Conjunctive Enhancement of Enzymatic Thrombolysis and Prevention of Thrombotic Reocclusion With the Selective Factor Xa Inhibitor, Tick Anticoagulant Peptide

Comparison to Hirudin and Heparin in a Canine Model of Acute Coronary Artery Thrombosis

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**Background.** Effective thrombolytic recanalization of an occluded coronary vessel is often limited by acute thrombotic reocclusion, which has galvanized the search for effective adjunctive or conjunctive antithrombotic agents.

**Methods and Results.** Recombinant versions of tick anticoagulant peptide (rTAP) and hirudin (rHIR) are highly selective and potent polypeptide inhibitors of factor Xa and thrombin, respectively. The comparative antithrombotic efficacies of rTAP, rHIR, and heparin, administered conjunctively with recombinant tissue-type plasminogen activator (rt-PA), on thrombolytic reperfusion and reocclusion, were determined in a canine model of occlusive coronary artery thrombosis with a superimposed critical stenosis. In this model, a platelet-rich occlusive thrombus was formed after damage to the intimal surface of the left circumflex coronary artery induced by electrolytic injury. Fifteen minutes after occlusion, the dogs received a systemic intravenous administration of either saline (control), heparin (200 units/kg bolus +2 units/kg/min, heparin (HEP) 200 or 100 units/kg bolus +1 unit/kg/min, HEP 100), rHIR (50 or 100 μg/kg/min, rHIR 50 or 100, respectively), or rTAP (100 μg/kg/min, rTAP 100) followed 15 minutes later by rt-PA (100 μg/kg bolus +10 μg/kg/min over 90 minutes). Infusions of the conjunctive agents were discontinued 60 minutes after termination of rt-PA. The incidence and time (mean±SEM) to thrombolytic reperfusion were determined for control (five of 12; 68.0±7.8 minutes), HEP 100 (six of eight; 40.1±8.3 minutes), HEP 200 (six of eight; 39.8±9.5 minutes), rHIR 50 (six of eight; 51.7±14.6 minutes), rHIR 100 (eight of eight; 19.5±4.2 minutes), and rTAP 100 (eight of eight; 22.8±10.0 minutes). The incidence and time to reocclusion after rt-PA were determined for control (four of five; 45.7±12.5 minutes), HEP 100 (four of six; 18.2±10.7 minutes), HEP 200 (five of six; 26.2±20.7 minutes), rHIR 50 (four of six; 47.3±21.6 minutes), rHIR 100 (six of eight; 89.8±5.9 minutes), and rTAP 100 (three of eight; 54.0±16.3 minutes). All of the dogs that reoccluded in the rHIR 100 group did so after termination of the inhibitor infusion, whereas two of the three dogs in the rTAP 100 group that reoccluded did so during the inhibitor infusion. Coronary artery blood flow was characterized by intermittent periods of reocclusion and recanalization in all groups except rTAP 100.

**Conclusions.** The potent antithrombotic effects of rTAP in this model directly implicate de novo thrombin formation as a major source of thrombin activity within the highly thrombogenic residual thrombus. These findings suggest that direct inhibition of prothrombinase activity may be an effective strategy in the development of a new class of conjunctive agents. (Circulation 1992;85:805–815)
Enzymatic recanalization of an occluded coronary vessel with recombinant tissue-type plasminogen activator (rt-PA) or streptokinase has been shown to reduce mortality significantly in patients suffering from acute transmural myocardial infarction. In spite of the overwhelming success of these agents, their clinical application has been associated with several significant and unpredictable limitations, including resistance to recanalization and acute thrombotic reocclusion in a high percentage of treated patients. Adjunctive therapy with currently available antiplatelet (aspirin) and anticoagulant (heparin) agents has shown some benefit in overcoming these difficulties, indicating the need for more potent and specific pharmacological agents.

The pathogenic mechanism resulting in arterial thrombosis after vessel damage is thought to involve platelet adherence and subsequent aggregation as well as activation of the coagulation cascade. The resulting formation of the serine protease factor Xa (FXa) and its assembly into active prothrombinase complexes on the surface of activated, adhered platelets or platelet microparticles result in the generation of locally high levels of thrombin through feedback activation and amplification. It is believed that thrombin has a central role in arterial thrombus development and stabilization through the generation of fibrin, the activation of factor XIII, and, most importantly, as the primary mediator of platelet activation.

The contributory role of platelets in arterial thrombosis has been well established in several experimental studies using highly selective antagonists of the platelet glycoprotein IIb/IIIa fibrinogen receptor. The underlying role of thrombin, however, was initially doubted because of the limited efficacy of heparin as an antithrombotic agent in experimental and clinical studies of arterial thrombosis/rethrombosis. Recently, several low-molecular-weight, direct inhibitors of thrombin have been shown in experimental models to be highly effective in preventing platelet-dependent arterial thrombosis and rethrombosis after thrombolytic reperfusion. The increased accessibility of direct thrombin inhibitors to thrombin bound within a platelet-rich clot and to subendothelial matrices and their resistance to endogenous heparin inactivators have been directly demonstrated to contribute to the improved efficacy of these inhibitors over heparin.

The potent antithrombotic effects of direct thrombin inhibition firmly established that the activity of the coagulation cascade is the primary source of thrombogenicity within a platelet-rich arterial thrombus. It has been proposed that preformed, clot-bound thrombin constitutes the primary source of thrombin activity that is manifested upon thrombolytic reperfusion. Previous studies using antithrombin III-independent inhibitors of thrombin could not directly discriminate between thrombin activity resulting from de novo thrombin formation and preformed thrombin within the vicinity of the residual thrombus. FXa is the catalytically active portion of the prothrombinase complex responsible for the generation of thrombin. A comparison of the in vivo antithrombotic effects of direct FXa and thrombin inhibition using highly selective inhibitors for each enzyme would be useful in further defining the source of thrombin activity within an arterial thrombus after thrombolytic recanalization.

We have previously reported on the isolation and characterization of tick anticoagulant peptide, a highly selective and potent 60-amino-acid inhibitor of FXa. The antithrombotic efficacy of a recombinant version of tick anticoagulant peptide (rTAP) has recently been demonstrated in a rabbit model of venous thrombosis and a baboon model of platelet-dependent arterial thrombosis. Hirudin is a 60–65-amino-acid polypeptide, originally isolated from the medicinal leech, that has been shown to be a highly selective and potent inhibitor of thrombin. The antithrombotic efficacy of natural and recombinant desulfatohirudin (rHIR) has been demonstrated in several experimental models of venous and arterial thrombosis. The high degree of selectivity of rTAP and rHIR for their cognate enzymes makes them ideal tools to investigate the in vivo antithrombotic effects of selectively inhibiting FXa and thrombin, respectively (see Figure 1).

In this study, we have evaluated the effects of rTAP, rHIR, and heparin on the efficacy of rt-PA-mediated thrombolysis and subsequent acute reclosure in a canine model of platelet-dependent coronary artery thrombosis. This animal model has been used extensively to investigate the antithrombotic effects of potential conjunctive agents. The results of the present study demonstrate that both rTAP and rHIR, but not heparin, significantly accelerated rt-PA-mediated thrombolysis. Each treatment had different effects on acute reclosure after thrombolytic recanalization, with the majority of dogs in the rHIR and heparin treatment groups.

FIGURE 1. Schematic of the specific points of inhibition by recombinant tick anticoagulant peptide and recombinant hirudin after either intrinsic or extrinsic activation of the blood coagulation enzymatic cascade. FXa, blood coagulation factor Xa.
Drugs and Solutions

All drugs were prepared on the day of the experiment in sterile saline. Purified rTAP was prepared as described previously. The material was judged essentially homogeneous by a number of analytical criteria, and all experiments used a single lot of the recombinant inhibitor. A hybrid of hirudin isoform HV-146 containing an Ile-Val instead of Val-Val at positions 1 and 2 was used in this study. rHIR was produced in Saccharomyces cerevisiae and purified to homogeneity using ion-exchange and reverse-phase high-performance liquid chromatography (RP-HPLC) (D. Lehman, manuscript in preparation). The purified material was judged essentially homogeneous by a number of analytical criteria, including amino acid composition, sequence analysis, and analytical RP-HPLC. rHIR was kinetically characterized as a stoichiometric inhibitor of purified human α-thrombin using the chromogenic substrate S-2238 (Kabi) with a dissociation constant (Kd) determined to be 0.54±0.04 pmol/l at pH 7.5. In addition, the in vitro anticoagulant properties of rHIR were indistinguishable from authentic rHIR variant, HV-2 (Sigma Chemical Co.), using pooled normal human plasma (George King Biomedical) in activated partial thromboplastin time (aPTT) and thrombin time clotting assays.

Surgical Preparation

Male or female purpose-bred mongrel dogs (11–15 kg) were anesthetized with sodium pentobarbital (35 mg/kg i.v.) and ventilated with room air by a positive-pressure ventilator (Harvard Apparatus, South Natick, Mass.). The right femoral artery and vein were cannulated for the measurement of mean arterial pressure (Statham P23ID, Gould Inc., Cleveland, Ohio) and for drug administration, respectively. The left external jugular vein and cephalic vein also were cannulated for the continuous infusion of 5% dextrose in saline and for drug administration, respectively. The heart was exposed via a left thoracotomy at the fifth intercostal space. The left circumflex coronary artery (LCx) was isolated proximal to the first obtuse marginal branch and dissected for a distance of approximately 2 cm. The vessel was instrumented, proximal to distal, as follows: electromagnetic flow probe (Model 501, Carolina Medical Electronics Inc., King, N.C.), stimulation electrode, adjustable mechanical occluder (Goldblatt clamp), and a silk snare. The stimulation electrode was constructed from a 26-gauge stainless steel hypodermic needle tip attached to a 30-gauge Teflon-insulated silver-coated copper wire. The adjustable occluder was tightened sufficiently around the artery to eliminate the reactive hyperemic response without affecting resting LCx blood flow. Continuous records of systemic blood pressure and mean and phasic LCx blood flow were displayed on a model 7E polygraph (Grass Instrument Co., Quincy, Mass.). Zero flow and hyperemic flows were determined by occluding the LCx distal to the flow probe for 20 seconds with the snare. The process of thrombotic occlusion of the LCx was initiated 30 minutes after surgical preparation by the application and maintenance of 150 μA continuous anodal direct current to the LCx stimulation electrode until coronary artery blood flow decreased to and remained at zero. Direct electrical stimulation was delivered by a Grass constant-current unit (model CCU1A), a Grass stimulus isolation unit (model SIU5), and a Grass stimulator (model S48) connected to the intraluminal LCx stimulation electrode.

Experimental Protocol and Treatment Groups

Figure 2 depicts the general experimental protocol used in the present studies. Dogs were randomly assigned to one of the six conjunctive treatment groups listed in Figure 2 and described below, with at least eight dogs assigned to each group. Specific protocols for the individual treatment groups are as follows. In all treatment groups, rt-PA (Activase, Genentech) was administered as a 100-μg/kg i.v. bolus at 30 minutes after LCx occlusion, followed by a maintenance infusion of 10 μg/kg/min i.v. for 90 minutes. Conjunctive therapies, administered intravenously, were initiated 15 minutes after LCx occlusion (i.e., 15 minutes before rt-PA) and were maintained for a period of 165 minutes (i.e., terminating 60 minutes after the end of rt-PA therapy). A 60-minute observation period followed the termination of conjunctive therapy. Specific conjunctive therapies for the individual treatment groups were: control, intravenous infusion of saline at a rate of 0.15 ml/min; HEP 100, HEP administered as a bolus of 100 units/kg i.v. followed by a 1-unit/kg/min i.v. infusion; HEP 200, heparin administered as a bolus of 200 units/kg i.v. followed by a 2-unit/kg/min i.v. infusion; rTAP 100, rTAP administered as a 100-μg/kg/min i.v. infusion; rHir 50, rHir administered as a 50-μg/kg/min i.v. infusion; and rHir 100, rHir administered as a 100-μg/kg/min i.v. infusion.

Criteria for Reperfusion and Reocclusion

Thrombotic occlusion of the LCx coronary artery was judged complete when coronary artery blood flow (CABF) decreased to and remained at zero. Five minutes after LCx occlusion, anodal current to the LCx stimulation electrode was terminated, and the rt-PA bolus and infusion regimen was initiated at having intermittent periods of reocclusion and reperfusion. In contrast, the majority of dogs in the rTAP treatment group remained patent. The potent antithrombotic effects of rTAP in this model establish de novo thrombin formation mediated by FXa as a major source of thrombin in an occlusive arterial thrombus. These findings indicate that direct inhibition of prothrombinase activity may be an effective strategy in the development of a new class of conjunctive agents.
30 minutes after LCx occlusion as described above. The criterion for reperfusion was defined as the reestablishment of at least 50% of control LCx CABF (flow immediately before delivery of anodal current to the vessel) for a period of at least 5 minutes or sustained LCx CABF for a period of at least 15 minutes. Times to reperfusion were quantified for all treatment groups as the time after the initiation of rt-PA infusion. Reperfusion was defined as the reestablishment of zero LCx CABF after the termination of rt-PA. In some cases, intermittent flow reductions occurred after thrombolytic reperfusion in this model. Thus, the first intermittent flow reduction that achieved zero flow constituted reperfusion. Times to reocclusion were expressed as times after the termination of rt-PA infusion. Residual thrombus mass was determined at the end of each experiment after ligation of the LCx proximal to the electromagnetic flow probe and distal to the Goldblatt clamp, careful dissection of the vessel, and blotting of the thrombus to remove excess blood.

**Plasma Concentrations of rtAP and rHIR**

Plasma levels of rtAP were measured as described previously\(^{37}\) in an assay using purified human FXa and the chromogenic substrate Spectrozyme Xa (American Diagnostica). The plasma levels of rHIR were determined similarly using purified human α-thrombin and the chromogenic substrate S-2238 (Kabi).

**Hemostatic Parameters**

*Ex vivo platelet aggregation.* Blood was withdrawn from the femoral artery into a plastic syringe containing one part 3.8% trisodium citrate to nine parts blood. Platelet-rich plasma (PRP) was obtained by centrifugation at 150g for 10 minutes at room temperature. Platelet count was adjusted to 300,000/ml. Aggregation studies were performed in an aggregometer (Chrono-Log Corp., Havertown, Pa.) using 0.25 ml PRP in a siliconized cuvette stirred at 1,000 rpm. PRP was prewarmed for 3 minutes at 37°C before addition of agonist. Adenosine diphosphate (10 μmol/l in the presence of 1 μmol/l epinephrine) and collagen (10 μg/ml in the presence of 1 μmol/l epinephrine) were added to the cuvette, and the change in light transmittance was measured to the point where the tracing reached a plateau. Platelet aggregation is expressed as percent of light transmittance, with platelet-poor plasma representing 100% transmittance. Sampling times for ex vivo platelet aggregation responses are the same as shown in Figure 6.

**Ex vivo clotting assay and bleeding time determination.** Effects on the intrinsic coagulation cascade were assessed by determination of the aPTT. Arterial blood (2 ml) was withdrawn into a syringe containing 0.2 ml of 3.8% trisodium citrate solution. The blood was centrifuged for 10 minutes at 2,000g. The plasma was removed and stored on ice for later assay. The aPTTs were determined with an automated clot timer (Electra 800, Medical Laboratory Automation, Mt. Vernon, N.Y.) and commercially available reagents (American Dade, Aquada, Puerto Rico). Buccal mucosal template bleeding times were measured with a Simplate bleeding time device (Organon Teknika Corporation, Durham, N.C.). Uniform incisions were made with the Simplate on the mucous membrane of the inner upper lip of the dog, and the duration of bleeding was timed. Sampling times for aPTT and template bleeding time determinations are shown in Figure 6.

**Statistical Analysis**

Data are expressed as mean±SEM. Among-group comparisons were conducted with a one-way ANOVA followed by a Dunnett’s test for multiple comparisons or with a Fisher’s exact test. Within-group comparisons at multiple time points were conducted with a one-way ANOVA with repeated measures followed by a Dunnett’s test for comparisons to baseline values.
TABLE 1. Effect of Conjunctive Treatments on Thrombolytic Reperfusion and Acute Reoclusion After Recombinant Tissue-Type Plasminogen Activator

<table>
<thead>
<tr>
<th>Conjunctive treatment groups</th>
<th>Control</th>
<th>rTAP 100</th>
<th>rHIR 100</th>
<th>Heparin 100</th>
<th>Heparin 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence of reperfusion*</td>
<td>5/12</td>
<td>8/8†</td>
<td>8/8†</td>
<td>6/8</td>
<td>6/8</td>
</tr>
<tr>
<td>Time to reperfusion (min)‡</td>
<td>68.0±7.8</td>
<td>22.8±10‡</td>
<td>19.5±4.2‡</td>
<td>51.7±14.6</td>
<td>40.1±8.3</td>
</tr>
<tr>
<td>Incidence of reoclusion</td>
<td>4/5</td>
<td>3/8</td>
<td>6/8</td>
<td>4/6</td>
<td>5/6</td>
</tr>
<tr>
<td>Time to reoclusion (min)§</td>
<td>45.7±12.5</td>
<td>54.0±16.3</td>
<td>89.8±5.9</td>
<td>47.3±21.6</td>
<td>18.2±10.7</td>
</tr>
<tr>
<td>Incidence of reoclusion during conjunctive treatment</td>
<td>...</td>
<td>2/8</td>
<td>0/8</td>
<td>2/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Thrombus mass (mg)</td>
<td>11.6±1.3</td>
<td>3.8±1.1‡</td>
<td>4.0±0.9‡</td>
<td>9.9±2.4</td>
<td>7.5±2.0</td>
</tr>
</tbody>
</table>

rt-PA, recombinant tissue-type plasminogen activator; rTAP, recombinant tick anticoagulant peptide; rHIR, recombinant hirudin. (See text for dosages.)

*The criterion for reperfusion after initiation of rt-PA is defined in “Experimental Methods.”
†p≤0.05 vs. the control group by Fisher’s exact probability test.
‡p≤0.05 among group comparisons to the control group by ANOVA followed by Dunnett’s test for multiple comparisons.
§The time to reocclusion after termination of rt-PA infusion; excludes preparation remaining patent for the duration of the protocol.

Results

Conjunctive Effects on rt-PA-Mediated Reperfusion and Acute Reoclusion

In total, 52 dogs were randomized into the following treatment groups: control (n=12), HEP 100 (n=8), HEP 200 (n=8), rTAP 100 (n=8), rHIR 50 (n=8), and rHIR 100 (n=8) as described in “Experimental Methods.” The effects of the conjunctive interventions on thrombolytic recanalization of the occluded coronary vessel are summarized in Table 1. The relatively low incidence of reperfusion in the control group (five of 12) reflects the highly resistant nature of the occluding thrombus in this preparation at this dose of rt-PA. The administration of both low- and high-dose heparin had only modest effects on the incidence and time to reperfusion compared with the control group (Table 1). rHIR given at a dose of 50 μg/kg/min i.v. also did not significantly affect the time to reperfusion but slightly improved the incidence compared with control. In contrast to these groups, all of the dogs in both the rTAP 100 and rHIR 100 treatment groups achieved reperfusion. The times to reperfusion were also dramatically accelerated in these groups compared with the control (Table 1).

Table 1 also summarizes the effects of the conjunctive treatments on reoclusion and the residual thrombus mass measured at the end of each experiment. In this model, the residual, uncontrolled thrombogenicity of the reperfused vessel resulted in a high incidence (four of five [80%]) of reoclusion in the control treatment group. Neither low- nor high-dose heparin significantly altered the incidence (four of six [67%] and five of six [83%], respectively) or time to the first reoclusion event. The apparent increased rates of reoclusion in the two heparin treatment groups compared with control were not statistically significant. Administration of rHIR was not beneficial with respect to the overall incidence of reoclusion at either dosing regimen (four of six [67%] for rHIR 50 and six of eight [75%] for rHIR 100), although the time to reoclusion in the rHIR 100 group was delayed (Table 1). All of the dogs that reoclusioned in the rHIR 100 group did so after termination of the inhibitor infusion. Treatment with rTAP at 100 μg/kg/min resulted in the lowest incidence of reoclusion compared with the other treatment groups (three of eight [38%]), with five of eight dogs remaining patent for the duration of the protocol (Table 1). The time to reoclusion in the three remaining dogs was not significantly different from the control group, with two of the three dogs reocluding during the inhibitor infusion. Residual thrombus mass was significantly reduced in the high-dose heparin as well as the rTAP 100 and rHIR 100 treatment groups.

Extent and Duration of Left Circumflex Coronary Artery Blood Flow

Integrated LCx CABF profiles reflecting the extent and duration of reperfusion after thrombolysis are shown in Figure 3. There was a rapid and full restoration of LCx CABF during the rt-PA infusion in both the rTAP 100 and rHIR 100 groups which gradually decreased after termination of rt-PA and the conjunctive treatment (Figure 3A). In comparison to these groups, the effects of rHIR at 50 μg/kg/min (Figure 3A) as well as both low- and high-dose heparin (Figure 3B) on the integrated LCx CABF profiles were less pronounced than rHIR 100 and rTAP 100 during the protocol. It is interesting to note, however, that although the extent of LCx CABF in the rTAP 100 and rHIR 100 treatment groups was greater than either heparin group during and immediately after administration of rt-PA, it was essentially the same at the end of the protocol for rTAP 100, rHIR 100, and HEP 100 (see Figure 3). It remains unclear why there was no increased benefit of the HEP 200 treatment regimen over the lower dose of heparin on both the extent of LCx CABF and the patency status of the vessel (see below).

Figure 4 summarizes the patterns of LCx reperfusion/reoclusion events for the rTAP 100 and rHIR 100 treatment...
groups shows a distinct difference between the two agents despite the equivalent integrated LCx CABF profiles shown in Figure 3A. Intermittent periods of reocclusion and reperfusion of LCx CABF were observed in six of eight dogs in the rHIR 100 group after initiation of rt-PA, whereas only one of eight dogs in the rTAP 100 group displayed this pattern of reocclusion during rt-PA treatment. Despite some improvement in the integrated LCx CABF in the HEP 100 and rTAP 50 treatment groups compared with control (Figure 3), the majority of the preparations displayed a pattern of intermittent reocclusion and reperfusion throughout the experimental protocol.

Plasma Levels of rHIR and rTAP
The plasma levels of rHIR and rTAP during the course of the experimental protocol are shown in Figure 5. Plasma levels reached a maximum of 2.55±0.29 μmol/l (rTAP 100), 2.35±0.17 μmol/l (rHIR 100), and 1.06±0.69 μmol/l (rHIR 50) 130 minutes after initiation of the inhibitor infusion. Plasma inhibitor levels declined rapidly after termination of the inhibitor infusion, achieving final concentrations of 0.518±0.1 μmol/l (rTAP 100), 0.360±0.076 μmol/l (rHIR 100), and 0.120±0.043 μmol/l (rHIR 50) 60 minutes after termination of the inhibitor infusions.

Hemostatic Parameters
The effects of the conjunctive interventions on aPTT are summarized in Figure 6A. In the control group, aPTT was essentially unaltered. Maximal increases in aPTT of 2.9±0.2- and 10.1±1.2-fold over baseline values were observed in the HEP 100 and HEP 200 treatment groups, respectively. In the rTAP 100 group, aPTT was elevated slightly to 1.5±0.1-fold, whereas maximal increases of 2.1±0.1- and 12.9±1.4-fold over baseline values were observed in the rHIR 50 and rHIR 100 treatment groups, respectively. The maximal elevations in aPTT in the HEP 200 and rHIR 100 treatment groups are reported as underestimates because of the use of a 150-second maximum limit in the determination of the aPTT (Figure 6A).

The effects of the conjunctive interventions on the buccal mucosal template bleeding times are shown in Figure 6B. In the control group, template bleeding time was elevated modestly to a maximal 1.9±0.3-fold over the baseline value. Maximal increases in template bleeding times of 2.2±0.2- and 2.6±0.5-fold over baseline values were observed in the HEP 100 and HEP 200 treatment groups, respectively. Template bleeding times were elevated to a maximal 2.7±0.4-fold over the baseline value in the rTAP 100 group and to maximal values of 2.9±0.3- and 5.1±0.7-fold in the rHIR 50 and rHIR 100 groups, respectively. The maximal elevation in template bleeding time in the rHIR 100 treatment group is reported as an underestimate because of the use of a 15-minute maximum limit in the determination of the bleeding time.

Total platelet count and ex vivo platelet aggregation in response to adenosine diphosphate and collagen were unaffected in the six conjunctive groups over the course of the experimental protocol (data not shown). Also, no significant differences were detected in plasma fibrinogen, plasminogen, and α2-antiplasmin levels among the treatment groups (data not shown).

Hemodynamic Parameters
Sinus heart rates were unchanged in the six treatment groups over the course of the experimental protocol (data not shown). Mean arterial pressures were reduced modestly in all treatment groups over the course of the protocol (data not shown).
Discussion

Resistance to rt-PA-mediated recanalization of an infarct-related coronary artery and acute reclosure of the reperfused vessel are the major factors that have limited the value of this thrombolytic agent in the treatment of acute myocardial infarction. Elucidation of the mechanisms responsible for these shortcomings is paramount in designing new and effective conjunctive agents to improve the outcome of thrombolytic therapy.

In the present study, we compared the antithrombotic efficacy of rTAP, rHIR, and heparin when administered conjunctively with rt-PA in a canine model of acute occlusive coronary thrombosis with a superimposed critical stenosis. In this model, an occlusive, platelet-rich thrombus forms after electrolytic damage to the vessel wall, which exposes the highly thrombogenic subendothelial surface. The resulting thrombus is highly resistant to thrombolysis with rt-PA, as reflected by the low incidence of reperfusion in the control group. This aspect of the model, coupled with the rapid, acute thrombotic reocclusion after rt-PA administration, makes it an appropriate preparation to investigate the effects of potential conjunctive agents on both reperfusion efficacy and reocclusion.

The acceleration of rt-PA-mediated reperfusion observed in the rHIR 100 treatment group was expected from previous studies with this and other direct thrombin inhibitors. The effects of direct thrombin inhibition probably reflect the pivotal role of thrombin in clot extension and stabilization as well as reclosure of early reperfusion channels. The equivalent effects of rTAP on reperfusion efficacy at a comparable dose of hirudin (as measured by the plasma level of each inhibitor) clearly implicate thrombin generation as a major source of thrombin activity during thrombolysis, in addition to preformed thrombin bound to the clot, which becomes exposed concomitantly with clot dissolution.

The high incidence and rapid rate of acute reocclusion after termination of rt-PA in the control treatment group (Table 1) was comparable to the results obtained in other experimental models of coronary thrombosis. The administration of rTAP and rHIR had very different effects on acute reocclusion and vessel patency after termination of rt-PA. Although the incidence of acute reocclusion was similar in the two rHIR groups, the extent and pattern of LCx CABF was significantly improved in the rHIR 100 group relative to the rHIR 50 group. The patency of the reperfused vessel was maintained during infusion of rHIR in the rHIR 100 group but did not persist after termination of the inhibitor, when numerous intermittent periods of reocclusion and recanalization were observed. The overall incidence of reocclusion in the rTAP 100 group was lower than in the comparable rHIR group (Table 1), although not significantly, with five of eight dogs remaining patent for the entire protocol. In contrast to rHIR, however, two of the three dogs that reoccluded did so during rTAP infusion. The lack of direct thrombin inhibition by rTAP at the plasma levels attained in this study suggests that acute reocclusion in these two dogs during the rTAP 100 infusion may be a result of a higher level of clot-bound thrombin activity remaining in the residual thrombus after recanalization as compared with the remaining dogs within this group. This observation may have important clinical implications, because it would appear from these studies that both clot-bound, preexisting thrombin and active thrombin generation may need to be inhibited to effectively eliminate the risk of acute reclosure following enzymatic thrombolysis.

Although direct inhibition of thrombin would be predicted to prevent the formation of new prothrombinase complexes by abolishing feedback activation, the continued generation of thrombin by established complexes within the residual clot would remain a potent source of thrombogenicity, assuming that the original thrombogenic stimulus is no longer contributing to prothrombinase formation. This implies that the period of treatment with a direct thrombin inhibitor such as rHIR would have to be long enough to suppress thrombin activity until the pool of established prothrombinase activity is neutralized by endogenous anticoagulant mechanisms. This reinforces the importance of directly inhibiting prothrombinase activity as well as preformed thrombin to neutralize thrombin activity rapidly and completely within the newly recanalized vessel. This concept too could have clinical implications, because a heightened hemorrhagic risk would be expected to exist for the duration of conjunctive antithrombotic therapy regardless of the agent used. Therefore, conjunctive treatments that require the shortest period of administration to maintain vessel patency after thrombolysis may have a safety advantage over regimens that require a longer duration of treatment.

The effects of conjunctive heparin administration during or after thrombolysis is a matter of considerable controversy. The results from both clinical and experimental studies indicate that this anticoagulant may be most effective in preventing or delaying acute reclosure if administered intravenously at the time of or shortly after the thrombolytic agent. Heparin was administered in this study before rt-PA as an initial intravenous bolus followed by a continuous infusion. This protocol was designed to ensure that heparin levels would be maximal during reperfusion and remain constant for the duration of the protocol. Two doses of heparin were chosen: one that maximally elevated ex vivo aPTT approximately twofold (low dose) and the other approximately eightfold (high dose) over baseline values. In humans, the low-dose regimen would be within the commonly used therapeutic range, whereas the high-dose regimen is beyond the bounds of accepted clinical practice. In this model, both doses of heparin had a modest effect on enhancing thrombolytic...
reperfusion but failed to prevent or significantly delay reocclusion. Heparin was not without antithrombotic effect, however, as evidenced by the improved LCx CABF and vessel patency status in the HEP 100 treatment group. The different antithrombotic profile of heparin compared with rTAP and rHIR may be a reflection of the inability of the heparin–antithrombin III complex to directly access FXa assembled in the prothrombinase complexes and thrombin enmeshed within the residual thrombus. In addition, the platelet-rich environment of the thrombus in this model may result in locally high levels of platelet-derived factors such as platelet factor IV, which has been shown to neutralize the anticoagulant effect of heparin.

The antithrombotic effects of direct thrombin inhibitors as well as antiplatelet agents acting through antagonism of the glycoprotein IIb/IIIa receptor have often been linked with mild to moderate elevations in template bleeding times. Infusions of rTAP in this model resulted in a maximal elevation of template bleeding time of approximately threefold, in contrast to the more than fivefold increase in the comparable rHIR 100 treatment group. The difference in the extent of bleeding time elevations between the two groups was not correlated with the antithrombotic efficacy of each inhibitor at these doses if the overall incidence of acute reocclusion is used as the measure of efficacy (Table 1). It appears from the results presented here and elsewhere using activated protein C that inhibitors of thrombin generation may provide adequate antithrombotic efficacy with minimal alterations in primary hemostasis. Although a significant correlation has been reported between the incidence of spontaneous bleeding and template bleeding time in patients being treated for acute myocardial infarction with rt-PA, it remains to be seen whether the effects of conjunctive agents on template bleeding time in experimental models are predictive of what will be observed clinically.

The antithrombotic efficacy of rTAP in this model was not correlated with a significant elevation in ex vivo aPTT. This was in sharp contrast to the rHIR 100 and the HEP 200 treatment groups, in which the ex vivo aPTT was maximally elevated approximately 13- and 10-fold over baseline values, respectively. The dramatic difference between rTAP, rHIR, and heparin would appear to be paradoxical in view of the relative antithrombotic efficacy of these agents. The effects of these agents on ex vivo aPTT, however, appear to be directly related to the nature of this
clotting assay, which is highly sensitive to inhibitors of uncomplexed, soluble thrombin. One possible explanation for the poor anticoagulant effect of rTAP using the ex vivo aPTT may be related to the kinetically slow nature of the FXa inhibition by this inhibitor. Over the time course of the aPTT assay, enough thrombin would be generated from uninhibited FXa to rapidly form fibrin, which serves as the end point of this assay. The anticoagulant effect of rTAP with this assay, therefore, appears to be poor compared with kinetically fast inhibitors of soluble thrombin. These data indicate that it may not be appropriate to use the aPTT assay to compare the efficacy of different antithrombotic agents in preventing arterial thrombosis.

In conclusion, we have clearly shown that potent and selective inhibition of FXa by rTAP administered conjunctively with rt-PA can significantly accelerate thrombolytic reperfusion and prevent acute reocclusion in a canine model of acute occlusive coronary artery thrombosis. The antithrombotic effects of rTAP were far superior to standard heparin and comparable to the direct thrombin inhibitor rHIR. The results of this study need to be put into perspective regarding the ability of the experimental preparation used to simulate the anatomic and pathophysiological correlates of coronary thrombosis in humans. The potent antithrombotic effects of rTAP in this model, however, strongly suggest that direct inhibitors of prothrombinase activity may be an effective means to control abnormal arterial thrombosis.

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