Intramuscular administration of human tissue-type plasminogen activator in rabbits and dogs and its implications for coronary thrombolysis*

BURNET E. SOBEL, M.D., JEFFREY E. SAFFITZ, M.D., PH.D., LARRY E. FIELDS, M.D., DONALD W. MYEARS, M.D., STANLEY J. SARNOFF, M.D., ALICE K. ROBISON, PH.D., DWAIN A. OWENSBY, M.D., PH.D., AND KEITH A. A. FOX, M.B., CH.B.

With the technical assistance of John Botz, Denise Nachowiak, Richard Rodriguez, and Joseph R. Williamson, M.D.

ABSTRACT To determine whether sustained plasma concentration of human tissue-type plasminogen activator (t-PA) can be induced promptly after intramuscular injection with enhancers of absorption devoid of deleterious local and systemic effects, we studied 250 rabbits and 13 dogs. In rabbits with t-PA injected directly into exposed muscle followed by local electrical stimulation at the site, early absorption was increased markedly by addition of 0.63M methylamine plus 0.079M hydroxylamine to the excipient. Elevations peaked within 5 min and increased with dose of t-PA, concentration of methylamine, and volume of injection medium. The enhancers were effective with percutaneous injections in the absence of local electrical stimulation as well. They did not elicit any obviously deleterious local or systemic effects. In separate experiments in rats, intramuscular injections of 0.63M methylamine plus 0.079M hydroxylamine induced local egress of intravascular radiolabeled albumin within the injection site and endothelial gaps in venules detected with colloidal carbon—changes consistent with direct effects on vascular permeability. In dogs, percutaneous intramuscular injection of t-PA in excipient without enhancers did not lead to early elevations of human t-PA in plasma, although late elevations were seen. When the enhancers were used, early elevations occurred as well, with functional activity documented by fibrin plate assays of serially obtained plasma samples and by sequential coronary angiography delineating thrombolysis after experimentally induced coronary thrombosis. The results indicate that intramuscular administration of t-PA with selected enhancers of absorption is a feasible approach for rapid induction of fibrinolysis.


THROMBOLYSIS induced by intravenous administration of activators of the fibrinolytic system early after the onset of ischemia aborts myocardial infarction,1,2 improves ventricular performance,3 and prolongs life.4,6 Its efficacy depends on the rapidity of implementation early after the onset of ischemia. Immediate intramuscular administration of lifesaving medication, sometimes with the use of autoinjectors, has proven feasible for emergency treatment of severe allergic reactions and potentially lethal arrhythmias. In a preliminary study, we found that intramuscular administration of tissue-type plasminogen activator (t-PA) for coronary thrombolysis might be feasible as well.7 Prompt implementation of such treatment by paramedical personnel and patients themselves under adequate medical surveillance might facilitate pharmacologic recanalization. In our preliminary study, absorption of t-PA in rabbits was shown to be enhanced markedly, although only briefly, by hydroxylamine hydrochloride coupled with local electrical stimulation at the injection site. Under these conditions, early peak blood levels were attained within 5 min. However, in the high concentrations that were required, this agent could elicit methemoglobinemia, hypotension, tachycardia, or local injury.

This study was undertaken to develop and evaluate
an approach potentially suitable for clinical use. Our objective was to promptly elicit functionally active and therapeutic blood levels of t-PA that could be sustained for several hours after intramuscular injection without local electrical stimulation under conditions in which hemodynamics and oxygenation were unaffected and local tissue injury was minimal. Analogs of hydroxylamine, vasodilators, and other agents alone and in combination with low concentrations of hydroxylamine were evaluated in 250 rabbits and 13 dogs to determine their effects on enhancement of absorption of human t-PA given intramuscularly, hemodynamics, and possible confounding effects of enhancers on detectable human t-PA antigen. One combination of agents, methylamine hydrochloride with low and physiologically well-tolerated concentrations of hydroxylamine, was found to promote rapid absorption of t-PA with fibrinolytic and persistent coronary thrombolytic activity attained within 5 min after intramuscular injection and maintained for at least 6 hr in dogs without induction of extensive myonecrosis at the site of injection, methemoglobinemia, tachycardia, or hypotension. An additional 31 rabbits and six dogs were studied with injections of media with or without enhancer but without t-PA. After the most favorable conditions had been defined in experiments involving exposure of skeletal muscle at the injection site, intramuscular injections were performed percutaneously without local electrical stimulation as opposed to directly into exposed muscle with local electrical stimulation.

To delineate mechanisms that might account for the absorption-enhancing effects of the agents employed, their effects on vascular permeability were evaluated directly and independently in rats and rabbits with intravascular radiolabeled tracers, including chromium-51 (\(^{51}\)Cr)–labeled erythrocytes, cobalt-57 (\(^{57}\)Co)–labeled EDTA, and iodine-125 (\(^{125}\)I)–labeled bovine serum albumin (BSA) as well as with colloidal carbon. Histopathologic findings were characterized conventionally.

The results indicate that sustained, therapeutic blood levels of t-PA can be obtained promptly after intramuscular injection without local electrical stimulation and under conditions devoid of deleterious local or systemic side effects and that injections in large laboratory animals induce sustained coronary thrombolytic effects that persist for as long as 6 hr.

**Materials and methods**

**Materials.** t-PA in concentrations of 0.5 to 50 mg/ml produced by recombinant DNA technology (rt-PA) was provided by Genentech, Inc., South San Francisco (lots BH004DAX, H9017, and 4869-42). Intramuscular or intravenous administration of excipient alone had no effect on functionally or immunologically detectable plasma t-PA in rabbits or dogs (n = 31 rabbits and six dogs). Intramuscular administration of t-PA in excipient alone yielded virtually no elevation of plasma t-PA within the first 30 min in either species. Several potential enhancers of absorption selected because of their known interaction with protease inhibitors or their known rapid absorption were evaluated alone or in combination in concentrations of 0.015M to 1.20M, including diethanolamine, diethylamine, dimethylamine, ethanolamine, ethylamine, histamine, hydroxylamine, methoxyamine, and methylamine. Adenosine and hydralazine were evaluated also because of their potential value as vasodilators augmenting local blood flow and hence absorption of t-PA. Hypertonic saline (0.63M) and hypertonic saline with methylamine were evaluated as well. Hylauronidase (Sigma, type IVS, 1000 U/mg) was included in the injection medium in some experiments (1 mg/mL) because of its potential utility for facilitating diffusion and absorption. None of the agents affected either immunologically detectable t-PA or functional activity of t-PA despite incubations in vitro with t-PA in plasma or phosphate-buffered saline, pH 7.4 at 37°C for 1 hr before injection.

**Experimental animals.** To characterize effects of enhancers on absorption of t-PA in small animal species under the diverse conditions that required evaluation, experiments were performed first in 250 nonfasted, male, New Zealand White rabbits weighing 1.9 to 2.5 kg. Rabbits were selected rather than larger animals because the total amount of t-PA that was available was limited. Some experiments employed direct injection into exposed muscle with or without local electrical stimulation as previously described. Others (n = 21) were performed with percutaneous injections. These experiments employed larger concentrations and amounts of t-PA, percutaneous injection, and no electrical stimulation. For the short-term studies in rabbits, animals were anesthetized with 10 mg/kg sodium pentothal and 50 mg/kg α-chloralose and instrumented for monitoring of arterial blood pressure via the carotid artery and acquisition of serial blood samples via the jugular vein. In most experiments skin and subcutaneous tissues overlying the sartorius muscles bilaterally were incised, the muscle was exposed, t-PA or excipient alone was injected directly into the exposed muscle, and serial blood samples were acquired sequentially throughout a 30 min interval via the indwelling jugular venous catheter. In some animals, skeletal muscle blood flow was augmented at the injection site by electrical stimulation of the muscle with 2.0 msec, 9 to 14 V pulses at a rate of 5/sec for 30 min as previously described. In 21 rabbits and in each of 13 dogs studied subsequently, injections were made percutaneously without exposure of the muscle and without electrical stimulation so that the factors favoring absorption that had been defined could be tested under conditions simulating those applicable clinically. For these experiments, concentrations of t-PA in the injection medium were adjusted within the range of 5 mg/ml (rabbits) and 50 mg/ml (dogs) permitting administration of 4 mg/kg (rabbits) and 10 mg/kg (dogs) with two injections of 1 or 2 ml, respectively.

For studies in dogs, animals weighing approximately 20 kg were anesthetized with 12.5 mg/kg Pentothal plus 60 mg/kg α-chloralose after anaesthesia with 1 mg/kg morphine sulfate subcutaneously, ventilated with room air with a Harvard respirator via an endotracheal tube, and monitored hemodynamically as previously described. Injections of 2 ml aliquots of t-PA or excipient alone were administered intramuscularly into the sartorius muscle manually and percutaneously via syringe through a 21-gauge stainless-steel needle. For studies of coronary throm-
bolysis, coronary thrombosis was first induced with a thrombogenic copper coil and documented angiographically, as was coronary thrombolysis elicited with t-PA.

To assess the short-term histologic effects of intramuscular injection of excipient with t-PA, injection sites in rabbits and dogs were excised immediately after the final blood sample had been collected and compared with those of the contralateral sartorius muscle into which excipient without t-PA had been injected simultaneously and in equal volume. Tissues were fixed immediately in sodium phosphate-buffered 10% formalin and processed conventionally for light microscopy.

For morphologic studies of longer-term local effects of injections in rabbits, injection sites were marked on the epimysial surface with tissue tonne green, skin incisions were closed, and the animals were allowed to recover after anesthesia and administration of excipient with or without enhancer, t-PA, or both. Control and test tissue specimens (i.e., 1 cm thick blocks with approximately 4 cm² cross-sectional area injected with excipient alone and contralateral muscle injected with excipient plus enhancer with or without t-PA) were obtained at necropsy, 48 to 96 hr after intramuscular injection, fixed, and prepared as serial 5 µm sections for microscopy.

For studies of effects of enhancers on permeation of radiolabeled tracers, male Sprague-Dawley rats weighing 200 to 450 g were used.

**Assay of plasma samples.** Immunoradiometrically detectable human t-PA antigen was measured in serial blood samples collected via an indwelling jugular venous catheter in citrate Vacutainer tubes at 0° to 4° C with a final concentration of citrate of 10 mM. Plasma was separated at 4° C by centrifugation for 10 min at 1600 g and stored at −20° C until assayed. t-PA antigen in rabbits was assayed with a two-site immunoradiometric (IRMA) procedure as previously described after binding of t-PA to anti-t-PA immunoglobulin G absorbed to wells in a microtiter plate and subsequent binding of 125I-anti-t-PA to bound t-PA. After removal of excess 125I-anti-t-PA, the amount of bound 125I-t-PA was determined by gamma scintillation spectrometry. Anti-t-PA antiserum and purified human melanoma t-PA used as reference standards were provided by Prof. Désiré Collen. For convenience, human t-PA antigen in dogs was assayed with a commercially available enzyme-linked immunosorbent (ELISA) procedure (American Diagnostica) standardized with the IRMA procedure. Because endogenous t-PA in rabbits or in dogs does not cross-react with human t-PA in the ELISA assay used, it did not influence results.

t-PA functional activity was assayed with fibrin plates and by a modified, microtiter amidolytic chromogenic procedure. Zones of fibrinolysis on plates were measured by planimetry. Plates prepared with human fibrinogen (KabiVitrum), thrombin (Sigma), and CaCl₂ (0.05M) were exposed to serial dilutions of euglobulin fraction samples prepared by dilution of citrated plasma (1:20) with distilled water, adjustment of pH to 5.8 for dogs and 6.2 for rabbits with acetic acid, centrifugation, and solubilization of precipitates in imidazole-buffered saline (pH 7.4) containing 0.8% BSA. They were incubated at 37° C for 18 hr. Quantitative analyses of plasma t-PA functional activity were performed with a modified microtiter amidolytic procedure. Both procedures were standardized with respect to the International Reference Preparation for t-PA (IRP-t-PA).

Effects of intramuscularly administered t-PA on fibrinolytic activity in vivo were monitored over 6 hr in dogs by sequential analyses of fibrinogen, plasminogen, and α₂-antiplasmin in plasma as previously described.

**Assessment of changes in vascular permeability.** For studies of effects of the enhancers of absorption on an index of the permeability of the microvasculature that was entirely independent of t-PA, vascular permeability was characterized with intravascular radiolabeled tracers. Two small animal species (rabbits and rats) were used to conserve radiolabeled material. The extent to which the enhancers of absorption of t-PA affected vascular permeability of the site of intramuscular injection of the enhancers was reflected by egress of intravascular tracers into the extracellular space. 51Cr-labeled erythrocytes (51Cr-RBC) and 57Co-labeled EDTA were used as markers of the intravascular and extracellular spaces, respectively. Permeability at the intramuscular injection site of enhancers to intravascular 125I-labeled BSA (125I-BSA) was determined by measuring the tissue-to-blood isotope ratio (TBIR) of 125I/51Cr (TBIR-125I/51Cr). In an analogous manner, the TBIR of 57Co/51Cr (TBIR-57Co/51Cr) was used to estimate the size of the extravascular space potentially available to 125I-BSA in tissue. Free 125I was excluded from the injectate by Sephadex gel filtration, and radioactivity in plasma and tissue fractions and homogenates was shown to be more than 99% protein bound as reflected by precipitation with 5% trichloroacetic acid.

In the initial experiments of this type, male Sprague-Dawley rats weighing 250 to 400 g were anesthetized with sodium pentobarbital (40 mg/kg). The left femoral vein and right carotid artery were exposed and cannulated. 51Cr-RBCs (150 µCi in 0.6 to 0.8 ml of a buffer suspension with hematocrit of 40%) were injected into the femoral vein 5 min before intramuscular injection of excipient with or without enhancer. In some rats, colloidal carbon (1 ml of a 2% suspension) was injected via the femoral vein for microscopic localization of alterations in endothelial junctions potentially produced by the intramuscular injection of excipient with or without enhancer. 125I-BSA (13 µCi) and 57Co-EDTA (10 µCi) (30 to 50 µl of each) were injected intravenously, and immediately thereafter 0.1 ml of excipient with or without absorption enhancers was injected intramuscularly into exposed sartorius muscles bilaterally. Approximately 6 min after the intramuscular injection, 2 ml of blood was withdrawn from the carotid arterial cannula into a heparinized syringe for quantification of blood levels of the three radiolabeled tracers. One minute later the heart was removed to arrest the circulation, and both injection sites were excised. Radioactivity in blood and skeletal muscle was quantified with a three-channel gamma scintillation spectrometer with automated correction for background and spillover. In animals given colloidal carbon, excised injection sites were fixed in 10% formalin in 0.05M potassium phosphate buffer (pH 7.4) containing 0.8% BSA. They were cut into small blocks and rehydrated in 0.67% formalin to a final concentration of 10% formalin.

Additional experiments were performed in male New Zealand White rabbits anesthetized with 150 mg/kg α-chloralose and instrumented similarly. 51Cr-RBCs (600 µCi in 4 ml of a buffer suspension with hematocrit of 40%) and 125I-BSA (39 µCi) were injected via the jugular venous catheter 15 and 10 min before intramuscular injections of t-PA excipient. Subsequently, 0.1 ml of t-PA excipient was injected intramuscularly into one exposed sartorius muscle and 0.1 ml of excipient with absorption enhancer into the contralateral muscle. Fluorescein, 0.01%, was included in the injection medium to facilitate later identification of the injection site. At selected intervals after intramuscular injections, a sample of blood was withdrawn from the carotid artery, the heart was arrested by bolus injection of saturated potassium chloride, both injection sites were delineated immediately with the use of ultraviolet light and excised, and radioactivity in blood and tissue was quantified by gamma scintillation spectrometry.

**Physiologic effects of enhancers of absorption of t-PA injected intramuscularly.** Effects of the agents tested for enhancement of absorption on heart rate, arterial pressure, and respiratory rate were evaluated in rabbits. Serial determinations...
of arterial blood gases and pH as well as hemoglobin and methemoglobin were performed. Hydroxylamine elicited massive methemoglobinemia, hypoxemia, transient tachycardia, and hypotension in the high concentrations required to maximize absorption in injection volumes needed for solubilizing large amounts of t-PA. However, on the basis of observations in rabbits subjected to local electrical stimulation, 0.63M methylamine alone and 0.63M methylamine plus 0.079M hydroxylamine were considered to be particularly promising enhancers of absorption of t-PA.

Conditions used in the longer-term experiments performed subsequently were selected on the basis of information acquired from studies of several groups of rabbits undertaken first to define the effects of absorption of t-PA of volume of injection medium per se (0.06 to 4.0 ml, n = 13); of pH, total t-PA dose, and concentration of a given enhancer of absorption with injection volume held constant (four 1 ml injections per rabbit) and electrical stimulation used (n = 27); and of electrical field stimulation at the injection site (n = 22). Judging from the results of these short-term experiments and from histologic findings (see below), methylamine hydrochloride (0.63M) alone or in combination with low concentrations of hydroxylamine (0.079M) was selected for further evaluation in intact rabbits and dogs to enhance intramuscular absorption of t-PA injected percutaneously.

Statistical analysis. Group data are expressed as means ± SE. Differences between groups were assessed by means of analysis of variance.

Results

Intramuscular injection of t-PA, excipient alone, or excipient supplemented with any of the enhancers of absorption without exogenous human t-PA did not elicit immunoradiometrically detectable t-PA in plasma within a 30 to 60 min interval of observation in any of 31 rabbits or six dogs. Elevation of functional t-PA activity in plasma was not detectable despite sham operation, electrical field stimulation at the injection site, or administration of enhancers of absorption in the absence of administration of exogenous t-PA.

Absorption of intramuscularly injected t-PA with media supplemented with potential enhancers of absorption: Short-term experiments with local electrical stimulation and injections into exposed muscle. Short-term effects in rabbits of hydroxylamine and several potential enhancers of absorption selected from among its analogs at a concentration of 0.63M in the injection medium and of hydralazine (0.88 mM), adenosine (7mM), or hyaluronicidase (1000 U/ml) in concentrations chosen to avoid hemodynamic perturbations were evaluated in 120 rabbits. In the absence of an absorption-enhancing agent, the highest plasma t-PA concentration occurred 5 min after injection and was barely detectable (8 ± 2 [SD] ng/ml, n = 3). Low peaks were seen also with ethyamine (16 ng/ml maximum, n = 3) and hyaluronidase (38 ng/ml, n = 2). Hydralazine or adenosine independently elevated peak t-PA levels but only trivially (n = 3). Histamine (0.65 mg/kg) was ineffective, elevating t-PA to only 18 ng/ml (n = 5). Among the remaining agents tested, only diethylamine (88 ng/ml, n = 3), methylamine (224 ± 32 ng/ml, n = 16), and hydroxylamine (371 ± 44 ng/ml, n = 9) elicited marked augmentation of absorption of t-PA. Methylamine 0.63M plus 0.315M hydroxylamine increased plasma t-PA maximally to an average peak of 562 ± 130 ng/ml (n = 9) 5 min after injection. In each of these experiments four separate injection sites were used because of the modest mass of rabbit muscle. The total amount of t-PA in the entire 4 ml total volume injected was verified by analysis to be consistent (2.5 ± 0.26 mg/kg body weight). Saline in the same concentration as that of hydroxylamine or methylamine (0.63M) augmented absorption of t-PA by less than 30% of that induced by either amine (n = 6). The addition of 0.63M NaCl to 0.63M methylamine in the injection medium did not augment absorption of t-PA appreciably beyond that elicited by methylamine alone. With hydroxylamine as the sole enhancer of absorption and the amount of t-PA injected held constant (2.4 mg/kg), peak plasma t-PA occurred within 5 min after injection and increased as a function of the total volume of injection from 0.06 ml to 1 ml and 4 ml (in 1 ml aliquots in four sites) from 41 ± 12 to 119 ± 33 and 356 ± 48 ng/ml, respectively (n = 13). With the volume of injection medium containing 0.63M methylamine held constant (four 1 ml injections in each rabbit), peak plasma t-PA increased monotonically with doses ranging from 2 to 13 mg/kg from an average of 180 to 936 ng/ml occurring within 5 min at each dose (averages of results of duplicate experiments at each dose, n = 26). Additional experiments (n = 22) demonstrated that hydralazine elicited effects comparable to those of electrical stimulation, that the two were not additive, and that each increased peak t-PA compared to that with 0.63M methylamine as the sole enhancer by an average of 96%.

With the exception of hydralazine and adenosine, which elicited modest decreases in arterial pressure and increases in heart rate (both less than 10%), the agent that exerted the most consistent effects on arterial pressure or heart rate monitored over the 30 min interval after injection was hydroxylamine (table 1). Methoxyamine induced methemoglobinemia, hypoxemia, and hypotension as well (data not shown). Hydroxylamine elicited an initial 10 to 15 mm Hg decrease in mean arterial pressure with a 30% increase in heart rate immediately after injection. These hemodynamic effects peaked in 2 min and abated in approximately 5 min. Subsequently, mean arterial pressure declined markedly associated with profound hypoxemia.
TABLE 1
Hemodynamic effects of methamphetamine and hydroxylamine in rabbits

<table>
<thead>
<tr>
<th>Injector medium</th>
<th>Baseline HR</th>
<th>Baseline MAP</th>
<th>15 min HR</th>
<th>15 min MAP</th>
<th>1 hr HR</th>
<th>1 hr MAP</th>
<th>2 hr HR</th>
<th>2 hr MAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>2</td>
<td>275</td>
<td>96</td>
<td>96</td>
<td>91</td>
<td>91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.63M methamphetamine</td>
<td>8</td>
<td>282</td>
<td>89</td>
<td>88</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.63M hydroxylamine</td>
<td>6</td>
<td>271</td>
<td>91</td>
<td>81</td>
<td>52*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (control)</td>
<td>2</td>
<td>82</td>
<td>108</td>
<td>92</td>
<td>99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.63M methamphetamine</td>
<td>2</td>
<td>171</td>
<td>116</td>
<td>101</td>
<td>103</td>
<td>100</td>
<td>84</td>
<td>85</td>
</tr>
<tr>
<td>0.63M hydroxylamine</td>
<td>2</td>
<td>106</td>
<td>104</td>
<td>143</td>
<td>115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.63M methamphetamine +</td>
<td>3</td>
<td>78</td>
<td>118</td>
<td>102</td>
<td>98</td>
<td>91</td>
<td>94</td>
<td>109</td>
</tr>
<tr>
<td>0.079M hydroxylamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Baseline values are averages of heart rate (HR) and mean arterial blood pressure (MAP). Results after injection are expressed as percentages of control values to normalize for animal-to-animal variation at baseline. Injections of 1 ml in each of four exposed sites were performed followed by local electrical field stimulation. Hemodynamic changes were more marked in rabbits because of the higher ratio of injection volume and hence the amount of methamphetamine, hydroxylamine, or both to body weight in rabbits than in dogs. Methemoglobin 1 hr after injection exceeded 11% in dogs given 0.63M hydroxylamine but was undetectable or less than 0.6% in dogs given 0.63M methamphetamine alone or 0.63M methamphetamine plus 0.079M hydroxylamine. It was as high as 48% in rabbits given hydroxylamine (but absent with methamphetamine) because of the larger ratio of amine given to body weight.

\*p < .05 compared with baseline values.

(gross cyanosis) and methemoglobinemia averaging 48%. None of the animals injected with saline, excipient, t-PA and excipient, or t-PA and excipient supplemented with methamphetamine (0.63M) or with 0.63M methamphetamine plus 0.079M hydroxylamine, the combination selected as the most promising for enhancement of absorption while devoid of deleterious hemodynamic effects, exhibited significant hemodynamic changes or hypoxemia. Hydroxylamine, methamphetamine, and all of the other agents did not alter immunoradiometrically detectable t-PA in plasma samples supplemented with human t-PA and incubated for 1 hr at 37°C whether they were assayed immediately or stored for as long as 1 month at 0°C to 4°C.

Effects of hydroxylamine and methamphetamine on vascular permeability in skeletal muscle. To elucidate mechanisms by which the enhancers elicited early peaks of plasma t-PA after intramuscular injections, intramuscular injection of either 0.63M hydroxylamine or 0.63M methamphetamine without t-PA was evaluated in rats and rabbits. Both elicited a prompt and significant increase in the permeation of 125I-albumin from the intravascular to the extravascular space (figure 1). Injection of t-PA excipient alone resulted in TBIR-125I/51Cr values of approximately 1.6 in rabbits (n = 14) and 2.3 in rats (n = 6). These values remained constant for 30 min after injection (n = 16). Similar control values were seen with rat skeletal muscle that was not subjected to intramuscular injection.\* Control TBIR-125I/51Cr values exceed 1.0 in part because of the physiologic permeation of albumin and the lower hematocrit in the microvasculature compared with that in large vessels. The inclusion of hydroxylamine or methamphetamine in the in-

FIGURE 1. 125I-BSA permeation assessed with respect to TBIR-125I/51Cr in tissue surrounding intramuscular injection sites in rabbits and rats. Methamphetamine (MA) or hydroxylamine (HA) were compared with excipient (Ex) by excising injection sites bilaterally and quantifying radioactivity with gamma scintillation spectrometry.

Vol. 75, No. 6, June 1987
jection medium resulted in elevation of TBIR-\textsuperscript{125}I/\textsuperscript{51}Cr values evident within 2 min and increasing linearly over the subsequent 28 min of observation (n = 16). Eight minutes after intramuscular injection in rabbits (n = 30), TBIR-\textsuperscript{57}Co/\textsuperscript{51}Cr was approximately 10 times greater than control TBIR-\textsuperscript{125}I/\textsuperscript{51}Cr, indicating that \textsuperscript{125}I-BSA had potential access to the extravascular space and that blood flow was sufficient for delivery of tracer. No difference was observed in \textsuperscript{51}Cr-RBC counts per gram wet weight in sites (n = 9) injected with methylamine (1145 ± 479 cpm) compared with values in sites injected with excipient alone (1205 ± 333 cpm) (n = 9), indicating that this agent did not elicit gross injury to vessels associated with hemorrhage. However, \textsuperscript{51}Cr radioactivity per gram wet weight was higher in sites injected with hydroxylamine (n = 5) than in control sites (n = 5) (2926 ± 899 cpm after hydroxylamine compared with 976 ± 465 cpm after excipient alone; p = .001).

Light microscopic examination of intramuscular injection sites in animals given colloidal carbon intravenously delineated a distinctive pattern of carbon labeling in rats given hydroxylamine or methylamine. As shown in figure 2, carbon labeling was confined exclusively to small, postcapillary venules. Thus both hydroxylamine and methylamine elicited gaps in endothelium of postcapillary venules—the presumed sites through which \textsuperscript{125}I-albumin leaves the vascular space and intramuscularly injected t-PA enters the vascular space when absorption of t-PA is facilitated by hydroxylamine, methylamine, or the two in combination.

**Histopathologic effects of injections with electrical stimulation**

**In rabbits.** Injection of t-PA in excipient alone (n = 4) produced only minor skeletal muscle trauma detectable histologically and manifested by localized interstitial hemorrhage and microfocal myonecrosis in a pattern consistent with mechanical trauma per se (i.e., the insertion of the needle and injection of an inert vehicle into the muscle). These changes were indistinguishable from those seen with injection of excipient alone or of isotonic saline (n = 6).

Hydroxylamine (0.63M in 1.0 ml injected in one site) caused substantial muscle necrosis (n = 27 animals) readily apparent 48 hr after injection. Injection of this agent elicited discrete zones of skeletal muscle necrosis surrounded by narrow mantles of interstitial hemorrhage (figure 3). The extent of necrosis was proportional to the volume of the injection but not influenced by the presence or absence of t-PA. Even the most extensive injury seen was confined to the immediate vicinity of the injection site. In contrast, 0.63M methylamine elicited considerably less local injury in volumes of injection ranging from 0.06 to 1.0 ml/site (n = 23). In most cases the injury apparent 48 hr after injection was no greater than that seen after injection with t-PA excipient alone. In some sites injected with a 1 ml volume of 0.63M methylamine, modest myonecrosis and interstitial hemorrhage juxtaposed to the needle track were evident.

**In dogs.** Injection of 0.063M methylamine (n = 8) or hydroxylamine (0.63M methylamine plus 0.079M hydroxylamine) (n = 8) in dogs caused only modest morphologic alterations, including interstitial edema, hemorrhage, and acute inflammation. Focal myonecrosis was minimal or absent (figure 3). The extents of interstitial edema, hemorrhage, and inflammation were related to the volume of injection. In all cases such abnormalities were confined to the immediate vicinity of the injection site. The extent of morphologic abnormality and myonecrosis was no greater in sites

![Figure 2](http://circ.ahajournals.org/)

**FIGURE 2.** Photomicrograph of rat skeletal muscle after a 0.1 ml intramuscular injection of 0.63M methylamine. Before injection, colloidal carbon was injected intravenously as described in Materials and Methods. Tissue in the immediate vicinity of the injection site was removed and fixed in formalin 6 min after injection of methylamine. A small venule (small arrow) is heavily labeled with carbon particles, indicating that gaps have formed between vascular endothelial cells. A small arteriole (large arrow) shows no labeling.
injected with methylamine or the combination of methylamine and a low concentration of hydroxylamine than in skeletal muscle injected with excipient alone. Histologic effects after percutaneous injections without electrical stimulation corresponded to those seen with each enhancer of absorption with injections followed by electrical stimulation.

Results of percutaneous intramuscular injections. In ag-

FIGURE 3. Photomicrographs illustrating skeletal muscle damage induced by intramuscular injection with local electrical stimulation of 0.63M hydroxylamine in rabbits (top panels) or 0.63M methylamine plus 0.079M hydroxylamine in dogs (bottom panels). Discrete zones of necrotic skeletal muscle (N) surrounded by hemorrhage and acute inflammatory infiltrate were evident in some injection sites exposed to 0.63M hydroxylamine. The photomicrographs in the top panels are typical of the most extensive damage observed in rabbits given 1.0 ml of 0.63M hydroxylamine. Although myonecrosis was present in most injection sites after hydroxylamine, the damage was generally less extensive than that illustrated in the top panels. As shown in the bottom panels, illustrating injury caused by intramuscular injection of 1.0 ml of 0.63M methylamine plus 0.079M hydroxylamine in dog skeletal muscle, interstitial edema, hemorrhage, acute inflammation, and focal necrosis of small groups of skeletal muscle cells (arrows) resulted. The damage shown in the bottom panels is illustrative of the most extensive injury observed after injection of 0.63M methylamine alone or 0.63M methylamine plus 0.079M hydroxylamine. In general, less extensive injury without discernible muscle necrosis was seen compared with that seen with hydroxylamine alone. The extent of injury after intramuscular injection of 0.63M methylamine or 0.63M methylamine plus 0.079M hydroxylamine was no different from that seen after injection of excipient alone without either amine.
aggregate, the results from the first 229 rabbits studied indicated that 0.63M methylamine or 0.63M methylamine plus 0.079M hydroxylamine facilitated absorption of t-PA after direct injection into exposed muscle followed by local electrical stimulation at the injection site without inducing deleterious local effects or physiologic derangements. Accordingly, additional studies were performed in 21 rabbits injected percutaneously without exposure of the muscle and without electrical field stimulation. The amount of t-PA in the injection medium was increased to yield a total dose of 4 mg/kg. Two 1 ml injections were administered to each animal. Peak plasma t-PA levels occurred within 5 min and averaged 134 ± 21 with 0.63M methylamine plus 0.079M hydroxylamine (n = 8), the combination of enhancers of absorption found to be most effective. Saline (0.63M) or excipient alone did not elicit substantial elevations within the 30 min interval of observation after injection. These results confirmed the feasibility of achieving therapeutic blood levels of t-PA promptly after intramuscular injection without the need for electrical stimulation or exposure of the muscle through skin incisions. They indicated that peak blood levels could be obtained within 5 min after injection with specific enhancers of absorption that increase vascular permeability.

Absorption of t-PA injected intramuscularly and its functional consequences in dogs. Effective coronary thrombolysis with t-PA requires early and sustained elevation of t-PA in plasma. To determine whether both could be accomplished and to assess the functional impact of intramuscular t-PA on the fibrinolytic system and on coronary thrombi, additional experiments were performed in dogs.

Initial results indicated that intramuscular administration of large amounts of t-PA (10 mg/kg) in two simultaneous injections of 2 ml each in the absence of enhancers of absorption did not elevate plasma t-PA values early after injection. Thus values 15 min after injection averaged only 44 ng/ml (n = 2) despite the large amount of t-PA administered (figure 4). Sustained elevations 90 min or more after injections averaging 339 ± 42 and 622 ± 86 (SD) ng/ml were seen, however, in each of the two dogs (n = 10 observations per dog). These persisted for the entire 6 hr interval of observation. Considering results from the 250 rabbits studied and these two dogs, it appeared likely to us that the response of t-PA given intramuscularly was biphasic. Early absorption appeared to be dependent on the presence of the enhancer of absorption. Later plasma elevations appeared to reflect the slow ingress of t-PA in the circulation relatively independently of the presence or absence of an enhancer. To test this hypothesis we performed experiments in dogs given 10 mg/kg t-PA intramuscularly with excipient alone, 0.63M methylamine, or 0.63M methylamine plus 0.079M hydroxylamine. Elevations of t-PA within 10 min were trivial without enhancer, averaging only 32 ng/ml (n = 3); modest with 0.63M methylamine, averaging 126 ng/ml (n = 3); and marked with 0.63M methylamine plus 0.079M hydroxylamine, averaging 297 ng/ml (n = 3). As shown in figure 5, intramuscular
injection of t-PA with 0.63M methylamine plus 0.079M hydroxylamine resulted in both an early, enhancer-dependent peak and sustained elevations persisting for the entire 6 hr of observation, whereas with excipient alone only the late elevations were apparent (figure 4). The elevations of plasma t-PA antigen were accompanied by elevation of functional activity generally consistent with the measured ratio of functional-to-antigenic activity of the t-PA injected. Thus the ratio of functional activity to t-PA antigen in plasma after intramuscular, injection of t-PA with methylamine and hydroxylamine averaged 0.31 ± 0.02 (SD) IU/ng (n = 44 samples obtained over 6 hr after injection from three dogs). The magnitude and persistence of elevations of plasma t-PA are consistent with bioavailability of approximately 50% of injected t-PA in the 6 hr interval studied. Circulating fibrinogen did not decline detectably, although α₂-antiplasmin decreased by 70% over 6 hr reflecting persistent elevation of functional t-PA activity.

Effects on coronary thrombolysis. As indicated in Materials and Methods, coronary thrombi were induced in dogs with percutaneously inserted, indwelling coronary arterial copper coils as previously described. The coils are markedly thrombogenic and consistently induce persistent coronary thrombosis within 2 ± 1 (SD) min (n = 17 control dogs given no t-PA [data not shown]). Full heparinization fails to lead to recanalization of the occluded coronary artery.

To determine whether 10 mg/kg t-PA administered intramuscularly with 0.63M methylamine plus 0.079M hydroxylamine elicited coronary thrombolytic effects rapidly with biological activity persisting over prolonged intervals, we studied two dogs with coronary thrombosis induced with copper coils. Occlusive clots were confirmed angiographically, and thrombolysis was initiated with two simultaneous, percutaneous intramuscular injections of 2 ml each of t-PA (10 mg/kg total dose) in 0.63M methylamine plus 0.079M hydroxylamine 7 to 10 min after documented thrombosis. In both dogs, clot lysis occurred within 60 min despite the persistent presence of the intracoronary thrombogenic copper coil (figure 6). Furthermore, coronary thrombolytic effects were evident throughout a 6 hr interval of observation after intramuscular administration of t-PA only once even though no heparin was given, manifested by repetitive recanalization after the anticipated, episodic reocclusion induced by the indwelling, thrombogenic copper coil (figure 7). These results indicate that (1) substantial elevations of plasma t-PA can be obtained within minutes after intramuscular injection when enhancers of absorption are employed; and (2) thrombolytic effects are persistent for at least 6 hr after injection.

Discussion

The results of this investigation indicate the following:

(1) Intramuscularly administered t-PA is absorbed slowly in the absence of enhancers of absorption, with plasma levels rising substantially only slowly but persisting for at least 6 hr.

(2) Inclusion of hydroxylamine in injection medium results in prompt absorption of t-PA with marked ele-
viation of plasma t-PA occurring within 5 min after injection.

(3) Concentrations of hydroxylamine required to enhance early absorption optimally result in local injury, methemoglobinemia, and hemodynamic derangements especially marked in rabbits compared with dogs because of the greater amount of agent given per kilogram of body weight.

(4) Prompt absorption of t-PA is facilitated by meth-

FIGURE 7. Coronary angiograms obtained 2 (top) and 6 (bottom) hr after intramuscular injection of t-PA from the same dog as that from which angiograms in figure 6 had been obtained. Although the thrombo-
genetic copper coil (arrow) remained within the coronary artery through-
out, thrombolysis was evident at the time of acquisition of both angiograms. As was the case with the three other dogs studied in this fashion that were not exposed to any heparin (data not shown), episodic reocclusion followed by repeated recanalization was seen.

ylamine—an agent that does not exhibit deleterious local effects or systemic derangements seen with high concentrations of hydroxylamine.

(5) The combination of methylamine with a low concentration of hydroxylamine augments initial absorption beyond that seen with methylamine alone without deleterious local or systemic effects.

(6) Substantial initial absorption of t-PA is elicited with percutaneous intramuscular injections in intact rabbits and dogs without local electrical stimulation.

(7) t-PA absorbed after intramuscular injection with the enhancers identified is functionally active throughout 6 hr of observation in dogs, judging from functional assay of t-PA activity in plasma, consumption of α2-antiplasmin in vivo, and sequential coronary angiograms demonstrating repetitive clot lysis even when a thrombogenic intracoronary copper coil remains in place and no heparin is given.

(8) The enhancers of absorption facilitate prompt absorption of t-PA by inducing endothelial gaps in postcapillary venules and increasing vascular permeability reflected by the deposition of labeled tracers and colloidal carbon.

Hydroxylamine was selected as a potentially useful agent for enhancing the absorption of t-PA because of its prompt efficacy in reversing cyanide toxicity after intramuscular administration, its known interactions with protease inhibitors, and its potentially favorable interactions with inhibitors of t-PA.7 To obviate effects of hydroxylamine that might be deleterious in patients with compromised cardiovascular status, we evaluated numerous analogs and found methylamine to be devoid of apparent toxicity. This agent enhanced absorption of t-PA considerably although somewhat less than hydroxylamine. Both agents were devoid of mutagenicity or genotoxic effects on mammalian cells at maximal tolerated concentrations in standard Environmental Protection Agency test systems and both exhibited very high mean lethal doses exceeding 1 g/kg body weight in rats (Survival Technology, Inc., Bethesda, MD, data on file). Hydroxylamine degrades rapidly to ammonia and is exhaled. Thus a persistent body burden should not be encountered. These considerations and the lack of toxicity demonstrated in this study suggest that the agents will be well tolerated in patients and support the feasibility of clinical studies.

Concentrations of t-PA in plasma obtained early after intramuscular injection in this study are in excess of those demonstrated previously to be sufficient to lyse coronary thrombi in dogs2,10,11 and are consistent with those effective clinically.12 They are in excess of those demonstrated to prevent reocclusion in dogs13
and in patients. Some of the variability of concentrations of plasma t-PA encountered in dogs and rabbits is undoubtedly attributable to variation in the depth of anesthesia from animal to animal. Although hemodynamic perturbations may induce variability in patients, clearance of t-PA from the circulation is remarkably independent of profound changes in blood pressure or hepatic blood flow. Thus variability of plasma levels of t-PA after intramuscular injections in patients, even those with severely compromised ventricular performance, may be less marked than that seen in animals.

In most studies of intravenously administered t-PA in patients, plasma concentrations of t-PA elicited are substantially higher than those induced in this investigation even though lower concentrations of plasma t-PA are effective for coronary thrombolysis. Lower concentrations are particularly likely to be sufficient when treatment can be initiated very soon after the onset of thrombosis, as would be the case for clinical applications of the approach explored in this study. The half-life of clearance of t-PA from patients is approximately twice as long as that in dogs. Accordingly, and in keeping with the exponential nature of clearance of t-PA, substantially higher blood levels for a given dose can be expected in human subjects compared with dogs. Should it be necessary to reduce the total amount of t-PA given to each patient because of economic or other considerations, relative augmentation of plasma concentrations in patients compared with dogs may be helpful. Several approaches to this objective appear feasible, including (1) use of an autoinjector rather than a conventional syringe, (2) augmentation of the volume of the injection medium, (3) sequential injections, and (4) local vasodilators. Results from this study indicate that inclusion of vasodilators in the injection medium in concentrations devoid of systemic effects will augment absorption and simulate effects of local electrical stimulation.

The sustained blood levels of t-PA induced by intramuscular injection are likely to be beneficial in preventing early reclosure of successfully recanalized vessels. Thus intramuscular administration may not require early supplemental intravenous t-PA.

t-PA is an effective coronary thrombolytic agent with a remarkably low toxic-to-therapeutic ratio judging from its pharmacodynamic profile and its biochemical properties. The clinical efficacy of coronary thrombolysis is clearly dependent on the rapidity with which it can be induced after the onset of thrombosis and ischemia. The possibility that patients at high risk of acute myocardial infarction may benefit from intramuscular injection of t-PA by paramedical personnel, spouses, or even the patients themselves under appropriate medical guidance was entertained because such an approach offers promise for rapid clot lysis with the potential for interruption of infarction early in its course.

Intramuscular t-PA must be evaluated meticulously. Although induction of antibodies and untoward reactions to rt-PA have not been problems in clinical trials involving several thousand patients given intravenous t-PA, potential antigenicity of intramuscularly administered material must be considered and excluded. The absence of local, deleterious effects such as hemorrhage or myonecrosis with the methyamine and hydroxylamine enhancer medium in this study is encouraging, but definitive exclusion of toxic effects of the enhancers of absorption will be required. Delineation of the range of plasma levels encountered after intramuscular absorption of t-PA initially and over time is essential, as is the exclusion of protracted elevations of circulating plasma t-PA levels that could lead to fibrinogenolysis. Nevertheless, our results underscore the feasibility of administration of intramuscular t-PA as a potentially valuable initial approach to coronary thrombolysis for incipient or early myocardial infarction attributable to thrombosis.

We appreciate the secretarial assistance provided by Lori Dales.

References
5. GISSI: Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Lancet 2: 397, 1986
10. Van de Werf F, Bergmann SR, Fox KAA, de Geest H, Hoyng CF,
Sobel BE, Collen D: Coronary thrombolysis with intravenously administered human tissue-type plasminogen activator produced by recombinant DNA technology. Circulation 69: 605, 1984


Intramuscular administration of human tissue-type plasminogen activator in rabbits and dogs and its implications for coronary thrombolysis.
B E Sobel, J E Saffitz, L E Fields, D W Myears, S J Sarnoff, A K Robison, D A Owensby and K A Fox

Circulation. 1987;75:1261-1272
doi: 10.1161/01.CIR.75.6.1261

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/75/6/1261