Matrix Metalloproteinases and TIMPs Are Associated With Blood-Brain Barrier Opening After Reperfusion in Rat Brain

G.A. Rosenberg, MD; E.Y. Estrada, BS; J.E. Dencoff, BS

Background and Purpose—Reperfusion disrupts cerebral capillaries, causing cerebral edema and hemorrhage. Middle cerebral artery occlusion (MCAO) induces the matrix-degrading metalloproteinases, but their role in capillary injury after reperfusion is unknown. Matrix metalloproteinases (MMPs) and tissue inhibitors to metalloproteinases (TIMPs) modulate capillary permeability. Therefore, we measured blood-brain barrier (BBB) permeability, brain water and electrolytes, MMPs, and TIMPs at multiple times after reperfusion.

Methods—Adult rats underwent MCAO for 2 hours by the suture method. Brain uptake of 14C-sucrose was measured from 3 hours to 14 days after reperfusion. Levels of MMPs and TIMPs were measured by zymography and reverse zymography, respectively, in contiguous tissues. Other rats had water and electrolytes measured at 3, 24, or 48 hours after reperfusion. Treatment with a synthetic MMP inhibitor, BB-1101, on BBB permeability and cerebral edema was studied.

Results—Brain sucrose uptake increased after 3 and 48 hours of reperfusion, with maximal opening at 48 hours and return to normal by 14 days. There was a correlation between the levels of gelatinase A at 3 hours and the sucrose uptake (P<0.05). Gelatinase A (MMP-2) was maximally increased at 5 days, and TIMP-2 was highest at 5 days. Gelatinase B and TIMP-1 were maximally elevated at 48 hours. The inhibitor of gelatinase B, TIMP-1, was also increased at 48 hours. Treatment with BB-1101 reduced BBB opening at 3 hours and brain edema at 24 hours, but neither was affected at 48 hours.

Conclusions—The initial opening at 3 hours correlated with gelatinase A levels and was blocked by a synthetic MMP inhibitor. The delayed opening, which was associated with elevated levels of gelatinase B, failed to respond to the MMP inhibitor, suggesting different mechanisms of injury for the biphasic BBB injury. (Stroke. 1998;29:2189-2195.)

Key Words: blood-brain barrier ▪ brain edema ▪ cerebral ischemia ▪ reperfusion injury ▪ matrix metalloproteinases ▪ type IV collagenases
MMPs and TIMPs in Reperfusion Injury

Reverse Zymography
Reverse zymography was performed as recently described. Polyacrylamide minigels (15%) are prepared: 5 mL Protogel (30% ultrapure, National Diagnostics); 2.5 mL of 1.5 mol/L Tris-HCl, pH 8.8; 1.67 mL porcine gelatin at 15 mg/mL; 0.83 mL distilled water; 0.2 mL of 10% SDS; 6.4 μL of purified gelatinase A (0.252 μg/μL; a gift from Dr W.G. Stetler-Stevenson at the National Cancer Institute); 50 μL of 10% ammonium persulfate; and 5 μL of N,N,N’-tetra-methylthlenediamine (TEMED). Gels were allowed to polymerize for 1.5 to 2 hours before the stacking gel was added (5.6 mL distilled water; 4.15 mL 0.5 mol/L Tris, pH 6.8; 1.66 mL Protogel 30%; 125 μL (NI SDS; 10 μL TEMED; and 200 μL 10% APS). The stacker was allowed to polymerize for 1 hour before samples were loaded.

Tissue samples were thawed and were mixed 1:1 with nonreducing SDS loading buffer (New England Biolabs) before loading on gels. Prepared samples were not boiled or exposed to reducing agents. Prestained rainbow-colored molecular weight markers (Amersham Life Science) and HT1080 fibrosarcoma media, which contains TIMP-1 and TIMP-2, were run in every gel to determine molecular weights of TIMPs. After loading, gels were electrophoresed at 150 V for 2.5 hours. Following electrophoresis, all gels were agitated (2 × 30 minutes) in 2.5% Triton X-100 to remove the SDS. Gels were rinsed 3 times in distilled water, then incubated for 16 hours at 37°C in 50 mL of 50 mmol/L Tris-HCl (pH 7.6) containing 0.2 mol/L NaCl, 5 mmol/L CaCl₂, 0.02% Brij-35, and 0.02% Azide. Finally, all gels were stained for 1 hour in Coomassie G-250. Gels were then destained for approximately 1 hour in 10% acetic acid. Zones of TIMP activity were seen as dark bands against a clear background.

Materials and Methods

MCAO With Reperfusion
The study was approved by the University of New Mexico Animal Care Committee and conformed to the National Institutes of Health Guidelines for use of animals in research. Adult Wistar rats, weighing 280 to 320 g, were anesthetized with 1.5% halothane in 70% nitrous oxide and 30% oxygen. Temporary MCAO was done by insertion of an intraluminal nylon suture with a bulb on the end. Neck vessels were exposed through a midline incision, and branches of the right external carotid artery were isolated and ligated. A 6-0 silk suture was loosely tied around the external carotid artery stump. A 4-0 monofilament nylon suture was introduced into the external carotid and advanced into the MCA. A silk suture around the stump was tied down onto the thread with the end of the thread protruding slightly. Reperfusion was achieved by slowly pulling the thread back.

BBB Permeability
Brain uptake of sucrose was measured in 55 rats that had undergone 2 hours of MCAO with reperfusion for 3, 6, 15, 24, 48, 120, or 336 hours. BBB permeability was measured by a modification of the brain uptake method. Ten minutes before death, the rats were anesthetized with 1.5% halothane in 70% N₂O and 30% O₂ and infused intravenously over 30 seconds with 10 μCi of 14C-sucrose (Dupont/New England Nuclear). Samples of blood were drawn from the femoral vein, and the heart was stopped with an intracardiac injection of a saturated potassium chloride solution. The brain was removed and frozen in 2-methylbutane cooled to −80°C. A 5-mm tissue section was removed from a region approximately 5 mm away from the tip of the frontal lobe, which is the main site of infarction. A piece of tissue, which contained cortex and caudate from the region of the infarct, was obtained from a 5-mm coronal section. A section was similarly taken from the noninfarcted hemisphere. For BBB, measurements tissue was dissolved in Aquasol (New England Nuclear), and brain and blood samples were counted for radioactivity in a liquid scintillation counter. The ratio of brain sucrose content to that in the blood was calculated. For zymography, tissues are taken from a contiguous 5-mm coronal section directly posterior to the one used for BBB measurements. Similar regions of the infarcted and noninfarcted sides were studied.

Quantitative Zymography
The zymographic method for measurements of MMPs in rat brain has been described previously. Briefly, tissue samples were weighed and dissolved in Triton X-100. The tissue was centrifuged and the supernatant removed for zymography in SDS gels with gelatin. Electrophoresis was run at 150 V for 1 hour. The sample gels were placed in Tritton X-100 for 30 minutes to remove the SDS and restore the proteins to activity. After incubation, the gels were stained with Coomassie G-250 dye. The molecular standards and HT1080 conditioned media, which contains gelatinases A and B, were used to calibrate the molecular weights. Protein content in the samples was measured by the BCA method with a kit (Pierce Co).
The artery was occluded with an intraluminal thread for 2 hours and then withdrawn to reperfuse the brain. Adult rats were injected with 14C-sucrose at the end of the reperfusion period and killed 10 minutes later. Samples from brain and blood were collected, and brain sucrose uptake was calculated as a percentage of sucrose in brain to that in blood (Sucrose Space %). The sucrose spaces in the ischemic sides were compared by ANOVA with Bonferroni corrections. A significant increase in the uptake of sucrose occurred at 3 and 48 hours compared with all other times. *Statistically significant increases with P<0.05.

**Results**

**BBB Permeability and Brain Water and Electrolytes**

Uptake of sucrose into the brain was significantly increased after 3 hours of reperfusion and was maximal at 48 hours (Figure 1). By 3 hours of reperfusion, the brain water was increased, reaching maximal levels by 24 and 48 hours, with sodium and potassium paralleling the changes in water content (Table 1). Significant increases in water content were found at 3, 24, and 48 hours. Sodium was significantly increased, reaching maximal levels by 24 and 48 hours, with potassium was significantly decreased at 24 hours and increased on the nonischemic side at 48 hours. Potassium was increased in the ischemic side compared with sham-operated controls at 24 and 48 hours, whereas the sodium was increased, plotting the individual values for gelatinase B at the same time in the zymogram.

**MMPs and TIMPs**

Representative zymograms and reverse zymograms from reperfused tissues are shown in Figure 2. Elevated levels of gelatinase B at 92-kDa are seen in the reperfused tissue at 48 hours (Figure 2A). Normally, MMP-9 is undetectable in rat brain. All tissue samples showed bands at 72 kDa from MMP-2, which is constitutively expressed. Reverse zymograms showed dark bands at 28 kDa and 21 kDa from TIMP-1 and TIMP-2, respectively (Figure 2B). To allow comparison over multiple time points from samples run on different gels, the amount of gelatinase B in the ischemic side was normalized by dividing it by the nonischemic side. Optical density measurements showed a significant increase in gelatinase B ratios by 48 hours (Figure 3A). The increase in gelatinase B began by 15 hours but was not significant at that time. After 48 hours, there was a drastic reduction in the levels of gelatinase B. Gelatinase A ratios were maximal at 5 days (Figure 3B).

Although the mean levels of gelatinases A and B were not increased at 3 hours, plotting the individual values for gelatinase A against sucrose space revealed a strong correlation at 3 hours (P<0.0003) and lower significance for

**TABLE 1. Water and Electrolytes in Cerebral Tissue After 2-Hour MCAO With Various Times of Reperfusion**

<table>
<thead>
<tr>
<th>Occlusion/Reperfusion</th>
<th>Water, %</th>
<th>Sodium, mEq/L</th>
<th>Potassium, mEq/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ischemic</td>
<td>Nonischemic</td>
<td>Ischemic</td>
</tr>
<tr>
<td>2/3 (n=9)</td>
<td>81.16±0.33 †</td>
<td>78.80±0.21</td>
<td>308±23.0</td>
</tr>
<tr>
<td>2/24 (n=9)</td>
<td>83.07±0.48 †</td>
<td>79.34±0.27</td>
<td>459±45†</td>
</tr>
<tr>
<td>2/48 (n=6)</td>
<td>83.42±0.53 †</td>
<td>79.04±0.24</td>
<td>591±44†</td>
</tr>
<tr>
<td>Sham* (n=6)</td>
<td>76.62±0.09</td>
<td>78.38±0.06</td>
<td>216±4</td>
</tr>
</tbody>
</table>

*Significance levels were †P<0.001; ‡P<0.01; and §P<0.05.
gelatinase B ($P<0.04$) (Figure 4). No correlation was found at 24 hours for either of the enzymes (data not shown). The marked increase in gelatinase B at 48 hours coincided with the maximal increase in sucrose uptake.

Ratios of TIMP-1 in the 2 sides showed a statistically significant elevation at 48 hours that was absent at 5 and 14 days (Figure 3C). Ratios of TIMP-2 were more erratic, with maximal levels seen at 5 days, reaching significance compared with the 3-, 6-, and 24-hour values. Maximal ratios were seen at 5 days when the ratios for gelatinase A were also increased (Figure 3D).

Effect of Treatment With a Metalloproteinase Inhibitor

The synthetic metalloproteinase inhibitor BB-1101 did not affect blood glucose level, mean arterial blood pressure, or temperature for the 24 hours during which these parameters were measured (Table 2). BB-1101 significantly decreased uptake of sucrose at 3 hours in the ischemic hemisphere and at 48 hours in the nonischemic hemisphere (Figure 5A). It also lowered the sucrose space at 48 hours in the ischemic side, but the results did not achieve statistical significance.

Brain water was significantly reduced by the BB-1101 at 24 hours (Figure 5B). No effect of the agent was seen at 48 hours, however. Administration of the drug after either a 1- or 2-hour delay failed to affect the cerebral edema at 24 or 48 hours, suggesting that the agent was acting to alter an early event (data not shown).

Discussion

Reperfusion for 3 and 48 hours after 2-hour occlusion of the MCA opened the BBB, with the maximal opening at 48 hours. The initial opening corresponded with increased levels of gelatinase A, whereas the second one occurred when gelatinase B was markedly increased. Maximal levels of gelatinase A were seen at 5 days after reperfusion, during the time when the repair process had begun. An inhibitor to metalloproteinases blocked the initial opening of the BBB

TABLE 2. Effect of BB-1101* on Blood Glucose, Temperature, and Mean Arterial Blood Pressure at 0, 4, and 24 Hours After Injection

<table>
<thead>
<tr>
<th></th>
<th>0 Hours</th>
<th>4 Hours</th>
<th>24 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dL</td>
<td>160±16</td>
<td>200±15</td>
<td>170±5</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>37.7±0.12</td>
<td>37.6±0.24</td>
<td>38.0±0.0</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>79.0±1.0</td>
<td>81.0±3.7</td>
<td>87.5±4.8</td>
</tr>
</tbody>
</table>

Dose of BB-1101 was 30 mg/kg IP at 10 minutes after start of the experiment.

*Five nonfasted rats used for each measurement.
and also the edema at 24 hours, suggesting that they were related to a metalloproteinase. Tissue inhibitor to metalloproteinase-1 was markedly increased at 48 hours, and TIMP-2 was maximally increased at 5 days. These results show that MMPs and TIMPs are dramatically affected by reperfusion but contribute in a complex manner to reperfusion injury.

Matrix-degrading proteases are important in many normal and pathological processes, including motility of developing cells, spread of cancer cells, inflammatory responses, and tissue repair after injury. The balance between the proteases and the inhibitors determines whether there is proteolytic breakdown of extracellular matrix or inhibition of proteolysis with buildup of extracellular matrix. Normally, gelatinase A is present in brain tissue in a latent form, which is activated by a membrane-bound protease, membrane-type metalloproteinase (MT-MMP). Attachment of TIMP-2 to MT-MMP is required for the activation MMP-2. Because the activation reaction proceeds on the membrane surface, proteolysis is spatially constrained. Gelatinase A is constitutively expressed, making it available for early tissue injury. Along with the serine protease, plasminogen activator, MMP-2 is also a critical factor in the controlled proteolysis during blood vessel regrowth. On the other hand, gelatinase B is a proinflammatory protease that is released during the neuroinflammatory response by the stimulation of the cytokines and immediate early genes. The gelatinase B promoter region contains both AP-1 and nuclear factor-κB sites, which respond to a wide variety of proinflammatory stimuli. Released in a proform, gelatinase B requires an activation step, which has not been reported for the in vivo situation, but may involve stromelysin, other proteases, or free radicals.

Several brain cells produce gelatinases under resting and stimulated conditions. Astrocytes normally produce latent gelatinase A. Microglial cells produce gelatinase B after stimulation by proinflammatory agents. Cerebral capillaries produce gelatinases. Neutrophils also produce gelatinases, elastase, and cathepsins, which may contribute to the disruption of the tissue in the secondary inflammatory response.

Intracerebral injection of activated gelatinase A opened the BBB, and TIMP-2 blocked the gelatinase-induced opening. Tumor necrosis factor-α, which is formed in ischemic tissue, increased gelatinase B at 24 hours when the BBB was maximally opened, and an inhibitor to metalloproteinases, Batimistat, reduced the capillary injury. Cerebral capillaries are surrounded by a basal lamina composed of type IV collagen, fibronectin, and laminin, which are attacked by the proteases, including gelatinases. Ischemia/reperfusion injury in monkeys causes loss of laminin in the basal lamina, which adds evidence to the concept that proteolytic disruption of the basal lamina contributes to the BBB injury.

The initial rise in capillary permeability may have been due to hyperemia that accompanies the early stages of reperfusion. Treatment with the hydroxymate-type synthetic inhibitor to MMPs reduced this initial increase in capillary permeability at 3 hours and modified the permeability at 48 hours on the nonischemic side. There was a correlation between the levels of gelatinases at 3 hours and the increases in the BBB that suggests that they are linked. One possible explanation for the early effect is that the inhibitor blocked the activation of the endogenous MMP-2 by MT-MMP. BB-1101 is a potent inhibitor of MT-MMP (K. Miller, personal communication, 1998). We have observed by immunohistochemistry that the MMP-2 is normally found around the cerebral vessels in astrocytic processes that abut on the capillary wall and that MT-MMP is also present (S. Mun-Bryce, J. Wallace, unpublished data, 1998). Another possibility is that the inhibitor blocked the conversion of tumor necrosis factor-α from a latent to an active form, preventing it from damaging the capillary. The delayed opening of the BBB seen at 48 hours was associated with a large increase in gelatinase B, which may have been endogenously from astrocytes and microglia or exogenous from the invading neutrophils and macrophages. However, the MMP inhibitor failed to interfere with the BBB damage or edema at that time.

A marked increase in TIMP-1 was seen around the time of maximal rise in the gelatinase B. At 5 days, the BBB was closed, and the levels of both MMP-9 and TIMP-1 had fallen drastically. TIMP-2 was maximal at the time of maximal increases in the levels of MMP-2. TIMP-1 inhibits the action of MMP-9, and TIMP-2 inhibits MMP-2; both have been implicated in other functions, but the role of TIMPs in brain is uncertain.

Brain water content rose significantly from the onset of reperfusion, while the changes in capillary permeability fluctuated. A composite graph showing the changes in multiple parameters is shown in Figure 6. At 24 hours when the
brain water was high, the sucrose space had returned to normal, suggesting that the cytotoxic component of the brain edema predominated. Sodium content was high and potassium low at that time, as expected for cytotoxic edema. However, by 48 hours the water content remained high with elevated levels of sodium and potassium. Furthermore, the sucrose space was maximal. Thus, vasogenic edema seemed to also contribute to the water changes at the later time.

We found that the synthetic metalloproteinase inhibitor prevented the increase in brain water at 24 hours but not at 48 hours. The initial opening of the BBB was also limited by the inhibitor, suggesting that it was affecting the initial increase in capillary permeability. The inability of the inhibitor to act at 48 hours suggests that the second phase of injury was related to the neuroinflammatory response, which involves the infiltrating leukocytes and the endogenous microglial cells. Free radicals and other proteases, such as elastase, which are not inhibited by the MMP inhibitor, may be important because they are released by neutrophils and macrophages and are disruptive to the capillary.38 Another possible explanation of the failure of the agent to affect the edema at 48 hours is that the delayed BBB opening is beneficial by helping to remove fluid from the injury site. Such a mechanism has been proposed earlier to explain the finding of increased capillary permeability without cerebral edema after an ischemic injury.39 Our results suggested that the MMP inhibitor reduced the capillary permeability at 48 hours, while the edema was slightly increased, which is consistent with the notion that the opening of the BBB then may be beneficial, but further studies are needed to clarify this point.

Our results suggest that the balance between the MMPs and their inhibitors play a role in reperfusion injury. Inhibition of the metalloproteinases altered the early damage to the capillary and controlled the brain edema at 24 hours. However, the failure of the MMP inhibitors to affect the delayed injury suggest a multifactorial process. Because of the complex manner in which the increases and reductions in the proteases and inhibitors are woven together during the reperfusion process, attempts at altering the patterns with drugs will be complicated.
Breakdown of blood-brain barrier (BBB) after cerebral ischemia–reperfusion may involve a multitude of molecular events, including the engagement of such mediators as free radicals, cytokines, and others. In the preceding article by Rosenberg and colleagues, a time-dependent correlation of postischemic BBB breakdown and the expression of matrix metalloproteinases (MMPs) was noted. An increase in sucrase space reflecting the extent of BBB breakdown was correlated with gelatinase A expression at 3 hours after ischemia. The enhanced proteolytic activity, suggesting a perturbed balance of proteases and their inhibitors, may contribute to the posts ischemic opening of the BBB. Rosenberg and associates also found an increase in tissue gelatinase and urokinase expression in rat brain. Lab Invest. 1994;71:417–422.

Dr Rosenberg’s laboratory was the first to apply zymograms and reverse zymograms to determine changes in the activities of MMPs and their inhibitors in the rat stroke model. This study, together with earlier works by the same group, provide interesting findings on the activation of a complex biochemical cascade that may contribute to ischemic brain edema.

It should also be noted that in the present study, the correlation of MMP activities and BBB breakdown, based on sucrase space and brain water content, was not as tight as expected. This may be partly caused by the sampling method applied to this study. Lack of BB-1101 effects on the ultimate outcomes at 48 hours, especially on the brain water content, suggests the need to measure MMP activities following BB-1101 treatment to possibly maximize the intended therapeutic effects.

In summary, the Rosenberg group pioneered in the demonstration of an activated MMP pathway in the setting of cerebral ischemia with or without reperfusion. MMP expression may contribute to the postischemic BBB breakdown after ischemic stroke. However, additional studies are needed before the roles of MMPs in the pathogenesis of ischemic brain edema can be fully defined.
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*Stroke*. 1998;29:2189-2195
doi: 10.1161/01.STR.29.10.2189

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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