Thrombolytic Activity of a Novel Plasminogen Activator, LY210825, Compared With Recombinant Tissue-Type Plasminogen Activator in a Canine Model of Coronary Artery Thrombosis

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LY210825, a recombinant tissue-type plasminogen activator (rt-PA), which contains the kringle-2 and serine protease functional domains of native tissue-type plasminogen activator, was previously produced by site-directed mutagenesis in a Syrian hamster cell line. We studied the thrombolytic potential of this molecule in a canine thrombosis model. Male hounds (16–22 kg) were anesthetized; a 2.0-cm segment of the left circumflex coronary artery (LCX) was isolated proximal to the first main branch, and the dogs were instrumented with an electromagnetic flow probe to measure coronary blood flow. An occlusive thrombus was formed after injury of the intimal surface of the LCX with an electrical current applied by a needle-tipped anode placed distal to the electromagnetic flow probe. After 1 hour of occlusion, either LY210825 or rt-PA was administered intravenously according to the following protocols: 1) a 1-hour infusion of either 0.25 mg/kg LY210825 or 0.4 mg/kg rt-PA, 2) single injections of 0.15–0.6 mg/kg LY210825, and 3) a single injection of 0.45 mg/kg LY210825 and a 3-hour infusion of 1.0 or 1.7 mg/kg rt-PA. Plasma half-lives of LY210825 and rt-PA were 58±7 and 3.3±0.3 minutes, respectively. LY210825 produced more rapid reperfusion of the LCX than did rt-PA. In the third study, 90% of the rt-PA-treated vessels reoccluded within 1 hour after cessation of drug, whereas only 25% of the LY210825-treated vessels reoccluded following a 4-hour washout period. There were significant, but relatively small, reductions produced by both plasminogen activators on plasma fibrinogen and plasminogen (25–35% decreases). Because of its longer plasma half-life, LY210825 could be administered intravenously as a single injection. In a canine model of coronary artery thrombosis, LY210825 was a more effective thrombolytic agent than was rt-PA. (Circulation 1990;82:930–940)

Since the approval of streptokinase and recombinant tissue-type plasminogen activator (rtPA) for intravenous administration in acute myocardial infarction, there has been significant growth in the acceptance of thrombolytic therapy for the early restoration of blood flow to ischemic myocardium. Streptokinase and rt-PA must be administered by intravenous infusion to ensure thrombolytic efficacy. Recent reviews on coronary thrombolysis suggest that the ideal properties for a new thrombolytic agent are that it be fibrin specific, have a

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available or being tested in clinical trials that is capable of being administered as a single injection and providing sustained plasma concentrations is anisoylated, plasminogen-streptokinase activator complex (APSAC).  

Since the production of rt-PA, investigators have produced variant or modified recombinant forms of t-PA and urokinase in an attempt to learn more about the molecular biology of these serine proteases.  

A major emphasis in the design of variant forms has been to increase fibrin specificity (more thrombus selectivity), increase circulating plasma half-life, and decrease the bleeding liability compared with the endogenous natural enzymes. Most of the research and development has involved in vitro analyses of the variant plasminogen activators in an attempt to determine fibrin specificity and plasminogen activating activity.

There is a sparsity of data describing the thrombolytic efficacy of variant plasminogen activators in animals. Lau et al. 11 have described a variant that contains an asparagine to glutamine point mutation that prevents N-glycosylation at residue 451 of the native t-PA molecule. This variant demonstrated a biphasic clearance with component half-lives in the rabbit of 0.9 minutes and 27 minutes compared with a monophasic 3-minute half-life for t-PA. Thrombolysis in a rabbit venous thrombosis model was not different from that observed with native t-PA. Larsen et al. 12 described a deletion variant that had the finger region epidermal growth factor region, and glycosylation at three sites removed (t-PA-ΔFE3X). This molecule was thrombolytic in a rabbit model of venous thrombosis 13 and in a canine model of copper coil–induced coronary artery thrombosis. 14 In the rabbit and dog, t-PA-ΔFE3X was found to possess an initial half-life of 14 minutes and a second phase half-life of 72–125 minutes; each phase represented approximately 50% of the circulating antigen. After a single injection, t-PA-ΔFE3X was found to be a more effective thrombolytic agent than t-PA in the canine model of thrombosis. Lucero et al. 15 recently reported that a variant containing the finger, kringle-1, kringle-2, and serine protease (FK1K2SP) functional domains of t-PA possessed a plasma half-life in rabbits of 25 minutes. The FK1K2SP variant demonstrated specific activity and affinity for the plasma inhibitor, PAI-1, similar to that demonstrated by t-PA.

In 1986, van Zonneveld et al. 16 described a variant t-PA (LK2) that contained the kringle-2 and serine protease functional domains of t-PA. This molecule was expressed in an Ltk mouse cell line and extracted from crude culture medium. Recently, Burck et al. 17 described the expression, purification, and biochemical characterization of a mammalian cell-derived t-PA variant, LY210825. This truncated variant, produced in a Syrian hamster cell line, contained only the kringle-2 and serine protease functional domains of native t-PA. The LK2 variant, in addition to containing the kringle-2 and serine protease domains, lacked the first 3 N-terminal amino acids of LY210825 and contained the last eleven amino acids of kringle 1 (t-PA). LY210825 demonstrated fibrin binding, plasminogen activating activity, and in vitro thrombolysis. The objective of this investigation was to determine in a canine model of coronary artery thrombosis the thrombolytic efficacy of LY210825 in comparison to rt-PA, emphasizing time to reperfusion and prevention or delay of reocclusion. Additional objectives were to determine whether LY210825 possessed a longer plasma half-life than rt-PA and whether this plasminogen activator demonstrated thrombolytic efficacy when administered as a single injection.

Methods

Surgical Preparation and Instrumentation

The canine model of coronary artery thrombosis used in this study is a modification of the procedures described by Romson et al. 18 and Schumacher et al. 19 Seven- to nine-month-old male, mixed-breed hounds (16–20 kg, Hazleton-LRE, Kalamazoo, Mich.) were anesthetized with sodium pentobarbital (30 mg/kg iv.), intubated, and ventilated with positive pressure (Harvard respirator) with room air. Tidal volume and respiratory rates were adjusted to maintain blood Po2, PCO2, and pH within normal limits. Subdermal needle electrodes were inserted for the recording of a lead II electrocardiogram (ECG).

A left mediolateral neck incision was performed, and the left jugular vein and common carotid artery were isolated. A precalibrated Millar transducer (model MPC-500, Millar Instruments, Houston, Tex.) was inserted into the carotid artery for the continuous measurement of mean arterial blood pressure (MAP). The jugular vein was cannulated to obtain blood samples during the experiment. In addition, the femoral vein of one hind leg was cannulated for administering thrombolytic agents.

A left thoracotomy was performed at the fifth intercostal space, and the heart was suspended in a pericardial cradle. A segment (1–2 cm) of the left circumflex coronary artery (LCX) was isolated proximal to the first major diagonal ventricular branch. A 26-g needle-tipped wire anodal electrode (teflon-coated, 30-g silver-plated, copper wire) 3–4 mm in length was inserted into the LCX and placed in contact with the intimal surface of the artery (confirmed at the end of the experiment). The stimulating circuit was completed by placing the cathode in a subcutaneous site. An adjustable plastic occluder was placed around the LCX over the electrode. A precalibrated electromagnetic flow probe (Carolina Medical Electronics, King, N.C.) was placed around the LCX proximal to the anode for measurement of coronary blood flow. The occluder was adjusted to produce a 20% reduction in the height of the pulsatile coronary blood flow waveform; this did not affect mean coronary blood flow, but provided for 40–50% inhibition of the hyperemic response that resulted from a 10-second mechanical occlusion of the LCX.
All hemodynamic and ECG measurements were recorded and analyzed with a data acquisition system (M3000, Modular Instruments, Malvern, Penn.).

**Thrombus Formation and Drug Regimens**

Thrombogenesis was initiated by applying 100 μA of direct current to the anode, producing endothelial cell injury. The current was maintained for 60 minutes and then discontinued. Thrombus formation proceeded in a spontaneous fashion until the LCX was totally occluded (determined as zero coronary blood flow and appearance of ST segment elevation). The occluding thrombus was allowed to age for 1 hour, and then thrombolytic therapy was initiated. The first study was conducted to determine the thrombolytic efficacy of LY210825 (0.25 mg/kg, n=4) compared with that of rt-PA (0.4 mg/kg, n=5) when infused for 1 hour. The second study assessed the thrombolytic potential of single injections of LY210825 (0.15, 0.3, and 0.6 mg/kg, n=3 per dose). Last, various doses of single injections of LY210825 were compared with 3-hour infusions of rt-PA: 1) LY210825, 0.45 mg/kg single injection (1 minute), n=8; 2) rt-PA, 1.0 mg/kg, 3 hour infusion, n=4; and 3) rt-PA, 1.7 mg/kg, 3-hour infusion, n=6. Vegetable\-treated groups were included in studies 1 (n=4) and 3 (n=6) as controls for spontaneous reperfusion. Coronary blood flow was observed for 2-4 hours after the administration of the plasminogen activators. Reocclusion of coronary arteries after successful thrombolysis was defined as zero coronary blood flow that persisted for 30 minutes or longer. At the end of the experiments, the LCX was dissected longitudinally and the residual thrombus was removed and weighed.

**Hematologic and Coagulation Assays**

Whole blood cell counts and analyses of hemoglobin and hematocrit levels were determined with a 40-μl citrated blood sample processed with a hematology analyzer (Cell-Dyn 900, Sequoia-Turner, Mountain View, Calif.). Duplicate blood samples (3.8% citrate and 3.8% citrate+trasylo/; 500 KIU/ml) were drawn for determining plasma plasminogen, α2-antiplasmin (citrated samples) and fibrinogen, and plasminogen activator antigen and functional activity concentrations (citrate+trasylo/; samples). Trasylo/; was added to prevent in vitro fibrinogenolysis. Fibrinogen level was determined in plasma by a thrombin clotting time assay which involved the addition of 100 μl bovine thrombin (10 NIH units/ml) to 200 μl diluted dog plasma (0.1 ml plasma diluted with 0.9 ml 3% bovine serum albumin). Clotting times were recorded with a Fibrometer (BBL) or CoA Screener (American LABOr Co., Largo, Fla.) Standard curves were constructed with purified dog fibrinogen in 3% bovine serum albumin for absolute estimations of fibrinogen or from serial dilutions of the prestimulation (predrug) plasma sample for relative changes in plasma fibrinogen concentrations. Plasminogen level was determined by the chromogenic procedure that uses excess streptokinase to activate the plasma. \(^{21,22}\) α2-Antiplasmin was determined by a chromogenic assay where known amounts of plasmin were added to the test plasma to measure enzyme inhibition capacity of the sample. \(^{23}\) Plasminogen and α2-antiplasmin assays were performed immediately after the plasma samples were prepared.

**Plasminogen Activator Antigen and Functional Enzyme Activity Assays**

A polyclonal antibody was used to immunologically capture plasminogen activator molecules for concurrent antigen and plasminogen activator activity determinations. Antigen concentrations were determined with a modification of a t-PA enzyme-linked immunosorbent assay (American Diagnostica, Greenwich, Conn.). Materials were: 96 well plates (Costar No. 3590 EIA plates), ELISA reagents (American Diagnostica), goat anti-human uterine t-PA polyclonal antibody (No. 381), normal goat immunoglobulin (No. 397), horseradish peroxidase conjugated goat anti-human t-PA (No. 112), single-chain t-PA activity standard (No. 115), and PET (phosphate buffered saline/EDTA/Tween) buffer (IMUBIND-5 kit, No. 122). Ortho-phenylene diamine (OPD) was obtained as 10-mg tablets (Sigma Chemical Company, St. Louis, Mo.). Plasminogen was human glu-type (No. 400, American Diagnostica). Plasmin substrate was H-D-Val-Leu-Lys-pNA (American Diagnostica). Bovine serum albumin (No. A=A-7888) was obtained from Sigma Chemical Company.

**Antigen Assay.** EIA (enzyme-immunoassay) plates were coated overnight with 200 μl polyclonal anti-t-PA antibody (8.8 μg/ml). After washing the plates four times with PET buffer, 200 μl nonspecific goat IgG (8.8 μg/ml) was added to the wells. Plasma samples were added to designated wells (10 μl plasma appropriately diluted with 3% bovine serum albumin in saline), and the plates were allowed to stand at room temperature for 3 hours. The plates were then washed four times with PET buffer, and 200 μl OPD/H₂O₂ (0.042/0.04% in H₂O₂) was added to each well and allowed to stand at room temperature for 30 minutes in the dark, at which time 50 μl 3N H₂SO₄; was added to stop the horseradish peroxidase reaction. Optical density values (at 490 nm) were determined with a Vmax plate reader (Molecular Devices, Palo Alto, Calif.). Data were analyzed by use of a nine-point standard curve with appropriate reference t-PA concentrations from the same EIA plate. All data were subjected to a curve-fitting program (SOFTmax, Molecular Devices) so that correlation coefficients were greater than 0.97. Standard deviations among the unknowns were 10% or less. The useful range of sample concentrations as applied in 10-μl amounts in this system was 1.0–40.0 ng/ml in the diluted sample or reference standard.

**Functional activity of immunologically captured antigen.** To measure the plasminogen activating activity of the antigen captured in the antigen assay procedure (above), a second EIA plate was prepared as above and treated exactly the same except this second plate was treated with plasminogen and S-2251.
rather than the horseradish peroxidase–second antibody reagent for antigen determination. After immunoadsorption of 10–μl samples and washing as above, 40 μl human glu-plasminogen (0.1 mg/ml in 0.3 mol/l Tris + 0.15 mol/l NaCl, pH 7.4) was added to each well, and 100 μl substrate S-2251 (0.75 mmol/l in H₂O) was added to each well. The activity was measured by the increase in optical density (at 405 nm) at 4 hours by fixed-time point readings with the Vmax plate reader. Each plate contained a series of native t-PA reference concentrations for a nine-point standard curve that allowed the SOFTmax program to assign a value for each unknown in international units per milliliter.

**Drugs and Data Analysis**

LY210825 was expressed, purified, and characterized as described by Burck et al. Briefly, LY210825 was expressed in an adenovirus-transformed Syrian hamster cell line, grown in suspension culture, and purified from culture medium with affinity chromatography. LY210825 has the following characteristics: 358 amino acids make up the kringle-2 and serine protease functional domains of native t-PA, had more than 95% single-chain content, and had a fibrinolytic specific activity = 0.9–1.2 × 10⁶ IU/mg. The t-PA used for comparison in this study was recombinant, had more than 95% single-chain material, and had a fibrinolytic specific activity = 580,000 IU/mg (rt-PA, Activase, Genentech, South San Francisco, Calif.).

Animals were rejected if 1) their vessels were totally occluded before 45 minutes or had not occluded by 90 minutes from initiation of electrolysis, 2) their whole blood platelet count was 150,000/μl or less, and 3) they required more than three internal defibrillation countershocks (30–50 J) for resuscitation from ventricular fibrillation. Five dogs were not used in any of the drug studies because of the following reasons: 1) vessels in two dogs did not occlude within 90 minutes from the initiation of anodal stimulation, 2) two dogs could not be resuscitated from occlusion ventricular fibrillation (>3 countershocks), and 3) one dog would not stop bleeding from around the puncture wound after insertion of the needle electrode.

Data comparisons between LY210825- and rt-PA–treated groups were performed with single-factor analysis of variance followed by Dunnett’s multiple range test to determine the level of significance between groups. A two-factor repeated measures analysis of variance was performed to determine significant differences between different time points during the experiment. Fisher’s exact test was used to determine significant differences on the incidence of reocclusion between LY210825 and rt-PA treatments. These animal studies conformed to the guiding principles of the American Physiological Society for the use of animals in research.

**Results**

**One-Hour Infusion Study**

A study was performed to assess the thrombolytic potential of LY210825 and determine whether this truncated form of rt-PA possessed a longer plasma half-life compared with rt-PA. All groups demonstrated similar times to occlusion: Vehicle, 71 ± 11 minutes (n=5); LY210825, 72 ± 6 minutes (n=4); and rt-PA, 55 ± 12 minutes (n=5, mean ± SEM). Table 1 illustrates that a 1-hour infusion of LY210825 (0.25 mg/kg) lysed coronary thrombi and resulted in shorter times to reperfusion than did rt-PA (0.4 mg/kg), 34 ± 3.2 versus 50 ± 5.2 minutes (p < 0.01), respectively. The doses of plasminogen activators used were essentially equivalent in the amount of active material infused: LY210825 was 225,000 IU/kg (calculated using a specificity activity of 0.9 × 10⁶ IU/mg material used for this study), and rt-PA was 232,000 IU/kg. After cessation of the drug infusion, patency of the LCX was monitored until the end of the experiment (2 hours). All LY210825-treated vessels maintained flow until the end of the experiment (i.e., no reocclusion), whereas all of the rt-PA–treated vessels reoccluded within 30 minutes after cessation of drug. LY210825–treated vessels maintained more average flow during the reperfusion period than did corresponding rt-PA–treated vessels (15.1 ± 6.9 versus 2.0 ± 1.4 ml/min, p < 0.01), respectively (Table 1). Prestimulation coronary blood flow was 39.6 ± 9.4 ml/min for LY210825 and rt-PA, respectively. Table 1 also demonstrates that thrombus mass of the LY210825–treated group was

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**Table 1. Summary of the Pharmacological Profile of Plasminogen Activators LY210825 and rt-PA Infused for 60 Minutes in the Anesthetized Dog**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time to reperfusion (min)</th>
<th>Average CBF during reperfusion (ml/min)</th>
<th>Thrombus wt (mg)</th>
<th>Incidence of reocclusion (min)</th>
<th>Half-life (min)</th>
<th>AUC (μg/ml·min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n=5)</td>
<td>&gt;180</td>
<td>0</td>
<td>30.8 ± 5.6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LY210825 (n=4)</td>
<td></td>
<td></td>
<td>34 ± 3.2*</td>
<td>15.1 ± 6.9*</td>
<td>4/0§</td>
<td>58 ± 6.7*</td>
</tr>
<tr>
<td>0.25 mg/kg/hr</td>
<td></td>
<td></td>
<td>12.0 ± 7.4*</td>
<td></td>
<td>3.3 ± 0.3</td>
<td>197 ± 43*</td>
</tr>
<tr>
<td>rt-PA (n=5)</td>
<td></td>
<td></td>
<td>50 ± 5.2*</td>
<td>2.0 ± 1.4</td>
<td>5/5</td>
<td>29.3 ± 3.7</td>
</tr>
<tr>
<td>0.40 mg/kg/hr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.3 ± 0.3</td>
<td>56 ± 6</td>
</tr>
</tbody>
</table>

Values are mean ± SEM where appropriate.

CBF, coronary blood flow; AUC, antigen area under the curve; rt-PA, recombinant tissue-type plasminogen activator.

*p < 0.01 compared with vehicle; 'p < 0.01, §p < 0.001 for LY210825 vs. rt-PA values.
Figure 1. Plot of circulating plasma antigen concentrations of LY210825 and recombinant tissue-type plasminogen activator (rt-PA) during a 1-hour infusion period and a 2-hour period of reperfusion after cessation of drug administration in dogs. ○, LY210825 (0.25 mg/kg infused for 60 minutes); ●, rt-PA (0.40 mg/kg infused for 60 minutes). Each point represents the mean±SEM.

significantly smaller than that of the rt-PA–treated group (which was identical to the vehicle-treated group). Plasma antigen determinations demonstrated that peak concentrations were the same for both plasminogen activators; however, LY210825 had a significantly longer plasma half-life than did rt-PA (58±6.7 versus 3.3±0.3 minutes, respectively; Figure 1, Table 1). Because of the longer plasma half-life, LY210825 also demonstrated a larger antigen area under the curve during the experiments. LY210825 and rt-PA produced similar reductions in systemic fibrinogen; peak declines were 25±8% (predrug, 2.9±0.3 mg/ml) and 20±10% (predrug, 3.0±1.0 mg/ml), respectively.

Single Injection of LY210825

Figure 2 illustrates that the longer plasma half-life of LY210825 allows this molecule to be administered as a single injection. Nine dogs received a single injection of LY210825 at doses of 0.15 mg/kg (n=3), 0.3 mg/kg (n=3), and 0.6 mg/kg (n=3). The antigen area under the curve for these doses increased in a linear, dose-dependent manner, whereas the plasma half-lives of the three LY210825 doses were similar (approximately 60 minutes). Thrombolytic efficacy determinations for LY210825 in this study were as follows. 1) Time to reperfusion: 43 (n=2), one did not reperfuse, 20±5, and 13±2 minutes for doses of 0.15, 0.3, and 0.6 mg/kg, respectively. 2) Average coronary blood flow during the 3-hour reperfusion period: 0.6±1, 11.3±2.4, and 16.4±3.6 ml/min at doses of 0.15, 0.3, and 0.6 mg/kg, respectively. Baseline coronary blood flows were equal to 31.7±2, 23.5±6, and 28.2±4 ml/min at doses of 0.15, 0.3, and 0.6 mg/kg, respectively. Residual thrombus mass was 12.1±3.5, 7.4±3.5, and 5.5±0.8 mg for doses of 0.15, 0.3, and 0.6 mg/kg, respectively. Prestimulation plasma fibrinogen values were 3.1±1.4, 1.9±0.2, and 2.5±0.6 mg/ml for doses of 0.15, 0.3, and 0.6 mg/kg, respectively. Fibrinogen declined with time and demonstrated peak reductions at the end of the experiment of 21±11%, 24±15%, and 68±24% (one dog depleted) for 0.15, 0.3, and 0.6 mg/kg, respectively. A single injection of rt-PA (0.6 mg/kg) failed to produce any signs of reperfusion when administered to three dogs (data not shown).

Single Injection of LY210825 Compared With 3-Hour Infusion of rt-PA

Functional activity determinations. Because of the short half-life of rt-PA, there is a consequent smaller antigen area under the curve compared with that of LY210825 when the two agents are administered at equivalent activity/kg doses (Figure 1, Table 1). A
study, therefore, was designed to compare the thrombolytic efficacies of LY210825 and rt-PA under conditions intended to maximize the plasma concentration of functionally active rt-PA relative to that observed with single injections of LY210825. Figure 3 is a representative schematic of the infusion regimens of rt-PA used in an attempt to provide plasma concentrations of functionally active plasminogen activator similar to those observed with a single injection of LY210825. The total amount of fibrinolytically active plasminogen activator administered for LY210825 was 540,000 IU/kg compared with 580,000 and 986,000 IU/kg for 1.0 and 1.7 mg/kg rt-PA, respectively. Total doses of 1.0 and 1.7 mg/kg rt-PA administered during a 3-hour period provided plasma concentrations of functionally active plasminogen activator that produced areas under the curve equivalent to 45,521 ± 4,601 (n = 4) and 89,214 ± 7,782 IU/ml·min (n = 6), respectively. A single injection of 0.45 mg/kg LY210825 provided a significantly (p < 0.01) greater area under the curve of functionally active plasminogen activator equivalent to 200,933 ± 19,549 IU/ml·min (n = 8).

**Pharmacological assessment.** Table 2 demonstrates that baseline (prestimulation) heart rate, mean arterial pressure, and coronary blood flow were similar among all groups. Thrombolytic therapy produced significant decreases in mean arterial pressure at the end of the reperfusion period for LY210825 and rt-PA, probably because of reperfusion arrhythmias and loss of blood (e.g., surgical wounds, blood sampling). Time to occlusion of the LCX was similar among all groups: 70 ± 6, 62 ± 3, and 56 ± 2 minutes for 1.0 and 1.7 mg/kg rt-PA and LY210825, respectively. Significant return and maintenance of coronary blood flow was observed after thrombolysis with LY210825 and rt-PA. After a single injection of LY210825, however, more rapid thrombolysis was observed compared with both doses of rt-PA. Vessels exposed to LY210825 demonstrated a significant earlier time to reperfusion of 17±2 minutes compared with 65±9 and 43±6 minutes for 1.0 and 1.7 mg/kg rt-PA, respectively (Table 3). When the drug infusion of either 1.0 or 1.7 mg/kg rt-PA was stopped, all but one vessel reocluded; reocclusion time for 1.0 mg/kg was 18±6 minutes (four of four; two of four vessels had reoccluded before the end of drug infusion), and reocclusion time for 1.7 mg/kg was 51±5 minutes (five of six vessels reoccluded) (Table 3). Only two of eight vessels exposed to LY210825 reoccluded within the 4-hour reperfusion and wash-out period. Figure 4 shows the differences between a single injection of LY210825 and a 3-hour infusion of rt-PA. The average coronary blood flow maintained the 3-hour reperfusion period was similar for both LY210825 and rt-PA groups; peak coronary blood flow was approximately 20 ml/min immediately after reperfusion. In addition, the LCX vessels from the LY210825-treated group maintained patency for a significantly longer period of time after cessation of drug administration. Table 3 also demonstrates that there were no significant differences in residual thrombus mass at the end of the experiments.

**Effects of rt-PA and LY210825 on Canine Hematology and Coagulation Proteins**

**Canine hematology.** Prestimulation values for hematocrit, hemoglobin, and circulating platelets were similar among the rt-PA- and LY210825-treated canine groups (Table 4). A 3-hour infusion of rt-PA had

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**Figure 3. Schematic of drug infusion protocol for a 3-hour infusion of recombinant tissue-type plasminogen activator (rt-PA).** The total doses of rt-PA infused for 3 hours were 1.0 and 1.7 mg/kg. Washout period lasted for 1 hour after cessation of rt-PA infusion.

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**Table 2. Effects of LY210825 and rt-PA on Hemodynamics in the Anesthetized Dog**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Heart rate (beats/min)</th>
<th>MAP (mm Hg)</th>
<th>CBF (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>180 min</td>
<td>180 min</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 6)</td>
<td>149±5</td>
<td>138±7</td>
<td>144±8</td>
</tr>
<tr>
<td>rt-PA</td>
<td>147±11</td>
<td>146±13</td>
<td>146±13</td>
</tr>
<tr>
<td>1.0 mg/kg (n = 4)</td>
<td>158±8</td>
<td>155±9</td>
<td>155±4</td>
</tr>
<tr>
<td>1.7 mg/kg (n = 6)</td>
<td>0.45 mg/kg (n = 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY210825</td>
<td>161±11</td>
<td>164±11</td>
<td>155±9</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

MAP, mean arterial pressure; CBF, coronary blood flow; Prestim, baseline values of heart rate, MAP, and CBF determined before anodal stimulation began; Predrug, values of heart rate, MAP, and CBF determined at the end of the 1-hour occlusion period, immediately before drug administration.

*p < 0.01 for predrug and 180-minute vs. prestimulation values; †p < 0.03 for 180-minute vs. predrug values; §p < 0.01 for rt-PA and LY210825 vs. vehicle group.
significant, but relatively small, effects on hemoglobin and hematocrit levels. Likewise, single injections of LY210825 similarly increased hemoglobin and hematocrit levels, but the increases were not significantly different from those caused by rt-PA. Both plasminogen activators did not affect circulating platelet counts.

Coagulation proteins. Table 5 illustrates that rt-PA and LY210825 caused decreases in circulating α2-antiplasmin and fibrinogen (ranges, 35–51% and 22–35%, respectively). Circulating plasminogen was the least affected; only the high dose of rt-PA demonstrated significant activation of systemic plasminogen. These effects of the plasminogen activators on circulating coagulation proteins were not sufficient to cause profuse bleeding from the surgical wounds. All preparations, however, demonstrated bleeding from surgical sites.

Discussion

The present investigation describes the thrombolytic potential of a novel t-PA variant, LY210825. This molecule, derived from a Syrian hamster cell line, contains the kringle-2 and serine protease functional domains of native t-PA. LY210825 had a longer plasma half-life compared with rt-PA, produced rapid reperfusion of a coronary thrombus, and prevented reocclusion of the LCX after a single injection of the thrombolytic.

The objective of the initial study was to determine the thrombolytic activity of LY210825 compared with rt-PA at equivalent doses of plasminogen activator. LY210825 produced more rapid reperfusion and maintained flow better than did rt-PA, even though circulating peak antigen concentrations for both LY210825 and rt-PA were similar during a 1-hour infusion. One possible reason for the improved efficacy may be because LY210825 demonstrated a 15–20-fold increase in circulating half-life compared with rt-PA (60 minutes versus 3 minutes). The longer half-life would allow for a period of extended thrombolysis. Functional domain or glycosylation changes have been shown to extend circulating half-lives of variant t-PAs in rabbits and dogs. LY210825 has had the finger, EGF, and kringle-1 functional domains removed by site-directed mutagenesis. Kalyan and coworkers observed that deletion of the finger and EGF domains of t-PA produced an extension in circulating half-life from 2 to 50 minutes in mice but caused an appreciable decrease in fibrin binding. Similar observations were made by Lucore et al. in the rabbit with a variant deficient in the EGF domain (a 25-minute half-life). Therefore, the lack of the EGF domain in LY210825 probably contributed to its longer plasma half-life.

Thrombolytic efficacy in the face of minimal systemic fibrinogenolysis suggests that LY210825 main-

FIGURE 4. Plot of thrombolytic efficacy for a single injection of LY210825 and two 3-hour infusions of recombinant tissue-type plasminogen activator (rt-PA). First value for coronary blood flow begins at the time of reperfusion (see Table 3). "Prestimulation coronary blood flow. Solid portion of bar, injection or infusion time for each thrombolytic agent (1 minute for LY210825 and 3 hours for rt-PA); open portion of bar, washout period after the administration of each thrombolytic agent. Each point on the plot represents mean ± SEM. Numbers in parentheses represent number of patent vessels at the end of the experiment. *p<0.01; rt-PA (1.0 mg/kg) versus rt-PA (1.7 mg/kg) and LY210825.
TABLE 4. Hematologic Profile of Dogs Receiving Plasminogen Activators

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hemoglobin (gm/dl)</th>
<th>Hematocrit (%)</th>
<th>Platelets (×10^3/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prestim 30 min 180 min</td>
<td>Prestim 30 min 180 min</td>
<td>Prestim 30 min 180 min</td>
</tr>
<tr>
<td>rt-PA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 mg/kg (n=4)</td>
<td>12.2±0.7 13.9±0.8* 14.0±0.9*</td>
<td>36±2 40±2* 41±3*</td>
<td>293±35 258±67 288±17</td>
</tr>
<tr>
<td>1.7 mg/kg (n=6)</td>
<td>11.5±0.3 12.2±0.3* 14.2±0.3*†</td>
<td>33±1 35±1* 41±1**</td>
<td>280±25 287±17 303±21</td>
</tr>
<tr>
<td>LY210825</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.45 mg/kg (n=8)</td>
<td>11.1±0.3 12.6±0.3* 13.2±0.4*†</td>
<td>32±1 36±1* 39±1*†</td>
<td>233±9 248±19 269±18</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
rt-PA, recombinant tissue-type plasminogen activator; prestim, baseline values determined for hemoglobin, hematocrit, and platelets before anodal stimulation began.
*p<0.03 for 30- and 180-minute vs. prestimulation values; †p<0.05 for 180-minute vs. 30-minute values.

tains significant fibrin selectivity and binding capacity. The finger domain,10 as well as kringle-2,28 has been shown to be involved in the binding of t-PA to fibrin. The kringle-2 has been conserved in the construction of LY210825, and as a result LY210825 appears to maintain fibrin binding capacity similar to rt-PA.17

As a result of the longer half-life for LY210825, this thrombolytic agent can be administered as a single intravenous injection. The minimum effective dose was found to be 0.15 mg/kg, which demonstrated a time to reperfusion of 43 minutes in two dogs; however, maintenance of flow during reperfusion was poor (2.2% of baseline; one dog did not reperfuse). When the dose was increased to 0.3 mg/kg, time to reperfusion was significantly decreased to 20±5 minutes; all three dogs maintained flow for the 3 hours of reperfusion (48% of pretreatment blood flow). Time to reperfusion was further reduced by increasing the dose of LY210825 to 0.6 mg/kg, and flow maintenance was similar to that observed for dogs receiving 0.3 mg/kg. In a canine model of copper coil–induced coronary artery thrombosis, Cambier et al14 observed similar times to reperfusion with a nonglycosylated t-PA variant (t-PA-ΔFE3X) that had a longer half-life and that contained both kringles and the serine protease domains. The highest dose (0.15 mg/kg) used by Cambier et al14 was the lowest effective dose used in the present study. Direct comparisons of dose and efficacy cannot be made between these two studies because the copper-coil model develops a fibrin-rich red thrombus (homogeneous presence of platelets), whereas the electrolytic injury model develops a thrombus that has a platelet-rich fibrin head and red tail.18 Red thrombi have been shown to be less resistant to lysis by t-PA than are platelet-rich thrombi.29,30

In the present investigation, three dogs were given a single injection of rt-PA (0.6 mg/kg), and no reperfusion was observed. This observation was similar to that observed by Gold et al31 in that repeated (two to four) single injections of rt-PA (0.45 mg/kg per injection) did not produce significant or sustained reperfusion. They found that it was necessary to combine a single injection rt-PA (0.45 mg/kg) with the 7E3-Fab monoclonal platelet GPIIb/IIIa antibody (0.4–0.8 mg/kg) to produce consistent reperfusion and maintenance of flow (flow maintenance was not quantified during a 100-minute period of reperfusion). Recently, several investigators demonstrated thrombolysis with single injections of rt-PA in humans,32 in a canine femoral artery red thrombus model,33 and in a rabbit venous thrombosis model.34 Because of the large difference between circulating half-lives of LY210825 and rt-PA, experiments were designed to compare the thrombolytic efficacy of a single injection of LY210825 to a 3-hour infusion of rt-PA. An attempt was made to produce circulating concentrations of plasminogen activator from the rt-PA doses that would be functionally equivalent to the LY210825 doses. A single injection of LY210825 (0.45 mg/kg) produced more rapid reperfusion than did either infusion of rt-PA: 17 minutes (LY210825) versus 65 and 43 minutes (1.0 and 1.7 mg/kg rt-PA, respectively) (Table 3). The single injection of

TABLE 5. Effects of LY210825 and rt-PA on Plasma α2-Antiplasmin, Plasminogen, and Fibrinogen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α2-Antiplasmin (% basal)</th>
<th>Plasminogen (% basal)</th>
<th>Fibrinogen (% basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal Reperfusion End</td>
<td>Basal Reperfusion End</td>
<td>Basal Reperfusion End</td>
</tr>
<tr>
<td>rt-PA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 mg/kg (n=4)</td>
<td>100 83±7 65±15</td>
<td>100 94±2 89±8</td>
<td>100 88±3* 69±19*</td>
</tr>
<tr>
<td>1.7 mg/kg (n=6)</td>
<td>100 79±7 49±4*†</td>
<td>100 97±2 90±3*</td>
<td>100 83±5* 67±12*</td>
</tr>
<tr>
<td>LY210825</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.45 mg/kg (n=8)</td>
<td>100 68±5 53±2**†</td>
<td>100 99±1 94±5</td>
<td>100 78±9* 74±11*</td>
</tr>
</tbody>
</table>

Values are mean±SEM where appropriate.
rt-PA, recombinant tissue-type plasminogen activator.
*p<0.03 for reperfusion and end values vs. basal values; †p<0.01 for end values vs. reperfusion values.
LY210825 provided larger plasma concentrations of functionally active plasminogen activator compared with rt-PA. This was observed even though the amount of functionally active plasminogen activator administered was approximately twofold higher for rt-PA (1.7 mg/kg) than for LY210825: 986,000 versus 540,000 IU/kg, respectively. Rapid elimination of rt-PA, even during a 3-hour infusion, apparently continued to contribute to the lower active antigen concentrations. Substantially larger doses of rt-PA would have had to be administered to provide equivalent plasma concentrations of functionally active plasminogen activator.

Acute reocclusion of coronary arteries occurs in 5–20% of patients having undergone successful thrombolysis. Attempts have been made to delay or prevent reocclusion in in vivo thrombolysis studies with rt-PA. These studies have required sustained infusions of the plasminogen activator (to enhance thrombolysis) or a “cocktail” (to inhibit thrombus formation) approach to delay or prevent reocclusion.

Fox et al. demonstrated that subtherapeutic doses of rt-PA could prevent thrombogenesis in the canine model of copper coil–induced thrombus. Once the infusion of rt-PA was stopped, however, all vessels occluded. In a study of 68 patients, Johns et al reported that a maintenance infusion of 0.2 mg/kg rt-PA for 4 hours prevented acute symptomatic coronary artery reocclusion after successful thrombolysis with a 90-minute infusion of 1.0 mg/kg rt-PA. Adjunctive pharmacological intervention has been studied with a variety of agents that affect platelet function. Golino et al demonstrated adjunctive effectiveness in the canine model of copper coil thrombosis with a combination of thromboxane and serotonin receptor antagonists administered with rt-PA. In these studies, neither receptor antagonist alone was effective in preventing reocclusion. Other investigators, however, have demonstrated that thromboxane receptor antagonists administered alone were effective in preventing spontaneous reocclusion after either rt-PA or streptokinase-induced thrombolysis. The 7E3 monoclonal platelet GPIIb/IIIa antibody has been shown to be effective in preventing reocclusion after thrombolysis with rt-PA. Fitzgerald et al have demonstrated that aspirin and prostacyclin, as well as the 7E3 platelet GPIIb/IIIa antibody, also were effective adjuncts to rt-PA–induced thrombolysis in the canine model. All of these studies included heparin anticoagulation as part of the thrombolytic protocol. Since neither heparin nor any type of adjunct was used in the present study, the ability of a single injection of LY210825 to not only induce rapid reperfusion but to also prevent reocclusion becomes even more significant.

The single injection of LY210825 provided immediate concentrations of the plasminogen activator that were in the range required for thrombolysis. Because of the longer half-life of LY210825, necessary sustained concentrations of the plasminogen activator were maintained so that the LCX remained patent even after only a single injection. Conversely, even though rt-PA provided timely reperfusion and maintained blood flow during drug infusion, all but one LCX in the rt-PA groups reoccluded as soon as the rt-PA infusion was stopped (Table 3, Figure 4). The time at which the six patent vessels in the LY210825 group would have reoccluded could not be determined because of the time limit of the reperfusion and washout phase of the experimental protocol. A single injection of rt-PA administered before the infusion was begun may have decreased the time to reperfusion, but the total dose would have been much greater than 1.7 mg/kg.

LY210825 and rt-PA had similar effects on blood pressures, hematocrit levels, and hemoglobin levels (Tables 2 and 4). Changes in these parameters observed at the end of the experiments most likely reflect hemocoagulation, blood loss due to indiscriminant lysis of hemostatic plugs by the plasminogen activators as well as volume depletion from blood sampling. There was no effect on platelet count by either plasminogen activator. The plasma proteins, α2-antiplasmin, plasminogen, and fibrinogen were reduced (Table 5); however, the reductions were not indicative of significant systemic fibrinolysis in response to LY210825 or rt-PA. These data indicate that although LY210825 has a longer plasma half-life than rt-PA, it has the capacity for excellent thrombolytic efficacy without the liability of compromising the systemic coagulation cascade (i.e., potential lower risk of peripheral or cerebral bleeds).

These data demonstrate that the novel recombinant t-PA variant, LY210825, is an effective thrombolytic agent. LY210825 possesses the kringle-2 and serine protease functional domains of native t-PA and, as a result, is fibrin specific and capable of thrombolysis. LY210825 demonstrated a 15- to 20-fold longer plasma half-life than rt-PA. Consequently, a single injection of LY210825 (2.5- to 3-fold lower dose than rt-PA) produced more rapid reperfusion than did a 3-hour infusion of rt-PA and prevented reocclusion without additional pharmacological therapy (e.g., heparin, GPIIb/IIIa antibody). LY210825 was as fibrinogen sparing as was rt-PA. This investigation demonstrates that in a canine model of coronary artery thrombosis, LY210825 is a superior thrombolytic agent compared with rt-PA. Because LY210825 can be administered easily and safely as a single injection, this novel thrombolytic may be useful in prehospital settings where physician or trained paramedic emergency care is available.

Acknowledgments

We thank Dr. Eugene Braunwald for his stimulating discussions and constructive criticism of the experiments described in this investigation. We thank Dr. August Watanabe for his critical review of the manuscript.
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**KEY WORDS** • plasminogen activator, recombinant tissue-type • plasminogen activator, tissue-type • thrombolysis • thrombosis • LY210825
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C V Jackson, V G Crowe, T J Craft, J L Sundboom, B W Grinnell, J L Bobbitt, P J Burck, J F Quay and G F Smith

Circulation. 1990;82:930-940
doi: 10.1161/01.CIR.82.3.930

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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