Matrix Metalloprotease 3 Exacerbates Hemorrhagic Transformation and Worsens Functional Outcomes in Hyperglycemic Stroke

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Background and Purpose—Acute hyperglycemia worsens the clinical outcomes and exacerbates cerebral hemorrhage after stroke. The mediators of hemorrhagic transformation (HT) in hyperglycemic stroke are not fully understood. Matrix metalloproteinase 3 (MMP3) plays a critical role in the tissue-type plasminogen activator–induced HT. However, the role of MMP3 in exacerbating the HT and worsening the functional outcomes in hyperglycemic stroke remains unknown.

Methods—Control/normoglycemic and hyperglycemic (blood glucose, 140–200 mg/dL) male Wistar rats were subjected to middle cerebral artery occlusion for 90 minutes and either 24 hours or 7 days reperfusion. MMP3 was inhibited pharmacologically (UK 356618, 15 mg/kg IV at reperfusion) or knocked down in the brain by shRNA lentiviral particles (injected intracerebroventricular). Neurovascular injury was assessed at 24 hours, and functional outcomes were assessed at 24 hours, day 3, and day 7. MMP3 activity was measured in brain homogenate and cerebral macrovessels. Localization of MMP3 within the neurovascular unit after hyperglycemic stroke was demonstrated by immunohistochemistry.

Results—Hyperglycemia significantly increased MMP3 activity in the brain after stroke, and this was associated with exacerbated HT and worsened functional outcomes. MMP3 inhibition significantly reduced HT and improved functional outcomes.

Conclusions—MMP3 plays a critical role in mediating cerebrovascular injury in hyperglycemic stroke. Our findings point out MMP3 as a potential therapeutic target in hyperglycemic stroke. (Stroke. 2016;47:843-851. DOI: 10.1161/STROKEAHA.115.011258.)

Key Words: blood glucose ▪ brain ischemia ▪ hyperglycemia ▪ intracranial hemorrhages ▪ stroke
Materials and Methods

Animal Models and Study Groups

The animals were housed at the Georgia Regents University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. This study was conducted in accordance with the National Institute of Health guidelines for the care, and the use of animals in research and all protocols were approved by the institutional animal care and use committee. All the Stroke Therapy Academic Industry Roundtable (STAIR) and RIGOR recommendations and guidelines about randomization, blinding, and statistical analysis were followed in this study.14,15

Study 1: Determine the Effect of Hyperglycemia on MMP3 Activity in the Brain and Localization Within the Neurovascular Unit During the Acute Phase of Ischemic Stroke

Male Wistar rats (Harlan Laboratories Inc., Indianapolis, IN) were randomly assigned in a block size of 2 rats per cage to 2 different groups: (1) control/normoglycemic (blood glucose [BG], 90–120 mg/dL) and (2) mild hyperglycemia (BG, 140–200 mg/dL). Animals were subjected to 90-minute middle cerebral artery occlusion (MCAO) by the suture occlusion method and 24 hours of reperfusion as previously described.8 In all groups, animals with reduction in cerebral blood flow <40% from baseline were excluded. A 30% glucose solution was used to achieve blood glucose levels of 140 to 200 mg/dL. Acute hyperglycemia was achieved through intraperitoneal injection of 2-mL glucose solution 15 minutes before MCAO. A second dose was given just after the stroke surgery to maintain hyperglycemia through 90 minutes of ischemia. At 24 hours, brains and macrovessels (Circle of Willis vessels) were isolated and processed for MMP3 activity assay and immunolocalization studies.

Study 2: Determine the Effect of MMP3 Inhibition on Reducing HT and Improving Outcomes in Hyperglycemic Stroke

Study 2.1: The Effect of Pharmacological Inhibition of MMP3 on Short-Term and Delayed Functional Outcomes

Male Wistar rats were randomly assigned in a block size of 3 rats per cage to the following groups: (1) control/normoglycemic (BG, 90–120 mg/dL), (2) mild hyperglycemia (BG, 140–200 mg/dL), and (3) mild hyperglycemia+MMP3 inhibitor. In this study, we used a potent and highly selective MMP3 inhibitor (UK 356618, Tocris Bioscience). The drug was intravenously injected through the jugular vein (15 mg/kg) at reperfusion. The dose was determined according to a dose titration study and a dose response curve to achieve an inhibition of MMP3 activity of ≥50% from nontreated animals (data not shown). Acute hyperglycemia and stroke were induced as in Study 1 section. For short-term outcomes, neurovascular injury and functional outcomes were measured at 24 hours. To assess delayed outcomes, same experimental design was used and additional group of animals were randomly assigned to 3 groups, and the neurobehavioral outcomes were assessed at days 1, 3, and 7.

Study 2.2: MMP3 Knockdown in the Brain via Stereotaxic Injection

Male Wistar rats were randomly assigned in a block size of 4 into 4 different groups. All animals were anesthetized with isoflurane and immobilized on a stereotaxic device 2 weeks before MCAO. MMP3 shRNA lentiviral particles (5 μL of 1x108 TU/mL, SMART choice lentiviral rat MMP3 shRNA, cat no. SH-092769-02, GE Healthcare Dharmacon Inc) or an empty vector was injected in both hemispheres >10 minutes/ side via a 30-gauge needle into the lateral ventricles. The stereotaxic coordinates were +0.9 mm anterior, ±1.5 mm lateral, and −4 mm ventral relative to bregma. The study groups were (1) normoglycemia+empty vector, (2) hyperglycemia+empty vector, (3) normoglycemia+MMP3 shRNA, and (4) hyperglycemia+MMP3 shRNA. MMP3 knockdown was confirmed by Western blot analysis.

Sample Size and Power

Power analysis was based on the preliminary data for MMP3 activity for normoglycemic animals after stroke (mean±SD, 250±50 MFU). MMP3 activity for hyperglycemic animals was assumed to be 50% greater than that of normoglycemia (375 MFU), and the addition of the MMP3 inhibitor to hyperglycemic animals was hypothesized to reduce the activity to normoglycemic levels. A sample size of 6 animals per group was predicted to yield at least 95% power to detect this difference as significant using 1-way ANOVA at α=0.05. In some experiments, vessels could not be harvested and extra animals were included which resulted in n=7 to 9 as indicated in figure legends (Figure 1).

Evaluation of Neurobehavioral and Functional Outcomes

Neurobehavioral tests (Bederson score, beam walk, and grip strength) were assessed on a 14-point scale by a blinded investigator as previously described.8

Evaluation of Infarct Size, Edema, HT, and Hemoglobin Content and Mortality

Infarct size, HT, edema, and hemoglobin content were measured by a blinded investigator. Macroscopic HT was scored in coronal brain slices B to E using a 4-point rubric (0, no hemorrhage; 1, dispersed individual petechiae; 2, confluent petechiae; 3, small diffuse hemorrhage or hematoma; and 4, large diffuse hemorrhage or hematoma), and the total score for each animal was reported. The infarct size was measured after staining the brain slices with 2,3,5-triphenyl tetrazolium chloride as previously described.13,14 Edema was calculated as a percent (%) area increase in the ischemic hemisphere versus the contralateral hemisphere. Hemoglobin content (μg hemoglobin/mg total protein) was measured using brain homogenates prepared from 2,3,5-triphenyl tetrazolium chloride sections with Quanti-Chrom kit (BioAssay Systems, Hayward, CA; 2007) and reported as excess hemoglobin (the hemoglobin values for the sham animals were subtracted from hemoglobin values obtained for the stroke animals) in the ischemic hemisphere normalized to sham animals.8 Dead animals were counted for mortality rates.

Evaluation of MMP3 Protein Expression

MMP3 in brain homogenate was analyzed by Western blot analysis. In brief, equal volumes of homogenized brain tissues (30-μg total protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. MMP3 was determined by using anti-MMP3 antibody (1:500; 14351, cell signaling). Primary antibodies were detected using horseradish peroxidase-conjugated antibody and enhanced chemiluminescence. Band intensity was quantified by densitometry software (Alpha Innotech, Santa Clara, CA).

Evaluation of MMP3 Enzymatic Activity

The enzymatic activity of MMP3 was determined using a fluorescence resonance energy transfer peptide and immunocapture assay as previously described elsewhere with minor modifications.15 Briefly, 50-μg total protein of macrovascular or brain homogenates were incubated at 4°C for 2 hours with rabbit polyclonal anti-MMP3 antibody (cat no. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX). A/G agarose...
beads were then added and allowed to incubate overnight at 4°C. The beads were then washed and samples were transferred to black 96-well plate and 100 μL of 2 mmol/L 5-FAM/QXL 520 fluorescence resonance energy transfer peptide (cat no. 60580-01; AnaSpec, San Jose, CA) in assay buffer were added per well. Plates were incubated for 8 hours at 37°C, then relative fluorescence units were read and monitored at excitation/emission wavelengths of 485 of 528 nm in a Synergy HT multimode microplate fluorescence reader (BioTek, Winooski, VT) running Gen5 data analysis software.

Immunohistochemistry and Colocalization Studies
Brain coronal sections for immunohistochemistry were prepared after the standard methods. Briefly, at 24 hours, brains were isolated, fixed in 4% paraformaldehyde for 48 hours and cryoprotected in 30% sucrose at 4°C. Cryostat sections were incubated overnight with rabbit polyclonal anti-MMP3 antibody (1:500, cat no. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. For the colocalization studies, brain sections were then incubated with either anti-NeuN antibody (ABN78, EMD Millipore) as neuronal marker, isolectin B4 (IB4, I21413, Invitrogen Life Technologies) as endothelial marker, glial fibrillary acidic protein (GFAP; Ab5804, Millipore) as marker for astrocytes or normoglycemia 2 (0.5±7, Millipore) as a pericyte marker. This was followed by reaction with fluorescent-conjugated secondary antibody; goat anti-rabbit (1:1000, Invitrogen Life Technologies). Images were acquired from regions B, C, and D (+3 to −3 mm from bregma) as regions of interest in the regions of interest with maximal brain infarct area. Three slides were obtained from each brain with 9 fields tested in the regions of interest within the ischemic border zone. Brain sections were examined using Axiovert200 Microscope (Carl Zeiss Micro-Imaging, Thornwood, NY).

Statistical Analysis
Data are presented as mean±SEM. A 2 hyperglycemia (no versus mild hyperglycemia) by 6 Time (baseline, 15-minute pre-MCAO, 45-minute MCAO, 90-minute MCAO, 30-minute reperfusion, and 24 hours) (repeated measures ANOVA) was used to determine changes in blood glucose over time dependent on hyperglycemia status. Stroke outcomes included MMP3 activity in brain homogenate and macrovessels, percent infarct size and edema, HT index, excess hemoglobin, and the functional outcome composite score and grip strength. The effect of addition of the MMP3 inhibitor was determined using a 1-way ANOVA with 3 groups (control, hyperglycemia, and hyperglycemia+MMP3 inh). The effect of hyperglycemia and protein knockdown by shRNA was assessed using a 2 hyperglycemia (no versus yes) by 2 shRNA (no versus yes) ANOVA, where a significant interaction would indicate a differential effect of shRNA on stroke outcomes dependent on hyperglycemia status. SAS 9.3 (SAS, Inc, Cary, NC) was used for all analyses. Statistical significance was determined at α <0.05 and a Tukey post hoc test was used to compare means from significant ANOVAs. Pearson product moment correlations were used to assess the linear association between BG area under the curve, MMP3 activity, and the behavior composite score.

Results

Effect of Hyperglycemia on MMP3 Activity
The use of 30% glucose solution achieved and maintained blood glucose levels of 140 to 200 mg/dL through 90 minutes of ischemia, which is significantly higher than control normoglycemic animals (80–120 mg/dL; Figure 1A). Acute mild hyperglycemia significantly increased MMP3 activity in cerebral macrovessels and brain homogenates, and the use of MMP3 inhibitor (UK 356618) at reperfusion significantly reduced MMP3 activity in the brain (Figure 1B and 1C). MMP3 activity in the brain (r=0.73) and macrovascular (r=0.87) homogenates correlated positively with blood glucose (area under the curve) levels (P<0.05).

Spatial Expression of MMP3 in Neurovascular Unit in Acute Hyperglycemic Stroke
MMP3 staining was evident in the brains of normoglycemic and hyperglycemic animals after stroke and colocalized with NeuN-positive neurons, isolectin-positive cerebral vessels, and normoglycemia 2-positive pericytes in the peri-infarct region. However, MMP3 was not detected in GFAP-positive astrocytes (Figure 2).

Effect of Early Inhibition of MMP3 on Short-Term and Delayed Outcomes in Hyperglycemic Stroke
Mild hyperglycemia did not increase infarct size (Figure 3A) but exacerbated vascular injury as indicated by greater edema and HT when compared with the control group (Figure 3B–3D) at 24 hours. The pharmacological inhibition of MMP3 significantly reduced HT, edema, and hemoglobin content in ischemic hemispheres without a reduction in infarct size in hyperglycemic animals at 24 hours (Figure 3A–3D). This was associated with better neurobehavioral scores and improved grip strength at 24 hours (Figure 4A and 4B). MMP3 activity in the brain (r=−0.74) and macrovascular (r=−0.57) homogenates inversely correlated with the composite score indicating that as MMP3 increases, neurological deficit worsens (P<0.05). Hyperglycemia significantly worsened the functional outcomes 24 hours after stroke and impaired recovery at 7 days. Early inhibition of MMP3 significantly reduced the hyperglycemia-induced neurobehavioral deficits and significantly improved functional outcomes 3 and 7 days after stroke (Figure 4C). The mortality rate was 10% to 15%, and there was no significant difference in mortality between different groups.

Effect of Knocking Down MMP3 in the Brain on Stroke Outcomes
Injection of MMP3 shRNA lentiviral particles significantly knocked down the expression of MMP3 in the brain compared with animals injected with the empty vector and sham animals (Figure 5A). MMP3 knockdown significantly reduced brain edema after stroke without affecting the infarct size (Figure 5B and 5C). Furthermore, knocking down MMP3 significantly reduced HT and hemoglobin content in the ischemic hemispheres in hyperglycemic animals, and this was associated with significant increase in neurobehavioral composite score and grip strength (Figure 6).

Discussion
The goal of this study is to fill a gap in knowledge about the mechanisms mediating the hyperglycemia-induced neurovascular injury and elucidate the role of MMP3 in the development of HT in hyperglycemic stroke. Accordingly, this study was designed to investigate the impact of (1) hyperglycemia on MMP3 activity and localization in the brain after stroke and (2) hyperglycemia-induced elevation in MMP3 activity on HT and functional outcomes.

MMP3 is a zinc endopeptidase that has broad substrate specificity and can target most of the components of the neurovascular unit causing degradation of the basal lamina and tight junction proteins. Suzuki et al showed that MMP3...
is a critical mediator of the tPA-induced cerebral hemorrhage, which was significantly reduced in an MMP3, but not an MMP9 knockout mouse model of normoglycemic animals. We have shown in this study that hyperglycemia significantly increased MMP3 activity in the brain after stroke. This hyperglycemia-induced increase in MMP3 activity was associated with a significant increase in cerebrovascular bleeding, HT, and worse functional outcomes without an increase in the infarct size. This can be related, in part, to the proteolytic ability of MMP3 to degrade tight junction proteins, leading to disruption of the blood–brain barrier, swelling of the brain, and cerebral hemorrhage. These findings also clarify the role of vasculature in mediating neurobehavioral deficit without an increase in neuronal injury.

Previous studies showed that MMP3 is expressed in the neurons in the acute phase after stroke and the administration of tPA induced its expression in endothelial cells; however, it was not detected in the astrocytes. In our study, we detected MMP3 in the neurons after stroke, and we did not see an increase in its expression in the neurons with hyperglycemia. However, the expression of MMP3 in cerebral vessels was more evident in hyperglycemic animals. MMP3 was localized to the pericytes surrounding the cerebral vessels and small portions of the vessels in normoglycemic animals. With hyperglycemia, MMP3 showed more staining along the vessel walls and surrounding pericytes. This spatial expression pattern of MMP3 in cerebral vessels and the surrounding pericytes in the peri-infarct region after stroke provides support to our hypothesis that MMP3 increases HT in hyperglycemic stroke.

We used 2 methods to block MMP3 activity. Acute pharmacological inhibition was achieved by single-dose administration at reperfusion. Given that there was no information on the in vivo use of MMP3 inhibitor UK 356618 in the literature, we first performed a series of dose-finding studies. We selected a dose that resulted in ≥50% reduction in MMP3 activity. Administration of the drug to sham-operated animals did not reduce MMP3 activity, suggesting that either the inhibitor does not cross the blood–brain barrier in the absence of ischemic injury or the MMP3 activity in sham animals was below the threshold of the drug action, as MMP3 showed low activity in the brains of sham animals compared with control and hyperglycemia-stroked animals (Figure I in the online-only Data Supplement). To address the concerns about the specificity of the inhibitor, we next used a molecular approach to knockdown MMP3 expression by stereotaxic injection of MMP3 shRNA lentiviral particles directly into the lateral ventricles in the brain. We achieved ≈50% reduction in MMP3 expression, similar to the decrease observed in MMP3 activity with the inhibitor. Interestingly, both approaches, whether MMP3 pharmacological inhibition or focal knockdown, significantly reduced HT and improved functional outcomes. These results provide a supporting evidence to our hypothesis that MMP3 is a critical mediator of vascular injury in hyperglycemic stroke.

MMPs are known to play a dual temporal role after stroke. They may exacerbate the neurovascular injury during the acute phase, whereas they may be involved in mediating vascular remodeling, improving outcomes, and promoting recovery during the late phase of injury. It was previously shown by Yang et al19 that MMP3 is expressed in cerebral vessels and surrounding pericytes, and it plays an important role in mediating recovery and vascular remodeling during the recovery phase 3 weeks after stroke. In the same study, they showed that the early inhibition of MMPs after ischemia promoted the long-term recovery.
Figure 2. Localization of matrix metalloproteinase 3 (MMP3) in the neurovascular unit in hyperglycemic (HG) stroke at 24 hours. A, MMP3 colocalizes with NeuN-positive neurons and (B) isolectin-positive cerebral vessels and (C) NG2-positive pericytes in the peri-infarct region in both control and HG groups. However, MMP3 was not seen in (D), glial fibrillary acidic protein (GFAP)-positive astrocytes. n=6 per group.
Zhao et al. also showed that MMPs may mediate plasticity and neurovascular remodeling at 7 to 14 days after stroke. They also showed that delayed inhibition of MMPs (7 days after stroke) increased the ischemic injury and impaired the functional recovery at 14 days after stroke. Here, we found that hyperglycemia worsened both short-term and delayed outcomes. Interestingly, the early inhibition of MMP3 did not only reduce the hyperglycemia-induced HT and improve early functional outcomes but also significantly promoted recovery and improved delayed outcomes.

Although this study can be considered the first to investigate the role of MMP3 in hyperglycemic stroke, the use of...
only male and young healthy animals is a limitation of this study. It is also to be acknowledged that colocalization studies can be strengthened by additional staining for basal lamina proteins such as laminin, and more specific endothelial cells and pericytes markers, such as RECA1 (rat endothelial cell antigen) and PDGF-R (platelet derived growth factor receptor), respectively, to determine exact localization of MMP3 in the vessel wall. Previous studies showed a robust expression

Figure 5. Matrix metalloproteinase 3 (MMP3) knockdown in the brain. A. Stereotaxic injection of MMP3 shRNA lentiviral particles significantly knocked down the expression of MMP3 in the brain compared with sham or animals injected with empty vector (EV; \( \alpha P<0.05 \) vs sham and EV). MMP3 knockdown significantly reduced the hyperglycemia (HG)-induced (B) brain swelling without affecting (A) infarct size at 24 hours. *\( P<0.05 \) vs control EV and control shRNA, #\( P<0.05 \) vs HG EV, n=6 to 7 per group.

Figure 6. Matrix metalloproteinase 3 (MMP3) focal knockdown in rat brain and stroke outcomes. Knocking down MMP3 in rats’ brains significantly reduced the hyperglycemia (HG)-induced increase in (A) hemorrhagic transformation (HT) and (B) hemoglobin (Hb) content in ischemic hemispheres and improved the HG-induced neurological deficits as indicated by a significant increase in (C) neurobehavioral composite score and (D) grip strength at 24 hours. *\( P<0.05 \) vs control empty vector (EV) and control shRNA, #\( P<0.05 \) vs HG EV later, n=6 to 7 per group.
of MMP3 in brain endothelial cells,19,20 and the strong lectin staining along the vessels’ walls observed in this study support the conclusion that activation of MMP3 in hyperglycemic conditions contributes to vascular injury. Another point is that we investigated only the effect of MMP3 without looking for other MMPs, such as MMP2 and MMP9. However, it was previously reported by our group that acute hyperglycemia significantly increased MMP9 activity in the MCA in a similar model of stroke.7 MMP2 and MMP9 were the most extensively studied MMPs implicated in mediating HT after stroke in both clinical and preclinical studies.10,21–23 Accordingly, several experimental studies attempted to pharmacologically inhibit MMP9, using broad spectrum MMPs inhibitors such as BB-94, GM6001, and minocycline, and they showed improved outcomes with MMP9 inhibition.9,10,24–26 However, minocycline was the only one able to show promising results clinically.22,28 In 2010, Fagan et al28 showed that administration of minocycline was safe and well tolerated ≤10 mg/kg alone or in combination with tPA. One year later, Switzer et al28 showed that administration of minocycline was associated with reduced MMP9 plasma levels in tPA-treated patients in the Minocycline to Improve Neurological Outcome in Stroke (MINOS) trial. The group interpreted this as minocycline may reduce the tPA-induced HT and improve outcomes in patients with acute ischemic stroke subjected to thrombolytic therapy. However, these studies focused only on MMP9 without much focus on MMP3. This experimental study is the first to demonstrate the impact of selective inhibition of MMP3 on outcomes in hyperglycemic stroke. In conclusion, the significant reduction in HT and improvement in functional outcomes achieved with the pharmacological inhibition and focal knockdown of MMP3 strongly support our hypothesis in demonstrating the deleterious role of MMP3 in mediating HT during the acute phase in hyperglycemic stroke. Taken together, this study points out MMP3 as a potential therapeutic target for reducing cerebral bleeding and improving clinical outcomes in hyperglycemic stroke.

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Disclosures
None.

References


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**Supplemental figure I.** MMP3 activity in Sham and Control animals.

Control (stroked normoglycemic) animals showed significant increase in MMP3 activity in both A, brain homogenates and B, cerebral macrovessels compared to sham animals. The use of MMP3 inhibitor caused a slight yet insignificant reduction in MMP3 activity in control animals. However, no reduction in activity was seen in sham animals after treatment. *p<0.05 vs control untreated, n=4-6/gp.