Acceleration of Recombinant Tissue-Type Plasminogen Activator–Induced Reperfusion and Prevention of Reocclusion by Recombinant Antistasin, a Selective Factor Xa Inhibitor, in a Canine Model of Femoral Arterial Thrombosis

Michael J. Mellott, Marie A. Holahan, Joseph J. Lynch, George P. Vlasuk, and Christopher T. Dunwiddie

Antistasin is a 119-amino acid protein initially isolated from salivary glands of the Mexican leech, Haementeria officinalis, that exhibits potent anticoagulant properties resulting from selective inhibition of blood coagulation factor Xa. The comparative antithrombotic efficacies of recombinant antistasin (rATS), standard heparin (Hep), and aspirin (ASA) administered adjunctly with recombinant tissue-type plasminogen activator (tPA) on thrombolytic reperfusion and reocclusion were determined in a canine model of femoral arterial thrombosis. An occlusive thrombus was formed by insertion of a thrombogenic copper coil into the femoral artery, and blood flow velocity was monitored directly and continuously by Doppler flowmetry. Sixty minutes after occlusion, dogs received an intravenous infusion of either saline (vehicle) or rATS (0.31, 1.25, or 2.5 μg/kg/min), intravenous boluses of Hep (100 units/kg+50 units/kg/hr or 200 units/kg+150 units/kg/hr), or a single intravenous bolus of ASA (2.0 mg/kg), followed 45 minutes later by tPA (0.8 mg/kg i.v. over 90 minutes). The saline and rATS infusions were discontinued 60 minutes after termination of tPA, and the last Hep boluses were given 105 minutes after termination of tPA. All dogs achieved reperfusion. The time to reperfusion in the ASA group was similar to that in the vehicle group (50±9 versus 50±6 minutes, respectively). Reperfusion times were slightly decreased by the low and high doses of Hep (34±6 and 31±4 minutes, respectively) and the rATS doses of 0.31 and 1.25 μg/kg/min (37±4 and 36±5 minutes, respectively). However, the time to reperfusion was dramatically reduced with the 2.5 μg/kg/min rATS dose (15±3 minutes, p<0.05). After termination of the tPA, the femoral arteries of all vehicle-treated dogs reoccluded within 24±2 minutes. Reocclusion was unaffected by ASA (30±6 minutes) and slightly delayed with low-dose Hep (52±12 minutes). High-dose Hep decreased the incidence of (four of eight) and prolonged the time to (83±24 minutes) reocclusion with an associated elevation in the activated partial thromboplastin time to 8.3-fold control. In stark contrast, reocclusion was prevented in all dogs both during rATS infusions and 2 hours after they were terminated. rATS caused a dose-dependent elevation in the activated partial thromboplastin time, resulting in clotting times 1.8-2.9-fold and 3.9-fold control at doses of 0.31, 1.25, and 2.5 μg/kg/min, respectively. Slight elevations in bleeding time (≤1.6-fold) were observed with high-dose Hep and rATS at doses of 1.25 and 2.5 μg/kg/min. Thus, in this model of arterial thrombosis, rATS as compared with Hep and ASA significantly accelerated reperfusion and completely prevented acute reocclusion, suggesting that specific factor Xa inhibition represents an effective pharmacological approach to adjunctive thrombolytic therapy. (Circulation Research 1992;70:1152-1160)

Key Words • arterial thrombosis • factor Xa inhibition • antistasin • copper coil • heparin

The efficacy of thrombolytic agents in achieving recanalization of occluded coronary arteries after acute myocardial infarction has been established.1–3 Despite their efficacy, the therapeutic benefit of thrombolytic therapy can be compromised by a delay in the time to reperfusion. Animal and clinical studies indicate that the time to flow restoration determines infarct size and the degree of preservation of left ventricular function and may influence mortality.4–11 After the administration of the thrombolytic agent, reocclusion also remains a persistent clinical problem.12–14 Consequently, different therapeutic approaches with adjunctive anticoagulant and antiplatelet agents have been investigated for their ability to promote recanalization and prevent reocclusion. Nonetheless, despite full heparin (Hep) anticoagulation alone or in combination with antiplatelet agents such as aspirin (ASA), the antithrombotic efficacy is not uniformly effective.15–19

The primary role of platelet aggregation in arterial thrombus formation has been demonstrated in animal

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models through the use of monoclonal antibodies and small Arg-Gly-Asp–containing peptides and proteins derived from snake venoms that block the platelet glycoprotein IIb/IIIa receptor complex.20–27 The contributing role of thrombin in the formation of arterial thrombi had been questioned on the basis of the limited efficacy of heparin in the prevention of these phenomena. However, the more recent analysis of small, direct, and highly selective thrombin inhibitors including hirudin, a tripeptide chloromethyl ketone (PPACK), and MCI-9038 (Argatroban Genentech, South San Francisco, Calif.) has demonstrated the utility of direct thrombin inhibition in preventing platelet-mediated arterial thrombosis.28–33 Although both of these approaches have demonstrated efficacy in preventing thrombus formation, they are associated with impairment of normal hemostasis as reflected by a significant prolongation of template bleeding time and/or coagulation time. The recent demonstration of the antithrombotic efficacy of activated recombinant protein C in a baboon model of arterial thrombosis suggests that interruption of the coagulation cascade by selective inhibition of thrombin generation rather than inhibition of preformed thrombin may prevent intravascular thrombosis without compromising normal hemostasis.34

Activation of the coagulation cascade via either the intrinsic or extrinsic branches results in the formation of factor Xa. Factor Xa in combination with its cofactor Va and calcium then assembles into a prothrombinase complex on an appropriate phospholipid surface and catalyzes the formation of thrombin from prothrombin. The central position of factor Xa in the coagulation cascade makes it an attractive target for pharmacological intervention. Antistasin is a 119–amino acid protein initially isolated from salivary glands of the Mexican leech, Haementeria officinalis.35,36 It exhibits potent anticoagulant properties resulting from the stoichiometric and highly selective inhibition of factor Xa.37 In this study we compared the effects of recombinant antistasin (rATS),38,39 Hep, and ASA on tissue-type plasminogen activator (tPA)–induced reperfusion and acute reocclusion in a canine model of femoral arterial thrombosis. The results demonstrate that rATS significantly accelerated reperfusion as compared with Hep and ASA and completely prevented acute reocclusion at plasma concentrations that did not impair primary hemostasis.

Materials and Methods

Experimental Protocol

Sixty-four adult mongrel dogs of either sex weighing 7–10 kg were used in the study. The dogs were anesthetized with sodium pentobarbital (35 mg/kg i.v.), intubated, and ventilated with room air through a respirator (Harvard Apparatus, South Natick, Mass.). Throughout the experiment, blood Po2, Pco2, and pH were monitored and adjusted to physiological levels, as needed. Surgical preparation began with the isolation of the left carotid artery and jugular vein. The left jugular vein was cannulated with a double lumen catheter to administer drugs separately. The right brachial artery was cannulated with a Tygon catheter to measure arterial blood pressure. Both femoral arteries were isolated, leaving intact the most prominent muscular side branch. A cuff-type Doppler flow probe was placed on both femoral arteries just distal to the side branch. Mean and phasic femoral arterial blood flow velocities (FABFVs) were measured with a pulsed Doppler flowmeter (Hartley, Houston, Tex.). Arterial blood pressure, heart rate, and FABFV were recorded continuously on a physiological recorder (Hewlett-Packard Co., Palo Alto, Calif.).

After a stabilization period of 30 minutes, an arterial thrombus was formed by the placement of a thrombogenic copper coil into either femoral artery, as described previously.33,40 A shortened 8F polyurethane pigtail catheter (USCI, Billerica, Mass.) was inserted into the left carotid artery and advanced to either femoral artery. A Teflon-coated guidewire (Cook Co., Bloomington, Ind.) was then passed through the hollow catheter and extended several centimeters beyond the end of the catheter. The hollow catheter was then removed while the guidewire was held in place. A coil (8.0 mm long), equal in diameter to the outside diameter of the femoral artery (range, 2.3–2.8 mm), was placed over the guidewire and advanced by the catheter to the femoral artery, where it was secured 6.0 mm distal to the intact muscular side branch. The guidewire was removed, and 2–3 ml saline was flushed down through the catheter and femoral segment ensuring nearly full recovery of blood flow. The Doppler flow probe was repositioned proximal to the copper coil and just distal to the intact side branch.

Dogs were assigned randomly to one of eight treatment groups with eight dogs in each group (Figure 1). All drugs were administered intravenously. Two groups of dogs received a continuous infusion of 0.1 ml/min saline (control and vehicle); two groups of dogs received Hep as an initial bolus of either 100 or 200 units/kg, followed by hourly boluses of 50 or 150 units/kg, respectively; one group of dogs received a 2 mg/kg bolus of ASA; and three additional groups of dogs received rATS as a continuous infusion of either 0.31, 1.25, or 2.5 μg/kg/min. All drug administrations were initiated 1 hour after occlusive thrombus formation, and the rATS infusions were maintained until 1 hour after the tPA infusion was terminated for a total infusion time of 195 minutes. Supplemental Hep boluses were given at hourly intervals with the final Hep boluses administered 105 minutes after termination of the tPA infusion. This protocol ensured that Hep levels, as determined by ex vivo activated partial thromboplastin time (APTT) values, were maintained throughout the observation period. Forty-five minutes after the drug administration was started (105 minutes after thrombus formation), all dogs except the control group received an infusion of 0.8 mg/kg i.v. human recombinant tPA (Activase, Genentech, South San Francisco, Calif.; specific activity, 580,000 IU/mg) over 90 minutes, representing an average total dose of 6.8 mg recombinant tPA per dog. An additional 120-minute observation period extended beyond the termination of the drug infusions. The coil and thrombus were removed at the end of the experimental period (375 minutes after thrombus formation), and thrombus mass was determined by subtracting the weight of the coil from the weight of the coil plus thrombus. All procedures were reviewed and approved by the Institutional Committee for the Care and Use of Laboratory Animals and complied with federal regulations.
Canine Femoral "Copper Coil" Protocol

FIGURE 1. Diagram showing experimental protocol and treatment groups. Sixty minutes after occlusive thrombus formation, adjunctive therapies were initiated; aspirin as a single bolus, heparin as an initial bolus followed by hourly maintenance boluses (arrows), and saline or recombinant antistasin (rATS) as a continuous intravenous infusion for 195 minutes. Forty-five minutes later, tissue-type plasminogen activator (tPA) was administered as an intravenous infusion over 90 minutes (0.8 mg/kg total dose).

Test Compounds

All solutions were prepared on the day of the experiment. Purified rATS was prepared as described previously from cultures of baculovirus-infected Sf9 cells. The preparation was judged >98% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, reversed-phase high-performance liquid chromatography, and amino acid composition analysis. All experiments were done from a single lot of rATS dissolved in saline. Hep (sodium injection, USP; Upjohn Co., Kalamazoo, Mich.) was prepared from a stock solution of 1,000 units/ml. ASA (acetylsalicylic acid; Sigma Chemical Co., St. Louis, Mo.) was initially dissolved in 1.0 ml ethanol, diluted with 2.0 ml of 50 mM Tris buffer (pH 9.4), and brought to a final volume of 10.0 ml with saline.

Hematological Evaluations

Four milliliters of arterial blood was withdrawn into a plastic syringe containing 0.4 ml of 3.8% trisodium citrate solution. The blood was centrifuged at 5°C for 10 minutes at 2,000g. The plasma was removed and stored on ice for immediate assay or frozen at −70°C. The APTT was determined with an automated APTT kit (Organon Teknika Corp., Durham, N.C.). Buccal mucosa bleeding times were measured with a Simplate bleeding time device (Organon Teknika), as previously described. Whole-blood platelet counts were determined on a Series 9000 blood analyzer (Serona Baker, Allentown, Pa.).

Plasma Recombinant Antistasin Concentrations

Plasma rATS levels were measured using a purified human factor Xa chromogenic substrate-based assay and a standard curve constructed from purified rATS dilutions made up in control, preinfusion plasma as described previously.

Ex Vivo Platelet Aggregation

Arterial blood was withdrawn into a plastic syringe containing one part 3.8% trisodium citrate to nine parts blood. Platelet-rich plasma was obtained by centrifuging the blood at 150g for 15 minutes. Platelet counts were adjusted to 300,000/mm³ with platelet-poor plasma. The platelet-rich plasma samples were primed with epinephrine (final concentration, 1 μM) 1 minute before the agonist was added. Aggregation to ADP (BioData; Hatboro, Pa.; 2.5, 5.0, and 10.0 μM) and arachidonic acid (BioData; 100, 250, 500, and 1,000 μM) was measured as a percent change in light transmission.

Reperfusion and Reocclusion

The time to reperfusion was defined as the reestablishment of FABFV to at least 50% of control "coil" FABFV (FABFV measured immediately after placement of the coil) for a period of 5 minutes or any measurable continuous flow for 15 minutes after the tPA infusion was started. The time to reocclusion was defined as the first incidence of FABFV returning to zero flow after the tPA infusion was stopped.

Statistical Analysis

Results are expressed as the mean±SEM. Comparisons between control values and values obtained at different time points within a treatment group were made by an analysis of variance followed by Dunnett’s test. Analysis of variance followed by Newman-Keuls test was used to perform multiple comparisons between treatment groups.

Results

Arterial Reperfusion and Reocclusion

After insertion of the copper coil, an occlusive thrombus was formed in all dogs in all groups in 9.1±0.3 minutes as indicated by a progressive decline in FABFV...
TABLE 1. Reperfusion and Reocclusion in a Canine Model of Femoral Arterial Thrombosis

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of reperfusion</th>
<th>Time to reperfusion (min)</th>
<th>Incidence of reocclusion</th>
<th>Time to reocclusion (min)</th>
<th>Thrombus mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>8/8</td>
<td>50±6</td>
<td>8/8</td>
<td>24±2</td>
<td>25±2</td>
</tr>
<tr>
<td>Aspirin 2 mg/kg</td>
<td>8/8</td>
<td>50±9</td>
<td>8/8</td>
<td>30±6</td>
<td>25±3</td>
</tr>
<tr>
<td>Heparin 100 units/kg+50 units/kg/hr</td>
<td>8/8</td>
<td>34±6</td>
<td>8/8</td>
<td>52±12</td>
<td>20±2</td>
</tr>
<tr>
<td>Heparin 200 units/kg+150 units/kg/hr</td>
<td>8/8</td>
<td>31±4</td>
<td>4/8*</td>
<td>83±24*</td>
<td>10±2*</td>
</tr>
<tr>
<td>rATS 0.31 μg/kg/min</td>
<td>8/8</td>
<td>37±4</td>
<td>0/8*</td>
<td>...</td>
<td>7±1*</td>
</tr>
<tr>
<td>rATS 1.25 μg/kg/min</td>
<td>8/8</td>
<td>36±5</td>
<td>0/8*</td>
<td>...</td>
<td>5±1*</td>
</tr>
<tr>
<td>rATS 2.5 μg/kg/min</td>
<td>8/8</td>
<td>15±3*</td>
<td>0/8*</td>
<td>...</td>
<td>5±1*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. rATS, recombinant antistasin. *p<0.05 vs. vehicle.

The maximal extent of FABFV achieved for the vehicle, ASA, low-dose Hep, high-dose Hep, and rATS doses of 0.31, 1.25, and 2.5 μg/kg/min were 90±6%, 76±10%, 89±6%, 79±7%, 78±7%, 85±6%, and 89±5% of control values, respectively. Reductions in the incidence of reocclusion after the termination of the tPA therapy, as well as beneficial alterations in the times to thrombolytic reperfusion and reocclusion afforded by adjunctive therapies, were reflected in the FABFV profiles. Figure 2 depicts the FABFV profiles of the groups treated with tPA alone, Hep, and ASA. After termination of the tPA infusion, a maintenance of FABFV was observed with both doses of Hep, reflecting the progressive delay in the time to reocclusion in the two Hep treatment groups, as well as the prevention of reocclusion in four of the eight animals in the group treated with the higher dose of Hep (200 units/kg+150 units/kg/hr). All rATS treatment groups displayed a pronounced maintenance of FABFV throughout the entire experimental period.

FIGURE 2. Graph showing effects of heparin and aspirin on femoral arterial blood flow velocity. Restoration of femoral arterial blood flow velocity during and after tissue-type plasminogen activator (tPA)–induced thrombolysis for those groups receiving vehicle (△), heparin 100 units/kg+50 units/kg/hr (○), heparin 200 units/kg+150 units/kg/hr (□), or aspirin (●). The bracket indicates the period of tPA infusion. Arrows denote the times at which heparin boluses (H) were given. *p<0.05 vs. vehicle.

to zero. Control FABFV was measured immediately after coil insertion and was not significantly different among any of the groups. Arterial blood flow was restored in all of the dogs in all groups that received tPA (vehicle, ASA, Hep, and rATS), whereas none of the dogs in the control group, which did not receive tPA, achieved reperfusion during the entire experimental period. The administration of ASA, at a dose sufficient to completely abolish ex vivo platelet aggregation to arachidonic acid, failed to accelerate the time to reperfusion in comparison to the vehicle group (50±6 versus 50±6 minutes, respectively) (Table 1). The times to reperfusion were decreased modestly in the two groups that received Hep. Similar decreases were observed with rATS administered at 0.31 and 1.25 μg/kg/min. In contrast, the time to reperfusion was dramatically reduced in the group that received rATS at a dose of 2.5 μg/kg/min in comparison to the vehicle-treated group (15±3 versus 50±6 minutes, respectively; p<0.05).

The time to reocclusion was defined as the first incidence of FABFV returning to zero after the tPA infusion was stopped. The femoral arteries of all vehicle-treated dogs reoccluded within 24±2 minutes (Table 1). ASA neither reduced the incidence of (eight of eight) nor delayed the time to (30±6 minutes) reocclusion. Hep, administered at 100 units/kg+50 units/kg/hr, slightly delayed the time to reocclusion (52±12 minutes) but had no effect on the incidence of reocclusion. High-dose Hep (200 units/kg+150 units/kg/hr) decreased the incidence of reocclusion (four of eight) and significantly delayed the time to reocclusion (83±24 minutes). All three doses of rATS (0.31, 1.25, and 2.5 μg/kg/min) were fully effective in preventing reocclusion in 100% of the dogs after termination of the tPA therapy. It is noteworthy that arterial patency was also maintained for the complete 120-minute period after the termination of the rATS infusions.

The thrombogenic mass associated with the copper coil was significantly reduced with the administration of the high dose of Hep (200 units/kg+150 units/kg/hr) and with all three doses of rATS versus the vehicle-treated group (Table 1).

Femoral Arterial Blood Flow

After initial tPA-mediated reperfusion, FABFV gradually increased and was maintained in all animals during the 90-minute tPA infusion (Figures 2 and 3).

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reflecting the complete prevention of reocclusion after the tPA infusion was terminated (Figure 3).

**Ex Vivo Clotting Assays, Platelet Counts, Platelet Aggregations, and Bleeding Times**

Negligible elevations in APTT values (<1.2-fold control values) were observed in the groups that received vehicle or ASA (Table 2). Hep administered at 100 units/kg+50 units/kg/hr maximally elevated APTT to 1.8-fold control values, while the administration of Hep at 200 units/kg+150 units/kg/hr caused a peak elevation in APTT to 8.3-fold control. Maximal elevations in APTT in the groups treated with 0.31, 1.25, or 2.5 μg/kg/min rATS were 1.8-, 2.9-, and 3.9-fold control, respectively. Only slight elevations in bleeding times were observed in any of the treatment groups (Table 2). The administration of ASA (2 mg/kg i.v.) was sufficient to completely abolish arachidonic acid-induced ex vivo platelet aggregation throughout the entire experimental protocol. The administration of Hep or rATS had no effect on ADP-induced ex vivo platelet aggregation. None of the vehicle or treatment groups exhibited any significant change in platelet counts during the course of the study.

**Plasma Recombinant Antistasin Concentrations**

Plasma rATS concentrations were determined 60, 120, and 180 minutes after the rATS administration was started and 45 and 105 minutes after the infusion was stopped (Figure 4). Concentrations in the plasma increased in a dose-dependent manner to maximum levels of 29.3±2.9, 58.1±4.9, and 89.4±11.6 nM (achieved at 180 minutes after the infusion was started) for the 0.31, 1.25, and 2.5 μg/kg/min doses, respectively. At 105 minutes after the infusion was stopped, the concentrations decreased to 23.5±3.4, 36.9±2.9, and 41.5±1.2 nM for the 0.31, 1.25, and 2.5 μg/kg/min doses, respectively. Therefore, levels as low as 25 nM in the plasma are sufficient to prevent reocclusion.

**Hemodynamics**

Changes in mean arterial blood pressure and heart rate throughout the experimental period are shown in

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**TABLE 2. Activated Partial Thromboplastin Times and Bleeding Times**

<table>
<thead>
<tr>
<th>Group</th>
<th>Minutes after thrombus formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>11.1±0.8</td>
</tr>
<tr>
<td>BT (min)</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Aspirin (2 mg/kg)</td>
<td></td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>10.9±0.8</td>
</tr>
<tr>
<td>BT (min)</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td>rATS (0.31 μg/kg/min)</td>
<td></td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>10.1±0.5</td>
</tr>
<tr>
<td>BT (min)</td>
<td>2.2±0.6</td>
</tr>
<tr>
<td>rATS (1.25 μg/kg/min)</td>
<td></td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>10.1±0.2</td>
</tr>
<tr>
<td>BT (min)</td>
<td>2.1±0.4</td>
</tr>
<tr>
<td>rATS (2.5 μg/kg/min)</td>
<td></td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>10.1±0.4</td>
</tr>
<tr>
<td>BT (min)</td>
<td>2.1±0.6</td>
</tr>
</tbody>
</table>

| Heparin (100 units/kg+50 units/kg/hr) |      |      |      |      |      |      |
| APTT (sec)                     | 10.6±0.2 | 19.0±1.0* | 18.4±0.8* | 18.0±0.9* | 18.1±0.8* | 18.2±1.0* |
| BT (min)                       | 2.3±0.2 | 2.4±0.2 | 2.5±0.2 | 2.5±0.2 | 2.5±0.1 | 2.3±0.2 |
| Heparin (200 units/kg+150 units/kg/hr) |      |      |      |      |      |      |
| APTT (sec)                     | 9.7±0.4 | 34.0±3.2* | 46.0±8.0* | 55.9±7.5* | 67.4±11.6* | 80.6±11.9* |
| BT (min)                       | 2.2±0.2 | 2.4±0.3 | 3.0±0.5* | 2.9±0.4* | 2.7±0.3 | 2.6±0.4 |

Values are mean±SEM. APTT, activated partial thromboplastin time; BT, bleeding time; rATS, recombinant antistasin; 60–255 minutes, rATS infusion; 105–195 minutes, tissue-type plasminogen activator infusion (0.8 mg/kg, total dose); heparin bolus administration, 60, 120, 180, 240, and 300 minutes.

*p<0.05 vs. 0 minutes (pretreatment).
FIGURE 4. Graph showing recombinant antistasin (rATS) concentrations in plasma. The rATS was administered as a continuous intravenous infusion from 0 to 195 minutes, and the levels of rATS in plasma were determined for doses of 2.5 (○), 1.25 (●), and 0.31 (▲) μg/kg/min.

Table 3. Dogs in all groups had similar control values for mean arterial pressure and heart rate. At the termination of the experimental protocol, moderate but significant reductions in mean arterial pressure were observed in the treatment groups that received Hep 200 units/kg+150 units/kg/hr and rATS 0.31 and 2.5 μg/kg/min. It is noteworthy that the reduction in blood pressure with rATS was not dose dependent, with no hypotension observed in the treatment group that received 1.25 μg/kg/min rATS. The latter observation suggests that the reduction in mean arterial pressure in this model may be random and unrelated to treatment.

TABLE 3. Mean Arterial Blood Pressure and Heart Rate

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>195</th>
<th>255</th>
<th>375</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>MAP (mm Hg)</td>
<td>130±3</td>
<td>133±3</td>
<td>131±3</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td>172±12</td>
<td>181±7</td>
<td>189±6</td>
</tr>
<tr>
<td>Aspirin (2 mg/kg)</td>
<td>MAP (mm Hg)</td>
<td>138±3</td>
<td>137±5</td>
<td>134±5</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td>163±10</td>
<td>174±10</td>
<td>187±8</td>
</tr>
<tr>
<td>Heparin (100 units/kg+50 units/kg/hr)</td>
<td>MAP (mm Hg)</td>
<td>138±4</td>
<td>133±3</td>
<td>125±3</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td>165±11</td>
<td>169±13</td>
<td>177±12</td>
</tr>
<tr>
<td>Heparin (200 units/kg+150 units/kg/hr)</td>
<td>MAP (mm Hg)</td>
<td>137±5</td>
<td>139±5</td>
<td>126±4</td>
</tr>
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<td></td>
<td>HR (bpm)</td>
<td>191±9</td>
<td>187±14</td>
<td>188±15</td>
</tr>
<tr>
<td>rATS (0.31 μg/kg/min)</td>
<td>MAP (mm Hg)</td>
<td>125±6</td>
<td>123±5</td>
<td>114±5</td>
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<td>HR (bpm)</td>
<td>171±8</td>
<td>183±13</td>
<td>195±11</td>
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<td>rATS (1.25 μg/kg/min)</td>
<td>MAP (mm Hg)</td>
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<td>HR (bpm)</td>
<td>179±3</td>
<td>201±4</td>
<td>207±5</td>
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<tr>
<td>rATS (2.5 μg/kg/min)</td>
<td>MAP (mm Hg)</td>
<td>132±4</td>
<td>127±4</td>
<td>117±4*</td>
</tr>
<tr>
<td></td>
<td>HR (bpm)</td>
<td>177±5</td>
<td>186±8</td>
<td>190±7</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP, mean arterial blood pressure; HR, heart rate; bpm, beats per minute; rATS, recombinant antistasin.

*p<0.05 vs. 0 minutes (pretreatment).

Discussion

Antistasin is a cysteine-rich, 119–amino acid peptide originally isolated from the Mexican leech, Haementeria officinalis.35,36 It has been shown to be an extremely potent and selective inhibitor of blood coagulation factor Xa.37 These properties make rATS a valuable tool to investigate the in vivo antithrombotic and antihemostatic effects of selective factor Xa inhibition. Previously, the antithrombotic properties of rATS have been demonstrated in a rabbit model of venous thrombosis and a rhesus monkey model of mild disseminated intravascular coagulation.39,43 In this report, we compared the individual effects of rATS, Hep, and ASA administered as adjuncts to thrombolytic therapy with tPA in a canine model of femoral arterial thrombosis. In this model, an occlusive thrombus is formed by insertion of a thrombogenic copper coil into the femoral artery. Previously, histological evaluation of thrombus produced in response to copper coils has shown thrombus to consist of platelets and fibrin, with platelets constituting the greatest fraction of the thrombus.40 The progressive decline in FABFV to zero in 9.1±0.3 minutes after coil insertion resembles that seen in another well-defined model of platelet-rich arterial thrombosis characterized by endothelial damage and fixed partial vascular obstruction.44 The consistent restoration of FABFV by tPA and the ability of specific thrombin inhibitory agents to prevent rethrombosis after lysis while agents with vasodilatory properties are ineffective strongly suggest that the primary mechanisms mediating initial occlusion and reocclusion in this model are thrombotic and not vasoplastic.43 The results of our study demonstrate that rATS dramatically accelerated tPA-induced reperfusion as compared with Hep and ASA and completely prevented acute reocclusion without impairment.
of primary hemostasis as reflected by modest elevations in bleeding times.

Hep and ASA are currently used clinically in the setting of acute thrombolytic therapy with the intention of enhancing the fibrinolytic efficacy of tPA and preventing acute reocclusion.2,13,17 The adjunctive efficacy of these agents in this setting, however, is currently a matter of considerable controversy.15-17 In this study, the administration of ASA at a dose sufficient to completely inhibit ex vivo platelet aggregation to arachidonic acid (2 mg/kg i.v.) provided no benefit to either accelerate reperfusion or prevent acute reocclusion. One pathway of thrombin-induced platelet aggregation is mediated by the synthesis of the potent proaggregatory endoperoxide thromboxane A2.45 In the presence of a cyclooxygenase inhibitor, thrombin-induced platelet aggregation is inhibited.46 However, this inhibition can be overridden by higher concentrations of thrombin, demonstrating a thromboxane A2-independent pathway of thrombin-induced platelet aggregation.47,48 Our results likely reflect the pivotal role of thrombin and suggest that it is a primary mediator of arterial thrombus formation and may explain the lack of effect by ASA in the present study. The favorable antithrombotic effects of a direct thrombin inhibitor (antithrombin III independent) in this model strongly reinforce this view.33

The administration of Hep at a dose that resulted in a 1.8-fold elevation in APTT (100 units/kg+50 units/kg/hr) caused a moderate decrease in the time to reperfusion but failed to inhibit acute reocclusion. There was no further improvement in the time to reperfusion when Hep was increased to a dose sufficient to elevate APTT to 8.3-fold control values, a level well above those achieved in the clinical setting. Furthermore, only a 50% reduction in the incidence of reocclusion was afforded by this higher dose of Hep. The effects of Hep demonstrated in this study support and confirm previous reports of the limited efficacy of this anticoagulant when used as an adjunctive therapy to thrombolytic agents.22,23,26 The reasons for the ineffectiveness of Hep as an antithrombotic agent are unclear; however, in vitro studies have demonstrated that active thrombin is incorporated into a developing clot49-51 and this clot-bound thrombin is protected from inactivation by the Hep-antithrombin III complex.52-54 Thrombolysis may result in reexposure of clot-bound thrombin or lead to the continued generation of new thrombin by prothrombinase complexes after reexposure of the original thrombogenic surface. The active, clot-bound thrombin, regardless of its source, may be available to induce platelet aggregation and blood coagulation and thus potentiate reocclusion that occurs after thrombolysis. The demonstrated antithrombotic potential of direct, low molecular weight thrombin inhibitors (PPACK, hirudin, and argatroban) that are capable of inhibiting fibrin-bound thrombin supports this view.28-33 In addition to the above process of thrombin protection, the Hep may be subjected to inactivation by endogenous inhibitors such as platelet factor 4, which may limit its effectiveness as an adjunct to thrombolytic therapy.55

In contrast to Hep and ASA, rATS administered as a continuous infusion of 2.5 μg/kg/min significantly reduced the time to tPA-induced reperfusion. This probably reflects the ability of rATS to limit clot extension by inhibiting continued thrombin generation and thus fibrin and platelet deposition at the site of the growing thrombus. Furthermore, thrombin activates factor XIII, which catalyzes the covalent cross-linking of fibrin, thereby increasing its resistance to lysis.56-58 Inhibition of thrombin generation would prevent the factor XIIIa-mediated cross-linking and possibly contribute to accelerated tPA-induced thrombolysis. All three doses of rATS administered in this study completely inhibited acute reocclusion in 100% of the dogs during the rATS infusions and for 120 minutes after they were terminated. This is reflected most dramatically by the complete maintenance of FABFV throughout the experimental protocol as well as an 80% reduction in final clot weights. The antithrombotic efficacy of rATS is attributed to the inhibition of factor Xa in the prothrombinase complex since rATS exhibits no detectable inhibition of thrombin.59 These results demonstrate that the principal mechanism of reocclusion after thrombolysis in this model is the continuous generation of new thrombin and not reexposure of preformed, clot-bound thrombin. Inhibition of thrombin generation may eliminate the strong, thrombin-mediated positive-feedback activation of factors V and VIII.59 This would result in reduced prothrombinase activity and might suppress the burst of thrombin that is thought to occur after activation of the coagulation cascade.60

The profound effects of rATS on reocclusion probably reflect its long circulating half-life. This is suggested by the slow rate of depletion of plasma rATS levels as well as the sluggish return of APTT values toward baseline after termination of the rATS infusions. Although we have not determined the clearance rate of rATS in the dog, preliminary results in the rhesus monkey suggest a circulating half-life approaching 10-12 hours (C.T. Dunwiddie, unpublished data). Therefore, because of the slow clearance rate of rATS and the relatively short observation period (120 minutes) inherent to acute animal models like the one used here, we are unable to determine whether the prolonged antithrombotic properties exhibited by rATS simply reflect a pharmacokinetic effect unique to this molecule or a mechanistic advantage of factor Xa inhibition over thrombin inhibition. This question is currently under investigation by using thrombosis models more amenable to long observation periods after termination of adjunctive therapies. Nonetheless, the lowest infusion dose of rATS, which reaches peak levels of only 29 nM in the plasma, is fully effective in preventing reocclusion for the full 2-hour observation period.

A variety of antiplatelet agents that block the platelet glycoprotein IIb/IIIa complex, like the highly selective, direct thrombin inhibitors mentioned above, have demonstrated antithrombotic efficacy in many animal models of thrombosis.20-27 Although both approaches prevent thrombus formation, they are associated with an impairment of normal hemostasis as reflected by a significant prolongation in the bleeding time. A recent study in a primate model of occlusive thrombus formation has demonstrated antithrombotic effects without compromising primary hemostasis with the administration of activated recombinant protein C.34 Likewise, rATS, at a dose that completely prevented reocclusion, resulted in maximal elevations in bleeding time and
APTT of only 1.2-fold and 1.8-fold control, respectively. These results suggest that inhibition of thrombin generation rather than direct inhibition of preformed thrombin may be fully antithrombotic without the associated effects on primary hemostasis.

The pronounced antithrombotic effects exhibited by rATS clearly implicate thrombin generation as a major source of thrombin activity during thrombolysis in this model. However, two limitations of this thrombolytic model require prudence when one attempts to extrapolate the beneficial results reported here to the clinical situation. First, this canine model uses a normal femoral artery rather than an atherosclerotic coronary artery, and second, adjunctive treatments were initiated after a 60-minute period of clot maturation rather than the 3–6-hour delay that occurs clinically.

In conclusion, the potent and selective factor Xa inhibitor rATS has been shown to significantly accelerate t-PA-induced thrombolysis and completely prevent reocclusion without impairing primary hemostasis in this canine model of femoral arterial thrombosis. The striking antithrombotic effects of rATS in this model suggest that specific factor Xa inhibition may represent a pharmacologically useful approach to adjunctive thrombolysis.

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