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PPACK-Desmodus rotundus salivary plasminogen activator (cDSPAα1) prevents the passage of tissue-type plasminogen activator (rt-PA) across the blood-brain barrier and neurotoxicity

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PPACK-*Desmodus rotundus* salivary plasminogen activator (cDSPAα1) prevents the passage of tissue-type plasminogen activator (rt-PA) across the blood-brain barrier and neurotoxicity

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The treatment of ischemic stroke remains one of the most challenging areas in medicine. To date, the only treatment approved by health authorities is early reperfusion by the thrombolytic agent, recombinant tissue-type plasminogen activator (rt-PA)[1]. Nevertheless, despite benefit from fibrinolysis, several limitations are linked to the use of rt-PA, including a clinically relevant risk of haemorrhage[1] and a highly suspected risk of neurotoxicity[2,3]. Desmoteplase (DSPA) is a highly fibrin-specific recombinant plasminogen activator (PA) isolated from the saliva of the vampire bat *Desmodus rotundus*[4]. Experimental data suggest that DSPA could display several advantages: i) in contrast to rt-PA, DSPA is devoid of pro-excitotoxic effects both in vitro and in vivo[5,6]; ii) intravenous DSPA significantly reduces the passage of co-administered rt-PA across the intact BBB and the attendant aggravation of excitotoxic damage[6]. In this context, the aim of the present study was to investigate, using a predictive in vitro model of BBB[7] (Fig. 1A), and an *in vivo* model of excitotoxicity, whether an inactive form of DSPA, clogged DSPA (cDSPA), could prevent the ability of rt-PA to cross the BBB and promote excitotoxic injuries. As previously demonstrated for native DSPA[6], we found that cDSPA did not alter the integrity of the BBB (passage of sucrose) alone or in combination with rt-PA (data not shown) and can cross the BBB, despite being catalytically inactive (Fig. 1B). Then, the passage of rt-PA alone or in combination with either DSPA or cDSPA was analyzed using both fluorogenic proteolytic (spectrozyme®) and zymography assays (Fig. 1C and D). Both DSPA and cDSPA reduced the passage of rt-PA by around 30% in our *in vitro* BBB model. The effects of intravenous injection of rt-PA and cDSPA were studied in a mouse model of excitoxicity induced by a striatal stereotaxic injection of NMDA (Figure 1E). Neither rt-PA nor cDSPA vehicles injected intravenously altered the extent of NMDA-induced striatal injury. As previously demonstrated[6], the systemic injection of rt-PA increased the striatal lesion volume seen with NMDA by almost

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60% (from ~10 to ~16 mm^3). Interestingly, intravenous co-administration of cDSPA with rt-PA, both at 1 mg/kg, suppressed the ability of exogenous rt-PA to enhance NMDA-induced lesion. No micro-haemorrhages were detected in animals treated with rt-PA and/or clogged-desmoteplase.

Endogenous tPA, released by vascular endothelial cells, is well-known to play a critical role as a thrombolytic enzyme that activates plasminogen to plasmin. Based on this activity, exogenous rt-PA-induced thrombolysis remains the only FDA-approved drug for the treatment of patients in acute ischemic stroke[1]. However, t-PA favors intracerebral hemorrhagic transformation, limiting the licensed use of this drug to a 3 hours post-ictus therapeutic window[1]. Accordingly, the balance between the beneficial effects of thrombolysis and the injurious cerebral effects of rt-PA may be improved by restricting the access of vascular rt-PA to the brain parenchyma. Previously, we and others have demonstrated both *in vitro* and *in vivo* that despite its common ability to cross the intact BBB trough a common transport system, in contrast to rt-PA, DSPA, is not pro-neurotoxic[6]. As a further characterization of the transport mechanism, our results show that a proteolytically inactive form of DSPA can restrict rt-PA trans-BBB passage. This is in agreement with the previous demonstration that PAs can cross the BBB independently of their proteolytic activity/domain[8]. We also demonstrate that clogged-desmoteplase prevents exogenous rt-PA-promoted excitotoxicity *in vivo*. This is in agreement with previous demonstrations that DSPA can prevent cerebral damage by rt-PA *in vivo*[5,6] a feature likely to contribute to the increased tolerance and safety of the clinical use of DSPA in the 3-9 hours time window post-onset of stroke symptoms[9]. Thus, while rt-PA remains the only approved thrombolytic drug to treat stroke patients, the interest in alternatives like DSPA is sustained. As intrinsic fibrinolytic activity of such an antagonist would not be required or might even be counterproductive,

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generation of a proteolytically inactive version of DSPA able to compete with rt-PA for transport at the BBB might open the way to a safer use of rt-PA as a thrombolytic in stroke.

Figure 1: (A) Schematic representation of the in vitro BBB model (B) biot-rt-PA or biot-cDSPAα1 were applied to the upper endothelial compartment. Two hours later, their activities or presence in the lower side medium were analyzed either by zymography assay or immunoblotting (n=3). (C,D) rt-PA alone or with DSPA or cDSPA was applied to the upper endothelial compartment. Two hours later, rt-PA activity was analyzed in the lower side medium, either by fluorogenic assay (spectrozyme®) (C) or zymography assay (D). Results are normalized to rt-PA (mean values ± SEM of 3 experiments per group; * $p<0.05$ Mann-Whitney test). (E) Effects of intravenous injection of tPA alone or in combination with cDSPA (1 mg/kg each) on the extent of neuronal death induced by the striatal administration of NMDA (10 nmol) in mice (mean ± SD; n=10 per group; *: $p<0.01$ compared to NMDA alone, $: $p<0.01$ compared to NMDA + tPA, Mann-Whitney test).

Reagents, Human recombinant rt-PA (Actilyse®) from Boehringer Ingelheim (Paris, France). DSPA was provided by PAION Deutschland GmbH (Aachen, Germany).

Production of cDSPA: cDSPA was generated by coupling the Phe-Pro-Arg-chloromethylketone modified tripeptide to DSPA.

Biotinylation: Since cDSPAα1 has no intrinsic enzymatic activity, we had to develop a method of detection for transport studies, based on biotinylation of the compound, allowing performing SDS-PAGE/immunoblotting against biotin (with a streptavidin secondary antibody). We used the FluoReporter®Biotin-XX Protein Labeling kit (Molecular Probes) according to the manufacturer's instructions.

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Blood-brain barrier (BBB) in vitro experiments: Transport across the BBB was studied using a previously characterized in vitro model[7], consisting of a co-culture of endothelial and glial cells and shown to closely mimic the in vivo BBB. rt-PA and/or desmoteplase were added to the upper side of the endothelial cells. Abluminal and luminal media were harvested after the 2 hours transport experiments.

Spectrozyme® assays were performed using a fluorogenic substrate (Spectrozyme XF444, American Diagnostica, Stamford, NJ, USA).

Zymography assay was performed by adding plasminogen (22.5µg) and casein (5mg) to a 12.6% SDS polyacrylamide gel.

Striatal excitotoxic lesions: Mice were anaesthetised with isoflurane and placed in a stereotaxic frame. Body temperature was maintained at 37 ± 0.5°C. The skull was exposed and an injection pipette was stereotaxically implanted in the right striatum according to the atlas of Paxinos & Watson (coordinates 0.5 mm posterior, +/- 2 mm lateral and -3 mm ventral to the bregma). NMDA (10 nmol) was injected in a volume of 0.3 µl. Excitotoxic treatment was followed after 10 minutes by intravenous injection of rt-PA (1 mg/kg), cDSPA (1 mg/kg), rt-PA + cDSPA (1 mg/kg each), tPA vehicle (L-Arg 35 mg/kg, phosphoric acid 10 mg/kg and polysorbate 80 0.2%) or cDSPA vehicle (glycine 4 mg/kg and mannitol 10.64 mg/kg).

Histological analysis: For volume analysis, one coronal section (20 µm) out of every 10 was stained with thionine and analysed with an image analyser (Scion Image, Scion Corporation, Frederick, Maryland, USA).

Statistical analysis: For *in vitro* and *in vivo* studies, data were expressed as mean ± SEM, and statistical analyses were performed using the Kruskal and Wallis test followed by a Mann-Whitney test for inter-group comparisons.

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References

1. NINDS. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Tissue plasminogen activator for acute ischemic stroke (1995) The National Institute of Neurological Disorders and Stroke tPA Stroke Study Group. N Engl J Med 333:1581-1587.

2. Tsirka SE, Gualandris A, Amaral DG, Strickland S (1995) Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. Nature 377:340-344.

3. Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET, Vivien D, Buisson A (2001) The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med 7:59-64.

4. Schleuning WD, Alagon A, Boidol W, Bringmann P, Petri T, Krätzschmar J, Haendler B, Langer G, Baldus B, Witt W, Donner P (1992) Plasminogen activators from the saliva of Desmodus rotundus (common vampire bat): unique fibrin specificity. Ann N Y Acad Sci 4:395-403.

5. Reddrop C, Moldrich RX, Beart PM, Farso M, Liberatore GT, Howells DW, Petersen KU, Schleuning WD, Medcalf RL (2005) Vampire bat salivary plasminogen activator (desmoteplase) inhibits tissue-type plasminogen activator-induced potentiation of excitotoxic injury. Stroke 36:1241-1246.

6. López-Atalaya JP, Roussel BD, Ali C, Maubert E, Petersen KU, Berezowski V, Cecchelli R, Orset C, Vivien D (2007) Recombinant Desmodus rotundus salivary plasminogen activator crosses the blood-brain barrier through a low-density lipoprotein receptor-related protein-dependent mechanism without exerting neurotoxic effects. Stroke 38:1036-1043.

For Peer Review

7. Cecchelli R, Dehouck B, Descamps L, Fenart L, Buée-Scherrer VV, Duhem C, Lundquist S, Rentfel M, Torpier G, Dehouck MP (1999) In vitro model for evaluating drug transport across the blood-brain barrier. Adv Drug Deliv Rev 36:165-178.

8. Benchenane K, Berezowski V, Ali C, Fernández-Monreal M, López-Atalaya JP, Brillault J, Chuquet J, Nouvelot A, MacKenzie ET, Bu G, Cecchelli R, Touzani O, Vivien D (2005) Tissue-type plasminogen activator crosses the intact blood-brain barrier by low-density lipoprotein receptor-related protein-mediated transcytosis. Circulation 111:2241-2249.

9. Hacke W, Albers G, Al-Rawi Y, Bogousslavsky J, Davalos A, Eliasziw M, Fischer M, Furlan A, Kaste M, Lees KR, Soehngen M, Warach S; DIAS Study Group (2005) The Desmoteplase in Acute Ischemic Stroke Trial (DIAS): a phase II MRI-based 9-hour window acute stroke thrombolysis trial with intravenous desmoteplase. Stroke 36:66-73.

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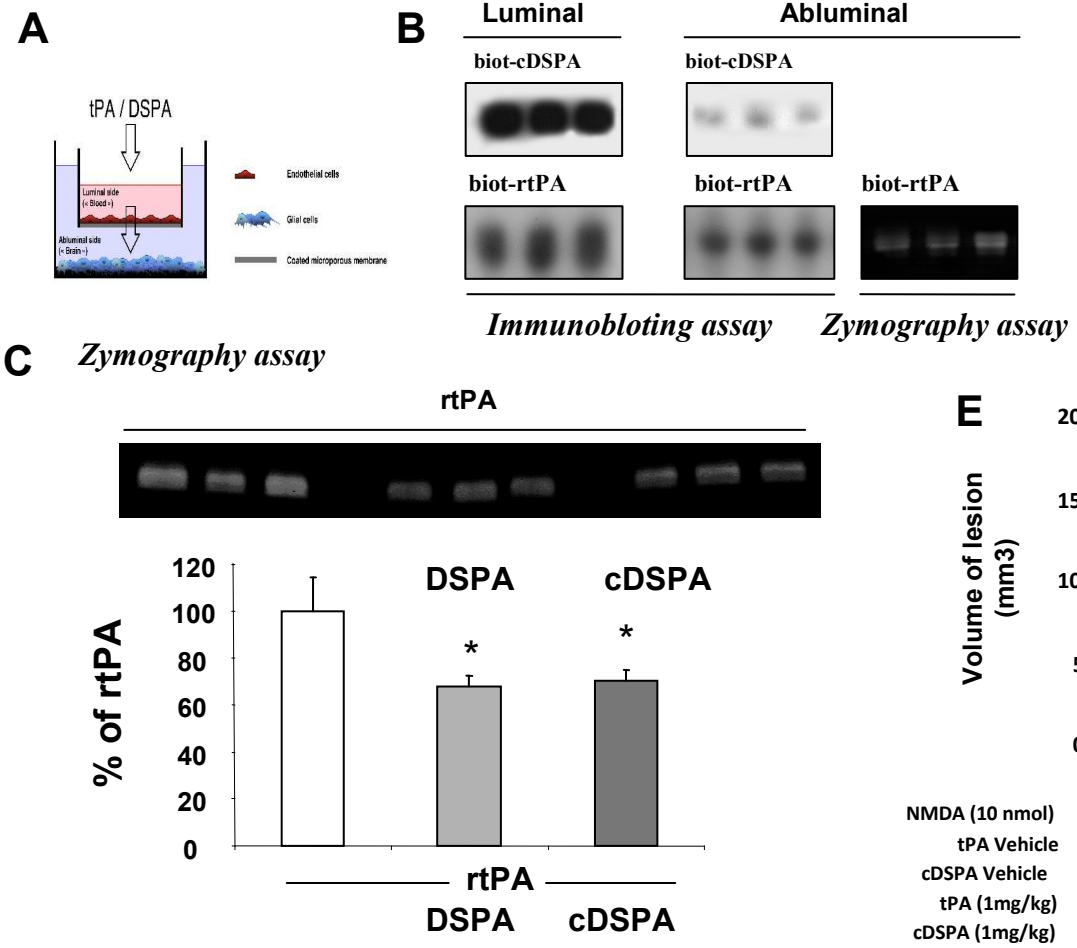
A
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(+ Blood +)
Abluminal side
(+ Brain -)
Endothelial cells
Glial cells
Coated microporous membrane
B
Luminal
Abluminal
biot-cDSPA
biot-cDSPA
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biot-rtPA
biot-rtPA
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- Based on its fibrinolytic activity, tissue type plasminogen activator (tPA) is the only treatment approved so far by the authorities, to treat ischemic stroke patients (NINDS, 1995).

- However, exogenous tPA can display deleterious effects on components of the neurovascular unit, including promotion of edema and blood-brain barrier (BBB) leakage (Aoki et al., Stroke, 2002, Yepes et al., J.Clin. Invest., 2003), which probably minimise its overall benefit.

- Moreover, tPA was shown to have the ability to cross both the healthy and injured BBB (Benchenane et al., Circulation, 2005), thus adding to the pro-neurotoxic effect of endogenously produced parenchymal tPA (Tsirka et al., Nature, 1995; Nicole et al., Nat. Med., 2001).

- Desmoteplase (DSPA) is a new thrombolytic agent derived from bat salivary glands (Hacke et al., Stroke, 2005), very close to tPA in terms of structural determinants, but previously demonstrated to :

  - cross the BBB as tPA does (Lopez-Atalaya et al., Stroke, 2007)

  - be devoid of pro-neurotoxic effect (Reddrop et al., Stroke, 2005; Lopez-Atalaya et al., J Cereb Blood Flow Metab, 2008)

- Although the use of DSPA to treat stroke patients is debated (see DIAS 2 data), we provide here the demonstration that :

  - a proteolytically inactive form of DSPA can prevent the passage of exogenous tPA across the BBB and its subsequent neurotoxic activity, thus demonstrating the therapeutic potential of competitive inhibitors of tPA's transport to the brain for stroke patients.

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