Oxygen Glucose Deprivation Switches the Transport of tPA Across the Blood–Brain Barrier From an LRP-Dependent to an Increased LRP-Independent Process

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Background and Purpose—Despite uncontroversial benefit from its thrombolytic activity, the documented neurotoxic effect of tissue plasminogen activator (tPA) raises an important issue: the current emergency stroke treatment might not be optimum if exogenous tPA can enter the brain and thus add to the deleterious effects of endogenous tPA within the cerebral parenchyma. Here, we aimed at determining whether vascular tPA crosses the blood–brain barrier (BBB) during cerebral ischemia, and if so, by which mechanism.

Methods—First, BBB permeability was assessed in vivo by measuring Evans Blue extravasation following intravenous injection at 0 or 3 hours after middle cerebral artery electrocoagulation in mice. Second, the passage of vascular tPA was investigated in an in vitro model of BBB, subjected or not to oxygen and glucose deprivation (OGD).

Results—We first demonstrated that after focal permanent ischemia in mice, the BBB remains impermeable to Evans Blue in the early phase (relative to the therapeutic window of tPA), whereas at later time points massive Evans Blue extravasation occurs. Then, the passage of tPA during these 2 phases, was investigated in vitro and we show that in control conditions, tPA crosses the intact BBB by a low-density lipoprotein (LDL) receptor-related protein (LRP)-dependent transcytosis, whereas OGD leads to an exacerbation of tPA passage, which switches to a LRP-independent process.

Conclusion—We evidence 2 different mechanisms through which vascular tPA can reach the brain parenchyma, depending on the state of the BBB. As discussed, these data show the importance of taking the side effects of blood-derived tPA into account and offer a basis to improve the current thrombolytic strategy. (Stroke. 2005;36:1059-1064.)

Key Words: blood–brain barrier ■ stroke ■ thrombolytic therapy ■ tissue plasminogen activator

Thrombolysis relies on the activation of plasminogen into plasmin and a subsequent cascade of events leading to the degradation of fibrin clots.¹ The activation of plasmin involves the serine proteases tissue plasminogen activator (tPA) or urokinase, which cleave plasminogen between Arg561 and Val562.¹ The ability of tPA to activate plasmin has been exploited through the therapeutic development of various forms of tPA.²⁻⁵ Recombinant tPA, Actilyse, is to date, the only approved emergency treatment for ischemic stroke.⁶

Apart from its fibrinolytic function, a growing body of data suggests that tPA also plays critical roles in many tissues, including the central nervous system.⁷ The involvement of tPA in several types of brain injury has been demonstrated. For example, hippocampal neurons of tPA-deficient mice are resistant to excitotoxin-induced and ischemia-induced neurodegeneration.⁸⁻⁹ Interestingly, after middle cerebral artery occlusion (MCAO), intravenous injection of tPA exacerbated ischemic damages.⁹ These observations question whether the current therapeutic strategy for stroke patients is actually optimal. In the case of blood–brain barrier (BBB) disruption, tPA could diffuse in the brain parenchyma and its deleterious effect¹⁰⁻¹⁴ would counteract its beneficial thrombolytic action. The knowledge of the kinetics of BBB disruption in stroke is therefore crucial, especially because some¹⁵ but not all¹⁶ studies report that tPA promotes BBB leakage. The situation might be even more complex, since we have recently shown that vascular tPA can cross the intact BBB, by transcytosis mediated by a member of the low-density lipoprotein receptor-related protein (LRP) family.¹⁴
In the present study, we have thus investigated the effect of ischemia on the passage of tPA through the BBB, both in vivo and in an in vitro model of BBB subjected to oxygen and glucose deprivation.

Materials and Methods
All experiments were performed in the framework of the French legislation that governs animal experimentation.

Materials
Human recombinant tPA (Actilyse) was purchased from Boehringer Ingelheim (Paris, France). Streptavidin-Alexa was from Molecular Probes (Leiden, the Netherlands). Receptor-associated protein (RAP) was provided by Dr G. G. Bu (Departments of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO). N-methyl-d-aspartate was from Tocris (Bristol, United Kingdom).

MCAO in Mice and Measure of BBB Permeability
Cerebral ischemia was performed by electrocoagulation of the middle cerebral artery in male Swiss mice (20–25 g) under sevoflurane-induced anesthesia.17 Injection of tPA (Actilyse) or its vehicle was performed 30 minutes after the onset of ischemia in the tail vein. Evans blue (100 μL; 2%) was injected at 0 minutes or 3 hours after ischemia. Three hours after the Evans Blue injection, animals were perfused with heparinized saline solution. Ipsilateral and contralateral hemispheres were dissected, weighed, homogenized in 500 μL of phosphate-buffered saline (0.1 mol/L) and centrifuged. Supernatants were diluted with 500 μL of trichloroacetic acid (TCA) (100%) overnight at 4°C. After a centrifugation at 21 000g for 30 minutes, Evans blue was quantified in the samples (absorbance at 620 nm). Results are expressed as the ratio between ipsilateral and contralateral hemispheres and expressed as mean±SD.

SDS-PAGE Plasminogen-Casein Zymography
Zymography assay was performed by adding plasminogen (4.5 μg/mL) and casein (1%) in 15% SDS-polyacrylamide gel. Migration was performed at 4°C. Gels were washed with Triton X-100 (2.5%) and incubated 2 hours at 37°C. Caseinolytic bands were visualized after Coomassie staining.

BBB Experiments
Briefly, bovine brain endothelial cells were isolated and characterized as described by Brillaut et al.18 After 3 weeks, coated filters were set in 6-well dishes containing glial cells. Endothelial cells were plated on their upper side in 1.5 mL of medium. Abluminal media were harvested at different times and expressed as mean±SD. tPA (1 mg/kg) was administered intravenously 30 minutes after MCAO induction. Statistical test is 2-way ANOVA (time effect: P<0.01; treatment effect: NS) (n=3).

Treatment with Biotinylated tPA and Confocal Microscopy
Biotinylated tPA was prepared as previously described.14 Biotinylated tPA was applied on the endothelial cells for 1 hour. Cells were then washed 3 times with phosphate-buffered saline and fixed with paraformaldehyde 4%. Individual cell nuclei were visualized by performing a DNA labeling with propidium iodide (5 μmol/L). Biotinylated tPA was revealed by using the streptavidin-alexa555 or 488. Stainings were then analyzed by 3-dimensional reconstruction with confocal microscopy. For confocal analyses, Nikon Eclipse (TE2000-E) inverted microscope and Nikon 20x, 1.4 numerical aperture epifluorescence oil immersion objective were used. Three-dimensional reconstruction was performed with Nikon EZ-C1.

Results
MCAO-Induced BBB Breakdown Occurs at Late Stages of Permanent Ischemia
The actual kinetics of BBB disruption after stroke remains controversial. In a model of cerebral ischemia induced by permanent MCAO in mice, we have quantified the extravasation of Evans Blue in the brain parenchyma, as an indicator of BBB breakdown. As shown in Figure 1, no significant Evans Blue leakage was observed in the brain parenchyma in the first 3 hours after the onset of injury. However, intense Evans Blue extravasation was seen between 3 hours and 6 hours after the onset of ischemia. Intravenous injection of tPA (1 mg/kg) 30 minutes after the onset of ischemia, neither changed this temporal profile nor exacerbated ischemia-induced BBB Evans Blue extravasation. Together with our previous finding that when administered in such conditions, tPA is promptly cleared from the vascular compartment (no residual activity after 1 hour),14 these data suggest that early tPA injection (at a time when BBB breakdown has not occurred yet) does not compromise BBB integrity.

Effect of tPA on OGD-Induced BBB Breakdown In Vitro
We have then investigated the effect of tPA on BBB integrity on a well-characterized in vitro model of BBB in control
conditions or during OGD, which is known to induce BBB breakdown. This model was previously characterized to closely mimic the in vivo situation, displaying for instance a high electrical resistance (up to 800 ohm/cm²), a very low permeability for sucrose and inulin, and constitutive tight junctions revealed by the presence of Occludin, ZO-1, claudin-1 and claudin-5 immunostainings.

In control conditions, BBB was impermeable to sucrose and inulin, and tPA (20 μg/mL, 4 hours of incubation at 37°C) failed to influence this basal permeability of the BBB for both sucrose (Figure 2A) and inulin (Figure 2B). However, when added during OGD (4 hours), tPA led to a significant increase in the OGD-induced BBB disruption, as estimated by the passage of sucrose and inulin (Figure 2A and 2B). As in our previous work, we have used a dose of 20 μg/mL of tPA, based on the observation that such a concentration can be reached in blood during thrombolysis.

OGD Potentiates the Passage of tPA Across the BBB In Vitro

We have next investigated the passage of tPA in control conditions and after 4 hours of OGD. Both zymography (Figure 3B) and fluorogenic assays (Figure 3C) showed that a significant amount of the tPA loaded in the luminal compartment (Figure 3A) was found in the abluminal compartment as early as 30 minutes after the application in control conditions and that this passage of tPA across the BBB was exacerbated after OGD in a time-dependent manner (Figure 3B and 3C). In these conditions, endothelial cells did not produce or release detectable amounts of tPA in the abluminal compartment (data not shown). Furthermore, our present data show that tPA remains active after its passage.

Exogenous tPA Is Found in Transcytotic Vesicles-Like Structures in Endothelial Cells

The passage of tPA across the BBB was confirmed by confocal microscopy by adding biotinylated tPA in the luminal part of the in vitro model of BBB. Two hours after tPA application, cell monolayers were washed and fixed. Immunostainings were performed with streptavidin-Alexa to reveal biotin. No staining was obtained in the absence of bio-tPA (data not shown). As shown in Figure 4A and 4B, exogenous tPA was found in the cytoplasm of the endothelial cells with a pattern suggesting the presence of tPA in transcytotic-like vesicles. OGD led to a large increase in labeling suggesting an increased transcytosis of tPA (Figure 4C and 4D). Altogether this confirms the ability of tPA to cross the intact BBB by a transendothelial pathway and that this passage is largely increased in OGD conditions.

Figure 2. Effect of tPA on the permeability of the BBB to sucrose and inulin in vitro. A and B, Effect of tPA application (20 μg/mL, 4 hours) during control or OGD conditions on BBB permeability. Results are mean±SD passage of [14C]sucrose (A) or [3H]inulin (B). Statistical tests are ANOVA with Bonferroni correction. Significant differences between control and OGD conditions are indicated as **P<0.01. Significant differences between control and tPA are indicated as §P<0.05 (n=3).

Figure 3. Passage of tPA across the BBB. A, Schematic representation of the in vitro model of BBB. B, tPA was applied in the luminal compartment. Temporal profiles of tPA activity that reaches the abluminal media, analyzed by casein–plasminogen zymography assay at different times. C, Same protocol as performed in (B) but the tPA activity was assessed with fluorogenic substrate instead of zymography assay. Data are expressed as a percentage of the tPA applied in the luminal compartment. Mean±SD. Statistical test is ANOVA followed by Bonferroni correction. In both conditions, there is a time effect. Post hoc analysis reveals significant differences between control and OGD conditions, indicated as **P<0.01.
The Passage of tPA After OGD Is Not Saturable

We have next investigated the dose-dependency of tPA passage in control and OGD conditions. In the concentration range of 0 to 30 μg/mL, there was a linear relationship between the concentration of loaded tPA and its passage in control conditions (Figure 5A and 5C). From 50 μg/mL, the passage of tPA reached a plateau, suggesting the implication of a saturable transporter. In contrast, after OGD, the passage of tPA displayed a linear profile for all the concentrations tested, suggesting a mechanism that is not mediated by a receptor (Figure 5B and 5C).

LRP Antagonist Does Not Prevent the Passage of tPA Through the Injured BBB but It Does in Control Conditions

Since we have previously shown that tPA crosses the intact BBB by LRP-mediated transcytosis, we have tested the implication of this pathway during OGD. Although, the LRP

Figure 4. Localization of exogenous tPA in endothelial cells by confocal microscopy. Biotinylated tPA (20 μg/mL) was applied on our in vitro model of BBB in control conditions or after OGD (4 hours). Two hours after application, cells were washed and fixed. Images are stainings with streptavidin–Alexa after 3-dimensional reconstruction by using confocal microscopy. A and B, Stainings with streptavidin–Alexa-488 (green) and propidium iodide (IP) (red) in control conditions. C and D, Staining with streptavidin–Alexa-488 in control conditions (C) and after 4 hours of OGD (D). Scale bar: A and B, 10 μm; B and C 30 μm.

Figure 5. Influence of OGD on the saturable passage of tPA through BBB in vitro. A, tPA was applied in the luminal compartment at different doses in control conditions or after OGD (4 hours). Two hours later, tPA activity in the abluminal media was analyzed by casein-plasminogen zymography assay (A) or by fluorogenic substrate assay (B). Data are expressed as mean±SD of abluminal tPA activity (n=3). R² are correlation coefficients to linear curve (for OGD conditions) and saturated curve (for control conditions).
of OGD leads to an increase in permeability to sucrose and breakdown in control conditions. We also show that 4 hours after intravenous injection of the thrombolytic agent, tPA. How-ever, tPA potentiates neuronal death both in vitro and in vivo, even when administered intravenously. From this, and despite clear overall benefit from its fibrinolytic action, arises a legitimate question as to whether exogenous tPA can contribute to brain damage. In other words, determining whether vascular tPA can invade the brain during stroke is a critical point.

A better understanding of the interactions between the brain parenchyma and the vascular compartment after stroke is a critical point to improve stroke therapy. To date, the only approved therapy for stroke remains early reperfusion by intravenous injection of the thrombolytic agent, tPA. However, tPA potentiates neuronal death both in vitro and in vivo, even when administered intravenously. From this, and despite clear overall benefit from its fibrinolytic action, arises a legitimate question as to whether exogenous tPA can contribute to brain damage. In other words, determining whether vascular tPA can invade the brain during stroke is a critical point.

Uncontrolled infiltration of deleterious biomolecules (such as tPA) through the compromised BBB could occur. This is supported, for instance, by the fact that a reduction of BBB disruption by BB-94, an inhibitor of MMP-9, counteracts the potentiation of tPA-induced mortality after cerebral ischemia. However, a more complex scheme appears, because we have recently shown that blood-derived tPA can cross the intact BBB and alter brain processes.

Here, we demonstrate in our in vitro model of BBB that tPA, at a concentration of 20 μg/mL, failed to induce BBB breakdown in control conditions. We also show that 4 hours of OGD leads to an increase in permeability to sucrose and inulin, confirming previous observations. Interestingly, when BBB has been damaged by OGD, co-application of tPA exacerbates this phenomenon. These results might seem in disagreement with our in vivo observations that tPA has no effect of Evans Blue extravasation after MCAO. In fact, the effect of tPA on BBB integrity could depend on previous injury-induced alteration. In our in vivo study, tPA was injected early after the onset of MCAO (30 minutes) at a time when BBB breakdown has not occurred yet. Moreover, we have previously shown that no tPA is detected any more 1 hour after its intravenous injection. Therefore, the short half-life of tPA restricts its action to a period of absence of BBB breakdown. The absence of tPA effect on BBB in vivo is thus in accordance with our in vitro results that tPA does not affect basal permeability of BBB. In other words, BBB may have not been altered enough by ischemia to be sensitive to tPA action. Moreover, the effect of tPA on BBB has not been found in all the studies that examined this issue. Interestingly, most of the studies showing an effect of tPA on BBB breakdown have been performed in hypertensive rats, which are more susceptible to BBB alterations after brain lesions than normotensive rats. In a recent article, Yepes et al have shown that tPA, in the absence of injury, can lead to an opening of the BBB by a mechanism involving LRP. This discrepancy with our results could be explained by the route of tPA administration (directly into the cerebrospinal fluid versus intravenously as performed in humans in our stroke model or on the luminal side of endothelial cells in our in vitro model). Taken together, these data suggest that because endothelial cells at the BBB are highly polarized, the effects of tPA could differ between the apical and basal sides of endothelial cells. Alternatively, the fact that in our hands RAP does not prevent the effect of tPA on the passage of sucrose through endothelial cells suggests that tPA could act by an indirect way to induce BBB breakdown.

The next step and the major point of our study was to investigate the capacity of the blood-derived tPA to reach the brain parenchyma in conditions of either intact or altered BBB. We confirm our previous observation that significant amounts of tPA can cross the intact BBB, but we observe a dramatic increase in the passage of tPA after OGD, which seems to occur by transcytosis, because tPA is found in transcytotic vesicle-like structures in endothelial cells. These results support our previous data that OGD-induced BBB permeability involves a transcellular rather than a paracellular pathway.

In the present study, we also evidence that although RAP prevents the passage of tPA across the intact BBB, it failed to prevent its passage during OGD. The absence of any detectable effect of RAP in the passage of tPA during OGD could be explained by an OGD-induced inhibition of receptor-mediated transports, as previously suggested and/or because unspecific endocytosis is so large that it bypasses the saturable LRP-dependent mechanism. In both control and OGD conditions, no effect of RAP was seen on the permeability of BBB to sucrose. Thus, although LRP has been shown to mediate the effect of tPA on MMP-9 mRNA induction, it does not seem to be involved in BBB permeability, especially in OGD-induced BBB breakdown. Our
results suggest 2 mechanisms by which tPA can cross the BBB, a moderate receptor-mediated transcytosis in control conditions and an exacerbated and unsaturable passage involving an unspecific transcellular pathway after ischemic conditions. Based on our in vitro data, it is possible to estimate that ∼30% of the tPA present in the luminal part can cross the injured BBB in 1 hour. Even if OGD cannot be compared with in vivo situations of stroke, these data suggest that the amount of blood-derived tPA that can reach the brain parenchyma could be compatible with deleterious reported effects of this serine protease on neurons. This might be the case, because the amount of tPA that crosses the intact BBB is high enough to potentiate excitotoxic lesion.\textsuperscript{14}

Altogether, these data suggest that preventing the interaction of tPA with LRP could be an interesting strategy to block the deleterious effect of tPA in vivo, but only as long as the integrity of the BBB is not altered. Interestingly, a recent MRI study shows that BBB disruption was found in 33% of stroke patients, 10 hours after ictus, and that the mean time between the onset of ischemia and the observation of BBB disruption is of ∼13 hours.\textsuperscript{25} This suggests that most patients eligible for thrombolysis could benefit from an adjunctive therapy preventing the interaction of tPA with LRP.

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