Vampire Bat Salivary Plasminogen Activator Promotes Rapid and Sustained Reperfusion Without Concomitant Systemic Plasminogen Activation in a Canine Model of Arterial Thrombosis

Michael J. Mellott, Inez I. Stabilito, Marie A. Holahan, Gregory C. Cuca, Shiping Wang, Ping Li, Jeffrey S. Barrett, Joseph J. Lynch, and Stephen J. Gardell

The efficacy of recombinant vampire bat salivary plasminogen activator (bat-PA) as a thrombolytic agent was compared with that of human tissue-type plasminogen activator (t-PA) in a canine model of arterial thrombosis. An occlusive thrombus was formed in the femoral artery by insertion of a thrombogenic copper coil; femoral arterial blood flow was monitored with a Doppler flow meter. Bat-PA and t-PA, when administered by 5-minute intravenous infusion (14 nmol/kg), reperfused seven out of eight and four out of eight dogs, respectively. The median reperfusion times in the bat-PA and t-PA groups were 24 and 113 minutes, respectively. The mean reperfusion times (±SEM) in the recanalized bat-PA- and t-PA-treated dogs were similar (20±5 and 11±2 minutes, respectively, p=NS). Maximal blood flow after reperfusion was greater with bat-PA than with t-PA (80±10% and 41±15% of control flow, respectively, p<0.05). Furthermore, the median reocclusion time was markedly delayed in the bat-PA group relative to the t-PA group (131 versus 34 minutes, respectively, p<0.05). Plasma fibrinogen and plasminogen were not significantly depleted by the administration of t-PA or bat-PA. However, plasma α2-antiplasmin activity was moderately depressed in the t-PA group relative to the bat-PA group (p<0.05). The clearance profile for t-PA was monoexponential, with a half-life (t1/2) of 2.4±0.3 minutes and a mean residence time of 3.5±0.4 minutes. The clearance profile for bat-PA was biexponential, with a t1/2α of 0.9±0.2 minutes, a t1/2β of 20.2±2.7 minutes, and a mean residence time of 21.3±4.3 minutes. The steady-state volume of distribution displayed by bat-PA was 16-fold greater than that of t-PA. Zymography of serial plasma samples from the bat-PA-treated dogs failed to demonstrate the apparent generation of a complex between bat-PA and plasminogen activator inhibitor-1; the corresponding complex with t-PA was observed in plasma samples from the t-PA-treated dogs. The sustained recanalization and improved blood flow in the bat-PA group relative to the t-PA group and the avoidance of fibrinogenolysis by bat-PA, despite its prolonged mean residence time, suggest that bat-PA may be superior to t-PA as a thrombolytic agent.

The potential salutary effect of thrombolytic therapy on myocardial salvage is realized by the prompt and persistent recanalization of the occluded coronary vessel. Consequently, the ability of tissue-type plasminogen activator (t-PA) to promote more rapid reperfusion than streptokinase may yield significant clinical benefit.1-3 On the other hand, t-PA-mediated reperfusion appears to be subject to early reclosure more frequently than streptokinase-mediated reperfusion. The increased incidence of this untoward event during use of t-PA may be due to the relatively short plasma half-life (t1/2) of t-PA and the occurrence of a less severe systemic lytic state.4

The currently approved regimen for t-PA-mediated thrombolysis calls for a loading intravenous dose followed by a 3-hour continuous intravenous infusion5; approximately 60% of the 100 mg total dose of t-PA is administered during the first hour. Variations

From the Departments of Pharmacology (M.J.M., I.I.S., M.A.H., J.J.L.), Cellular and Molecular Biology (G.C.C., S.W.), Drug Metabolism (J.S.B.), and Biological Chemistry (P.L., S.J.G.), Merck Sharp & Dohme Research Laboratories, West Point, Pa., and Rahway, N.J. Address for correspondence: Dr. Stephen J. Gardell, Department of Biological Chemistry, WP 26-431, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

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of this standard dosing regimen have been evaluated to investigate whether the incidence of early reocclusion could be decreased. For example, a maintenance infusion of a subthrombolytic dose of t-PA has been shown to delay reocclusion. The attractiveness of this approach is diminished by the increased requirement for t-PA. In addition, studies have suggested that serious bleeding complications are provoked by a prolonged infusion of t-PA.8

An alternative approach to achieve sustained levels of t-PA in the circulation is to use a t-PA variant that exhibits a prolonged plasma t½. For a variety of purposes, a multitude of molecular variants of native t-PA containing substitutions, deletions, or insertions that range in size from a single amino acid to entire structural domains have been constructed.9-11 Certain of these t-PA variants display a longer t½ than does the native protein. This characteristic undoubtedly contributes to the enhanced thrombolytic efficacy and delayed reocclusion that have been observed when they are used as thrombolytic agents.13-16 Nevertheless, as these t-PA variants circulate for a longer time, the accompanying systemic effects are invariably compounded and generate a more profound systemic lytic state. Hence, the increased plasma residence time displayed by these t-PA variants may heighten the risk of serious bleeding complications.

Our search for solutions to the shortcomings that currently limit the value of thrombolytic therapy led to the isolation and characterization of a vampire bat salivary plasminogen activator, bat-PA.17-18 The predicted amino acid sequence of bat-PA is homologous to t-PA.19 Bat-PA's structure is composed of several domains that were previously identified in t-PA:19; these include a fibronectin finger–like domain, an epidermal growth factor–like domain, a single kringle domain (unlike t-PA that contains two kringles), and a protease domain. Nevertheless, the ability of bat-PA to activate plasminogen is strictly dependent on the presence of a fibrin cofactor. Bat-PA's requirement for fibrin is much more rigid than that of t-PA. We also showed that bat-PA is quiescent in human plasma but that levels of activity comparable to those of t-PA are manifest in the presence of fibrin.20 When evaluated in a rabbit model of femoral arterial thrombosis, bat-PA administered by bolus intravenous injection relative to equimolar t-PA was superior with respect to the incidence of reperfusion, yet bat-PA caused only a minor perturbation of the plasma levels of fibrinogen, plasminogen, and α2-antiplasmin.21

In the present study, we evaluated the thrombolytic activities of bat-PA and t-PA in a canine model of femoral arterial thrombosis. A potentially desirable feature of this animal model is that the occlusive thrombus is enriched in platelets, such as are typically found in arterial thrombi. Furthermore, this canine thrombosis model allowed us to assess the tendency for reocclusion after bat-PA– or t-PA–mediated reperfusion.

Methods

Surgical Preparation and Induction of Arterial Thrombi

Sixteen adult male or female mongrel dogs (8–11 kg) were anesthetized with sodium pentobarbital (35 mg/kg i.v.), intubated, and ventilated with room air with a positive-pressure ventilator (Harvard Apparatus, South Natick, Mass.). The left jugular vein and right brachial artery were cannulated for drug administration and measurement of arterial pressure, respectively. The right femoral artery was isolated and fitted with a cuff-type Doppler flow probe. Mean and phasic femoral arterial blood flow velocities (FABFVs) were measured with a pulsed Doppler Flowmeter (C. Hartley, Houston, Tex.). Arterial blood pressure, heart rate, and FABFV were recorded continuously on a Hewlett-Packard physiological recorder (Hewlett-Packard Co., Palo Alto, Calif.). After a stabilization period of 30 minutes, an arterial thrombus was formed in the right femoral artery by placement of a thrombogenic copper coil, as described previously.22,23 A shortened 8F polyurethane pigtail catheter (Cook Co., Bloomington, Ind.) was inserted into the left carotid artery and advanced to the right femoral artery. A Teflon-coated guidewire (Cook Co.) was then passed through the hollow catheter so that its tip extended beyond the end of the hollow catheter. The hollow catheter was withdrawn while the guidewire was held in place. A coil (8.0 mm long), with an outside diameter equal to that of the femoral artery (as measured by calipers; range, 2.5–2.9 mm), was placed over the guidewire, followed by placement of the hollow catheter. The catheter was used to advance the coil into the femoral artery, where it was secured approximately 6.0 mm distal to the intact muscular side branch. The guidewire was quickly removed. Before the hollow catheter was withdrawn, the femoral arterial segment containing the coil was flushed with saline, ensuring nearly full recovery of FABFV. The Doppler flow probe was repositioned proximal to the coil.

Thrombogenic coils are constructed of 24-gauge bare copper wire wrapped around tubing adapters of different diameters (Becton Dickinson, Rutherford, N.J.). There were no significant differences between any of the treatment groups with respect to the femoral artery or coil diameters.

Plasminogen Activators

Recombinant t-PA (Activase), predominantly one-chain material, was obtained from Genentech, South San Francisco, Calif. t-PA was dissolved in buffer supplied by the manufacturer to yield a final concentration of 2 mg/ml (equivalent to approximately 28 nmol/ml). Recombinant bat-PA (form 2),17,18 corresponding to DSPA22 described elsewhere,24 was produced by heterologous expression of the bat-PA-2 cDNA in CV1-P cells by use of a microcarrier bead process in spinner flasks (J.-S. Tung et al, unpublished observations). The expressed protein was purified
from the conditioned media by affinity chromatography by using immobilized Erythrina trypsin inhibitor (American Diagnostica Inc., Greenwich, Conn.). Bat-PA was dialyzed into 0.05 M sodium acetate and 0.01% Triton X-100 (pH 5.0), lyophilized, and stored at −80°C. The concentration of resolublized bat-PA was determined by amino acid composition analysis and verified by active-site titration with 4-methylumbelliferyl-p-guanidinobenzoate.17

For comparative purposes, the PAs used in this study were administered on a molar rather than a weight basis because of their dissimilar molecular mass values (bat-PA and t-PA are approximately 54 and 70 kd, respectively). The specific activities of bat-PA and t-PA were 15,500 and 33,000 IU/nmol, respectively, using the PA activity assay calibrated with one-chain t-PA activity standard (American Diagnostica Inc.).

Experimental Protocol and Treatment Groups

Thirty minutes after the formation of an occlusive arterial thrombus, t-PA (n=8) or bat-PA (n=8) was administered as a 5-minute intravenous infusion in the dogs randomized to the study. This dosing schedule was selected because of the potential advantages of administering PAs by bolus or accelerated infusion (see “Discussion”). The PA dose used in this study, 14 nmol/kg, was selected on the basis of our previous investigation in rabbits.21 The 14 nmol/kg dose of t-PA corresponded to approximately 1 mg/kg. Blood samples for the determination of functional levels of plasma fibrinogen, plasminogen, and α2-antiplasmin and whole-blood platelet counts were obtained before administration of the PA and at 15, 30, 60, 90, 120, 180, and 240 minutes after administration. At 240 minutes after PA administration, the residual thrombi were excised from the dogs, separated from the copper coils, blotted dry, and weighed.

Criteria for Reperfusion and Reocclusion

Reperfusion was defined by either of two criteria: continuous FABFV for 15 minutes or reestablishment of at least 50% of “control coil FABFV” (FABFV after placement of the coil) for a period of at least 5 minutes. Reocclusion was defined as the first incidence of FABFV that returned to zero.

Statistics

Incidence-of-reperfusion data were analyzed by Fisher’s exact test. Reperfusion and reocclusion time data were analyzed by Gehan’s generalized Wilcoxon test.25 Comparisons between t-PA and bat-PA at similar time points were made with a two-tailed unpaired Student’s t test. Comparisons between control values and values obtained at different time points within a treatment group were made with a one-way analysis of variance followed by a Dunnett’s test. In all cases, p<0.05 was considered significant.

Fibrinogen, Plasminogen, and α2-Antiplasmin Determinations

Arterial blood samples for determination of plasma levels of functional fibrinogen, plasminogen, and α2-antiplasmin were drawn into EDTA-precocated tubes containing 5 μl of 1 μM d-Phe-Pro-Arg-CH2Cl (Calbiochem Corporation, La Jolla, Calif.) per milliliter of blood (to prevent fibrinogen degradation). The samples were centrifuged at 4°C and 1,500g for 10 minutes, and the platelet-free plasma was removed and stored in liquid nitrogen until assayed. Functional fibrinogen concentration was determined by a modification of the thrombin time (Clauss method26) (American Dade, Aguada, Puerto Rico). Plasminogen levels were measured by using a chromogenic substrate for plasmin (Spectrozyme Pl, American Diagnostica Inc.) and urokinase (Calbiochem). Plasma for this assay was acidified and neutralized to destroy plasmin inhibitors before testing. The levels of α2-antiplasmin activity were determined essentially by the Coatest Antiplasmin method (KabiVitrum AB, Stockholm, Sweden) employing the chromogenic substrate S-2251. The results for plasminogen and α2-antiplasmin are expressed as a percentage of activity relative to control (pre-PA infusion) activity.

Plasminogen Activator Activity Assay

Solutions (190 μl) containing human fibrinogen (0.132 mg/ml; American Diagnostica Inc.), human glutamic acid–plasminogen (0.66 μM; American Diagnostica Inc.), and human thrombin (0.26 units/ml; Sigma Chemical Co., St. Louis, Mo.) in 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 0.15 M NaCl, and 0.24 mM Triton X-100 (pH 7.5) were placed in the wells of a microtitration plate and incubated for 30 minutes at 37°C. Ten microliters of a2-antiplasmin (200 units/ml; American Diagnostica Inc.) was added, and the clots were incubated for an additional 30 minutes at 37°C. Thirty microliters of 3.33 mM Spectrozyme Pl (American Diagnostica Inc.) and 20 μl of a diluted plasma sample were added, and the release of p-nitroaniline from the chromogenic substrate was measured spectrophotometrically with a THERMOmax microplate reader (Molecular Devices, Menlo Park, Calif.).

Pharmacokinetic Properties

In separate studies, two groups of four anesthetized dogs received t-PA or bat-PA (14 nmol/kg) as a 5-minute intravenous infusion via the cannulated left jugular vein. Arterial blood samples (0.9 ml) were drawn from the right brachial artery into syringes containing 0.1 ml of 3.8% trisodium citrate. The samples from the dogs that received t-PA were obtained at 0 (predose), 1–10, 12, 15, 20, 25, 30, 40, 50, 60, 75, 90, and 120 minutes. The blood samples from the dogs that received bat-PA were obtained at 0 (predose), 1–6, 8, 10, 12, 15, 20, 25, 30, 40, 50, 60, 75, 90, 120, 150, and 180 minutes. A 0.5-ml aliquot of each blood sample was acidified by the addition of
0.25 ml 1 M acetic acid, pH 4.0, and centrifuged. The plasma was removed immediately and stored at −80°C until assayed for residual PA activity.

Individual concentration–time profiles from each dog were analyzed in a noncompartmental fashion based on statistical moment theory. Terminal disposition-rate constants (λ) were estimated by regression of the terminal log-linear concentration time points in PC SAS (SAS Institute Inc., Cary, N.C.). Terminal disposition t1/2 was calculated as the quotient of the natural log of 2 and λ. The area under the curve extrapolated to infinity (AUC∞) was calculated from the sum of the log–linear trapezoid, AUC0–t∞, and the extrapolated AUCt–∞, obtained by dividing the last measured t-PA or bat-PA concentration by λ. The volume of distribution at steady state (Vdss) was estimated by the following equation:

\[
Vd_{ss} = \frac{k_{bolus} \times AUMC}{AUC^2} = \frac{k_{bolus}^2}{2 \times AUC}
\]

where t is the infusion time, AUMC is the area under the moment curve, and k0 is the zero-order infusion rate. Both AUC and AUMC refer to the respective profiles extrapolated to infinity.

Curve-fitting techniques were employed on the bat-PA and t-PA data to resolve the plasma profiles into their exponential components. Initial estimates were obtained by the method of residuals in the program RSTRTP (MicroMath Scientific Software, Salt Lake City, Utah). These values were then used in the nonlinear least-squares algorithm of the same program to obtain final parameter estimates. From biexponential fits to individual concentration–time profiles, the α and β phases of the bat-PA-administered dogs were resolved, and the contribution of each to the total disposition was calculated.

Zymographic Analysis of Bat Plasminogen Activator and Tissue-Type Plasminogen Activator in Canine Plasma Samples

Bat-PA and t-PA (and immunologically related complexes) in the canine plasma samples were isolated by adsorption to rabbit anti–bat-PA immunoglobulin G (IgG) and mouse anti–t-PA IgG (PAM-2; American Diagnostica Inc.), respectively, coupled to cyanogen bromide–activated Sepharose 6B (Pharmacia LKB, Piscataway, N.J.). Fifty microliters of a 50% slurry of the immobilized antibodies was added to 80 μl of the corresponding plasma samples; 10 μl Erythrina trypsin inhibitor (1 mg/ml); and 420 μl of 0.05 M tris(hydroxymethyl)aminomethane HCl, 0.1 M NaCl, 1 mM EDTA, 0.05% Tween 80, and 0.02% NaN3 (pH 7.5). The samples were mixed for 4 hours at 4°C and centrifuged, and the immunoadsorbed material was released by treatment with 25 μl 1% sodium dodecyl sulfate. The samples were electrophoresed on a 12.5% acrylamide denaturing gel and analyzed by fibrin zymography essentially as described elsewhere.

**Results**

**Arterial Thrombus Formation, Reperfusion, and Reocclusion**

An occlusive thrombus, as indicated by a progressive decline of FABFV to zero, occurred 11±1 minutes after placement of the copper coil in the 16 dogs randomized to the study. Maximal FABFVs before and immediately after coil placement were each similar in the bat-PA– and t-PA–treatment groups. The intravenous administration of t-PA or bat-PA, 14 nmol/kg over 5 minutes by intravenous bolus injection, resulted in successful reperfusion in four of eight (50%) dogs and seven of eight (88%) dogs, respectively (Table 1). The median reperfusion times in the t-PA– and bat-PA–treatment groups were ≥131 and 24 minutes, respectively. The t-PA– and bat-PA–treated dogs in which blood flow was restored displayed mean reperfusion times of 11±2 and 20±5 minutes, respectively (p=NS). Maximal FABFV was significantly less (p<0.05) after administration of t-PA (41±15% of control flow at 20 minutes) relative to bat-PA (80±10% of control flow at 60 minutes) (Figure 1).

Reocclusion of the recanalized femoral artery occurred in 100% of the dogs that had been successfully reperfused with either t-PA or bat-PA (Table 1). Nevertheless, the reocclusion time was significantly delayed in the bat-PA–treatment group relative to the t-PA–treatment group (median reocclusion times were 131 and 34 minutes, respectively, p<0.05; mean reocclusion times were 144±14 and 37±6 minutes, respectively, p<0.05). Furthermore, all dogs that met reperfusion criteria after administration of bat-PA (n=7) demonstrated cyclic periods of reflow after initial reocclusion (Figure 2). In contrast, reestablishment of flow was not observed in any of the successfully reperfused t-PA–treated dogs (n=4) after the initial reocclusion event. Residual thrombus mass at the end of the experiment was significantly greater in the t-PA–treated dogs (50±3 mg) versus the bat-PA–treated dogs (39±4 mg) (p<0.05; Table 1).

**Table 1. Reperfusion and Reocclusion in a Canine Model of Femoral Arterial Thrombosis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>t-PA</th>
<th>Bat-PA</th>
</tr>
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<tbody>
<tr>
<td>Incidence of reperfusion*</td>
<td>4/8</td>
<td>7/8</td>
</tr>
<tr>
<td>Reperfusion time (minutes)†</td>
<td>≥131</td>
<td>24</td>
</tr>
<tr>
<td>Incidence of reocclusion*</td>
<td>4/4</td>
<td>7/7</td>
</tr>
<tr>
<td>Reocclusion time (minutes)‡</td>
<td>34</td>
<td>131‡</td>
</tr>
<tr>
<td>Thrombus mass (mg)§</td>
<td>50±3</td>
<td>39±4</td>
</tr>
</tbody>
</table>

*The criteria for reperfusion and reocclusion after administration of tissue-type plasminogen activator (t-PA) or bat plasminogen activator (bat-PA) are defined in "Methods." †The reperfusion and reocclusion times are the median values relative to the start of administration of the fibrinolytic agent. The end point of the trial (≥240 minutes) was the reperfusion time assigned to those dogs that failed to reperfuse. ‡p<0.05 vs. t-PA group. §Values for the thrombus mass are reported as mean±SEM.
FIGURE 1. Plot showing restoration of femoral arterial blood flow velocity (FABFV). Dogs received 14 nmol/kg tissue-type plasminogen activator (•; n=8) or bat plasminogen activator (○; n=8) by constant intravenous injection over 5 minutes. The reestablishment of blood flow in the occluded vessel was monitored with a pulsed Doppler flow meter.

Whole-blood platelet counts did not vary significantly over the course of the experimental period after administration of either t-PA or bat-PA (data not shown). Heart rate and mean arterial blood pressure also did not vary significantly over the course of the experimental period (data not shown).

Plasma Fibrinogen, Plasminogen, and α2-Antiplasmin

Figures 3, 4A, and 4B summarize the effects on functional levels of plasma fibrinogen, plasminogen, and α2-antiplasmin, respectively, after administration of t-PA or bat-PA. Whole-blood platelet counts did not vary significantly after administration of either t-PA or bat-PA (data not shown). Heart rate and mean arterial blood pressure also did not vary significantly over the course of the experimental period (data not shown).

Plasma Fibrinogen, Plasminogen, and α2-Antiplasmin

Figures 3, 4A, and 4B summarize the effects on functional levels of plasma fibrinogen, plasminogen, and α2-antiplasmin, respectively, after administration of t-PA or bat-PA. There were no significant decreases in plasma fibrinogen, plasminogen, and α2-antiplasmin from control values after the administration of bat-PA. The observed decreases in fibrinogen and plasminogen after administration of t-PA were also not significantly different from control values. However, the level of α2-antiplasmin decreased moderately but significantly in the t-PA group relative to the bat-PA group (p<0.05) at all postadministration time points except at 15 minutes (Figure 4B). For example, the α2-antiplasmin values in the dogs that received t-PA were 67±4%, 68±4%, 68±4%, and 70±4% of control at 30, 60, 90, and 120 minutes, respectively. The corresponding α2-antiplasmin values in the dogs that received bat-PA were 84±3%, 88±2%, 87±2%, and 86±2% of control.

Pharmacokinetics of Tissue-Type Plasminogen Activator and Bat Plasminogen Activator

The plasma level of PA activity progressively increased during a 5-minute constant intravenous injection of t-PA (14 nmol/kg), to yield a peak t-PA concentration of approximately 79 nM (Figure 5). Immediately after the administration of t-PA was completed, plasma levels of t-PA activity rapidly returned to baseline values, as adequately described by a monophasic elimination profile. The peak value of plasma PA activity immediately after the 5-minute infusion of equimolar bat-PA corresponded to approximately 53 nM (Figure 5). The elimination profile of bat-PA activity was biphasic, with the plasma levels of bat-PA activity only slowly returning to baseline.

Table 2 summarizes the derived pharmacokinetic parameters for the disposition of t-PA or bat-PA after intravenous administration in dogs. The monoeponential elimination profile for t-PA displayed a t1/2 of 2.4±0.3 minutes, a plasma clearance rate of
24.9±3.3 ml/min/kg, and a mean residence time (MRT) of 3.5±0.4 minutes. The biphasic clearance profile for bat-PA displayed a $t_{\text{ma}}$ of 0.9±0.2 minutes, a $t^{\sigma/3}$ of 20.2±2.7 minutes, a plasma clearance rate of 21.6±5.6 ml/min/kg, and an MRT of 21.3±4.3 minutes. An approximate 16-fold difference was evident with respect to the $V_{\text{d}0}$ of bat-PA and t-PA ($393.5±73.0$ ml/kg and $24.1±8.8$ ml/kg, respectively).

Fibrin Zymography of Plasma Samples

The serial plasma samples from a representative dog that received t-PA were analyzed by fibrin zymography (Figure 6A). Only very early samples up to and including those at 20 minutes (lane 8) yielded lytic zones that comigrated with t-PA (lane 10) — a finding concordant with the relatively rapid loss of t-PA activity noted previously. Two weaker zymographic lytic zones that displayed apparent molecular weight values greater than t-PA were also observed. One of these comigrated with the t-PA–human plasminogen activator inhibitor–1 (PAI-1) complex (lane 11).

The relatively slow plasma elimination of bat-PA evident from the activity profile was confirmed by fibrin zymography of the serial plasma samples from a representative dog that received bat-PA (Figure 6B). The doublet zone of clearing displayed by purified bat-PA (lane 11) was due to carbohydrate heterogeneity (data not shown). A signal corresponding to uncomplexed bat-PA was observed in all samples, spanning 1 minute (lane 2) to 120 minutes (lane 10). Interestingly, there was no evidence for a complex that comigrated with the bat-PA–human PAI-1 complex (lane 12). However, two different active species that exhibited apparent molecular mass values greater than the bat-PA–human PAI-1 complex were observed in the serial plasma samples. The identity of the active species that displayed an apparent molecular mass value of approximately 70 kd is unknown.

Discussion

In this study, we compared the thrombolytic effects of bat-PA and t-PA in a canine model of arterial thrombosis. The results show that intravenous administration of bat-PA in comparison to equimolar t-PA caused a higher incidence of reperfusion, a greater restoration of arterial blood flow, and a prominent delay in reocclusion.

We previously demonstrated the superior fibrinolytic efficacy of bat-PA compared with t-PA in a rabbit model of arterial thrombosis. In the preceding model, the thrombus was formed by the injection of whole blood and thrombin into an isolated segment of the rabbit femoral artery; hence, the ensuing thrombus was relatively platelet poor. In the present

FIGURE 4. Line plots showing effects of bat plasminogen activator (bat-PA) or tissue-type plasminogen activator (t-PA) administration on plasma levels of (panel A) plasminogen and (panel B) $\alpha_1$-antiplasmin. Dogs with an occluded femoral artery received 14 nmol/kg of either bat-PA (•; n=8) or t-PA (○; n=8) by intravenous injection over 5 minutes. *p<0.05, t-PA group vs. bat-PA group.

FIGURE 5. Pharmacokinetics curves of bat plasminogen activator (bat-PA) and tissue-type plasminogen activator (t-PA) in the dog. Both plasminogen activators were administered as intravenous injections (14 nmol/kg) over 5 minutes. Blood samples were taken at indicated times and monitored for levels of plasminogen activator activity. These values were converted to plasma concentrations of t-PA (○) or bat-PA (●) by comparison of the activity values with standard curves. Each data point represents mean plasma concentration±SD, n=4.
Table 2. Pharmacokinetic Parameters of Bat Plasminogen Activator and Tissue-Type Plasminogen Activator After Intravenous Administration In Dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bat-PA</th>
<th>t-PA</th>
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<tbody>
<tr>
<td>$t_{1/2a}$ (minutes)</td>
<td>0.9±0.2</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td>$t_{1/2b}$ (minutes)</td>
<td>20.2±2.7</td>
<td></td>
</tr>
<tr>
<td>AUC (nM-min)</td>
<td>679.6±158.5</td>
<td>570.3±72.6</td>
</tr>
<tr>
<td>%AUC $\alpha$</td>
<td>72.1±11.2</td>
<td></td>
</tr>
<tr>
<td>%AUC $\beta$</td>
<td>27.9±11.2</td>
<td></td>
</tr>
<tr>
<td>$C_l$ (ml/min/kg)</td>
<td>21.6±5.6</td>
<td>24.9±3.3</td>
</tr>
<tr>
<td>$V_{d,1}$ (ml/kg)</td>
<td>393.5±73.0</td>
<td>24.1±8.8</td>
</tr>
<tr>
<td>MRT (minutes)</td>
<td>21.3±4.3</td>
<td>3.5±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SD of four experiments. Dogs received bat plasminogen activator (bat-PA) or tissue-type plasminogen activator (t-PA) (14 nmol/kg) by 5-minute constant intravenous infusion. Serial blood samples were drawn, and the plasma disappearance of plasminogen activator activity was monitored and converted to plasminogen activator concentration by using standard curves constructed with bat-PA or t-PA.

A plausible explanation for the sustained blood flow after bat-PA-mediated reperfusion pertains to its relatively slow disposition. Bat-PA displayed an approximate sixfold longer MRT than did t-PA. The slower elimination of bat-PA relative to t-PA arises from the protracted $\beta$-elimination phase, which displayed a $t_{1/2b}$ value of approximately 20 minutes and accounted for approximately 30% of the total disposition. We previously showed that the elimination of bat-PA was far slower than that for t-PA in rabbits as well. Likewise, a prominent $\beta$-elimination phase was observed for bat-PA in the rabbit studies.

Another possibility that might explain the delayed reocclusion in dogs treated with bat-PA as opposed to t-PA is that bat-PA may display an initial higher efficiency at lysing the thrombus. Lesser residual stenosis after bat-PA-mediated recanalization would necessitate that a longer period of time elapse before enough thrombotic material had reaccumulated to cause reocclusion.

The pharmacokinetic analysis of the levels of bat-PA activity in the canine plasma samples yielded a $V_{d,1}$ value that was approximately 10-fold greater than the predicted plasma volume. The large $V_{d,1}$ value displayed by bat-PA may reflect its initial and rapid partitioning into a peripheral pharmacokinetic compartment. Another explanation for the sizable $V_{d,1}$ value is that the plasma levels of bat-PA activity are abated by inactivation instead of sequestration. However, the estimated value for the peak plasma concentration of bat-PA as deduced from activity measurements was in close agreement with that derived from a similarly designed complementary study that monitored the elimination of radioiodinated bat-PA (data not shown). Hence, sequestration and not inactivation is the most likely explanation for the large $V_{d,1}$ displayed by bat-PA.

The conspicuous $\beta$-elimination phase exhibited by bat-PA may describe the release of bat-PA from the putative peripheral compartment into the plasma compartment. Consequently, the delayed reocclusion...
after bat-PA-mediated reperfusion may result from the slow release of sequestered bat-PA and the sustained presence of appreciable levels of bat-PA activity in the circulation. The nature of the putative extraplasma compartment is unknown. Binding sites for PAs (unrelated to PAI-1) on endothelial cells have been described and may be mediating the transient exodus of bat-PA from the plasma compartment. In any event, it is unclear why bat-PA and t-PA exhibit such dissimilar pharmacokinetic behavior.

The thrombolytic activities of several t-PA variants that are eliminated more slowly than native t-PA have been evaluated in a variety of canine thrombosis models. These "long-lived" variants were more effective thrombolytic agents than native t-PA—a fact clearly evident by the delayed times to reocclusion associated with their use. Nevertheless, the prolonged residence times displayed by these t-PA variants generally precipitated more profound systemic effects. Hence, bat-PA can be distinguished from these t-PA variants because the prolonged residence time is not accompanied by discernable consumption of plasminogen, α₂-antiplasmin, and fibrinogen. The remarkable fibrin selectivity of bat-PA compared with t-PA is not underscored in this study because of the relative insensitivity of canine blood to systemic activation by t-PA. However, the strict fibrin selectivity of bat-PA relative to t-PA has been demonstrated by in vivo studies carried out in rabbits and monkeys (M.J. Mellott et al, unpublished observations) as well as ex vivo studies with human plasma.

Fibrin zymography showed that the administration of t-PA to dogs resulted in the apparent formation of a t-PA–canine PAI-1 complex. The identity of the active species that exhibited an apparent molecular weight value greater than that of the putative t-PA–PAI-1 complex is unknown but probably represents a dissimilar t-PA–inhibitor complex. For example, complexes between t-PA and CI-esterase inhibitor or α₂-antiplasmin were observed after administration of t-PA to patients with acute myocardial infarction or to normal rabbits.

We showed previously, using a purified system, that the apparent second-order association rate constants for the interaction between human PAI-1 and t-PA or bat-PA were similar. However, zymographic analysis of the plasma samples from the bat-PA–treated dogs failed to show a species that comigrated with the bat-PA–human PAI-1 complex. The "slower-migrating" active species could represent the participation of inhibitors in canine plasma other than PAI-1. Our data suggest that bat-PA, unlike t-PA, may be sensitive to discrete structural differences between human and canine PAI-1. Alternatively, the complex between canine PAI-1 and bat-PA may have formed in vivo but was not recognized by the zymographic analysis. For instance, the bat-PA–canine PAI-1 complex may have dissociated or migrated anomalously during sodium dodecyl sulfate–polyacrylamide gel electrophoresis, or the complex might not display activity during fibrin zymography.

If bat-PA truly resists inactivation by canine PAI-1, then the apparent superiority of bat-PA relative to t-PA in our canine thrombosis model may be somewhat abated in humans because of anticipated inhibition by human PAI-1.

In conclusion, we suggest that the use of bat-PA as a thrombolytic agent could decrease the incidence of acute reocclusion, an untoward event that undermines the success of thrombolytic therapy. Early reocclusion is detrimental to the recovery of functional myocardium and gives rise to more complicated hospital courses and higher in-hospital mortality rates. Bat-PA, by virtue of its delayed elimination and protracted thrombolysis, could act conjunctively with antiplatelet and antithrombin agents to ameliorate the reocclusion problem. Importantly, a manifestation of bat-PA's remarkable fibrin specificity is that its prolonged residence time in plasma is not accompanied by appreciable activation of systemic plasminogen. Although not conclusively established, the ability of bat-PA to promote recanalization without precipitating plasminemia and fibrinogenolysis may decrease the severity of the bleeding complications that can jeopardize the safety of thrombolytic therapy. A related potential benefit of the fibrinogen-sparing nature of bat-PA would be minimization of the surgical bleeding that typically accompanies invasive procedures initiated soon after the cessation of thrombolytic therapy. Hence, patients who fail to reperfuse after administration of bat-PA, perhaps because of unfavorable pathomorphological or anatomic considerations, could immediately be eligible for emergency rescue procedures such as percutaneous transluminal coronary angioplasty.

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M J Mellott, I I Stabilito, M A Holahan, G C Cuca, S Wang, P Li, J S Barrett, J J Lynch and S J Gardell

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