Functional Occurrence of the Interaction of Tissue Plasminogen Activator With the NR1 Subunit of N-Methyl-D-Aspartate Receptors During Stroke

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Background and Purpose—Despite its fibrinolytic effect, tissue-type plasminogen activator (tPA) displays deleterious effects in the brain, including proexcitotoxicity, that can reduce the overall benefit from thrombolysis during stroke. We have proposed that tPA potentiates excitotoxicity by interacting with and cleaving the aminoterminal end of the NR1 subunit of N-methyl-D-aspartate receptors, leading to an increased calcium influx, Erk1/2 activation, and neurotoxicity. Because this mechanism is debated, our aim was to demonstrate its in vivo occurrence and relevance. Because tPA is released under ischemic conditions, we hypothesized that if it indeed processes NR1, then the released fragment should reactivate the immune system in animals that had been immunized long before with recombinant aminoterminal end of the NR1. This effect should be exacerbated in ischemic animals thrombolysed with recombinant tPA.

Methods—At a time when specific antibodies could not be detected any longer, mice previously vaccinated with recombinant aminoterminal end of the NR1 were subjected to thromboembolic stroke induced by injecting thrombin in the middle cerebral artery alone or with intravenous thrombolysis.

Results—Stroke performed 1 year after active immunization induced the reappearance of antibodies against the aminoterminal end of the NR1 in the plasma, an effect significantly increased when ischemia was followed by recombinant tPA-induced reperfusion. Moreover, immunization preventing the interaction of tPA with aminoterminal end of the NR1 reduced ischemic brain damages and extended the therapeutic window of tPA-induced thrombolysis.

Conclusion—We demonstrate that the tPA-dependent interaction and cleavage of the NR1 subunit of N-methyl-D-aspartate receptors occurs in vivo after stroke and that this interaction is a relevant therapeutic target for stroke treatment. (Stroke. 2010;41:00-00.)

Key Words: NMDA receptor ■ stroke ■ tPA

In the vascular compartment, in response to local thrombosis, the serine protease tissue-type plasminogen activator (tPA) converts fibrin-bound plasminogen into active plasmin, leading to fibrin blood clot degradation. Accumulating evidence also indicate that apart from this fibrinolytic activity, tPA plays critical roles in the brain parenchyma, including the control of some cognitive processes in the healthy brain as well as neuronal survival after injuries to the central nervous system.

In the brain parenchyma, tPA’s substrates or interaction partners are multiple, thus extending its mode of action below the sole activation of plasminogen. Indeed, the interaction between tPA and the low-density lipoprotein receptor-related protein, the N-methyl-D-aspartate receptor (NMDAR), annexin II in glial cells, and/or neurons activates cell signaling processes that result in different outcomes depending on the physiological or pathological contexts. This complexity is for instance illustrated by the observation that through positive modulatory effects on NMDAR-mediated glutamatergic neurotransmission, tPA can be considered as beneficial for learning and memory processes but injurious during excitotoxic injuries.

As the first cause of acquired disability in adults, stroke is a major drain on public healthcare funding in industrialized countries. Although during the last 15 years we have witnessed remarkable progress in the understanding of the pathophysiology of ischemic stroke, attempts to transfer therapeutic molecules from the bench to the bedside have been nothing but a succession of failures. Intravascular thrombolysis with recombinant tPA (rtPA; Actilyse) thus remains the only approved acute treatment by health authorities. Nevertheless, due to strict inclusion criteria, <10% of...
patients with stroke actually benefit from thrombolysis,\textsuperscript{3} thus leaving most patients without alternative acute treatment. The design of new therapeutic targets for stroke treatment is thus mandatory.

One of the main restrictions to the use of Actilyse in clinical settings is the time-dependent occurrence of symptomatic intracerebral hemorrhages\textsuperscript{4} that is responsible for the narrow therapeutic window of the drug (initially 3 hours poststroke onset, but recently extended to 4.5 hours\textsuperscript{6}). Besides, although debated in the clinical community, there is also a significant amount of experimental arguments indicating that Actilyse, like endogenous tPA, activates several noxious pathways, which endanger components of the neurovascular unit.\textsuperscript{3} Although the proexcitotoxic effect of tPA has been unequivocally demonstrated by many independent groups,\textsuperscript{7–11} its underlying mechanisms remain controversial. Since 2001, we have proposed that tPA interacts with the NR1 subunit of the NMDAR, leading to the cleavage of its aminoterminal domain at the arginine 260 and thus to a potentiation of NMDAR-mediated calcium influx, mitogen-activated protein–kinase activation, and neurotoxicity.\textsuperscript{8,12–15} Although this cleavage of the NMDAR NR1 subunit by tPA was confirmed by some groups,\textsuperscript{16–17} some authors argued that it is mediated by plasmin.\textsuperscript{9} Alternatively, it has also been proposed that tPA modulates NMDAR-mediated signaling through the NR2B subunit\textsuperscript{18} or lipoprotein receptor-related protein.\textsuperscript{11}

Because this putative interaction and cleavage of NR1 by a tPA-dependent mechanism could be a relevant therapeutic target for stroke treatment, we aimed at providing convincing evidence of its in vivo occurrence and mechanistic modality. Stroke is known to induce the production and/or release of tPA by endothelial cells, depolarized neurons, and activated glial cells.\textsuperscript{3} We thus postulated that if the cleavage of NR1 by a tPA-dependent mechanism indeed occurs in vivo after stroke, then thromboembolic stroke performed 1 year after primoinfection of animals with the recombinant form of the aminoterminal end of the NMDAR NR1 subunit (at a time when barely no anti-aminoterminal end of the NR1 [ATD-NR1] antibodies are detected any longer in the plasma) should lead to a new boost of the corresponding antibodies. This effect should also be exacerbated by rtPA-mediated thrombolysis.

Materials and Methods

Experiments complied with the European Directives and the French Legislation on Animal Experimentation and were approved by the local ethical committee.

Production of Recombinant Recombinant ATD-NR1

As previously described,\textsuperscript{12} the region of the aminoterminal domain of the NR1-1a subunit (amino acids 19 to 371) corresponding to the domain of interaction with tPA (designed rATD NR1) was produced from the full-length rat NR1-1a cDNA. Recombinant proteins were purified from inclusion bodies of isopropyl 1-thio-β galactopyranoside-induced bacterial cultures (Escherichia coli, M15 strain) on a nickel affinity matrix (Qiagen).

Active Immunization

Active immunization was performed on Swiss mice (CERJ, Le Genest Saint Isle, France) weighing 25 to 30 g. Mice were immunized by intraperitoneal injection of immunogenic mixtures: complete Freund adjuvant (first injection) and incomplete Freund adjuvant (once a week during 3 weeks) alone (control) or containing the rATD-NR1 (30 μg).

Preparation of Polyclonal Antibodies

Two weeks after the last inoculation, sera were collected and IgG fractions purified on hydroxyapatite columns (Proteogenix) to obtain αATD-NR1 or control polyclonal antibodies.

Thromboembolic Cerebral Ischemia

Volume Studies

Eleven days after the active immunization described previously, mice (28 to 30 g, n = 10 per group) were deeply anesthetized with isoflurane 5% and, thereafter, maintained with 2.5% isoflurane in a 70%/30% mixture of NO2/O2. Animals were subjected to focal cerebral ischemia by injection of rtPA (10 mg/kg; Actilyse; Boehringer Ingelheim) into the middle cerebral artery.\textsuperscript{19} Thrombolysis by rtPA (10 mg/kg; Actilyse; Boehringer Ingelheim) was initiated 4 hours postischemia (tail vein injection, 10% bolus, 90% perfusion during 40 minutes). Control groups received the same volume of saline under identical conditions. Cerebral blood velocity was determined by laser Doppler flowmetry using an optic fiber probe (Oxford Optronix) affixed on the skull above the middle cerebral artery downstream of the thrombin injection site. Cerebral blood velocity was continuously measured before the injection of α-thrombin (100% baseline), throughout the duration of ischemia, and until the end of the intravenous infusion of rtPA or vehicle. The postischemic cerebral blood velocity was expressed as the percentage of cerebral blood velocity measured during the last 5 minutes of rtPA or vehicle infusion over the baseline cerebral blood velocity. After 24 hours, brains were collected and frozen in isopentane. Then, coronal brain sections (20 μm) were stained with thionine and analyzed with an image analyser (Image J).

Detection of Circulating Antibodies

One year after the last injection of rATD-NR1, mice were subjected to the protocol of ischemia described previously (n = 5 per group). A blood sample was harvested from each mouse by a retro-orbital puncture 24 hours before and 1, 7, and 15 days after ischemia. Sera were centrifuged 15 minutes at 1500 g and then frozen at −80°C until immunoblotting.

Immunoblotting on rATD-NR1

rATD-NR1 (50 μg) was subjected to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinyl fluoride membrane. Membranes were blocked with TTBS (10 mmol/L Tris; 200 mmol/L NaCl; 0.05% Tween; pH 7.4) containing 5% dry milk and incubated overnight at 4°C with harvested sera of mice at different time postischemia (1:1000). After incubation with a goat secondary peroxidase-conjugated IgG antibody (1:750; Sigma Aldrich), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN). For titration assays, the same experiments were performed with 3 different dilutions of the sera (1:1000; 1:10 000; 1:100 000).

Immunoblotting on Protein Extracts

Ice-cold TNT buffer (50 mmol/L Tris–HCl pH 7.4; 150 mmol/L NaCl; 0.5% Triton X-100)–dissociated cells/tissues were centrifuged (10 000 g, 4°C, 15 minutes) and protein content assessed by the BCA method (Pierce). Proteins (20 μg) were then separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinyl fluoride membrane. Membranes were blocked with TTBS containing 5% dry milk and incubated overnight at 4°C with primary antibodies: our mouse αATD-NR1 or a goat
αCter-NR1 (1:200; Santa Cruz). After incubation with the appropriate peroxidase-conjugated secondary antibodies (1:5000), proteins were visualized with an enhanced chemiluminescence ECL-Plus detection system (Perkin Elmer-NEN).

**Immunocytochemistry**

Neuronal cultures were prepared on Labtek Chamber-Slide culture system (ThermoFisher Scientific, Rochester, NY) coated with poly-D-lysine (0.1 mg/mL) and laminin (0.02 mg/mL).8,15 Neurons were fixed during 30 minutes with 4% of paraformaldehyde in phosphate-buffered saline (0.1mol/L, pH 7.4). After washes with phosphate-buffered saline, they were incubated in the presence of mouse αATD-NR1 (1:2000) and goat αCter-NR1 (1:500). Secondary antibody F(ab')2 donkey antigoat IgG linked to TRITC (1:300; Jackson ImmunoResearch) was incubated overnight. Slides were then cover-slipped with antifade medium containing 4',6-diamidino-2-phenylindole and examined with a Leica DM6000 microscope. Images were digitally captured using a coolsnap camera and visualized with Metavue software (n=3).

**Immunohistochemistry**

Anesthetized mice were transcardially perfused with 0.9% NaCl followed by fresh 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. Cryomicrotome-cut brain sections were incubated in the presence of mouse αATD-NR1 (1:2000) or goat αCter-NR1 (1:500) polyclonal antibodies before an overnight incubation with the respective secondary antibodies, donkey F(ab')2 anti-IgG coupled to TRITC (1:300; Jackson ImmunoResearch) was incubated overnight. Slides were then cover-slipped with antifade medium containing 4',6-diamidino-2-phenylindole and examined with a Leica DM6000 microscope. Images were digitally captured using a coolsnap camera and visualized with Metavue software (n=3).

**Statistical Analyses**

Results are the mean±SEM. Statistical analyses were performed by the Kruskal-Wallis test followed by post hoc comparison with the Mann–Whitney test. Statistical significance was concluded for probability values <0.05.

**Results**

Because we have previously demonstrated that tPA interacted with ATD-NR1 to mediate its proneurotoxic effects,12 the corresponding rATD-NR1 was produced, purified, and used as an antigen for active immunization in mice (Figure 1A). Two weeks after the last inoculation, sera were harvested from control and immunized mice and immunoglobulins purified and tested for their ability to reveal rATD-NR1 by immunoblotting. Purified antibodies from immunized mice (termed αATD-NR1) recognized rATD-NR1, whereas those from control mice did not (Figure 1B). We then tested whether αATD-NR1 could recognize the full-length NR1 subunit of NMDAR in vitro and in vivo and thus performed immunoblotting on protein extracts harvested from primary cultures of cortical neurons and brain tissues. αATD-NR1 revealed a band a 120 kDa both in neurons and brain extracts similar to that revealed when using a commercially available antibody targeting the C-terminal end of the NMDAR NR1 subunit (Figure 1C–D). Parallel immunohistochemistry performed from corresponding cultures and tissues confirmed these observations with a positive neuronal immunostaining for both αATD-NR1 and αCter-NR1 antibodies (Figure 1E–F). Altogether, these data demonstrate the efficiency of our immunization protocol to initiate the endogenous production of antibodies directed against the interaction site of tPA on the NMDAR NR1 subunit. We postulated that, in previously vaccinated animals in which circulating antibodies targeting rATD-NR1 had re-
turned to undetectable levels with time, if the cleavage of NR1 and subsequent release of the ATD of NR1 actually occurs as a consequence of an ischemic episode, then memory B cells would reactivated the production of $\alpha$ATD-NR1.

To address this question, blood samplings for assessment of $\alpha$ATD-NR1 were performed on a regular basis and for up to 12 months in vaccinated animals. One year after the last inoculation of the immunogenic peptide, circulating $\alpha$ATD-NR1 was detected to very low levels (Figure 2A–B). At that time, the mice were thus subjected to thromboembolic stroke followed after 4 hours by an intravenous injection of either saline or tPA to promote thrombolysis (Figure 2C). Blood samples were collected before ischemia and then 1, 7, and 15 days after ischemia. The corresponding serum was assessed for their content in $\alpha$ATD-NR1 by testing their ability to reveal a fixed amount of immobilized rATD-NR1 ($10^7$ per line). Interestingly, 24 hours after, thromboembolic stroke had already led to a significant increase in the levels of circulating $\alpha$ATD-NR1 (47% when compared with the same animals before stroke onset), an effect enhanced by the intravenous injection of rtPA (106% when compared with nonischemic animals and +68.5% when compared with clotted animals; Figure 2D–E; $n=5$ per group; $P<0.05$). The levels of circulating $\alpha$ATD-NR1 continuously increased with time after stroke (+91% and +366% before versus 14 days after ischemia without and with thrombolysis respectively, +192% 15 days after stroke with thrombolysis versus stroke alone; Figure 2D–E). Controls experiments confirmed that sera from control immunized mice failed to reveal rATD-NR1 (data not shown). Dilution studies of the sera showed progressive declines in the signal intensity on immunoblots, thus confirming the specificity of the immune responses and that $\alpha$ATD-NR1 titer is higher in the ischemic animals when compared with control ones (Figure 2F).

Altogether, these data reveal in vivo that under ischemic stroke conditions, the interaction with and cleavage of NR1 occurs and is dramatically potentiated by endogenous and exogenous rtPA.

We then intended to confirm that this pathway is a relevant target for the development of therapeutic strategies to improve stroke treatment. We thus subjected control or vaccinated mice (2 weeks after the last inoculation) to thromboembolic stroke (Figure 3A). Lesion volumes were measured 24 hours after induction of ischemia. Although control animals showed a lesion volume of 25 mm$^3$ ±3.1, vaccination targeting the interaction of endogenous tPA with NR1 dramatically reduced brain damages (−43%, $n=10$; $P<0.05$; Figure 3B) without alterations of perfusion parameters (Figure 3C). These data confirm that endogenous tPA plays a critical role in the brain outcome after cerebral ischemia due to the cleavage of the N-terminal end of the NMDAR NR1 subunit. Then, we tested whether vaccination could also
preventing the deleterious effect of exogenous tPA when injected intravenously late after cerebral ischemia (Figure 4A).

Although intravenous injection of rtPA, 4 hours after thombin-induced clot formation, increased ischemic damages (H11001 32.6%; n = 10, P < 0.05; Figure 4B), vaccination not only completely prevented the deleterious effect of rtPA, but even reverted it into a beneficial effect (H11002 43% when compared with control animals, n = 10, P < 0.05 and H11002 66% when compared with rtPA-treated animals, n = 10, P < 0.05; Figure 4B). Vaccination did not influence the fibrinolytic activity of rtPA (Figure 4C).

Altogether our study unequivocally demonstrates that the cleavage of NR1-NMDAR indeed occurs in vivo after stroke, an effect potentiated by exogenous tPA, and playing a critical role in the evolution of ischemic brain lesions.

Discussion

Worldwide, 6 million deaths occur as a consequence of stroke, ranking this affliction second as a cause of death in some countries. Approximately 300 trials have been registered for acute ischemic stroke performed so far, of which >200 were considered as complete with no evidence of patient benefit apart from results of the rtPA studies. It is now well admitted that the primordial concept of acute stroke treatment, namely, the prompt restoration of cerebral blood flow, is a prerequisite to any subsequent successful therapies. However, new combination therapies, including thrombolytics and neuroprotective agents, are only emerging and not proven efficient yet. The identification of relevant targets for neuroprotection is thus 1 of the current challenges of stroke therapy.

One potential limitation of rtPA treatment, based on experimental models, is its proneurotoxic effects through a potentiation of NMDAR signaling. However, as already mentioned, although the proexcitotoxic effect of endogenous and exogenous tPA is much documented, its mechanism of action remains debated, especially regarding the putative plasmin-independent cleavage of the NMDAR NR1 subunit. A clear demonstration of the exact mechanism involved in the proneurotoxic effect of tPA is thus mandatory to define efficient adjunctive therapies for stroke. By using a protocol of vaccination followed by stroke-induced boost of the immune system, we aimed at proving that tPA leads to the cleavage of the ATD-NR1 to promote neurotoxicity in vivo after stroke. Our demonstration that cerebral ischemia induced a rebound of antibodies against the ATD-NR1 is a key argument in favor of our hypothesis. Interestingly and in agreement with the fact that exogenous rtPA can increase ischemic damages even when administered intravenously, the reinduction of αATD-NR1 antibodies was dramatically potentiated by the injection of the thrombolytic drug late after stroke onset. This could be explained by the passage of tPA across the blood–brain barrier.

The second key argument in favor of our hypothesis is the fact that active immunotherapy reduces ischemic damages...
after thromboembolic stroke and reverts the damaging effect of late thrombolysis by rtPA.

In addition to tPA, plasmin, thrombin, and matrix metalloproteinase 7 have been shown to cleave NR1. However, none of the proteolytic processing exerted by these proteases has been associated to increased N-methyl-d-aspartate-induced signaling and toxicity.\textsuperscript{9,11,23,24} In addition, we have previously demonstrated the specificity of anti-ATD-NR1 antibodies toward tPA-dependent behavior tasks\textsuperscript{13} and recent data showing the lack of effect of these antibodies in tPA-deficient mice (unpublished data). This supports further our hypothesis of the occurrence and relevance of the cleavage of NR1 by tPA.

Altogether, we provide clear evidence that the interaction between tPA and the ATD of the NR1 subunit of the NMDAR occurs in the ischemic brain, is deleterious, and thus is a relevant target for the development of innovative therapeutic strategies for stroke.

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Disclosures

None.

References

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