Vampire Bat Salivary Plasminogen Activator (Desmoteplase) Inhibits Tissue-Type Plasminogen Activator-Induced Potentiation of Excitotoxic Injury

Courtney Reddrop, BSc (Hons); Randal X. Moldrich, PhD; Philip M. Beart, DSc; Mark Farso, BSc (Hons); Gabriel T. Liberatore, PhD; David W. Howells, PhD; Karl-Uwe Petersen, MD; Wolf-Dieter Schleuning, MD, PhD

Background and Purpose—In contrast to tissue-type plasminogen activator (tPA), vampire bat (Desmodus rotundus) salivary plasminogen activator (desmoteplase [DSPA]) does not promote excitotoxic injury when injected directly into the brain. We have compared the excitotoxic effects of intravenously delivered tPA and DSPA and determined whether DSPA can antagonize the neurotoxic and calcium enhancing effects of tPA.

Methods—The brain striatal region of wild-type c57 Black 6 mice was stereotaxically injected with N-methyl-D-aspartate (NMDA); 24 hour later, mice received an intravenous injection of tPA or DSPA (10 mg/kg) and lesion size was assessed after 24 hours. Cell death and calcium mobilization studies were performed using cultures of primary murine cortical neurons.

Results—NMDA-mediated injury was increased after intravenous administration of tPA, whereas no additional toxicity was seen after administration of DSPA. Unlike DSPA, tPA enhanced NMDA-induced cell death and the NMDA-mediated increase in intracellular calcium levels in vitro. Moreover, the enhancing effects of tPA were blocked by DSPA.

Conclusions—Intravenous administration of tPA promotes excitotoxic injury, raising the possibility that leakage of tPA from the vasculature into the parenchyma contributes to brain damage. The lack of such toxicity by DSPA further encourages its use as a thrombolytic agent in the treatment of ischemic stroke. (Stroke. 2005;36:1241-1246.)

Key Words: excitotoxicity, tissue plasminogen activator

In addition to its well-established fibrinolytic role within the vascular system, tissue-type plasminogen activator (tPA) has been implicated as an effector within the central nervous system (CNS). Although tPA is produced by endothelial cells within the CNS, tPA is also expressed by both neuronal and microglial cells and contributes to neuronal plasticity and long-term memory formation. Besides these neurophysiological roles, tPA has been recognized as a significant factor in causing excitotoxic injury in vivo. tPA can also promote N-methyl-D-aspartate (NMDA)-mediated cell death and enhance hemoglobin-induced and oxygen-glucose deprivation–induced neurotoxicity in vitro. Intriguingly, one study indicated a non-proteolytic protective effect of tPA against zinc-induced neuronal injury, although the basis for this is unclear. In the context of ischemic injury, tPA has been shown to have a negative, positive, or no effect at all on infarct size. These contradictory results can be attributed, at least in part, to differences between the experimental ischemic models used and whether tPA was used as a thrombolytic. In the latter cases, tPA treatment usually had a beneficial effect. However, in models involving permanent occlusion of cerebral vessels, the presence of tPA was often detrimental.

The potentiating effect of tPA on neurotoxicity has a bearing on its use as a thrombolytic drug, particularly in the context of ischemic stroke. Currently, the therapeutic window for tPA administration to ischemic stroke patients is restricted...
to the first 3 hours after the onset of symptoms, because later use is not of proven benefit and is also associated with an enhanced risk of cerebral hemorrhage.\textsuperscript{14,15} Whether this is related to the neurotoxic effects seen in animal studies is unknown but remains a plausible hypothesis.

The highly fibrin-selective plasminogen activator derived from vampire bat saliva (Desmodus rotundus salivary plasminogen activator [DSPA]; desmoteplase),\textsuperscript{16} is equally effective as tPA at dissolving clots but does not promote the consumption of coagulation factors. However, in contrast to tPA, DSPA does not promote neurodegeneration when co-injected with excitotoxic agents into the mouse brain.\textsuperscript{17} The basis for the differential effect of tPA and DSPA remains to be determined, although it is possible that tPA acts on substrates other than plasminogen that are not recognized by DSPA but play a role in the cascade of events culminating in neuronal injury.

In this study, we have tested the hypothesis that DSPA is inactive at promoting excitotoxic injury when delivered intravenously. Because tPA and DSPA are structurally related, we have also investigated the ability of DSPA to antagonize the potentiating effects of tPA on NMDA-induced neuronal injury.

In this study, we have tested the hypothesis that DSPA is inactive at promoting excitotoxic injury when delivered intravenously. Because tPA and DSPA are structurally related, we have also investigated the ability of DSPA to antagonize the potentiating effects of tPA on NMDA-induced neuronal cell death.

Materials and Methods

Animals

Animal experiments were approved by the Austin and Repatriation Medical Centre Animal Welfare Committee and Animal Ethics Committees of Monash University.

NMDA-Induced Excitotoxicity and Intravenous Injection

Mice (c57/black6) were anesthetized with 40 to 60 mg/kg Nembutal via intraperitoneal injection. The left striatum was injected stereotaxically with 50 nmol/µL NMDA (Sigma) (1 µL) as described;\textsuperscript{17} 24 hours later, mice were administered a 100-µL solution containing 0.9% saline, tPA (Actilyse; Boehringer Ingelheim, Germany) or DSPAα1 (PAION Deutschland GmbH, Aachen, Germany), both at 10 mg/kg by tail-vein injection. Twenty-four hours after injection, mice were anesthetized, transcardially perfused with saline, then fixed with 4% paraformaldehyde.\textsuperscript{17}

Quantitation of the NMDA-Induced Lesion

Coronal sections of the brain were cut at 40-µm intervals. Using the ballistic light approach to identify the lesion,\textsuperscript{18} 2 consecutive sections that contained the largest lesion were selected together with 4 consecutive sections either side of this region, giving 10 consecutive sections for total analysis. The area of the lesion in each section was first determined using the Micro Computer Imaging Device and the volume (mm$^3$) within the assessed region then calculated. Sections were alternatively kept in a neutral phosphate-buffered saline solution for immunohistochemistry.

Immunohistochemistry

DSPA was detected in fixed coronal sections using a polyclonal anti-DSPA antibody,\textsuperscript{17} whereas γ-globulin was detected using an anti-mouse γ-globulin antibody (Chemicon, Melbourne, Australia), followed by incubation with avidin-biotin peroxidase complex (Vector Laboratories, Calif).

Neuronal Cell Cultures

Cells from the cerebral neocortex were cultured from Swiss white embryonic mice (day 15 to 16)\textsuperscript{19} and seeded at 0.12×10$^6$ or 0.3×10$^6$ cells/well for viability assays (96-well plates) or calcium and morphological experiments (24-well plates), respectively. Cultures were maintained serum-free in Neurobasal medium containing B27 supplement in a humidified CO$_2$ incubator (5% CO$_2$, 8.5% O$_2$; 37°C) for up to 9 days in vitro. Cultures were regarded as neuronal as they contained <5% glial fibrillary protein-positive cells.\textsuperscript{19}

Cytotoxicity Studies

After 8 to 9 days in vitro, medium was replaced with MEM (Gibco-BRL Life Technologies) and cultures were treated with NMDA (30 or 70 µmol/L) and tPA and/or DSPA (5 to 500 nM) for 24 hours. Cell death was determined using a Cytoxicity Detection Kit (lactate dehydrogenase [LDH] assay; Boehringer Mannheim). Negative and positive control cultures were exposed to MEM only and 0.1% Triton X-100, respectively. Results were expressed relative to Triton X-100-induced LDH release. Viability was alternatively quantified by the ability of surviving cells to reduce 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan.\textsuperscript{19}

Calcium Mobilization Studies

Neurons cultured for 6 to 9 days in vitro were loaded with 10 µmol/L Fluo-3/AM calcium-binding dye (Invitrogen) in HEPES-buffered saline (HBS).\textsuperscript{19} Where indicated, tPA or DSPA (500 nmol/L) was added alone or in combination to cells 5 minutes before addition of 70 µmol/L NMDA. Cellular fluorescence was measured in relative fluorescence units using the Fluoroskan Ascent fluorometer. Readings were taken at 485/530 nm (excitation/emission) after the wash to obtain basal values (5 minutes) and during treatment (5 minutes). For quantitation, background fluorescence levels in cells treated with HBS containing 1 mmol furosemide (HBSF; Sigma) alone were subtracted from all samples at each designated time point. Data were normalized to the 100% calcium increase produced with NMDA alone.

Statistical Analyses

Results are expressed as the mean±SEM and analyses were performed by 2-sample 2-tailed Student $t$ test. Differences in toxicity or calcium influx in cultures were analyzed using 1-way ANOVA with Student Newman Keuls post-hoc tests. $P<0.05$ was considered as significant.

Results

Effect of Intravenous Administration of tPA and DSPA on NMDA-Induced Striatal Injury

Mice were stereotaxically injected with NMDA, then 24 hours later administered with tPA, DSPA, or 0.9% saline by tail vein injection. Intravenous injection of tPA after NMDA injection resulted in a 30% increase in the lesion size compared with animals infused with saline ($n=13$, $P<0.05$; Figure 1). In contrast, mice administered with DSPA did not exhibit any change in the size of the lesion. Hence, tPA was able to traverse the compromised BBB and promote neuronal injury.

NMDA-Induced Injury Facilitates Leakage of DSPA

γ-Globulin was detected within the NMDA-induced lesion (Figure 2A), indicating that the BBB had been disrupted. To determine whether this also resulted in leakage of DSPA, NMDA-treated mice injected intravenously with DSPA 24 hours after injury, were assayed by immunohistochemistry. DSPA was detected within the lesion indicating that DSPA had permeated into the brain (Figure 2Bii). In contrast, only minimal staining for DSPA was seen after intravenous delivery of DSPA in mice given an intrastriatal injection of...
saline (Figure 2Cii), indicating that leakage was a consequence of the NMDA-induced injury. Experiments performed to confirm the presence of tPA within the damaged area were compromised because of cross-reactivity of anti-tPA antibodies between endogenous mouse tPA and human tPA. In situ zymographic experiments, however, demonstrated an increase in tPA proteolytic activity within the NMDA-induced lesion after intravenous injection of tPA compared with mice injected with saline (not shown).

**tPA Enhances NMDA-Mediated Cell Death In Vitro**

Addition of NMDA (30 and 70 µmol/L) to primary cultures of mouse cortical neurons for 24 hours resulted in significant cell death (Figure 3A). Addition of tPA significantly increased NMDA-induced cell death at the highest concentration (500 nmol/L). In contrast, DSPA (500 nmol/L) had no effect, and even appeared to reduce the degree of cell death, although this was not statistically significant. MTT analysis (Figure 3B) and morphological analyses (not shown) also confirmed that tPA, but not DSPA, potentiated NMDA-mediated cell death.

**Antagonistic Effect of DSPA on tPA-Potentiation of NMDA-Mediated Neurotoxicity**

DSPA may recognize the same substrates as tPA, but because of its greater dependence on a fibrin-cofactor and/or differences in affinity, cannot cleave them. If tPA and DSPA do recognize the same substrates, it is plausible that DSPA would antagonize the tPA effect. To test this, NMDA (70 µmol/L) was added to cultures of cortical neurons for 24...
hours in the presence of 500 nmol/L tPA, together with increasing concentrations of DSPA (5 to 500 nmol/L). DSPA inhibited tPA-dependent potentiation of NMDA-induced cell death at equimolar concentrations (Figure 4).

Enhancement by tPA on NMDA-Mediated Intracellular Calcium Increase Is Blocked by DSPA

We next compared changes in intracellular free calcium levels after NMDA treatment of neurons in the presence of tPA or DSPA, added alone or in combination. NMDA alone (70 μmol/L) caused a 30% to 40% increase in the concentration of intracellular calcium after 1 minute, an effect maintained over a 5-minute period (Figure 5A). These levels increased significantly when cells were pretreated with 500 nmol/L tPA (P<0.001) 5 minutes before adding NMDA (Figure 5A and 5B). In contrast, pretreatment with DSPA alone had an attenuating effect on the NMDA-induced increase in intracellular calcium. Pretreatment of cells with a combination of tPA and DSPA (each 500 nmol/L) produced significantly less increase in calcium levels after NMDA addition than cells pretreated with tPA alone (Figure 5B). We also noted that pretreatment with tPA and DSPA in the absence of NMDA tended to reduce background fluorescence (Figure 5B) and that the majority of this effect was caused by DSPA (not shown). Whether this is nonspecific or a genuine effect on background calcium levels remains to be determined. Even with the change in background fluorescence taken into account, DSPA still significantly reduced the potentiating effect of tPA on NMDA-induced increase in intracellular calcium.

Discussion

It is a widely supported hypothesis that ischemia promotes the release of glutamate from affected cells causing excitotoxic injury to neurons. That tPA can potentiate excitotoxic injury has been documented in a number of reports, and a recent study further suggests that tPA worsens endothelial and neuronal ischemic damage. tPA appears to enhance neuronal injury initiated by ischemic glutamate release and this may also be coupled with vascular effects including edema.

In contrast to tPA, DSPA does not promote neuronal injury when co-injected with excitotoxins into the mouse brain. Given the clinical routine of thrombolytic therapy, it was important to determine whether these agents could also cause harm via the intravenous route. We therefore investigated the neurotoxic effects of tPA and DSPA delivered intravenously into mice harboring an NMDA-induced striatal injury.
Results of experiments using cultures of mouse cortical neurons further demonstrated that tPA, but not DSPA, enhanced NMDA-induced neuronal cell death and increase in intracellular free calcium. Because tPA and DSPA share structural characteristics and are likely to bind similar substrates, we tested the hypothesis that DSPA might antagonize the enhancing effect of tPA on NMDA-induced cell death. Our findings show that the enhancing effect of tPA on NMDA-induced neurotoxicity and increase in intracellular free calcium were both inhibited by DSPA. Hence, DSPA used as a therapeutic agent may not only stimulate thrombolysis but also limit the potentially damaging effect of endogenous tPA. We also observed DSPA alone reduced the NMDA-mediated calcium increase, although whether this is entirely specific to NMDA remains to be determined as DSPA also had a slight attenuating effect on background calcium-associated fluorescence (not shown).

The mechanistic basis for the blocking effect of DSPA is presently unknown. tPA has been shown to cleave the NR1 subunit of the NMDA receptor independently of plasminogen. Although this remains controversial, an increase in calcium influx and cell death subsequent to the cleavage of this subunit would provide a plausible explanation for our findings. Given its structural similarity to tPA, DSPA may also bind to the NR1 subunit, but result in steric hindrance of NMDA binding to its receptor, hence blocking the ensuing pathways culminating in cell death. Alternatively, the inhibitory effect of DSPA could involve other effectors recognized by tPA, most notably the low-density lipoprotein receptor-related protein (LRP). An LRP-dependent effect of tPA within the CNS has been described, whereas ligand binding to LRP has been shown to modulate NMDA-dependent calcium uptake. Hence it is possible that tPA and DSPA have differential effects on LRP. Alternatively, DSPA may lack neurotoxicity because of the absence of its cofactor fibrin. However, fibrin deposition occurs within the parenchyma after ischemia, thereby providing DSPA with its cofactor. Our results therefore argue against a role of plasmin in the cause of NMDA-induced striatal injury, which is distinct from its requirement during kainate-induced injury in the hippocampus. If the tPA effects were plasmin-dependent, DSPA would be expected to produce similarly harmful consequences. A plasmin-dependent effect of tPA in our in vitro study is also unlikely as the cells are maintained under serum-free conditions.

DSPA lacks both the fibrin-binding kringle II domain and a plasmin-sensitive activation site in the connecting peptide that are present in tPA. However, DSPA contains a salt bridge between Lys156 and Asp194 that is shielded by the intact activation loop, which favors fibrin specificity. Whether these differences account for the different effects in promotion of neurotoxicity remains to be determined.

The function of tPA in the CNS is clearly distinct from its role in wound-healing and fibrinolysis. It is an open question, however, which function emerged first in the course of phylogeny. In any case, there was no evolutionary pressure to preserve a neuronal function for the vampire bat salivary protease, because DSPA has evolved to specifically promote fibrin dissolution to support the animals feeding habit. Like its structural cousin, thrombin, tPA appears to be a protease with pleiotropic functions associated with structural elements other than the classical active site. One of the most conspicuous sites is the lysine-binding kringle II domain of tPA, which is notably absent in DSPA.

Acknowledgments
This study was funded in part by PAION Deutschland GmbH, Aachen, Germany, and in part by grants awarded to R.L.M. (#236862), P.M.B. (Program #236805), and D.W.H. from the National Health and Medical Research Council of Australia.

References
Vampire Bat Salivary Plasminogen Activator (Desmoteplase) Inhibits Tissue-Type Plasminogen Activator-Induced Potentiation of Excitotoxic Injury
Courtney Reddrop, Randal X. Moldrich, Philip M. Beart, Mark Farso, Gabriel T. Liberatore, David W. Howells, Karl-Uwe Petersen, Wolf-Dieter Schleuning and Robert L. Medcalf

Stroke. 2005;36:1241-1246; originally published online May 5, 2005;
doi: 10.1161/01.STR.0000166050.84056.48
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/36/6/1241

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/