-PA-treated group and 89% (8/9) in the group treated with alfimeprase. A single dose of alfimeprase achieved thrombolysis in six vessels (6/9, 66.7%); two vessels reopened after a second local administration of alfimeprase, while the occlusive thrombus in one animal failed to undergo lysis and the vessel remained occluded for the duration of the study. For the coronary arteries in which thrombolysis was achieved, the concomitant administration of the GPIIb/IIIa platelet receptor inhibitor CRL-42796 did not have a significant effect on time to thrombolysis for either rt-PA or alfimeprase, 8.1 ± 1.9 min and 2.6 ± 1.0 min, respectively. Although the time to clot lysis did not differ significantly, the data suggested a trend for a more rapid restoration of blood flow in the alfimeprase-treated animals compared with rt-PA-treated animals when both lytic agents were administered in the presence of CRL-42796 (2.6 ± 1.0 min for alfimeprase + CRL-42796 vs. 8.1 ± 1.9 min for rt-PA + CRL-42796).

In the absence of adjunctive treatment, rethrombosis occurred within 1 h in each of the animals treated with alfimeprase, whereas patency was maintained in the rt-PA-treated animals, albeit at a very low patency score characterized by repetitive cyclic reductions in LCX coronary artery blood flow (Fig. 1). The patency score in the alfimeprase plus CRL-42796-treated animals significantly surpassed that achieved with the combined use of rt-PA and CRL-42796. The infusion of CRL-42796 prolonged the total reperfusion time, 132.0 ± 26.9 min for rt-PA-treated and 163.5 ± 26.8 min for the alfimeprase-treated group compared with their respective controls. Two hours after discontinuing the infusion of CRL-42796, each of the six vessels reoccluded in the rt-PA-treated group, and one of nine vessels in the alfimeprase-treated group remained patent. The flow patency scores for each of the four groups are summarized in Fig. 1 according to the previously published method (34). Graphic representations of the coronary artery flow patterns achieved with rt-PA or alfimeprase when used alone or in combination with CRL-42796 are shown in Fig. 2.

Local administration of rt-PA achieved rapid thrombolysis in which there was a pattern of repetitive cyclic flow variations and a gradual progression toward rithrombosis. In the presence of the platelet GPIIb/IIIa receptor antagonist CRL-2796, the subsequent local administration of rt-PA resulted in gradual restoration of coronary artery blood flow that ultimately progressed to rithrombosis.

When used as a singular intervention, the local administration of alfimeprase achieves rapid clot lysis followed by a rapid
restoration of an occlusive thrombosis. However, when administered in the presence of CRL-42796, alfimeprase resulted in a rapid restoration of coronary artery blood flow that had a normal flow pattern and did not exhibit signs of cyclic flow variations. The restoration of coronary artery blood flow was maintained beyond the known pharmacodynamic effects of the lytic agent that is susceptible to rapid inactivation by α2-macroglobulin (Fig. 2).

Bleeding time and ex vivo platelet aggregation responses. When administered in the absence of an adjunctive therapy, neither alfimeprase nor rt-PA, in the doses used, altered tongue-bleeding time. In contrast, the administration of CRL-42796 was associated with an increase in tongue bleeding time, which returned to baseline values 2 h after the infusion of CRL-42796 was discontinued (Table 2). As expected, CRL-42796 inhibited ex vivo platelet aggregation in response to AA and ADP. Ex vivo inhibition of platelet aggregation in response to the two-platelet agonists persisted for ~2 h after discontinuing the infusion of CRL-42796 (Table 3).

Protocol II: Effects of rt-PA and Alfimeprase on Myocardial Infarct Size

The initial study group consisted of 29 dogs. Seven (3 control, 3 alfimeprase treated, and 1 rt-PA treated) were excluded for infarct size comparison among the three groups because RMBF was >0.16 ml·min⁻¹·g tissue⁻¹. One animal from the alfimeprase-treated group was excluded from the study because of intractable ventricular fibrillation that required more than three attempts at electrical defibrillation.

RMBF determinations. RMBF values for control, rt-PA-treated, and alfimeprase-treated groups are summarized graphically in Fig. 3. RMBF in the distribution of the LCX coronary artery (ischemic region at risk) was determined with the use of radiolabeled microspheres administered 45 min after application of the occlusive ligature. RMBF was reduced significantly in the risk region compared with the RMBF in the LAD coronary artery perfused area (nonischemic or nonrisk region). The RMBF within the respective LCX and LAD regions were similar among the control, rt-PA, and alfimeprase groups.

Myocardial infarct size. The area at risk in the distribution of the LCX coronary artery is expressed as a percentage of the left ventricle and was found to be similar among the three experimental groups (Fig. 4). Infarct size, expressed as a percentage of the area at risk, is shown in Fig. 4 and is expressed as a weight percent of the risk region. Infarct size in the animals administered rt-PA was significantly larger (32.16 ± 3.95) compared with the alfimeprase-treated and placebo-treated groups. Thus, in the absence of significant differences in collateral blood flow in the area at risk among the three treatment groups, infarct size is significantly larger when reperfusion occurs in the presence of rt-PA compared with either the placebo- or alfimeprase-treated groups.

DISCUSSION

Plasmin-dependent thrombolytic agents have the potential to activate diverse proinflammatory events that contribute to the pathogenesis of ischemia-reperfusion injury (20). When incubated in the presence of plasmin and normal human serum, both endothelial cells and PMNs exhibit an increased staining

Table 2. Tongue bleeding time in each of the 4 groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time Point</th>
<th>Time</th>
<th>Value (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline + rt-PA</td>
<td>Baseline</td>
<td>2.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Saline + Alphimeprase</td>
<td>0.5 h after saline or CRL-42796 infusion</td>
<td>2.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CRL-42796 + rt-PA</td>
<td>2.5 h after saline or CRL-42796 infusion</td>
<td>2.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CRL-42796 + Alphimeprase</td>
<td>2 h after discontinuing saline or CRL-42796</td>
<td>2.5 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE (in min). *P < 0.05 vs. baseline from each group.

AJP-Heart Circ Physiol • VOL 290 • MARCH 2006 • www.ajpheart.org
for the membrane attack complex. However, staining was not
detected when the cells were incubated with plasmin in the
presence of heat-inactivated normal human serum (20), thereby
suggesting that plasmin-dependent lytic agents may contribute
to the extension of tissue injury associated with reperfusion.
The plasmin-dependent thrombolytic agents possess the poten-
tial to augment tissue demolition through activation of the
classical complement pathway and facilitation of a local in-
flammatory response within the area at risk (1, 16, 33).

In the present study, we examined the hypothesis that a
plasmin-dependent thrombolytic agent (rt-PA) would exacer-
bate the extent of myocardial injury on reperfusion of the
ischemic myocardium. The testing of the hypothesis was made
possible by comparing the extent of myocardial injury after
reperfusion of the ischemic myocardium coincident with the
administration of a plasmin-dependent lytic agent, rt-PA, or the
direct acting fibrin-specific proteolytic enzyme alfimeprase.
The latter, in contrast to rt-PA, has proteolytic activity against
the fibrinogen Aα chain and does not require the endogenous
fibrinolytic system (conversion of plasminogen to plasmin) for
achieving clot lysis. Both rt-PA and alfimeprase were adminis-
tered immediately proximal to the site of vessel occlusion at
the time of reperfusion. The dose of each lytic agent was
determined in
protocol I
and found to be effective in achieving
thrombolysis without inducing a systemic lytic effect.

In contrast to the plasmin-dependent thrombolytic agents,
alfimeprase does not require an endogenous fibrinolytic system
(conversion of plasminogen to plasmin). Thus alfimeprase

### Table 3. Percent ex vivo platelet aggregation in response to AA or ADP

<table>
<thead>
<tr>
<th></th>
<th>Saline+rt-PA (n = 6)</th>
<th>Saline+Alfimeprase (n = 6)</th>
<th>CRL-42796+rt-PA (n = 6)</th>
<th>CRL-42796+Alfimeprase (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA (0.65 mM)</strong></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>84.0±2.0</td>
<td>85.5±3.0</td>
<td>78.8±2.9</td>
<td>79.0±2.2</td>
</tr>
<tr>
<td>0.5 h after saline or CRL-42796 infusion</td>
<td>84.3±2.2</td>
<td>85.3±2.6</td>
<td>12.3±5.6*</td>
<td>9.3±2.6*</td>
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<tr>
<td>2.5 h after saline or CRL-42796 infusion</td>
<td>77.5±5.7</td>
<td>76.6±4.8</td>
<td>6.8±2.0*</td>
<td>8.4±2.3*</td>
</tr>
<tr>
<td>2 h after discontinuing saline or CRL-42796</td>
<td>68.7±7.6</td>
<td>76.7±9.0</td>
<td>19.6±4.7*</td>
<td>17.9±5.0*</td>
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<tr>
<td><strong>ADP (20 µM)</strong></td>
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<tr>
<td>Baseline</td>
<td>81.5±4.4</td>
<td>76.3±3.4</td>
<td>79.0±5.4</td>
<td>74.0±5.7</td>
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<tr>
<td>0.5 h after saline or CRL-42796 infusion</td>
<td>76.2±3.7</td>
<td>72.7±1.3</td>
<td>3.0±1.3*</td>
<td>3.9±1.2*</td>
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<tr>
<td>2.5 h after saline or CRL-42796 infusion</td>
<td>50.8±14.9</td>
<td>67.3±10.6</td>
<td>3.5±1.6*</td>
<td>5.1±2.8*</td>
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<tr>
<td>2 h after discontinuing saline or CRL-42796</td>
<td>35.0±11.7</td>
<td>54.0±14.0</td>
<td>9.4±6.1*</td>
<td>5.3±1.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in percent). AA, arachidonic acid. *P < 0.05, †P < 0.01 vs. baseline with each group.
differs from the plasminogen activators by its unique mechanism of action and is defined as a direct acting fibrinolytic agent. The advantages of alfimeprase include thrombolytic activity independent of plasmin activation (2), inability to activate platelets (39), and a clearance mechanism by α2-macroglobulin that limits the molecule’s pharmacodynamic action in the systemic circulation (39).

Although the present study was designed to compare the thrombolytic effects of rt-PA and alfimeprase in a canine model of occlusive coronary artery thrombosis (29, 34), the primary goal was to examine each agent in the setting of regional myocardial ischemia and reperfusion. The effective lytic dose of each agent was determined by direct intracoronary injection, immediately proximal to an occlusive arterial thrombus. The minimal dose of rt-PA needed to achieve reproducible thrombolysis was predetermined based on pilot studies and was selected to minimize induction of a systemic fibrinolytic state and excessive bleeding. The selected dose of rt-PA induced thrombolysis in <15 min in all of the vessels and was followed by reocclusion in 50% of the trials, within 3 h after completion of the infusion protocol. The clinical use of rt-PA by the intra-arterial route of administration has been discussed in a number of published articles (38) and has gained renewed interest for the management of patients with ischemic stroke (5, 8). Interestingly, the intracoronary dose of rt-PA determined in our pilot studies corresponds to the dose used in previously reported clinical trials (23, 28). The dose of alfimeprase of 0.5 mg/kg, used in the present study, was shown to be effective and well tolerated in patients with chronic peripheral arterial occlusion (25). In the current study, alfimeprase was administered as a single dose of 0.5 mg/kg, and the average time to clot lysis was significantly shorter than that observed with rt-PA. In contrast to rt-PA, vessel patency after alfimeprase was relatively brief (77.5 ± 31.9 vs. 3.2 ± 0.5 min) consistent with its short pharmacological half-life due to rapid inactivation by α2-macroglobulin (39). The results in the present study are in agreement with those reported previously in a rat carotid artery thrombosis model in which alfimeprase was compared with urokinase in terms of efficacy (time to clot lysis) and safety (hemorrhagic complications) (39).

Although capable of achieving clot lysis, currently available thrombolytic agents are used in combination with adjunctive therapies to maintain vessel patency. The present study used the adjunctive agent CRL-42796, a GPIIb/IIIa platelet receptor antagonist (13, 14). The platelet receptor antagonist was administered as a continuous intravenous infusion commencing before the administration of the respective lytic agents. The concomitant administration of CRL-42796 with either rt-PA or alfimeprase was effective in maintaining vessel patency for an extended period along with a significant decrease in the number of cyclic flow reductions. CRL-42796 has a relatively short pharmacological half-life that accounts for the fact that coronary artery reocclusion developed in each of the animals in both the rt-PA- and alfimeprase-treated groups after the infusion of GPIIb/IIIa platelet receptor antagonist was discontinued. It is important to note that the tongue bleeding time increased slightly in both the rt-PA- and alfimeprase-treated groups after the administration of CRL-42796. The tongue bleeding times for both groups returned to baseline values after the infusion of CRL-42796 was discontinued. The normalization of the bleeding time and the occurrence of reocclusion in each of the treatment groups suggests that systemic fibrinolysis was not a major factor influencing the outcome. Furthermore, ex vivo platelet aggregation remained suppressed 2 h after discontinuing the administration of CRL-42796 and at a time when coronary artery reocclusion had occurred and tongue bleeding time values had normalized, suggesting that determinations of ex vivo platelet reactivity may not reflect the status of in vivo platelet function.

Previous studies (10) suggest that rt-PA benefits the heart independent of its ability to lyse coronary artery thrombi. The study by Kloner et al. (15) is the first to suggest that rt-PA (0.21 mg/kg iv) neither protects the canine ischemic myocardium subjected to 2 h of occlusion nor prevented the development of the no-reflow phenomenon after 4 h of reperfusion. The authors noted that neutrophil infiltration was more prominent in the rt-PA-treated group and that infarct size was slightly larger, although it did not differ significantly from controls. In the current study, the dose of rt-PA was 0.022 mg/kg infused directly into the previously ligature-occluded coronary artery throughout the first hour of reperfusion. Compared with the diluent-treated control group, infarct size, expressed as a percentage of the area at risk, was significantly larger in the rt-PA treated group (18.46 ± 3.34% vs. 32.16 ± 3.95%). In contrast to an earlier study (15), the duration of coronary artery occlusion was limited to 60 min, resulting in a relatively smaller infarct in the control group, which in the presence of rt-PA was increased when determined 4 h after reperfusion. Unlike the results obtained with rt-PA, the intracoronary administration of alfimeprase at the time of reperfusion did not have a significant effect on myocardial infarct size compared with the diluent-treated control group. Thus doses of rt-PA or alfimeprase that can achieve a similar extent of initial thrombolysis, which can then be maintained by the adjunctive agent CRL-42796, exhibit significantly different effects on the viability of myocardial tissue in the heart subjected to 60 min of regional ischemia followed by 4 h of reperfusion.

Alfimeprase acts directly on fibrin to achieve fibrinolysis in contrast to rt-PA, which achieves its thrombolytic effect indirectly through the conversion of clot bound and free plasminogen to plasmin. In addition to its proteolytic effects, plasmin is known to activate the complement system (1, 12, 16, 33), which in turn can lead to an extension of reperfusion injury (18, 19). In addition, plasmin increases the synthesis of leukotriene B4 (41), cytokines (IL-1α, IL-1β, TNF-α), and tissue factor (TF) expression (35); and directly influences platelet reactivity (31, 32), neutrophils (30), and endothelial cells (9). Extension of tissue injury induced by plasmin and rt-PA was observed in rat brain tissue (24, 44). Thus the potential for plasmin to exacerbate a local inflammatory response may account for the extension of tissue injury observed in the rt-PA-treated group compared with the group treated with alfimeprase.

Although the doses for both lytic agents were shown to induce a similar extent of initial thrombolysis that was maintained by an adjunctive agent, the different administration procedure for rt-PA and alfimeprase (infusion vs. rapid injection) might be viewed as a potential limitation in the experimental design for an infarct size study. The bolus injection of alfimeprase and its rapid neutralization will cause “unequal exposure” of the myocardium to alfimeprase as opposed to rt-PA. However, this difference in duration of exposure of the
myocardial tissue to each lytic agent demonstrates the potential advantage of alfimeprase over rt-PA because the two lytic agents differ radically in their respective pharmacokinetic and pharmacodynamic properties. The additional duration of myocardial exposure to rt-PA is a consequence of its pharmacological actions, which determine the manner in which the drug must be administered to achieve successful thrombolysis. In the current model involving thrombolysis with rt-PA, the lytic agent achieves clot lysis in ~10 min. Therefore, we draw the readers attention to the fact that the extra ~ 50 min of rt-PA infusion beyond the time of flow restoration may have contributed to the overall injury in infarct size and might be considered as the limitation of our study even though the mode of rt-PA administration approximates that recommended for clinical use in patients undergoing lytic therapy for acute coronary syndrome. Furthermore, the lytic action of alfimeprase is terminated by plasma α2-macroglobulin that becomes saturated with prolonged administration beyond the time of blood flow restoration, thereby resulting in a systemic lytic effect that is directed primarily on fibrinogen (39). Hence, alfimeprase can be distinguished by its unique mode of action pharmacokinetics and can be defined as a direct fibrinolytic agent.

The pharmacological properties of rt-PA require that the drug be administered as a loading dose followed by an infusion to achieve restoration of blood flow. In the current study, the intracoronary dose of rt-PA was relatively small compared with the intravenous dose required to achieve thrombolysis. Thus it is reasonable to predict that a greater extent of myocardial injury plus an increased potential for bleeding would have resulted if rt-PA were administered as a full intravenous dose of 1–2 mg/kg.

In conclusion, the experimental data suggested that the local administration of alfimeprase induces a rapid thrombolytic effect in a dose that does not alter hemostasis or induce bleeding at remote sites. Termination of the lytic action is through inhibition of alfimeprase by α2-macroglobulin, thus limiting the lytic activity to the site of application. The rapid on-off action of alfimeprase requires the concomitant use of adjunctive therapy, such as a GPIIb/IIIa platelet antagonist, to maintain vessel patency. In contrast to rt-PA, a plasmin-dependent lytic agent, alfimeprase was not associated with an increase in myocardial infarct size after reperfusion. The data support the concept that plasmin may increase the extent of myocardial tissue injury associated with ischemia and reperfusion. The results of the present study are in agreement with findings by others who express the view that plasmin-dependent thrombolytic agents activate several enzymatic systems with proinflammatory effects, thus potentially contributing to the pathogenesis of ischemia-reperfusion injury (20, 22). Inflammation and activation of the coagulation cascade contribute significantly to the etiology of postthrombolytic complications that include obstruction of the microvasculature and reperfusion injury. The failure to achieve maximum benefit with the use of current thrombolytic agents may be due, in part, to the plasmin-induced procoagulant and proinflammatory effects that contribute to the extension of irreversible tissue injury on reperfusion. Alfimeprase, by virtue of its direct proteolytic action on fibrin, may possess an advantage over plasmin-dependent lytic agents in terms of limiting the cytotoxic effects associated with thrombolytic therapy and an ability to reestablish physiological level of tissue perfusion.

Early impairments in myocardial perfusion grade are known to be associated with mortality after thrombolysis for acute myocardial infarction (3). The results of this study indicate that a directly acting fibrin-specific lytic agent has the potential to be more effective than a plasmin-dependent thrombolytic agent in terms of tissue salvage, restoration of a physiological pattern of blood flow, and absence of hemorrhagic complications.

GRANTS

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