Closure of the Blood-Brain Barrier by Matrix Metalloproteinase Inhibition Reduces rtPA-Mediated Mortality in Cerebral Ischemia With Delayed Reperfusion

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Background and Purpose—Intravenous recombinant tissue plasminogen activator (rtPA) can be beneficial in ischemic stroke despite an increased risk of hemorrhage and potential neurotoxic effects. We hypothesized that rtPA-mediated adverse effects depend on the timing of reperfusion and injury to the blood-brain barrier (BBB).

Methods—Male Wistar rats had middle cerebral artery occlusion (MCAO) by intraluminal thread placement. Intervals of ischemia/reperfusion, respectively, in hours were 0/18, 1.5/16.5, 3/15, 6/12, 18/0, and 6/1. Animals received either rtPA or saline for 1 hour at the time of reperfusion or, for the 18/0 trial, starting 1 hour after MCAO. Outcome parameters were mortality, matrix metalloproteinase-2 and -9 (MMP-2 and -9) concentrations, tissue hemoglobin, and brain water content. We analyzed the permeability of the BBB by using the brain $^{14}$C[sucrose] uptake method. Effects of the MMP inhibitor BB-94 on the BBB without rtPA treatment and on mortality with rtPA were tested in animals with 6/1 and 6/12, respectively.

Results—In delayed reperfusion (6/12), rtPA increased mortality from 17% to 83% ($P < 0.01$) without significantly affecting other outcome parameters. In 6/1, sucrose uptake in the ischemic hemisphere was markedly increased (8.80 ± 1.14% vs 2.15 ± 0.26%; $P < 0.01$). This uptake was reduced by treatment with BB-94 (3.95 ± 1.48%, $P < 0.01$). Furthermore, BB-94 reduced rtPA-mediated mortality in 6/12 to 33% ($P < 0.05$).

Conclusions—rtPA-mediated mortality in delayed reperfusion is associated with early opening of the BBB. Closure of the BBB with BB-94 given before rtPA treatment reduced mortality, suggesting that treatment with MMP inhibitors might reduce the risk associated with thrombolysis. (Stroke. 2003;34:2025-2030.)

Key Words: blood-brain barrier ■ metalloproteinases ■ thrombolysis ■ rats

Intravenous recombinant tissue plasminogen activator (rtPA) can be beneficial in ischemic stroke when thrombolytic therapy is started within 3 hours after symptom onset. However, thrombolysis is associated with a markedly increased risk of symptomatic hemorrhage.1 Further improvements in safety require a better understanding of rtPA effects in ischemic stroke.

Besides its fibrinolytic activity, the plasminogen-plasmin system might be involved in degradation of extracellular matrix components2 and activation of matrix metalloproteinases (MMPs).3,4 Therefore, rtPA might potentiate the loss of microvascular integrity that is observed in focal cerebral ischemia and reperfusion.5 Another mechanism for rtPA toxicity might be the alteration of endothelial function after thrombolytic therapy, which would promote inflammation in ischemic stroke.6,7

Additionally, rtPA has been shown to promote neurodegeneration after intracerebral injection of excitotoxins.8 Because excitotoxins play an important role in focal cerebral ischemia, thrombolytic therapy with rtPA might aggravate neurodegeneration in human stroke. In experimental stroke, rtPA-mediated neurotoxicity was found by some9 but could not be confirmed by other10,11 groups.

To further elucidate the intravascular and extravascular effects of tPA/rtPA, we investigated mortality, MMP-2 and -9 generation, brain tissue hemoglobin concentration, brain water, and blood-brain barrier (BBB) integrity in rats with variable intervals of ischemia and reperfusion. Ischemia was achieved by mechanical middle cerebral artery occlusion (MCAO). Thereby the fibrinolytic effects of rtPA were minimized.

Because MMP-2 and -9 are upregulated in focal cerebral ischemia and play a role in BBB opening12 and thrombolysis-associated hemorrhage,13,14 we treated selected animals with the MMP inhibitor BB-94. When given before the onset of ischemia, this inhibitor has been shown to improve survival in rats with focal cerebral ischemia and delayed thrombolytic therapy.13 To investigate its protective
property in a situation similar to that of acute stroke patients, we studied the mechanism of protection and efficacy when treatment with BB-94 was started 2 hours after MCAO.

Materials and Methods

Animal Preparation

The study was approved by the University of New Mexico Animal Care Committee and confirmed to the National Institutes of Health guidelines for use of animals in research. We used a modified model of reversible MCAO in adult male Wistar rats (280 to 320 g, supplied by Harlan, USA) as previously described. Anesthesia was achieved with 1.5% halothane in 70% nitrous oxide and 30% oxygen. MCAO was induced by inserting a 4-0 monofilament nylon suture with a bulb on the end into the internal carotid artery through an isolated external carotid artery stump and advancing it until the bulb occluded the osmium of the MCA 18 mm from the bifurcation. To initiate reperfusion, the nylon suture was slowly retracted into the external carotid artery stump. For rtPA (Genentech) or saline treatment, the right femoral vein was exposed and cannulated.

Immediately after euthanasia with intravenous saturated KCl, the brain was removed and carefully examined for macroscopically visible edema and hemorrhage. To verify successful MCAO with consecutive infarction, a frontal 3-mm brain section was taken from all animals and stained with 2,3,5-triphenyltetrazolium chloride (TTC).

Experimental Groups

A total of 94 animals were studied. In a first set of experiments, the effect of rtPA on different outcome parameters was investigated in 73 rats with different intervals of ischemia and reperfusion adding up to 18 hours. Physiologic saline (10 mL/kg body weight) with or without rtPA (10 mg/kg body weight) was administered to the right femoral vein over a period of 1 hour with 10% given as a bolus. The relatively high dose of rtPA was necessary to achieve a fibrinolytic effect in rats similar to that of thrombolytic therapy in humans. Because rtPA has a short half-life, the drug or saline treatment was administered 30 minutes before reperfusion and continued for 30 minutes after release of the suture. This regimen assured that the reperfused tissue was exposed to the agent.

The different groups were defined by the following intervals of ischemia/reperfusion in hours: 0/18, 1.5/16.5, 3/15, 6/12, and 18/0. Animals with 0/18 were sham-operated, with the filament placed into the internal carotid artery without occluding the MCA. TTC staining, brain water quantification, and hemoglobin measurements were made on all animals including the ones that died during the reperfusion period. In the case of death, tissue processing for later analysis of these parameters was started within 1 hour. Measure-ments of hemoglobin concentrations, however, was only performed in the different groups shown in parentheses above the bars. When reperfusion was delayed to 6 hours, mortality was increased markedly in rtPA-treated animals (**P<0.01). Treatment with BB-94 reduced rtPA-associated mortality significantly (P<0.05). Control animals had MCAO without rtPA treatment.

Quantitative Zymography and Hemoglobin Quantification

For quantitative zymography and hemoglobin quantification, another adjacent 1.5-mm section was dissected. Again, this section was separated into 2 hemispheres with ventricular and interventricular sparing. Tissue samples were then dissolved in Triton X-100 and centrifuged. The supernatants were divided into 2 equal aliquots for quantitative zymography and hemoglobin quantification.

For zymography, supernatants underwent a gelatinase purification process with gelatin-Sepharose 4B (Pharmacia). Zymography was performed on the purified supernatants in sodium dodecyl sulfate gels containing gelatin, as previously described. Dried gels were scanned with a transparency scanner, and images were analyzed with commercially available software (AlphaEase, Alpha Innotech). The relative lysis of an individual sample was expressed as the integrated density value of its band and divided by the protein content of the sample.

Hemoglobin in brain tissue was quantified with a kit designed for quantitative, colorimetric determination of hemoglobin in plasma (Sigma No. 527-30). With this kit, hemoglobin concentrations as low as 5 mg/dL can be measured. To validate this method for brain tissue, we added whole rat blood with known concentrations of hemoglobin to brain homogenate supernatants of all sham-operated animals. Within the range of 6.25 to 100 mg hemoglobin per deciliter of brain homogenate supernatants, extinction at 570 nm showed a linear function (r=0.994). To measure relative hemoglobin concentrations in infarcted tissue, 7.5% dilutions of homologous supernatants in 3.3,5,5′-tetramethylbenzidine were prepared. A microplate was filled with 100 μL of these dilutions in duplicate, and 100 μL H2O2 per well was added. After exactly 15 minutes, extinction was measured with a microplate reader at 570 nm. Tissue hemoglobin was expressed as arbitrary units of optical density to allow for semiquantitative analysis.
RESULTS

Verification of MCAO

TTC staining of the frontal first 3-mm section of the brain verified infarction in 66% of animals with 1.5/16.5, with or without rtPA treatment. In 3/15, 88% of untreated and 80% of rtPA-treated animals showed infarction. This difference was not significant. Regardless of treatment, 100% of animals with 6/1, 6/12, or 18/0 showed infarction. TTC staining was negative in all sham-operated animals.

MCAO With Long-Term Reperfusion or Permanent MCAO

In the experiments lasting 18 hours, rtPA was well tolerated after 1.5 and 3 hours of ischemia. However, when reperfusion was delayed to 6 hours, rtPA increased mortality from 17% to 83% (P<0.01). In permanent ischemia, rtPA had no significant effect on mortality. Treatment with the MMP inhibitor BB-94 markedly reduced the mortality to 33% (P<0.05; Figure 1). The rtPA-treated animals died mainly between 6 and 12 hours after onset of reperfusion (Figure 2). Brains of all animals that had died during the reperfusion period showed macroscopically visible massive hemispheric edema without signs of subarachnoid or intracerebral hemorrhage.

Brain water was increased in the ischemic hemisphere of animals with 1.5/16.5 (P<0.01). When reperfusion was delayed to 3 hours (ie, 3/15), water uptake further increased (P<0.01). Delay of reperfusion to 6 hours (ie, 6/12) had no significant additional effect on water uptake. Water uptake in permanent ischemia (18/0) was significantly lower than that in animals with 3 or 6 hours of transient ischemia (P<0.05). Treatment with rtPA did not significantly affect water uptake (Figure 3A). Compared with sham-operated animals, the hemoglobin concentration in the ischemic hemisphere was increased in transient ischemia (P<0.01). Comparing 3 and 6 hours of ischemia, there was a nonsignificant trend for higher hemoglobin concentrations in delayed reperfusion. Treatment with rtPA did not affect hemoglobin concentrations (Figure 3B).

Zymograms revealed only inactive pro-forms of MMP-2 (72 kDa) and MMP-9 (92 kDa) (Figure 4A). Compared with sham-operated animals, the hemispheric MMP-2 ratio after 1.5 hours of ischemia was increased (P<0.01). This ratio significantly increased with delay of reperfusion (P<0.05; Figure 4B). Compared with sham-operated animals, the hemispheric MMP-9 ratio after 1.5 hours of ischemia was increased (P<0.01). In contrast to MMP-2, the MMP-9 ratio did not further increase when reperfusion was delayed to 3 or 6 hours (Figure 4C). Treatment with rtPA had no effect on MMP-2 or MMP-9 in transient ischemia up to 3 hours. In permanent ischemia, MMP-2 and MMP-9 ratios of saline-treated animals were significantly lower than in transient ischemia (P<0.05). Treatment with rtPA significantly increased the MMP-9 ratio but not the MMP-2 ratio in permanent ischemia (P<0.05; Figure 4B and 4C). There was no significant sucrose uptake in all surviving animals 18 hours after MCAO, regardless of the ischemic interval and whether animals were treated with rtPA or not (data not shown).

MCAO With Short-Term Reperfusion

Animals with 6/1 that received saline presented with massive opening of the BBB in the ischemic hemisphere. The sucrose uptake was 8.80±1.14% compared with 2.15±0.26% in sham-operated animals (P<0.01). Intraperitoneal administration of BB-94 at 2 and 5 hours after MCAO drastically decreased the BBB opening after 6/1 (sucrose uptake, 3.95±1.48%; P<0.01; Figure 5A).

Figure 2. Time course of death in animals with transient ischemia for 6 hours. Significant differences between rtPA- and saline-treated animals were found after 9 (**P<0.01) and 12 (***P<0.01) hours of reperfusion.

Figure 3. A. Brain water was increased in animals with 1.5 hours of ischemia (**P<0.01). Delay of reperfusion to 3 or 6 hours further increased water uptake (***P<0.01). In permanent ischemia, water uptake was lower than in transient ischemia for 3 and 6 hours (**P<0.05). Treatment with rtPA had no effect. B. Tissue hemoglobin, quantified in arbitrary units (AU) of optical density, was increased after 3 hours of ischemia (***P<0.01). There was a nonsignificant trend for a further hemoglobin increase after 6 hours of ischemia. Treatment with rtPA had no effect.
MMP-2 and MMP-9 ratios after 6/1 were significantly increased compared with sham (P<0.05) but were not affected by BB-94 (Figure 5B). Treatment with rtPA had no effect on MMP-2 and MMP-9 ratios after 6/1 (MMP-2, 1.78±0.51 vs 1.93±0.40; MMP-9, 1.79±0.56 vs 2.11±0.31). Compared with saline-treated animals, rtPA slightly but significantly decreased sucrose uptake in the ischemic hemisphere after 6/1, to 5.96±1.65% (P<0.05).

**Discussion**

We found a high mortality in rtPA-treated rats when reperfusion was delayed to 6 hours after MCAO. An MMP inhibitor given 2 and 5 hours after the onset of ischemia dramatically reduced mortality. The action of the MMP inhibitor was to reverse the massive BBB opening in the presence of elevated MMP-2 and MMP-9 concentrations after 6 hours of ischemia and 1 hour of reperfusion, suggesting an association between MMP-mediated early BBB dysfunction and rtPA-associated mortality.

Our results correspond to findings of a recent embolic stroke study on spontaneously hypertensive rats (SHR), in which 50% of the animals died within 24 hours when rtPA treatment was started 6 hours after the onset of ischemia; treatment with BB-94 given before ischemia reduced mortality to 9%.13 Our results show that in a mechanical stroke model, BB-94 is effective even when therapy is started 2 hours after onset of ischemia and that its major effect is to block the disruption of the BBB. Future studies are warranted to confirm the protective effect of BB-94 treatment started 2 hours after MCAO in embolic stroke.

Although associated with a very high mortality in delayed reperfusion, rtPA did not affect any of the other outcome parameters. Therefore, we can only provide evidence on what the rats did not die of and speculate on possible causes. In the present study on young, normotensive rats, hemorrhage did not appear to be involved in rtPA-associated mortality. This is in contrast to several previous studies investigating rtPA effects on hemorrhage in focal cerebral ischemia. In a rabbit model of embolic stroke, macroscopically visible bleeding occurred in 77% of rtPA-treated animals compared with 24% in the control group.14 Embolic stroke studies in SHR also showed increased hemorrhage, quantified by spectroscopically measured hemoglobin concentrations in ischemic tissue of rtPA-treated animals.13,20 Our findings suggest that depending on species, strain, ischemic model, and intervals of ischemia and reperfusion, detrimental rtPA effects might not necessarily involve hemorrhage.

Because rtPA was well tolerated in permanent and short-term transient ischemia, the observed mortality in delayed reperfusion with rtPA does not seem to be related to extracerebral injury. All rtPA-treated animals that died during the reperfusion period showed macroscopically visible massive hemorrhage.
hemispheric edema without signs of intracerebral or subarachnoidal hemorrhage. This suggests that death occurred owing to transfarinal herniation after development of malignant infarction, although the measured brain water did not seem to be affected by rtPA. This lack of effect might have been due to submaximal brain water concentrations in untreated animals. A further rtPA-associated increase in brain water might have been too small to be statistically significant. Besides, most of the rtPA-treated animals with delayed reperfusion died during the reperfusion period of 12 hours. Therefore, the measured brain water in these animals represented the content at the time of death and not the content at the end of the reperfusion period. Death itself did not seem to affect edema, because the water content in the nonischemic hemisphere remained stable (data not shown).

We did not monitor cerebral blood flow, but controlled sufficient MCAO by TTC staining, which showed reliable tissue infarction. Although the suture model is not expected to interact with rtPA, it remains possible that thrombosis persisted even after thread retraction. A recent study demonstrated that immediate and complete recanalization is achieved after 2 hours of mechanical MCAO.22 The exact pattern of cerebral blood flow after 6 hours of mechanical MCAO has not been investigated, but the prompt and massive uptake of intravenously administered sucrose observed in our animals with 6 hours of MCAO, even in the absence of rtPA, strongly suggests rtPA-independent substantial recanalization early in the reperfusion period.

For reasons that will need further investigation, rtPA treatment in our study had a slight protective effect on the BBB in delayed reperfusion. This finding is in contrast to a recent embolic stroke study on SHR. In that study, magnetic resonance imaging showed increased extravasation of contrast agent during delayed rtPA treatment in areas with subsequent hemorrhagic transformation.21 There are possible explanations for these opposing findings. First, different animal strains were used. Compared with young Wistar rats, the cerebral vasculature of SHR might be more susceptible to rtPA-mediated BBB disruption after focal cerebral ischemia and reperfusion. Second, different ischemic models might have different effects on the BBB. This has been shown in another stroke study on SHR, wherein BBB leakage was aggravated in thrombolytic compared with mechanical reperfusion.23

The lack of rtPA-associated aggravation of the BBB opening in our study argues against vasogenic edema as a factor in rtPA-associated mortality. A more likely explanation is that rtPA entered the brain through a compromised BBB and increased mortality by aggravating excitotoxic mechanisms. Excitotoxin-induced neurotoxicity has been shown to be mediated by rtPA.8 A recently suggested mechanism involves cleavage of the N-methyl-D-aspartate receptor NR1 subunit by rtPA, increasing its sensitivity to excitotoxins.23 To aggravate neurotoxicity in focal cerebral ischemia, rtPA has to reach the extravascular space. With a molecular weight of \(~70\) kDa, rtPA extravasation depends on BBB opening. Our study suggests that early opening of the BBB after initiation of reperfusion is an important prerequisite for rtPA-associated mortality. After 6 hours of ischemia and 1 hour of reperfusion, the BBB was severely injured. This opening could be substantially reduced by MMP inhibition. We found elevated levels of MMP-2 and MMP-9 after 6 hours of ischemia and 1 hour of reperfusion. Other proteases are expressed in ischemic brain tissues, including stromelysin-1 (MMP-3). Because BB-94 is a broad-spectrum MMP inhibitor, it might have inhibited MMP-2, -3 or -9, all of which are potentially toxic to the BBB.

MMP inhibitors have been developed for the treatment of metastatic cancer.24 Most are hydroxymates, which block the zinc in the active site of the enzymes.25 MMP inhibitors have been tested in clinical trials for cancer but have been found to have long-term side effects, such as shoulder pain due to fibrosis from inhibition of extracellular matrix turnover. However, short-term use of MMP inhibitors was safe in clinical trials, suggesting that they could be used to control BBB damage during the acute stages of injury.24

Several MMP inhibitors have been tested in other experimental models of neurologic diseases. Bacterial meningitis, cerebral infarction, experimental allergic encephalomyelitis, and experimental allergic neuritis can be treated with MMP inhibitors, which block the vascular damage.26 Although BB-94 will probably be unsuitable for clinical use because of its poor solubility in water, other agents are under development for use in cancer and arthritis that could be tested in treatment trials to control rtPA toxicity in cerebral ischemia. Our results suggest that opening of the BBB in delayed reperfusion is mediated by MMPs and permits extravasation of rtPA, which is associated with increased mortality. This mortality can be reduced by MMP inhibition, even if treatment is initiated 2 hours after onset of ischemia, a time point at which treatment of stroke patients is feasible. Maintaining the integrity of the vasculature with MMP inhibitors might increase safety in thrombolytic therapy.

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References


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