Blood-Brain Barrier Disruption and Matrix Metalloproteinase-9 Expression During Reperfusion Injury
Mechanical Versus Embolic Focal Ischemia in Spontaneously Hypertensive Rats

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Background and Purpose—Most experimental models of cerebral ischemia use mechanical methods of occlusion and reperfusion. However, differences between mechanical reperfusion versus clot thrombolysis may influence reperfusion injury profiles. In this study we compared blood flow recovery, blood-brain barrier (BBB) permeability, and matrix metalloproteinase-9 (MMP-9) expression in cortex after mechanical versus thrombolytic reperfusion in rat focal ischemia.

Methods—Male spontaneously hypertensive rats were used. Mechanical ischemia/reperfusion was achieved with the use of an intraluminal filament to occlude the middle cerebral artery for 2 hours. Thrombolytic reperfusion was achieved by administering tissue plasminogen activator at 2 hours after embolic focal ischemia. Regional cortical blood flow was monitored by laser-Doppler flowmetry. BBB permeability in cortex was measured by Evans blue dye leakage. Cortical MMP-9 levels were assessed with zymography and immunohistochemistry.

Results—Blood flow recovery during mechanical reperfusion was complete in both central and peripheral areas of ischemic cortex. However, after thrombolysis, reperfusion was incomplete, with moderate recovery in the periphery only. BBB permeability was mainly increased in the central regions of the ischemic cortex after mechanical reperfusion but was increased in both central and peripheral areas after thrombolysis. Overall, MMP-9 levels were higher after embolic versus mechanical ischemia/reperfusion, even though ischemic injury was similar in both models at 24 hours.

Conclusions—There are significant differences in the profiles of blood flow recovery, BBB leakage, and MMP-9 upregulation in mechanical versus thrombolytic reperfusion after focal ischemia. (Stroke. 2002;33:2711-2717.)

Key Words: brain edema ■ metalloproteinases ■ neuroprotection ■ stroke ■ tissue plasminogen activator

Thrombolysis with tissue plasminogen activator (tPA) is an effective therapy for acute thromboembolic stroke. Clinical1–3 and experimental4 studies have shown that early restoration of blood flow salvages ischemic brain. However, delayed reperfusion may also lead to negative sequela such as blood-brain barrier (BBB) breakdown and the development of hemorrhagic transformation and edema.2,5,6 These vascular complications of reperfusion injury pose a serious limitation for thrombolytic stroke therapy. However, the precise mechanisms that underlie these deleterious events remain to be fully elucidated.

The majority of experimental cerebral ischemia studies utilize mechanical methods of arterial occlusion and reperfusion.7 Recently, it is increasingly recognized that there may be critical differences in cerebral pathophysiology between mechanical models and embolic clot–based models of ischemia.8–12 In the context of reperfusion injury, profiles of tissue damage after clot thrombolysis may differ from those that occur after mechanical reperfusion.

In this study we examine 2 rat models of focal cerebral ischemia. A mechanical model of ischemia/reperfusion is compared with an embolic model with tPA-induced thrombolysis. Blood flow recovery and BBB leakage patterns are assessed in central and peripheral zones of cortical ischemia. Recent data strongly implicate a role for matrix metalloproteinase-9 (MMP-9) in the development of vascular damage in stroke.13 Therefore, we also compare profiles of MMP-9 upregulation in these 2 models.

Materials and Methods

Animal Models of Focal Cerebral Ischemia
All experiments were performed following an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male spontaneously hypertensive rats were anesthetized with halothane (1% to 1.2%) under spontaneous respiration in an air-oxygen mixture. Rectal temperature was maintained between 37°C and 38°C with a thermostat-controlled heating pad. The right femoral artery was
cannulated, and physiologic parameters, including rectal temperature, mean blood pressure, pH, P\textsubscript{O}\textsubscript{2}, and P\textsubscript{CO}\textsubscript{2}, were monitored. The tail vein was cannulated for tPA administration. In this study, 2 different models of focal ischemia were used: an embolic model that used homologous clots to occlude the middle cerebral artery and a mechanical model in which arterial occlusion was achieved with the use of a silicon-coated filament.

For the embolic model, homologous clots were made with the use of methods adapted and modified from Zhang et al.\textsuperscript{14–17} Femoral artery from a donor rat was withdrawn into PE-50 tubing, kept for 2 hours at room temperature, then maintained at 4°C for 22 hours. Four centimeters of clot was transferred, washed with saline, and shifted to a modified PE-50 catheter with a 0.3-mm outer diameter. A surgical incision was performed via the ventral surface of the neck to expose the internal and external carotid arteries. The external carotid artery, occipital artery, and pterygopalatine artery were ligated. Surgical clips were applied to the common and internal carotid arteries. The modified PE-50 catheter containing the clot was inserted gently from the external carotid artery into the distal internal carotid artery (just proximal to the origin of the middle cerebral artery). The clot was injected, and after 5 minutes the catheter was withdrawn. Reperfusion was achieved with tPA administered intravenously at 2 hours after ischemia.

For the mechanical ischemia model, a standard intraluminal method was used.\textsuperscript{18,19} Briefly, a silicon-coated 4.0 nylon monofilament was inserted from the external carotid artery and advanced into the internal carotid artery until the tip occluded the proximal stem of the middle cerebral artery. Reperfusion was achieved by withdrawal of filament.

**Laser-Doppler Flowmetry**

Regional cerebral blood flow (rCBF) was continuously monitored by laser-Doppler flowmetry (LDF). For placement of LDF probes, burr holes were created in the right parietal bone at the positions of ponsuema cortex (2 mm posterior and 4 mm lateral to bregma) and core cortex (2 mm posterior and 6 mm lateral to bregma). Changes of blood flow were monitored during ischemia and up to 3 hours after reperfusion.

**Measurement of Infarct Volumes**

Rats were killed 24 hours after induction of focal ischemia. Seven coronal sections per brain (2 mm in thickness) were prepared and stained with 2,3,5-triphenyltetrazolium chloride (Sigma). Infarct volume was quantified with a standard computer-assisted image analysis technique.\textsuperscript{15,16}

**Quantitative Evaluation of Evans Blue Dye Extravasation**

Vascular permeability was quantitatively evaluated by fluorescent detection of extravasated Evans blue dye.\textsuperscript{20} Briefly, 2% Evans blue dye in saline was injected intravenously as a BBB permeability tracer at 4 hours after middle cerebral artery occlusion. Rats were deeply anesthetized with halothane and then transcardially perfused with ice-cold PBS (pH 7.4). The brains were quickly removed and divided into sections in a manner similar to that used for the Evans blue dye study, ie, ischemic cortex was dissected and divided into central and peripheral ischemic zones. Ischemic tissue and matching tissue were frozen immediately in liquid nitrogen and stored at −80°C. Brain tissue extracts were prepared as previously described. Briefly, brain samples were homogenized in lysis buffer including protease inhibitors on ice. After centrifugation, supernatant was collected, and total protein concentrations were determined with the use of the Bradford assay (Bio-Rad Laboratories). Samples were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After separation by electrophoresis, the gel was renatured and then incubated with developing buffer at 37°C for 24 hours as previously described. After development, the gel was stained with 0.5% Coomassie blue R-250 for 30 minutes and then destained appropriately. MMP activity was quantified with standard gel densitometry techniques.\textsuperscript{20–24}

**MMP Zymogram**

Rats were deeply anesthetized at 6 hours after the onset of ischemic insult with halothane and then transcardially perfused with ice-cold PBS (pH 7.4). The brains were quickly removed and divided into sections in a manner similar to that used for the Evans blue dye study, ie, ischemic cortex was dissected and divided into central and peripheral ischemic zones. Ischemic tissue and matching tissue were frozen immediately in liquid nitrogen and stored at −80°C. Brain tissue extracts were prepared as previously described. Briefly, brain samples were homogenized in lysis buffer including protease inhibitors on ice. After centrifugation, supernatant was collected, and total protein concentrations were determined with the use of the Bradford assay (Bio-Rad Laboratories). Samples were loaded and separated by 10% Tris-glycine gel with 0.1% gelatin as substrate. After separation by electrophoresis, the gel was renatured and then incubated with developing buffer at 37°C for 24 hours as previously described. After development, the gel was stained with 0.5% Coomassie blue R-250 for 30 minutes and then destained appropriately. MMP activity was quantified with standard gel densitometry techniques.\textsuperscript{20–24}

**Immunohistochemistry**

To assess the spatial distribution of MMP-9 after transient focal ischemia, immunohistochemistry was performed as previously described.\textsuperscript{20} Briefly, rats were transcardially perfused with ice-cold PBS (pH 7.4) followed with ice-cold 4% paraformaldehyde in PBS (pH 7.4) at 6 hours after the induction of ischemia. The brains were removed, immersed with 4% paraformaldehyde in PBS overnight at 4°C, and cryoprotected in 30% sucrose in PBS at 4°C. Frozen coronal sections (30 μm thickness) were prepared with the use of a microtome. Sections were blocked with 5% normal goat serum, incubated overnight at 4°C with the MMP-9 rabbit polyclonal antibody (1:400, Chemicon), and followed by incubation with FITC-labeled anti-rabbit IgG secondary antibody (1:400). Immunostainings were analyzed with a fluorescence microscope (Olympus) interfaced with a digital charge-coupled device camera and an image analysis system.

**Statistical Analysis**

Quantitative data were expressed as mean±SEM. Statistical comparisons were conducted with the use of ANOVA followed by Tukey-Kramer tests for intergroup comparisons. Differences with P<0.05 were considered statistically significant.

**Results**

**Ischemic Injury Is Similar in Mechanical and Embolic Models**

Physiological parameters including rectal temperature, pH, blood gases, and blood pressure remained within normal range and were similar in mechanical and thrombolytic reperfusion groups (Table).

At 24 hours after focal ischemia, ischemic damage was similar in both groups. Infarct volumes were 341±18.6 mm\textsuperscript{3} in the mechanical model and 363±20.0 mm\textsuperscript{3} in the embolic

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**Physiological Variables in Mechanical and Thrombolytic Reperfusion Groups**

<table>
<thead>
<tr>
<th></th>
<th>Mechanical</th>
<th>Embolic+tPA</th>
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</thead>
<tbody>
<tr>
<td>Rectal temperature, °C</td>
<td>37.9±0.07</td>
<td>37.6±0.14</td>
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<tr>
<td>pH</td>
<td>7.38±0.01</td>
<td>7.37±0.01</td>
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<tr>
<td>P\textsubscript{O}\textsubscript{2}, mm Hg</td>
<td>43.0±0.95</td>
<td>44.1±2.64</td>
</tr>
<tr>
<td>P\textsubscript{O}\textsubscript{2}, mm Hg</td>
<td>114±0.95</td>
<td>116±9.60</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>177±5.53</td>
<td>171±3.64</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=6 per group).
model (Figure 1). These were not statistically different, suggesting that ischemic severity was similar in both models.

Thrombolytic Reperfusion Is Incomplete in Central Ischemic Cortex
LDF was performed to assess rCBF in central and peripheral regions of ischemic cortex. During the 2-hour ischemic period, perfusion dropped to approximately 20% and 60% in the central and peripheral ischemic zones, respectively (Figure 2). The degree of ischemia was the same in both mechanical and embolic occlusions. However, after reperfusion, patterns of rCBF restoration were significantly different.

In the mechanical model, filament withdrawal resulted in full recovery of rCBF with a hyperemic response in peripheral ischemic cortex (Figure 2). In contrast, thrombolytic reperfusion was poor in central ischemic areas, and no hyperemic response was observed in the peripheral ischemic cortex (Figure 2).

Cortical Evans Blue Dye Leakage in Ischemic Periphery Is Higher After Thrombolysis
The distribution of Evans blue dye leakage in rats that underwent thrombolysis was different from that observed after mechanical reperfusion. After mechanical reperfusion, Evans blue dye leakage appeared to be concentrated mostly in the central regions of ischemic cortex (Figure 3). In contrast, Evans blue leakage after thrombolysis was observed throughout the ischemic cortex (Figure 3). Quantitative analysis of Evans blue dye in each region demonstrated that leakage within the peripheral ischemic areas of the cortex was significantly higher after thrombolysis compared with mechanical reperfusion (Figure 4). Leakage of Evans blue dye in the ischemic striatum was lower than in the cortex, and there were no significant differences between the 2 models (Figure 4).

MMP-9 Upregulation Is Greater After Embolic Focal Ischemia Plus Thrombolysis
Gelatin zymography showed that MMP-9 upregulation was significantly higher after embolic focal ischemia in both central and peripheral areas of cortex compared with the model of mechanical focal ischemia (Figure 5A and 5B). MMP-2 levels were also slightly increased, but there was no statistically significant difference between the 2 models.

Fluorescent immunohistochemistry also demonstrated a higher upregulation of MMP-9 in the embolic than in the mechanical ischemia model (Figure 6). Assessment of sections at high magnification demonstrated that MMP-9 expression after clot embolization plus thrombolysis was intensely observed in the cerebral microvasculature within the ischemic territory. In addition to cellular staining, MMP-9 also appeared to show diffuse signals in the parenchyma. In contrast, mechanical ischemia/reperfusion induced MMP-9 expression mostly in the central ischemic areas with vascular-like staining; no diffuse signal was detectable in the parenchyma.

Discussion
In this study we explored potential differences between thrombolytic and mechanical reperfusion in a rat model of focal cerebral ischemia. The first critical finding was that cortical patterns of blood flow recovery were significantly different in these 2 models. Perfusion recovered almost immediately in the mechanical model. Indeed, in the periph-
eral zones of cortical ischemia, there was a transient hyperemic response in which blood flow increased significantly above normal levels within the first few minutes after the end of arterial occlusion. In contrast, reperfusion after tPA thrombolysis was poor in the ischemic center and showed only modest and slow recovery in the peripheral zones of ischemia. It is possible that since emboli were lysed by tPA from the proximal side, restoration of flow may occur slowly through collateral branches of the middle and anterior cerebral arteries, thus resulting in reperfusion in the border zone around the middle cerebral artery territory.

Recirculation after cerebral ischemia results in reoxygenation and subsequent free radical formation. Hence, differences in blood flow recovery in the 2 models may lead to differences in oxygen radical generation and reperfusion injury. It is somewhat puzzling that ischemic lesions in the 2 models were the same despite the fact that reperfusion was rapid in the mechanical model but slow and incomplete in the embolic plus tPA model. There may be 2 opposite effects. Reperfusing the brain quickly should be beneficial because one needs to restore oxygen and glucose delivery; on the other hand, reperfusing too rapidly may generate a sharper burst of oxidative stress. Hence, one might speculate that the benefits of rapid reperfusion in the mechanical model were somehow counterbalanced by the larger generation of free radicals, thus resulting in a final lesion outcome that was similar to the slower reperfusion in the embolic model. Further studies that more carefully measure and compare the generation of reactive oxygen radicals in mechanical versus thrombolytic reperfusion will be useful. Aside from potential differences in oxidative stress, we cannot exclude the possibility that in spontaneously hypertensive rats, tissue damage is already severe and maximal after 2 hours of transient ischemia, so that no significant differences in infarction are expected even with altered rates of reperfusion.

A critical parameter of tissue damage in reperfusion injury is the breaching of the BBB and development of edema and/or hemorrhage. In this study we assessed the integrity of the BBB during the first 4 hours of reperfusion and found that although the total amount of dye leakage in the ischemic hemisphere was similar (data not shown), the spatial patterns of leakage were quite different. After mechanical ischemia/reperfusion, dye leakage tended to be concentrated mostly in the central areas of ischemic cortex. In contrast, tPA reperfusion led to a more uniform pattern of leakage, and Evans blue dye was detected throughout the ischemic cortex. The precise reason for this difference is unclear but may be related to how rapidly blood flow recovers. Rapid reperfusion in the ischemic core after mechanical reperfusion may lead to a sudden generation of reactive oxygen radicals and hemodynamic surge that leads to pronounced BBB leakage in this area.

Recently, emerging data suggest that MMPs play an important role in the disruption of vascular integrity during reperfusion. In the context of reperfusion injury, MMP-9 may degrade critical components of the vascular matrix such as collagen IV, thus weakening vessels and causing leakage and rupture. In mouse focal cerebral ischemia, in situ measurements of free radical production colocalized with vascular sites of MMP activity during reperfusion. Furthermore, MMP-9 knockout mice were protected against BBB disruption and edema after transient focal cerebral ischemia. However, a major caveat is that this and most other studies of reperfusion injury are conducted in models in which arterial occlusion and reperfusion are achieved mechanically. The question arises regarding whether MMP-9 responses may be different in the more clinically relevant model of embolic clot-induced stroke. In this study we found that patterns of MMP-9 upregulation were different in embolic plus tPA focal ischemia compared with mechanically induced ischemia/
reperfusion. After thrombolysis, quantitative zymography showed that MMP-9 levels were elevated equally in both central and peripheral areas of ischemic cortex. However, in the mechanical model of reperfusion, MMP-9 was significantly elevated only in the ischemic center.

Overall, however, the striking finding here was that total hemispheric levels of MMP-9 in the embolic clot plus tPA model were significantly higher than in the mechanical model of ischemia/reperfusion. Higher MMP-9 levels are not simply explained by the severity of ischemic insult because infarct volumes were the same in both thrombolytic and mechanical reperfusion models. Furthermore, the degree of ischemia as measured by LDF showed similar reductions of blood flow during arterial occlusion. One speculative possibility may be related to the biological versus mechanical nature of the occluding methods. In the embolic model, focal ischemia was induced by homologous blood clots. Clot emboli contain many blood coagulation factors, such as thrombin, that may stimulate MMP-9 production. A second possibility is that tPA itself might promote MMP-9 expression in ischemic brain tissue. A recent study showed that after embolic focal ischemia in rats, delayed tPA administration significantly elevated the production of MMP-9 in ischemic brain tissue. Mechanisms that underlie the higher elevation of MMP-9 after tPA reperfusion remain to be fully elucidated. Insofar as proteolytic degradation of the vascular matrix can weaken vessels and lead to edematous leakage or hemorrhagic rupture, MMP-9 may play a pivotal role in clinical stroke, in which tPA is used for reperfusion therapy.

A question that arises from these data is whether patterns of Evans blue dye leakage match the distribution of MMP-9. In the peripheral zones of ischemic cortex, both Evans blue dye leakage and MMP-9 upregulation were significantly higher after thrombolysis than after mechanical reperfusion. These data are consistent with our hypothesis that MMP-9 may play a role in the degradation of vascular integrity. In the ischemic center, MMP-9 levels were also significantly higher in the thrombolytic versus mechanical model. However, although

Figure 5. Zymographic analysis in central and peripheral zones of ischemic cortex after 2-hour transient ischemia plus 4 hours of mechanical versus thrombolytic reperfusion. A, Representative zymogram shows greater upregulation of MMP-9 with thrombolytic than with mechanical reperfusion. PC indicates positive controls of MMP-9 and MMP-2 (arrows); S, sham control; F, filament model of ischemia/reperfusion; and E, embolic+tPA model of ischemia/reperfusion. B, Quantitative analysis of MMP-9 and MMP-2 optical densities (mean±SEM). MMP-9 but not MMP-2 upregulation was significantly greater in central and peripheral regions of ischemic cortex after thrombolytic reperfusion compared with mechanical reperfusion (*P<0.05).
mean levels of Evans blue dye leakage were somewhat greater after thrombolysis, the difference did not reach statistical significance. One reason for the lack of correlation between BBB leakage and MMP-9 in the ischemic center is that data from this zone may be more difficult to interpret. Because blood flow recovery after thrombolysis was poor in the central ischemic region, rates of leakage of Evans blue dye may be limited. Therefore, in the poorly perfused ischemic center, Evans blue dye leakage may not be an accurate measurement of BBB permeability. Indeed, if one were to pseudonormalize the data by calculating the ratio of Evans blue dye leakage (μg/g) versus the level of LDF perfusion (% preischemic baseline), the degree of Evans blue leakage per “unit” of perfusion in the ischemic center then becomes much higher in the embolic plus tPA model (1.1 μg/g per unit) compared with the mechanical reperfusion model (0.3 μg/g per unit). The same limitations will be even more pronounced in the severely ischemic striatum. Although we were unable to document reperfusion in striatum with surface-based LDF in this study, it is interesting to note that Evans blue dye leakage in striatum was lower than in cortex, even though damage was likely to be more severe. The technical caveat with the Evans blue method is that it is dependent on adequate dye delivery and blood flow levels.

Although the present data show significant differences in BBB leakage and MMP-9 profiles in the 2 models, a caveat is that we only looked at a single acute time point. This was based on prior studies showing that maximal BBB leakage after transient focal cerebral ischemia occurred during the first few hours after reperfusion. However, it has also been suggested that BBB disruption may follow biphasic temporal profiles. After an initial phase of leakage, there may be a transient “resealing” followed by a later secondary leakage.

Future studies should follow chronic responses to delineate potentially important differences in the later outcomes as well. Others have shown that MMP-2 and MMP-9 upregulation can follow biphasic and prolonged temporal patterns after transient focal ischemia.

In summary, our study demonstrated that there are characteristic differences in cerebral blood flow recovery, BBB leakage, and MMP-9 upregulation between thrombolytic and mechanical reperfusion after focal cerebral ischemia. More detailed studies using the thrombolytic reperfusion model may be warranted to more closely simulate the clinical scenario in stroke. These data may be critical to better understand the role of MMP-9 in the development of vascular complications after tPA stroke therapy.

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References


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