Matrix Metalloproteinase-12 Induces Blood–Brain Barrier Damage After Focal Cerebral Ischemia

Bharath Chelluboina, PhD; Jeffrey D. Klopfenstein, MD; David M. Pinson, PhD; David Z. Wang, DO; Raghu Venuganti, PhD; Krishna Kumar Veeravalli, PhD

Background and Purpose—Matrix metalloproteinases (MMPs) have a central role in compromising the integrity of the blood–brain barrier (BBB). The role of MMP-12 in brain damage after ischemic stroke remains unknown. The main objective of the current study is to investigate the effect of MMP-12 suppression at an early time point before reperfusion on the BBB damage in rats.

Methods—Sprague–Dawley rats were subjected to middle cerebral artery occlusion and reperfusion. MMP-12 shRNA–expressing plasmids formulated as nanoparticles were administered at a dose of 1 mg/kg body weight. The involvement of MMP-12 on BBB damage was assessed by performing various techniques, including Evans blue dye extravasation, 2,3,5-triphenyltetrazolium chloride staining, immunoblot, gelatin zymography, and immunofluorescence analysis.

Results—MMP-12 is upregulated ≈31-, 47-, and 66-fold in rats subjected 1–, 2-, or 4-hour ischemia, respectively, followed by 1-day reperfusion. MMP-12 suppression protected the BBB integrity by inhibiting the degradation of tight-junction proteins. Either intravenous or intra-arterial delivery of MMP-12 shRNA-expressing plasmid significantly reduced the percent Evans blue dye extravasation and infarct size. Furthermore, MMP-12 suppression reduced the endogenous levels of other proteases, such as tissue-type plasminogen activator and MMP-9, which are also known to be the key players involved in BBB damage.

Conclusions—These results demonstrate the adverse role of MMP-12 in acute brain damage that occurs after ischemic stroke and, thereby, suggesting that MMP-12 suppression could be a promising therapeutic target for cerebral ischemia. (Stroke. 2015;46:3523-3531. DOI: 10.1161/STROKEAHA.115.011031.)

Key Words: blood–brain barrier ■ degradation ■ ischemia ■ matrix metalloproteinase ■ reperfusion ■ tight junction ■ tissue-type plasminogen activator

The blood–brain barrier (BBB) is formed by the endothelial cells of cerebral microvessels, which are distinguished from peripheral endothelial cells by their lack of fenestrations, minimal pinocytic activity, and the tight junctions (TJs).1 In addition to endothelial cells, the BBB is composed of pericytes, astrocytes, neurons, and extracellular matrix.2 Endothelial cells and pericytes are surrounded by the extracellular matrix, which is composed of structural proteins, such as collagen type-IV, laminin, fibronectin, elastin, thrombospondin, and various proteoglycans. The TJs between the endothelial cells of the cerebral microvessels serve to restrict blood-borne substances from entering the brain. The BBB thus provides a dynamic interface between the peripheral circulation and the brain.

BBB disruption is an important contributing factor to brain injury that occurs after ischemic stroke. The expression of matrix metalloproteinases (MMPs) in the normal adult brain is very low or undetectable. In the injured brain, MMPs are expressed in various cell types, including resident and infiltrating inflammatory cells.3 However, the brain regions and cellular sources of expression differ for the specific MMPs, as well as the type, severity, and duration of injuries.4 Ischemia leads to induction of MMPs, which contribute to BBB extracellular matrix degradation. The induction of these MMPs may further perpetuate BBB TJ permeability, thereby leading to BBB leakage, leukocyte infiltration, brain edema, and hemorrhage.5 MMP-2 and MMP-9 are considered the central mediators of ischemic BBB disruption because of their ability to degrade components of microvascular basal lamina, especially collagen type-IV, and to disrupt TJ proteins.5–8

Recent investigations highlighted the possible pathological role for MMP-12 in the context of ischemic stroke.9,10 MMP-12 is upregulated several fold higher than any other MMPs tested after focal cerebral ischemia, and its suppression attenuated the ischemic brain damage.10 Also, MMP-12 is reported to activate other MMPs, such as pro-MMP-2 and...
pro-MMP-3, which, in turn, can activate pro-MMP-1 and pro-MMP-9.11 In addition, MMP-12 suppression after ischemia reduced MMP-9 activity in a rat model of focal cerebral ischemia and indicated that MMP-12 could be an upstream protease that can control the activity of other proteases, such as MMP-2 and MMP-9.10 Other potential mechanisms that could contribute to BBB integrity after MMP-12 suppression include reduced interferon (IFN)-α release from neuronal cells and tumor necrosis factor-α protein expression.2,13

Based on the reported data and our preliminary investigations, we hypothesize that MMP-12 suppression before commencement of reperfusion would inhibit TJ protein degradation and BBB disruption and, thereby, attenuate the brain damage that occurs after focal cerebral ischemia and reperfusion. We determined the effect of nonviral, nanoparticle-mediated intravenous/intra-arterial delivery of MMP-12 shRNA–expressing plasmid (M-12sh) on early brain damage in a rat model of middle cerebral artery occlusion (MCAO) followed by reperfusion. We report that a single dose of M-12sh formulation before instigation or immediately after reperfusion attenuates TJ protein degradation and preserves BBB integrity. To our knowledge, this is the first study to explore the deleterious role of MMP-12 on early brain damage after ischemic stroke.

Methods

Ethical Statement

All experiments were performed to comply with the Animal Research: Reporting In Vivo Experiments guidelines.14 The Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria approved all surgical interventions and postoperative animal care. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines. In addition, all the procedures that were performed on the animals were in compliance with the approved Institutional Animal Care and Use Committee protocol.

Experimental MCAO Model

After the animals reached a weight of 260±5 g, they were subjected to right MCAO procedure as previously described.13 After MCAO procedure, all animals were treated with appropriate analgesics and antibiotics and subjected to the approved postprocedural care.

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction and cDNA synthesis were performed as previously described.10 The forward and reverse primer sequences of rat MMP-12 used in this study have been reported earlier by our group.13 Reaction set-up for each cDNA sample was assembled using the IQ SYBR Green Supermix kit (Bio-Rad Laboratories, CA) as per the manufacturer’s instructions (see the online-only Data Supplement for further details).

Evans Blue Dye Extravasation

Leakage of Evans blue dye (EBD) in the ischemic brain tissue indicative of BBB disruption was analyzed 2 hours after MCAO (2h-I group) and 1 day after MCAO (sham group, 2h-I+1d-R group, M-12shIV+2h-I+1d-R group, and M-12shIVA+2h-I+1d-R group) using EBD (see the online-only Data Supplement for further details).

2,3,5-Triphenyltetrazolium Chloride Staining

Animals from various groups allocated for 2,3,5-triphenyltetrazolium chloride (Sigma) staining procedure were deeply anesthetized with pentobarbital and then decapitated. Brains were then removed rapidly and subjected to 2,3,5-triphenyltetrazolium chloride staining and infarct size measurement as described previously.10

Statistical Analysis

Results are expressed as the mean±SEM. Statistical comparisons were performed using Graph Pad Prism software (version 3.02). Quantitative data from either immunoblot analysis, gelatin zymography, or EBD extravasation assay were evaluated for statistical significance by 1-way ANOVA followed by Bonferroni’s multiple comparison test. Differences in the values were considered significant at P<0.05.

Results

MMP-12 Is Upregulated After Ischemia and Reperfusion

MMP-12 mRNA expression in the ischemic brains of rats was predominantly upregulated after ischemia with or without reperfusion. MMP-12 mRNA upregulation was also present in rats subjected to ischemia for 1 hour, the lowest ischemic duration tested, and, thereby, indicated that MMP-12 upregulation could occur early after ischemic injury. The upregulation was 72- to 3-fold in rats subjected to 1 or 2 hours of right MCAO as compared with sham-operated animals. Prominent upregulation of MMP-12 mRNA (≈25 fold versus sham) in ipsilateral brain tissue was noticed in rats after 4 hours of right MCAO (Figure 1A). MMP-12 mRNA expression was further increased after reperfusion of the ischemic brain. It was upregulated ≥31-, 47-, and 66-fold in rats subjected to 1-, 2-, or 4-hour ischemia, respectively, followed by 1-day reperfusion. These results demonstrated that it was not only the ischemia that contributes to MMP-12 mRNA upregulation, but also the reperfusion. In addition, MMP-12 mRNA levels were increased proportionally with the duration of ischemia. Protein expression of MMP-12 in the ischemic brains of rats was also increased after focal cerebral ischemia and reperfusion. Unchanged expression profile of GAPDH across various treatment groups confirmed the loading consistency for the gels. Unlike MMP-12 mRNA, the increase in MMP-12 protein expression was significant (P<0.05) in all the ischemic rat brains tested, except the samples obtained from animals subjected to a 1/2-hour right MCAO (Figure 1B). Immunofluorescence analysis also demonstrated a prominent increase in MMP-12 protein expression in the ischemia/reperfusion-induced rat brain sections (see Figure IA in the online-only Data Supplement). However, trends in protein expression were similar to those of mRNA expression. MMP-12 expression at 2-hour ischemia without reperfusion is colocalized with both microglia and neurons (see Figure IB in the online-only Data Supplement).

TJ Protein Expression Status After Ischemia and Reperfusion

The expression of TJ proteins, such as claudin-5, occludin, and zona occludens (ZO)-1, was evaluated by immunoblot analysis in the ischemic brains of rats that were subjected to various durations of ischemia with or without reperfusion as described in Table I in the online-only Data Supplement. The protein expression of claudin-5, occludin, and ZO-1 was prominent in
MMP-12 Knockdown After Cerebral Ischemia Reduces BBB Damage

Based on the objectives of the current study and the results presented in the previous sections of this article, we selected 2-hour ischemia and 1-day reperfusion as appropriate durations for the remaining experiments. M-12sh plasmids were constructed in pSilencer 4.1-CMV neo vector as described in the methods section of this article and synthesized from the overnight bacterial culture. A recent report from our laboratory has demonstrated the in vivo efficiency of M-12sh in rats when administered as a nonviral, nanoparticle formulation. In contrast to the previous study, wherein we administered M-12sh formulation intravenously via tail vein 1 day after reperfusion, in this study, the formulation was administered at the end of the 2-hour ischemia. Leakage of intravenously administered EBD into the ischemic brain tissue of rats that were subjected to a 2-hour ischemia without reperfusion demonstrates the disruption of BBB and provides a substantial support for the timing of treatment adopted in the present study (see Figure II in the online-only Data Supplement). To achieve the target delivery of our treatment and evaluate the differences with intravenous treatment, we injected the M-12sh formulation to a separate group of rats intra-arterially via the internal carotid artery with the help of a microcatheter, which was introduced into internal carotid artery after removal of the monofilament (see supplemental Figure III in the online-only Data Supplement). The reduced \((P<0.001)\) protein expression of MMP-12 in the ischemic brain tissue of rats after intravenous or intra-arterial delivery of M-12sh formulation demonstrated the in vivo efficiency of M-12sh (Figure 3A). There was no difference in intravenous and intra-arterial delivery of M-12sh for MMP-12 protein expression. Previous experiments demonstrated an absence of any difference between animals not treated with plasmids and those treated with scrambled sequence plasmids (vehicle controls). Thus, we did not use vehicle controls in these studies. Immunofluorescence analysis of the paraffin-embedded coronal brain sections obtained from various groups of rats also demonstrated a prominent decrease in the MMP-12 protein expression in the ischemia/reperfusion-induced rat brain sections after M-12sh treatment as compared with untreated rat brain sections (see Figure 1A in the online-only Data Supplement).

EBD extravasation into the ischemic brain was prominent in rats subjected to a 2-hour right MCAO and 1-day reperfusion (Figure 3B). Either intravenous or intra-arterial delivery of M-12sh formulation significantly \((P<0.05)\) reduced the percent EBD extravasation from \(33.09\pm4.58\) in untreated ischemia/reperfusion-subjected animals to \(15.93\pm4.5\) and \(11.48\pm5.57\), respectively. In addition, MMP-12 suppression achieved after intravenous administration of M-12sh formulation significantly \((P<0.05)\) reduced the infarct volume as evidenced by \(2,3,5\)-triphenyltetrazolium chloride staining (Figure 3C). These results demonstrated that the knockdown of MMP-12 after ischemia reduces the brain damage by protecting the integrity of the BBB. As expected, suppression of MMP-12 after focal cerebral ischemia preserved the protein expression of TJ proteins, such as claudin-5, occludin, and ZO-1 (Figure 4). The increase in protein expression of these TJ proteins in the ipsilateral brains of ischemia/reperfusion-induced rats is significant \((P<0.001)\) after MMP-12 knockdown compared with untreated rats. In addition, all these TJ proteins were colocalized with astrocytes in the ischemic brain regions of untreated, ischemia/reperfusion-induced rats (Figure 5A). However, the colocalization of astrocytes with the TJ proteins was absent in sham-operated and M-12sh-treated rats. Colocalization of TJ proteins with endothelial cells was seen in the ischemic hemisphere of sham-operated and M-12sh-treated rats (Figure 5B). However, the TJ protein expression was less associated with endothelial cells in the brain tissue of sham-operated rats. As expected, a decrease in expression of these TJ proteins is noticed after ischemia with or without reperfusion (Figure 2A). Although not significant, expression of TJ proteins was decreased in animals subjected to 1-hour ischemia (with or without reperfusion), the lowest ischemic duration tested in the present investigation. The decrease in protein expression was prominent and significant \((P<0.05)\) in rats subjected to 2- or 4-hour ischemia with or without reperfusion (Figure 2B). These results indicated that the degradation of TJ proteins in rats was initiated as soon as the initiation of ischemia induction.
ischemia, untreated rats. Altogether, these results indicate that the early release or increased expression of MMP-12 in the brain, in response to focal cerebral ischemia and reperfusion, degrades the TJ proteins, which constitutes the BBB and, thereby, contributes to the disruption of BBB and early brain damage.

**Influence of MMP-12 Knockdown on Other Proteases, Such as MMP-9 and Tissue-Type Plasminogen Activator**

Gelatin zymography revealed a prominent increase in MMP-9 activity after reperfusion in rats subjected to various durations of ischemia (Figure 6A). The increase was significant (P<0.001) in rats subjected to 2 hours of ischemia followed by 1-day reperfusion as compared with sham group (Figure 6B). Administration of M-12sh formulation by either intravenous or intra-arterial route before reperfusion significantly (P<0.001) reduced the increased MMP-9 activity. Although some reduction in MMP-2 was observed after MMP-12 suppression, especially when the formulation was administered by intra-arterial route, this reduction was not significant. Further, MMP-12 suppression did not affect MMP-3 protein expression as it did to the MMP-9 expression (see Figure IV in the online-only Data Supplement).

The protein expression of tissue-type plasminogen activator (tPA) was significantly (P<0.001) increased in rats subjected to focal cerebral ischemia and reperfusion (Figure 6C and 6D). Unchanged expression profile of GAPDH across various treatment groups confirmed the loading consistency for the gels. MMP-12 knockdown, irrespective of the route of administration, significantly (P<0.001) reduced the increased tPA expression. There was no significant difference in MMP-9 activity and tPA expression after MMP-12 knockdown compared with sham group.

**Discussion**

A pathogenetic role for MMP-12 after ischemic stroke has been recently reported by our group. Of all the MMPs, MMP-12 showed the highest upregulation at all the time points tested after focal cerebral ischemia. The results of the current study further demonstrated that it is not only the ischemia that induces MMP-12 expression in the ischemic brain, but also the reperfusion that contributes to predominant and significant elevation of MMP-12 (Figure 1). Although the pathological role of this matrix metalloelastase is reasonably documented in lung diseases, such as chronic obstructive pulmonary disease, emphysema, and asthma, this important protease has not been well-studied, despite its detrimental role in the context of ischemic stroke. MMP-12 suppression after ischemic stroke attenuated the brain damage in a rat model of transient focal cerebral ischemia. Unlike earlier studies, the current study is focused on suppressing MMP-12 at an early time point, rather than hours after reperfusion. In addition, formulation was administered by intra-arterial route to achieve target delivery of the formulation to ischemic brain. Despite our efforts, no different treatment outcome was noticed between intravenous and intra-arterial administrations. However, there is a possibility that a small dose of formulation by intra-arterial delivery could offer the same therapeutic benefit as a large dose of intravenous formulation, which was not studied in the current investigation. Although several research groups used adenoviral or lentiviral vectors to administer shRNA-expressing plasmids of target genes to attain the efficiency in vivo, M-12sh in this study were administered as a nonviral, nanoparticle-based formulation. In vitro and in vivo efficiency, as well as in vivo efficacy, of M-12sh formulation used in this study was already demonstrated by our group. In addition, we now demonstrate the in vivo efficiency and efficacy of the same formulation after intra-arterial administration. Because we recently reported the efficiency and specificity of plasmids expressing

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2.** Effect of cerebral ischemia with or without reperfusion on the expression of tight junction proteins. A, Representative immunoblots depicting the protein expression of tight junction proteins. B, Bar graphs represent the densitometry analysis of immunoblot. Error bars indicate SEM. n=6. *P<0.05 vs sham group. I indicates ischemia; and ZO, zona occludens.
MMP-12shRNA as compared with plasmids expressing a scrambled sequence shRNA (vehicle control), we did not use additional animals in the present study for vehicle control treatment. BBB disruption after ischemic stroke can be continuous or occur transiently in 2 distinct phases. In experimental studies, BBB opening is biphasic; the initial breakdown is most likely caused by oxidative stress and is followed by a partial BBB recovery before the second phase of increased BBB permeability leads to neutrophil infiltration through TJ redistribution. An important question addressed in this study is whether a 2-hour ischemia in rat is enough to cause BBB disruption. MMP-12sh formulation administered to rats at the end of 2-hour ischemia was able to reach the brain cells in the ipsilateral hemisphere, caused MMP-12 gene silencing, and thereby indicated opening of the BBB in these subjects. EBD extravasation into the ischemic brain regions of these animals further strengthened these results and provided rationale for the timing of treatment instituted in the current investigation. Based on EBD extravasation in Sprague–Dawley rats subjected to a 2-hour MCAO using an intraluminal suture, some degree of BBB disruption occurred at 3 to 4 hours, maximal disruption at 5 hours, and delayed disruption at 48 to 50 hours after reperfusion. In the same 2-hour MCAO model, the current study demonstrated the occurrence of some degree of BBB disruption at the end of 2-hour ischemia without reperfusion. Given the EBD staining of ischemic areas, despite the presence of monofilament that occluded the MCA at its origin, there must be minor collaterals supplying some blood to the ischemic area. In any case, this experiment demonstrates that 2-hour ischemia is sufficient to deliver the administered formulation to ischemic brain cells.

The BBB is not a static barrier but tightly regulated, allowing changes in vascular permeability. TJs play a key role in the functional preservation of the BBB. TJ-associated proteins claudin-5, occludin, and ZO-1, which have different molecular
structures and regulation characteristics, play important roles in the maintenance of structure and function of TJ. The expression of these TJ proteins directly correlates with BBB permeability and vasogenic edema. In this study, the decreased expression of TJ proteins is noticed as early as 1 hour after ischemia induction. As expected and discussed earlier, TJ protein degradation is predominant in rats subjected to 1-day reperfusion subsequent to a 1-, 2-, or 4-hour ischemia.

We hypothesize that MMP-12 by itself or by regulating the activity of other important MMPs, such as MMP-9 and MMP-2, could contribute to BBB disruption by degrading ≥1 extracellular matrix and TJ proteins. As expected, MMP-12 suppression in this study significantly reduced the EBD extravasation into the ischemic brain and TJ protein degradation and, thereby, protected the BBB integrity after ischemic stroke. MMP and serine protease gene families have been shown to degrade proteins of the basal lamina around the cerebral blood vessels. This attack on the basal lamina exposes the TJ proteins, such as claudin-5, occludin, and ZO-1. Occludin, a regulatory protein that can alter paracellular permeability assembles with claudin-5 into heteropolymers and intramembranous strands. Occludin also anchors the claudins to ZO and the plasma membranes of adjacent cells. MMPs, such as MMP-9 and MMP-2, have been shown to degrade TJ proteins after ischemia and reperfusion. Reports indicate that MMPs and tPA play critical roles in the BBB disruption during acute ischemic stroke. In the current study, MMP-12 suppression significantly reduced the activity of MMP-9 and protein expression of tPA, a serine protease that is well-known to contribute to BBB disruption after ischemic stroke (Figure 6). Therefore, the BBB

![Figure 4](image.png)

**Figure 4.** Effect of matrix metalloproteinase-12 (MMP-12) suppression on the expression of tight junction proteins in ischemic brains of rats. Representative immunoblots depicting the protein expression of tight junction proteins in the ischemic brain of rats subjected to ischemia and reperfusion with or without MMP-12 shRNA-expressing plasmid (M-12sh) treatment. Bar graphs represent the densitometry analysis of immunoblots. n=6. *P<0.05 vs sham group; **P<0.05 vs 2h-I+1d-R rats. IA indicates intravascular; IV, intravenous; and ZO, zona occludens.

![Figure 5](image.png)

**Figure 5.** Effect of matrix metalloproteinase-12 (MMP-12) suppression on colocalization of astrocytes or endothelial cells with tight junction proteins. A. Immunofluorescence analysis showing the protein expressions of astrocytes (GFAP, red) and tight junction proteins (green) in the ischemic hemisphere of rats subjected to a 2-h ischemia and 1-day reperfusion with or without treatments. Arrows in the merged images point the colocalization (yellow) of astrocytes with tight junction proteins. B. Immunofluorescence analysis showing the expression of endothelial RECA-1 (red) and tight junction proteins (green). Arrows in the merged images point the absence of colocalization (yellow) of endothelial cells with tight junction proteins. Nuclei were stained with DAPI (blue). Each image is the magnified portion of the confocal images obtained and is representative of at least 6 images obtained from 6 different animals.
protection offered after MMP-12 suppression in this study could be (1) a direct therapeutic effect of MMP-12 suppression, (2) mediated via MMP-9 and tPA, or (3) a combination of both. Recently, it was reported that the intracellular MMP-12 lead to IFN-α secretion, whereas the extracellular MMP-12 cleaved IFN-α receptor 2–binding site of systemic IFN-α.12 IFN-α is a master cytokine capable of inducing the production of other proinflammatory cytokines, such as interleukin-1, interleukin-2, interleukin-6, tumor necrosis factor, and IFN-γ. In our earlier study, MMP-12 suppression after ischemic stroke reduced tumor necrosis factor-α protein expression and inhibited apoptosis.10 Therefore, a possible reason for the decrease in tumor necrosis factor-α protein expression after MMP-12 suppression in our ischemic stroke model could be the suppression of IFN-α release. The release of IFN-α from MMP-12-expressing neuronal cells after cerebral ischemia and during reperfusion could also contribute to BBB damage.

Earlier studies have consistently shown that astrocytes are necessary for maintenance and maturation of the BBB.31 Further, astrocyte end-foot contacts have been shown to mediate regional cerebral blood flow.32 Astrocyte end-feet around the blood vessels were clearly separated from the TJ proteins, including claudin-5, occludin, and ZO-1 in sham-operated rats. Cross sections of blood vessels in these animals showed a continuous rim or line of TJ protein immunostaining in the endothelial cell clefts. In contrast, disrupted appearance of TJ proteins around the endothelial cells was noticed in the ischemic hemisphere at 24 hours after reperfusion (Figure 5). In addition,
GFAP-positive immunoreactivity in the region of the blood vessels was colocalized with degraded TJ proteins in the ischemic hemisphere. These results are completely in agreement with the earlier report wherein the authors clearly demonstrated colocalization of astrocytes around the blood vessels in the ischemic hemisphere with TJ proteins at 24 hours after reperfusion. Whether the astrocytes take up the degraded TJ proteins or synthesize them in response to the injury will require further study. Synthesis of TJ proteins by astrocytes in response to ischemia/reperfusion injury might occur at longer reperfusion times as a part of neurovascular remodeling process. Therefore, in this study, the appearance of TJ proteins in the astrocytes around the blood vessels at 24 hours after reperfusion could be attributed to the uptake of degraded TJ proteins by astrocytes. As expected, MMP-12 suppression after ischemia prevented TJ protein degradation and diminished the appearance of TJ proteins in astrocytes around the blood vessels.

To summarize, this study clearly demonstrates the pathological role of MMP-12 in causing the BBB disruption after ischemia and reperfusion. Although we could test only a single dose of M-12sh formulation, the tested dose was effective in demonstrating the early brain damage caused by MMP-12 after focal cerebral ischemia. Considering the multiphasic role played by MMPs, further investigations are warranted to determine whether the long-term suppression of MMP-12 after ischemic stroke is beneficial.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

MMP-12 induces blood-brain barrier damage after focal cerebral ischemia

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SUPPLEMENTAL METHODS

Animals and experimental design
Young adult male Sprague–Dawley rats were used in this study. A total of 137 rats weighing 200–240 g were procured from Harlan Laboratories and maintained in a specific pathogen free Laboratory Animal Care Facility of University of Illinois College of Medicine at Peoria. Animals were housed in a 12-h light/dark cycle at a controlled temperature and humidity with free access to food and water. After the animals reached a weight of 260±5 g, they were assigned to nine groups as described in Supplemental Table 1. Based on the literature and after considering 10-20% mortality associated with middle cerebral artery occlusion (MCAO) procedure, we needed these numbers of animals to obtain data from at least six animals for each experiment and attain a proper statistical analysis. Animals that did not show post-stroke symptoms after MCAO procedure were excluded from the study. Animals that showed post-stroke symptoms after MCAO procedure were randomly allocated to groups 2 to 9.

Antibodies
Anti-glial fibrillary acidic protein (GFAP) was obtained from Dakocytomation (Carpinteria, CA). Anti-MMP-12, anti-claudin-5, anti-occludin, anti-ZO-1, and anti-GFAP were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-claudin-5, anti-occludin, and anti-ZO-1 were also obtained from Life Technologies (Carlsbad, CA). Anti-tissue plasminogen activator (tPA) and anti-MMP-3, Iba1 and RECA-1 were obtained from abcam (Cambridge, MA). Anti-NeuN antibody was obtained from Millipore (Billerica, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Novus Biologicals (Littleton, CO).

Synthesis of shRNA expressing plasmids
We designed, constructed, and synthesized rat MMP-12 shRNA (M-12sh) plasmid by using pSilencer™ 4.1-CMV neo vector. The resultant vectors inserted with shRNAs were transformed into chemically competent E. coli cells (JM109 competent cells) and cultured overnight. M-12sh expressing plasmids were synthesized from the overnight bacterial culture by using QIAGEN plasmid mini/maxi kit according to the manufacturer’s protocol. Positive clones were confirmed by gene sequencing analysis.

Formulation preparation and administration
MMP-12 shRNA expressing plasmids were formulated as nanoparticles, which are sufficiently small to diffuse into the tissues and enter the cells by endocytosis. M-12sh expressing plasmids, synthesized by using QIAGEN plasmid maxi kit, were further purified by phenol-chloroform extraction procedure. In vivo-JetPEI, obtained from Polyplus transfection (Illkirch, France) contains a cationic polymer that efficiently condenses plasmid DNA. M-12sh expressing plasmids were formulated with in vivo-JetPEI and sterile water for injection using aseptic conditions according to the manufacturer’s instructions. M-12sh formulation was administered at a dose of 1 mg/kg body weight to designated groups of rats by either intravenously (IV) or intra-arterially (IA). In case of IV administration, formulation was slowly injected via tail vein at the end of the MCAO procedure, i.e., after reestablishing the blood flow to the ischemic brain. In case of IA administration, formulation was slowly injected directly into the ischemic brain via a micro catheter which was introduced into the right internal carotid artery after removal of the monofilament.
Quantitative real-time PCR

Samples were subjected to forty cycles at 95°C for fifteen seconds and 60°C for one min in iCycler IQ (Multi Color Real-Time PCR Detection System, Bio-Rad Laboratories, CA). Data were collected and recorded using the iCycler IQ software (Bio-Rad Laboratories, CA) and expressed as a function of the threshold cycle (Ct), which represents the number of cycles at which the fluorescent intensity of the Sybr Green dye is significantly above than that of the background fluorescence. The housekeeping gene, β-actin was used for normalization of MMP-12 expression. Average Ct values were normalized with average Ct values of β-actin. After normalization of the Ct values, fold differences were calculated by using the formula 2^-(ΔCt of Test) / 2^-ΔCt of controls.

Immunoblot analysis

In order to study the expression of various proteins in the ischemic brain tissues of sham, untreated and treated MCAO subjected rats with/without reperfusion; immunoblot analysis was performed. Briefly, ipsilateral brain hemispheres of rats from various groups were suspended in 0.2 mL of homogenization buffer, homogenized using a Tissue Tearor (Biospec Products, Inc.), and followed by sonication. Tissue homogenate was centrifuged at 15,000 x g for 30 min at 4°C, and the protein levels in the supernatant were determined using the BCA assay (Pierce, Rockford, IL). Samples [equal amount (30-50 μg) of total protein/well] were subjected to 8-14% SDS-PAGE based on the specifications of the protein, and the protein bands on the gel were transferred onto nitrocellulose membranes. The membranes were processed with primary antibodies followed by appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using chemiluminescence ECL Western blotting detection reagents on Hyperfilm-MP autoradiography film (Amersham, Piscataway, NJ). Immunoblots were re-probed and processed with GAPDH antibody and the measurements were normalized to loading controls.

Immunofluorescence analysis

Paraffin-embedded brain sections of various groups of animals were subjected to immunofluorescence analysis. Briefly, paraffin-embedded rat coronal brain sections were de-paraffinized, subjected to antigen retrieval, permeabilized, and processed with primary antibody. The sections were then washed in PBS and incubated with the appropriate fluorescent-labeled secondary antibodies, counterstained with DAPI, cover slipped, and observed using a confocal microscope (Olympus Fluoview). Negative controls (without primary antibody or using isotype specific IgG) were maintained for all the samples. Immunofluorescence analysis was also performed to identify the colocalization of astrocytes and endothelial cells with the tight junction proteins such as claudin-5, occludin and ZO-1 as well as MMP-12 with neurons and microglia.

EBD extravasation

Briefly, EBD (2% in saline, ~4ml/kg) was injected over two min into the tail vein and allowed to circulate for 60 min. After intravenous administration, EBD binds rapidly to serum albumin and its extravasation indicates the passage of albumin through the BBB into the parenchyma. Rats were deeply anaesthetized with pentobarbital and transcardially perfused with PBS followed by 10% buffered formalin. Rats were decapitated and brains were removed rapidly and placed in an adult rat brain matrix (Kent Scientific Corporation). Brains were then sliced into 2-mm-thick
coronal sections. EBD leaked area and hemisphere area of each section was traced and measured with National Institute of Health Image J software (version 1.46b). BBB disruption was quantified by using the formula: EBD extravasation (%) = \{\text{(volume of contralateral hemisphere) } – \text{(volume of EBD unstained ischemic hemisphere)}\} / \text{volume of contralateral hemisphere}. This formula accounts for the possible interference of brain edema.

**Gelatin Zymography**

Tissue lysates obtained from the ischemic brains of various groups of animals as well as the respective brain tissue of sham group animals with equal amounts of protein were subjected to 10% polyacrylamide gels that were co-polymerized with substrate (2 mg/mL gelatin) at 4°C. The proteins were renatured by washing the gel twice (each time for 30 min) with 2.5% Triton X-100 and then incubated in substrate buffer (50 mM Tris–HCl, 5 mM CaCl$_2$, pH 7.6) for 48 h at 37°C. After a rinse in water, the gel was stained with amido black and destained for appropriate color contrast.
### SUPPLEMENTAL TABLE I

**Description of experimental groups**

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Group Designation</th>
<th>Group Description</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Immunoblot/ Real-time PCR/Zymography</strong></td>
<td><strong>Immunofluorescence analysis</strong></td>
</tr>
<tr>
<td>1</td>
<td>sham</td>
<td>Rats subjected to the MCAO procedure without monofilament insertion</td>
<td>6</td>
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<tr>
<td>2</td>
<td>1h-I</td>
<td>Rats subjected to MCAO with a 1 hour monofilament insertion</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>2h-I</td>
<td>Rats subjected to MCAO with a 2 hour monofilament insertion</td>
<td>6</td>
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<tr>
<td>4</td>
<td>4h-I</td>
<td>Rats subjected to MCAO with a 4 hour monofilament insertion</td>
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<tr>
<td>5</td>
<td>1h-I+1d-R</td>
<td>Rats subjected to MCAO with a 1 hour monofilament insertion and 1 day reperfusion</td>
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<tr>
<td>6</td>
<td>2h-I+1d-R</td>
<td>Rats subjected to MCAO with a 2 hour monofilament insertion and 1 day reperfusion</td>
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<td>7</td>
<td>4h-I+1d-R</td>
<td>Rats subjected to MCAO with a 4 hour monofilament insertion and 1 day reperfusion</td>
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<tr>
<td>8</td>
<td>M-12shIV+2h-I+1d-R</td>
<td>Rats subjected to MCAO with a 2 hour monofilament insertion and 1 day reperfusion, and M-12sh IV treatment</td>
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<tr>
<td>9</td>
<td>M-12shIA+2h-I+1d-R</td>
<td>Rats subjected to MCAO with a 2 hour monofilament insertion and 1 day reperfusion, and M-12sh IA treatment</td>
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MCAO-middle cerebral artery occlusion; EBD-Evans blue dye; I-ischemia; R-reperfusion; IV-intravenous; IA-intra-arterial
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

**Supplemental Figure I**
MMP-12 protein expression in the rat ischemic hemisphere. **A,** Representative photographs of immunofluorescence analysis depicting MMP-12 protein expression (green fluorescence) in the ischemic brain regions of rats subjected to ischemia and reperfusion with or without M-12sh treatment. n=6. **B,** Immunofluorescence analysis showing the protein expressions of MMP-12 (green fluorescence) and NeuN/Iba1 (red fluorescence) in the ipsilateral brain regions of rats subjected a two-hour MCAO procedure. Yellow fluorescence in the merged images indicates the cellular localization of MMP-12. Nuclei were stained with DAPI. n=6.

**Supplemental Figure II**
Evans blue dye (EBD) extravasation in rats after focal cerebral ischemia. Representative photographs of coronal sections of brains after EBD extravasation in rats subjected to ischemia. Leakage of EBD (blue area in the right hemisphere) after ischemia indicates BBB disruption. n=6.
Supplemental Figure III
Insertion of monofilament or micro catheter into the rat internal carotid artery. A, Photomicrograph showing the bifurcation of rat right common carotid artery (CCA) into external carotid artery (ECA) and internal carotid artery (ICA). B, Photomicrograph depicting the insertion of monofilament into ICA via ECA. Yellow arrow is pointing the monofilament. C, Photomicrograph depicting the insertion of micro catheter into ICA via ECA after removal of the monofilament. Yellow arrow is pointing the micro catheter.

<table>
<thead>
<tr>
<th>M-12sh</th>
<th>Reperfusion</th>
<th>Ischemia</th>
<th>Sham</th>
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<td>-</td>
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<td>IA</td>
<td>1d</td>
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Supplemental Figure IV
Representative immunoblots depicting the protein expression of MMP-3 and MMP-9. GAPDH served as a loading control. n=3. IV-intravenous; IA-intra-arterial.