Vampire Bat Salivary Plasminogen Activator (Desmoteplase) 
A Unique Fibrinolytic Enzyme That Does Not Promote Neurodegeneration

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Background and Purpose—Tissue-type plasminogen activator (tPA) promotes excitotoxic and ischemic injury within the brain. These findings have implications for the use of tPA in the treatment of acute ischemic stroke. The plasminogen activator from vampire bat (Desmodus rotundus) saliva (D rotundus salivary plasminogen activator [DSPA]; desmoteplase) is an effective plasminogen activator but, in contrast to tPA, is nearly inactive in the absence of a fibrin cofactor. The purpose of this study was to compare the ability of DSPA and tPA to promote kainate- and N-methyl-D-aspartate (NMDA)–induced neurodegeneration in tPA−/− mice and wild-type mice, respectively.

Methods—tPA−/− mice were infused intracerebrally with either tPA or DSPA. The degree of neuronal survival after hippocampal injection of kainate was assessed histochemically. Wild-type mice were used to assess the extent of neuronal damage after intrastriatal injection of NMDA in the presence of tPA or DSPA. Immunohistochemistry and fibrin zymography were used to evaluate DSPA and tPA antigen or activity.

Results—Infusion of tPA into tPA−/− mice restored sensitivity to kainate-mediated neurotoxicity and activation of microglia. DSPA was incapable of conferring sensitivity to kainate treatment, even when infused at 10-fold higher molar concentration than tPA. The presence of tPA also increased the lesion volume induced by NMDA injection into the striatum of wild-type mice, whereas DSPA had no effect.

Conclusions—DSPA does not promote kainate- or NMDA-mediated neurotoxicity in vivo. These results provide significant impetus to evaluate DSPA in patients with ischemic stroke. (Stroke. 2003;34:537-543.)

Key Words: excitotoxins ▪ neuronal death ▪ stroke ▪ tissue plasminogen activator
exacerbate neuronal injury.\textsuperscript{11,12} The reason for this inconsistency is unclear. On the other hand, a number of animal stroke studies that have assessed tPA on the basis of its thrombolytic action have reported a protective effect of tPA.\textsuperscript{13,14} The protective effect of tPA under these conditions clearly relates to its ability to promote reperfusion.

Curiously, one prominent in vitro study yielded an unexpected protective effect of tPA with respect to excitotoxic injury.\textsuperscript{15} In this study tPA inhibited zinc-mediated neurotoxicity of rat neuronal cultures\textsuperscript{15} in a manner independent of its proteolytic action, suggesting that its effect was independent of plasminogen activation. However, other in vitro studies using cultured cortical neurons from tPA\textsuperscript{−−} mice subjected to oxygen/glucose deprivation showed that the absence of tPA reduced neuronal death, while addition of exogenous tPA exacerbated neuronal loss.\textsuperscript{16} Additionally, tPA has been shown to significantly amplify hemoglobin-induced neurotoxicity of rat neuronal cultures,\textsuperscript{17} raising the possibility that tPA may have negative effects in hemorrhagic ischemic stroke.

With respect to ischemic injury, it appears that tPA has both positive and negative effects depending on the stroke model used.\textsuperscript{15} While controversy still remains, a pattern is emerging that implicates tPA as a potential neurotoxic agent if permitted access to brain tissue after injury.\textsuperscript{19} This has led to the notion that prolonged or delayed use of tPA in ischemic stroke patients could exacerbate neurodegeneration and destruction of the blood-brain barrier and thereby contribute to the increased risk of hemorrhage seen in these patients.

The limited fibrin specificity of tPA and its possible neurotoxic effects have fueled the search for other plasminogen activators displaying greater fibrin dependence and selectivity but that lack detrimental effects within the central nervous system. One candidate enzyme has been found in the saliva of the blood-feeding vampire bat, Desmodus rotundus.\textsuperscript{4} Four distinct proteases have been characterized and are referred to as D rotundus salivary plasminogen activators (DSPAs). Full-length vampire bat plasminogen activator (DSPA\textsubscript{1}) is the variant most intensively studied and exhibits >72\% amino acid sequence identity with human tPA.\textsuperscript{20} However, 2 important functional differences are apparent. First, unlike tPA, the DSPAs exist as single-chain molecules and are not cleaved into 2 chain forms.\textsuperscript{20} Second, the catalytic activity of the DSPAs is critically dependent on a fibrin cofactor.\textsuperscript{21,22} This is particularly conspicuous for DSPA\textsubscript{1} because its catalytic efficiency increases 102 000-fold in the presence of fibrin yet only 8-fold by fibrinogen.\textsuperscript{21} When the effect of fibrinogen is taken into account, the catalytic efficiency of DSPA\textsubscript{1} is specifically increased 13 000-fold by fibrin. Since similar analyses have shown that the catalytic efficiency of tPA is specifically enhanced only 72-fold by fibrin,\textsuperscript{21} it is clear that DSPA\textsubscript{1} is substantially more fibrin dependent and fibrin specific than tPA. Furthermore, DSPA\textsubscript{1} as well as DSPA\textsubscript{2} has demonstrated faster and more sustained reperfusion than human tPA in animal models of arterial thrombosis.\textsuperscript{23} Moreover, the DSPAs have been shown to cause less fibrinogenolysis\textsuperscript{22} and antiplasmin consumption than tPA.

The aim of this study was to determine whether the exquisite fibrin dependence and specificity of DSPA\textsubscript{1} would render it less likely to confer the neurotoxic effects described for tPA. To address this, we compared the ability of DSPA\textsubscript{1} and tPA to promote kainate- and NMDA-mediated neurodegeneration in tPA\textsuperscript{−−} and wild-type mice, respectively.

**Materials and Methods**

**Animals**

Wild-type (c57/Black 6) and tPA\textsuperscript{−−} mice (c57/Black 6 background)\textsuperscript{24} were used for this study. These experiments were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia for live animal use. The experiments were approved by the Animal Ethics Committee, the Austin and Repatriation Medical Center Animal Welfare Committee, and the Physiology Animal Ethics Committee of Monash University.

**Protein Extraction From Brain Tissue**

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg) and then transcardially perfused with 0.1 mol/L PBS, and the brains were removed. The hippocampal region was dissected, weighed, and incubated in an equal volume (wt/vol) (approximately 30 to 50 µL) of NP-40 lysis buffer (0.5\% NP-40, 10 mmol/L Tris-HCl [pH 7.4], 10 mmol/L NaCl, 3 mmol/L MgCl\textsubscript{2}, 1 mmol/L EDTA) containing no protease inhibitors. Brain extracts were homogenized with the use of a hand-held glass homogenizer and left on ice for 30 minutes. Samples were centrifuged, and the pellet was discarded. Protein concentration was then determined (Bio-Rad reagent).

**Fibrin Zymographic Analysis of Proteases**

The proteolytic activity in samples or brain tissue extracts was determined by fibrin zymography\textsuperscript{25} with the use of either recombinant protein (up to 100 pmol) or brain tissue extracts (20 µg). Gels were incubated in a 37\°C humidified chamber until proteolyzed zones appeared.

**Kainate-Induced Excitotoxicity**

The kainate injury model was based on the approach previously described.\textsuperscript{5} Animals were injected intraperitoneally with atropine (4 mg/kg), then anesthetized with pentobarbital. Mice were then placed into a stereotactic frame, and a micro-osmotic pump (Alzet model 1007D) containing 100 µL of 0.1 mol/L PBS, recombinant human tPA (alteplase, 0.12 mg/mL; 1.85 µmol/L), or DSPA\textsubscript{1} (1.85 µmol/L; supplied by Berlex) was implanted subcutaneously between the shoulder blades. The pumps were connected via sterile tubing to a brain cannula and inserted through a burr hole made through the shoulder blades. The pumps were allowed to infuse the respective solutions at rate of 0.5 µL/h for a total of 7 days.

Two days after infusion of the proteases, mice were reanesthetized, and 1.5 nmol of kainate in 0.3 µL of PBS was then injected unilaterally into the hippocampus. The coordinates were as follows: bregma -2.5 mm, mediolateral 1.7 mm, and dorsoventral 1.6 mm. The excitotoxin was delivered over 1 to 2 minutes. After kainate treatment, the injection needle remained at these coordinates for an additional 2 minutes to prevent reflux.

**Brain Processing**

Five days after kainate injection, animals were transcardially perfused with 30 mL of PBS and then with 70 mL of a 4\% paraformaldehyde solution; they were postfixed in the same fixative for 24 hours, followed by 24-hour incubation in 30\% sucrose. Coronal sections (40 µm) were cut on a freezing microtome and either stained...
with thionin (BDH) to detect neurons or used for immunohistochemistry.

Quantification of Neuronal Loss
Quantification of neuronal loss in the CA1 to CA3 hippocampal subfields was performed as described. Five consecutive sections from the dorsal hippocampus from all treatment groups were prepared, and care was taken to ensure that the sections spanned the injection site and lesioned area. The hippocampal subfields (CA1 to CA3) on these sections were traced from camera lucida drawings of the hippocampus. The entire length of the subfields was measured by comparison to 1-mm standards traced under the same magnification. The length of tissue with viable pyramidal neurons (normal morphology) and length of tissue devoid of neurons (no cells present, no thionin staining) were determined. The lengths representing intact neurons and neuronal loss over each hippocampal subfield were averaged across sections.

NMDA-Induced Excitotoxicity
This method was performed as previously described with slight modifications. Anesthetized wild-type C57/Black 6 mice were placed in a stereotaxic frame. The left striatum was injected with a 1-μL solution containing 50 mmol/L NMDA either alone or in combination with 46 μmol/L (3 μg) tPA or 46 μmol/L DSPA. tPA and DSPA were also injected alone as controls. The injection coordinates were as follows: bregma −0.4 mm, mediolateral 2.0 mm, and dorsoventral 2.5 mm. The 1-μL solutions were delivered over a 5-minute period at 0.2 μL/min, and the needle remained for an additional 2 minutes after injection to minimize reflux of fluid. After 24 hours mice were transcardially removed, and the ipsilateral and contralateral hippocampi were assessed for proteolytic activity.

Immunohistochemistry
Sections were immersed in 3% H₂O₂/10% methanol for 5 minutes and then incubated with 5% normal goat serum for 60 minutes. Sections were incubated overnight with either an anti–MAC-1 antibody (1:1000; Serotec) to detect microglia or polyclonal anti–DSPA antibodies (provided by Schering AG) and then incubated in the appropriate biotinylated secondary antibodies (Vector Laboratories). This was followed by a final incubation in avidin/biotin complex (Vector Laboratories) for 60 minutes before visualization with 3,3′-diaminobenzidine/0.05% H₂O₂. Sections were then mounted on gelatin-coated slides.

Statistical Analyses
All values are expressed as mean±SEM. Statistical analyses were performed by 2-sample Student’s t test. The null hypothesis was rejected at P<0.05.

Results

tPA Activity and DSPA Activity Remain Stable After 7-Day Incubation at 37°C
To confirm that both DSPA and tPA retained proteolytic activity during the 7-day infusion period, aliquots of tPA and DSPA were incubated at 37°C for 7 days. Serial 10-fold dilutions from 100 pmol were then performed, and proteolytic activity was assessed using fibrin zymography. Similar dilutions of freshly prepared tPA and DSPA were assessed in parallel as controls. The degree of proteolytic activity present in freshly prepared samples of either tPA or DSPA (lanes 1 to 4 and lanes 9 to 12, respectively) was compared with that seen in samples of tPA or DSPA incubated at 37°C for 7 days (lanes 5 to 8 and lanes 13 to 16, respectively). tPA and DSPA proteolytic activity after infusion into the hippocampal region (Hipp) of tPA−/− mice. tPA−/− mice were infused with either tPA (mouse 1 and 2) or DSPA (mouse 3 and 4) for 7 days. Protein extracts prepared from both the ipsilateral (ipsi) and contralateral (contr) hippocampus were assessed for proteolytic activity by fibrin zymography. Extracts were also prepared from the cerebellum (cereb) as a negative control. As shown, DSPA and tPA proteolytic activity was recovered from the ipsilateral (lanes 1, 4, 7, 10) and contralateral (lanes 2, 5, 8, 11) regions of the hippocampus but was absent in the cerebellum (lanes 3, 6, 9, 12).
their activity in the brain but also had diffused bilaterally within the hippocampal region. No activity was detected in the extract prepared from the cerebellum from any tPA−/− mouse (lanes 3, 6, 9, and 12).

Immunohistochemical Assessment of DSPA
Coronal sections of brains of tPA−/− mice that had been infused with DSPA were assessed by immunohistochemistry. DSPA antigen was detected within the hippocampal and cortical regions, with the most pronounced staining apparent around the infusion site (Figure 2). This result confirms that the infused DSPA is soluble and had diffused within the mouse brain.

DSPA Infusion Does Not Restore Kainate-Mediated Neurodegeneration In Vivo

tPA−/− mice were infused intrahippocampally with either 1.85 μmol/L tPA or DSPA. Two days later animals were injected with kainate and then killed on day 7. The degree of neuronal staining was assessed in at least 4 consecutive coronal sections by thionin staining. Infusion of recombinant tPA clearly promoted kainate-mediated neurodegeneration within the ipsilateral hippocampal region. In contrast, infusion of the same concentration of DSPA into the hippocampal region of tPA−/− mice did not alter the sensitivity of these animals to kainate. Figure 3A shows representative thionin-stained sections of 3 mice infused with either protease. Of the 11 mice infused with DSPA, some minor neurodegeneration was seen in 2 of these animals, whereas marked neurodegeneration was seen in all 12 mice infused with tPA. Quantification of the extent of neuronal survival within the ipsilateral and contralateral sides of the hippocampus for all mice is shown in Figure 3B. Also as shown in Figure 3B, infusion of either protease alone followed by intrahippocampal injection of PBS did not promote neurodegeneration (n=3). On the basis of these findings, it is apparent that when tPA and DSPA are infused at equimolar concentrations, only tPA is capable of restoring sensitivity to kainate-induced neurodegeneration.

Titration of DSPA and tPA into Mouse Hippocampus

The kainate-injury experiments were repeated with one tenth the concentration of tPA (0.185 μmol/L) and a 10-fold higher amount of DSPA (18.5 μmol/L). As shown in Figure 4, infusion of 18.5 μmol/L DSPA caused only minor neuronal loss after kainate treatment, whereas the lower concentration of tPA (0.185 μmol/L) still restored sensitivity to kainate treatment Figure 4A. This experiment was performed with the use of 3 mice in each group, and the combined quantified data are presented in Figure 4B. The minor degree of neuronal loss seen with the higher concentration of DSPA is virtually identical to that seen when the lower concentration of DSPA is used, suggesting that this minor effect is not due to DSPA. Indeed, tPA−/− mice have been shown to display a minimal response to kainate injury. Taken together, these findings further highlight the relative inability of DSPA to promote sensitivity to kainate in tPA−/− mice.

Activation of Microglia

The restoration of kainate sensitivity in tPA−/− mice after tPA infusion has been shown to result in microglial activation. To assess the degree of microglial activation after tPA or DSPA infusion and subsequent kainate treatment, coronal sections of mice were subjected to immunohistochemistry for activated microglia with the use of the anti–Mac-1 antibody. As shown in Figure 5, 3 of the 4 tPA−/− mice infused with
DSPA before kainate treatment displayed minimal increase in Mac-1 staining (Figure 5). In contrast, all 4 mice infused with tPA before kainate injection produced a marked increase in microglial activation after 7 days (Figure 5). Infusion of either DSPA or tPA alone did not result in activation of microglia (data not shown). Higher magnification confirmed the specificity of staining for microglia (not shown).

**Effect of tPA and DSPA on NMDA-Mediated Neurodegeneration in Wild-Type Mice**

Injection of tPA into the striatum of normal mice enhances the neurodegenerative effects of the glutamate analogue NMDA. This approach was used to compare the effects of tPA and DSPA (46 μmol/L) at promoting NMDA-induced neuronal cell loss in wild-type mice. As shown in Figure 6A, injection of tPA with NMDA markedly increased the lesion area compared with that produced by NMDA alone. In contrast, coinjection of the same concentration of DSPA did not result in any detectable neurodegeneration (not shown). These experiments were performed with the use of 8 mice in each treatment group, and composite quantitative results are presented in Figure 6B. These results illustrate that the presence of tPA results in 2.15-fold increase in the NMDA-induced lesion size ($P<0.0001$). In contrast, DSPA produced no further increase in lesion size after NMDA treatment.

To verify that the injection of DSPA had indeed infused into the hippocampal region, immunohistochemistry was performed on coronal sections with the DSPA antibody. As shown in Figure 7, DSPA had indeed perfused into the striatal region.
Discussion

We have compared the effects of tPA and DSPA using 2 different animal models of neurodegeneration: (1) using the kainate model of neurodegeneration in the hippocampus of tPA−/− mice and (2) using NMDA-induced lesion of the striatum of wild-type mice. Data obtained with the kainate injury model confirmed initial reports that highlight the critical need for tPA to promote kainate neurotoxicity and to activate microglia. However, under the same experimental conditions, infusion of an equimolar concentration of DSPA did not restore sensitivity to kainate, nor did it promote microglial activation. Indeed, infusion of a 10-fold higher concentration of DSPA (18.5 μmol/L) into tPA−/− mice still did not restore sensitivity to kainate treatment, whereas a 10-fold lower concentration of tPA (0.185 μmol/L) was still effective. Hence, tPA is at least 100-fold more potent than DSPA at promoting neurodegeneration after kainate treatment.

Our second model of neurodegeneration was established to assess the potential effects of both tPA and DSPA at promoting NMDA-mediated neurodegeneration in wild-type mice. The lesion area after injection of NMDA into the mouse striatum was increased 2.15-fold in the presence of tPA. In contrast, coinjection of DSPAα1 did not exacerbate the neurotoxic effect of NMDA. Although our immunohistochemistry confirmed that DSPA was indeed present within the striatal region, similar experiments performed to visualize tPA after injection into the striatal region were unsuccessful because of excessive background staining of the antibodies. It could be argued that the greater lesion area produced in response to tPA+NMDA injection compared with DSPA+NMDA is a consequence of differential diffusion of the 2 proteases within the striatal region. However, tPA and DSPA are both highly soluble proteins with similar molecular weights and are unlikely to display differences in diffusion rates. Nonetheless, immunohistochemical data for tPA are required to formally rule out this possibility.

Our results have shown that DSPA does not enhance neurodegeneration even in the presence of plasma proteins that have diffused into the lesioned area as a consequence of NMDA-mediated neurodegeneration. Taken together, our findings establish that DSPAα1 is essentially an inert protease when present within the murine central nervous system and is incapable of promoting neurotoxicity induced by either kainate or NMDA.

The lack of effect of DSPA in this environment is most likely a consequence of the different biochemical properties of this vampire bat–derived protease. The catalytic activity of DSPA is exquisitely dependent on the presence of fibrin but not fibrinogen as a cofactor. Although not investigated directly in this study, it seems likely that fibrin formation does not occur to any significant extent after diffusion of fibrinogen into the brain following the breakdown of the blood-brain barrier. It also remains to be determined whether fibrin-activated DSPA would indeed act like tPA after excitotoxic injury. There has been much speculation that the neurotoxic effects of tPA are, at least in part, unrelated to its ability to activate plasminogen. tPA has been shown to cleave the NR1 subunit of the NMDA receptor in a plasmin-independent manner, and it is likely that other substrates are direct targets for tPA. Whether fibrin-activated DSPA also recognizes these different substrates remains to be determined.

This study defines DSPAα1 as a plasminogen activator that does not exhibit the inherent neurotoxic properties associated with tPA. It would be interesting to determine and compare the effects of DSPAα1 and tPA at promoting neuronal loss after excitotoxic or ischemic injury when these proteases are administered intravenously.

The inability of DSPA to promote neurodegeneration provides substantial impetus to assess the efficacy of this protease in stroke patients. In this context, it is of interest that a clinical trial using DSPA (desmoteplase) in acute stroke (DIAS trial) has recently commenced in Europe (sponsored by PAION GmbH, P.I. Werner Hacke).

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