Published in final edited form as:

J Neurosci Res. 2010 March ; 88(4): 764–773. doi:10.1002/jnr.22257.

Divergent Role for MMP-2 in Myelin Breakdown and Oligodendrocyte Death Following Transient Global Ischemia

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Abstract

Transient global ischemia causes delayed white matter injury to the brain with oligodendrocyte (OLG) death and myelin breakdown. There is increasing evidence that hypoxia may be involved in several diseases of the white matter, including multiple sclerosis, vascular dementia, and ischemia. Matrix metalloproteinases (MMPs) are increased in rat and mouse models of hypoxic hypoperfusion and have been associated with OLG death. However, whether the MMPs act on myelin or OLGs remains unresolved. We hypothesized that delayed expression of MMPs caused OLG death and myelin breakdown. To test the hypothesis, adult mice underwent hypoxic hypoperfusion with transient bilateral occlusion of the carotid arteries. After 3 days of reperfusion, ischemic white matter had increased reactivity of astrocytes and microglia, MMP-2 localization in astrocytes, and increased protein expression and activity of MMP-2. In addition, there was a significant loss of myelin basic protein (MBP) by Western blot and caspase-3- mediated OLG death. Treatment with the broad-spectrum MMP inhibitor, BB-94, significantly decreased astrocyte reactivity and MMP-2 activity. More importantly, it reduced MBP breakdown. However, MMP inhibition had no effect on OLG loss. Our results implicate MMPs released by reactive astrocytes in delayed myelin degradation, while OLG death occurs by an MMP-independent mechanism. We propose that MMP-mediated myelin loss is important in hypoxic injury to the white matter.

Keywords

BB-94; gelatinase; matrix metalloproteinase; caspase-3; MMP inhibition

The white matter contains oligodendrocytes (OLGs) and myelin covered axons both of which are essential for conduction of electrical impulses throughout the brain. In human, white matter comprises almost half of the brain tissue volume (Miller et al., 1980). Deep white matter is sensitive to various injuries including stroke (Pantoni et al., 1996), multiple sclerosis (MS) (Barnett and Prineas, 2004), and spinal cord injury (SCI) (Park et al., 2004). Ischemic models of in vivo white matter injury to the rodent have demonstrated various damaging pathways including glutamate excitotoxicity (Tekkok et al., 2007), inflammatory cytokines (Schmitz and Chew, 2008) and protease activation (Rosenberg et al., 2001).

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Following transient global ischemia in the mouse, there is evidence for a role of matrix metalloproteinases (MMPs) in delayed neuronal death (Lee et al., 2004; Walker and Rosenberg, 2009). In addition, MMP-2 and MMP-9 have delayed expression in the glial cells following global ischemia (Magnoni et al., 2004).

The gelatinases, MMP-2 and MMP-9, are increased in various models of ischemia. In focal ischemia, MMP-9 expression has been shown to be involved in neuronal and white matter injury to the mouse (Asahi et al., 2000, 2001b), but MMP-2 was not found to be involved in the acute injury (Asahi et al., 2001a). In global ischemia, MMP-9 is involved in acute hippocampal neuronal injury (Lee et al., 2004) but is not expressed in the microglia or vascular epithelium of the white matter (Ihara et al., 2001). MMP-2 and MMP-9 also contribute to caspase-mediated brain endothelial cell death following hypoxia-reoxygenation in vitro (Lee and Lo, 2004). With glial expression of MMP-2 and neuronal expression of MMP-9 in vivo (Magnoni et al., 2004), the roles of these two gelatinases in global ischemia appear to be distinct.

MMPs are involved in the breakdown of myelin basic protein (MBP) and myelin associated glycoprotein (MAG) in various models of MS (Gijbels et al., 1993; D'Souza and Moscarello, 2006). In an in vitro assay, MMP-2 was determined the most active enzyme in the degradation of MBP, followed by MMP-3 and MMP-9 (Chandler et al., 1995). MMP inhibition has been shown to reduce MMP-9 mediated MBP breakdown, glial activation, and cell death following spinal nerve injury (Kobayashi et al., 2008).

OLGs are sensitive to both in vitro and in vivo ischemia models (Petito et al., 1998; Yoshioka et al., 2000). There is evidence for an apoptotic mechanism of OLG death with caspase-3 positive cells in the white matter following SCI (Terayama et al., 2007) and in MS (Hisahara et al., 2003). The rat model of permanent global ischemia demonstrates white matter vulnerability (Tomimoto et al., 2003) and there are implications of a role for MMP-2 in white matter damage and blood brain barrier (BBB) breakdown following chronic hypoperfusion in the mouse (Nakaji et al., 2006). However, in the mouse model of transient global ischemia, the mechanisms of white matter damage are not understood.

Transient global ischemia leads to hypoxic hypoperfusion and has a delayed effect on the components of the white matter. We hypothesized that MMPs contributed to myelin breakdown and oligodendrocyte loss following transient global ischemia in the mouse. To test this hypothesis, we analyzed cell loss and expression of MMP-2. We measured MMP-2 activity, MBP expression and OLG cell bodies as determined stereologically after 3 days of reperfusion. We used MMP inhibition to reduce MBP and OLG loss. We report a divergent role for MMPs in myelin and OLG cell body injury. Myelin breakdown was associated with MMP-2 activity. However, OLG cell death by a caspase-3 mechanism was independent of MMPs.

Materials and Methods

Transient Global Cerebral Ischemia and Tissue Processing

All experiments were approved by the University of New Mexico (UNM) Animal Care Committee and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Transient bilateral common carotid artery occlusion (BCAO) was performed on the C57BL/6 mouse as described previously (Walker and Rosenberg, 2009). Briefly, both common carotid arteries were exposed, isolated, and clipped with metal aneurysm clips for 30 min. Cerebral blood flow was measured before, during, and after surgery with a Laser Doppler blood flow monitor (Moore Instruments Limited, Devon, UK) 3 mm caudal and 3 mm lateral to Bregma. Drug treated animals were administered 50 mg/kg BB-94 intraperitoneally as a suspension of 3 mg/ml in saline 30 min prior to surgery, 2 hr following surgery, and once daily. Controls received saline injections. Perfusion with PBS/0.1% Procaine followed by 2% PLP (2% paraformaldehyde, 0.1 M sodium periodate, 0.075 M lysine in 100 mM phosphate buffer at pH 7.4) was conducted intracardially. Brains were removed and immersed in 2% PLP overnight. The brains were cryoprotected with 30% sucrose in PBS, and frozen in OCT. They were stored at -80°C until sectioned. For activity assays and immunoblotting, animals were perfused with PBS/0.1% Procaine, brains were removed and quickly frozen in 2-methylbutane.

Immunohistochemistry (IHC)

For fluorescence IHC, 10 μm sections were rehydrated through alcohols and PBS containing 0.1% Tween-20 (PBT). Nonspecific binding sites were blocked with PBT containing 1% bovine serum albumin (BSA), and 5% normal serum. Slides were incubated overnight at 4°C with primary antibodies. Slides were washed in PBT and incubated for 90 min at room temperature with secondary antibodies conjugated with FITC and Cy-3 (1:500; Alexafluor 546 and Alexafluor 488, Invitrogen, Carlsbad, CA). Slides were incubated with 4'-6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Inc., Carlsbad, CA) prior to cover slipping to label cell nuclei. Slides were dehydrated through alcohols and xylene and cover slipped using DPX (Sigma-Aldrich). All IHC slides were viewed on an Olympus BX-51 bright field and fluorescence microscope (Olympus America Inc., Center Valley, PA) equipped with an Optronics digital camera and Picture Frame image capture software (Optronics, Goleta, CA). Primary antibodies used for IHC included glial fibrillary acidic protein (GFAP) for astrocytes (1:400; Sigma-Aldrich, St. Louis, MO), Iba-1 for microglia/macrophages (1:400; Wako Pure Chemical Industries, Richmond, VA), MMP-2 (1:200; Chemicon, Temecula, CA), MMP-9 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), MBP (1:1000; Chemicon), CC-1 (APC) (1:20; Calbiochem, Gibbstown, NJ), and caspase-3 (1:100; Cell Signalling Technology, Danvers, MA).

Fluorescence Intensity Analysis

To compare various reperfusion time points and regions of interest, slides stained with IHC were analyzed for fluorescence staining intensity. All slides to be compared were stained together. Images were taken at the same exposure time for each section and changed to grayscale. Using NIH Image J software, the region of interest was outlined and the total

mean fluorescence intensity of each region was recorded and compared to control staining and other reperfusion time points. Values are reported as fluorescence intensity.

Oligodendrocyte Stereology

To assess OLG injury following transient global ischemia, 20 μm sections taken every 100 μm were rehydrated, rinsed, blocked, and incubated with primary antibodies for GFAP and Iba-1 for two nights at 4°C. Slides were then rinsed, blocked, and incubated with biotin conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 90 min at room temperature. Slides were rinsed in PBT, quenched in 0.3% H_2O_2 in methanol, rinsed, and exposed to the Vector Elite ABC solution. Slides were then rinsed, exposed to 3,3'-diaminobenzidine (DAB) for one minute, rinsed and counterstained with cresyl violet acetate (CVA). Slides were then rinsed, dehydrated through alcohols and xylene, and coverslipped with DPX (Sigma-Aldrich Chemicals, St. Louis, MO). Stereology of DAB-negative, CVA-positive round OLG cell bodies $\sim 10 \mu\text{m}$ in diameter (Pantoni et al., 1996) was performed in the external capsule over a total distance of 3.0 mm using the optical fractionator function of StereoInvestigator software (Version 6, MicroBrightField Inc., Williston, VT) controlling a motorized stage equipped Olympus BX-51 microscope. The region of interest was delineated and overlaid with a grid size of $130 \times 130 \mu\text{m}$ and counting frame set at $50 \times 50 \mu\text{m}$ for stereological quantification of cells. Histological sections were blinded to the investigator as to animal identity and reperfusion time.

Gelatin Zymography

White matter of freshly isolated non-fixed tissue was collected by punch biopsy at -20°C . One mm diameter tissue punches from slide-mounted 300 μm thick sections were collected from the rostral to caudal extent of the corpus callosum and external capsule. Samples were homogenized in a lysis buffer containing 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM CaCl_2 , 0.05% Brij-35, 0.02% NaN_3 , and 1% Triton X-100. MMP-2 and -9 present in the homogenates were concentrated with gelatin-sepharose 4B beads (Amersham Bio-sciences, Sweden). 500 μg of total protein in 500 μL buffer was incubated with 50 μL Gelatin-Sephadex 4B beads for 1 hr at 4°C with gentle rotation. The beads were collected and the gelatinases eluted by incubating with 50 μL elution buffer (10% DMSO in PBS) for 30 min at 4°C with gentle rotation. Equal amount of samples (20 μL containing 200 μg of protein) were electrophoretically separated on 10% SDS-polyacrylamide gels co-polymerized with 1 mg/ml gelatin (Sigma-Aldrich) under non-reducing conditions. Gels were washed in 2.5% Triton X-100 for 20 min and then incubated for 72 hr with a developing buffer containing 50 mM Tris pH 7.6, 5 mM CaCl_2 , 0.2 mM NaCl, and 0.02% Brij-35 at 37°C. The gels were then stained with 0.125% Coomassie Blue R-250 for 1 hr in 10% acetic acid and 50% methanol. Gels were destained with 10% acetic acid until clear bands of gelatinolysis appeared on a dark blue background (2 days). The gels were dried and scanned for densitometry (Alpha Imager 2200; Alpha Innotech, San Leandro, CA). Molecular weights were determined by both protein standards (Bio-Rad Laboratories, Hercules, CA), and conditioned media from HT1080. HT1080 is conditioned media from HT1080 human fibrosarcoma cells, a source of MMP-2 and -9. Data are expressed as relative optical density normalized to HT1080.

Immunoblotting

White matter including corpus callosum and external capsule of freshly isolated non-fixed tissue were collected by punch biopsy at -20°C and homogenized in lysis buffer as described above. Protease inhibitors were added and samples were analyzed for protein expression by Western blot. Fifty μg of total protein was separated on 10% (12% for MBP) acrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked, and incubated overnight at 4°C with primary antibodies against MMP-2 (1:500; Santa Cruz Biotechnology), MBP (1:500; Chemicon), and caspase-3 (1:1000; Cell Signalling). Membranes were incubated with HRP conjugated secondary antibodies (1:2000; Cell Signalling) and blots developed using chemiluminescent detection with the West Pico Kit (Pierce Biotechnology, Inc., Rockford, IL). Protein bands were visualized on X-ray film and densitometric analysis was used for semiquantitation of band intensities with AlphaEase image software (Alpha Innotech Corp.). Actin staining (1:7500 Sigma-Aldrich) on the same PVDF membranes was used to normalize protein loading and transfer. Results are reported as protein expression normalized to actin band intensity for quantification.

MMP-2 Activity Assay

MMP-2 enzymatic activity was measured with a fluorimetric assay using a 5-FAM/QXL 520 FRET peptide Substrate VI (Cat.60573-01) [QXL520 -Pro-Leu-Gly-Met-Trp-Ser-Arg-Lys(5-FAM)-NH₂] (Anaspec, San Jose, CA). In the intact FRET peptide, the fluorescence of 5-FAM was quenched by QXL 520. Upon cleavage into two separate fragments by the MMP-2 present in the sample, the fluorescence of 5-FAM was recovered and monitored at excitation / emission = 490 nm / 520 nm at 60 min in room temperature using a luminescence spectrometer (PerkinElmer LS55). Homogenized white matter tissue samples with 100 μg protein were mixed with assay buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl₂, 20 μM ZnSO₄, and 0.05% Brij-35) and fluorogenic substrate (1 μM final concentration) to a final volume of 200 μl . FL WinLab software was used to express data as relative fluorescence units (RFU)/ μg protein/min.

Statistical Analysis

Statistical comparisons among groups were done using ANOVA with post-hoc analysis for multiple *t*-tests (Prism 4.0, GraphPad Software Incorporated). All data were presented as mean $\pm$ standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Results

Glial Inflammation Following Transient Global Ischemia

Cerebral blood flow was reduced during transient global ischemia to $11.2 \pm 1.1\%$ of original flow and returned to $44.9 \pm 6.3\%$ following clip removal. To investigate the change in astrocyte and microglial expression in the hypoxic white matter following global ischemia, we stained coronal sections with GFAP or Iba-1 at various reperfusion time points and analyzed the stained tissue for fluorescence intensity. Astrocytosis increased in the white matter region at 1 day ($p < 0.05$), 3 days ($p < 0.01$), and 7 days ($p < 0.01$) of reperfusion as compared to sham animals (Fig. 1A). In addition, the Iba-1 microglial staining increased in

the white matter at both 3 days ($p < 0.01$) and 7 days ($p < 0.05$) reperfusion as compared to sham animals (Fig. 1B).

MMP Activity in Astrocytes

Immunohistochemistry demonstrated constitutive expression of MMP-2 in the astrocytes of the sham animals, as well as increased reactive astrocytes at 3 days of reperfusion (Fig. 2A). MMP-9 was rarely seen in the sham animals with some co-localization in the astrocytes following 3 days of reperfusion (Fig. 2B).

Gelatin zymography of white matter tissue punches demonstrated an increase in MMP-9 (98 kDa) expression, which was not significant following 3 days of reperfusion, while MMP-2 (72 kDa) had a significant increase ($p < 0.05$) in expression at that time (Fig. 2C). We confirmed the expression of MMP-2 protein by Western blot, which demonstrated a significant increase in MMP-2 expression at 1 day ($p < 0.01$), 3 days ($p < 0.001$), and 7 days ($p < 0.01$) of reperfusion (Fig. 3A). We also performed a fluorimetric enzyme activity assay and found a significant increase ($p < 0.05$) in MMP-2 activity at 3 days of reperfusion (Fig. 3B). A similar fluorimetric assay for MMP-3 showed no change in activity at all time points (data not shown).

Myelin Basic Protein Breakdown

MBP is a critical component of the myelin sheath produced by OLGs. As other models of white matter injury have shown a vulnerability of this protein to increased protease activity, we decided to investigate the changes in MBP following transient global ischemia. Immunohistochemistry for MBP shows a decrease in the staining of the white matter tract following 3 days of reperfusion (Fig. 4A). To quantify the change in MBP expression, we performed Western blots on white matter tissue punches. We found a significant decrease in MBP expression at 3 days ($p < 0.01$) and 7 days ($p < 0.01$) of reperfusion for both the 21.5 kDa and 18 kDa isoforms of MBP (Fig. 4B), which corresponded with increased MMP-2 activity at 3 days of reperfusion.

Oligodendrocyte Death

Since MBP degradation occurred in our model, we investigated the effect of transient global ischemia on the loss of OLG cell bodies. Stereological quantification of OLGs in the white matter demonstrated a significant reduction in the CVA-positive cell bodies at 3 days ($p < 0.05$) and 7 days ($p < 0.05$) of reperfusion (Fig. 5A). To confirm the morphological analysis, we also performed immunostaining of the mature oligodendrocyte marker, CC-1, in the white matter. Quantification of the mean fluorescence intensity of CC-1 also showed a reduction in OLGs at 3 days of reperfusion ($p < 0.01$) (Fig. 5B).

Caspases have been implicated in OLG death. Immunohistochemistry showed co-localization of the executioner caspase-3 with CC-1 following 3 days of reperfusion, with minimal caspase-3 staining in the sham animals (Fig. 6A). This implicated an apoptotic mechanism of cell death in the OLGs. Quantification of caspase-3 by Western blot demonstrated a significant increase in the pro band at 3 days ($p < 0.05$) and 7 days ($p < 0.05$) of reperfusion as compared to the sham (Fig. 6B).

MMP Inhibition Protects Myelin Breakdown, Not Oligodendrocyte Death

MMP inhibition has demonstrated protective effects in various models, including MS, SCI, permanent global ischemia, and transient global ischemia. We have previously shown that treatment with the broad-spectrum MMP inhibitor, BB-94, protects hippocampal neuronal death following transient global ischemia in the mouse. Following treatment with BB-94 in our model, we found reduced astrogliosis and less MMP-2 activity (data not shown) in the white matter punches as compared to vehicle treated animals at 3 days of reperfusion (Fig. 7A).

Immunohistochemistry of the white matter tissue showed an increase in MBP staining following BB-94 treatment as compared to vehicle (Fig. 7B). In addition, Western blot analyses showed reduced breakdown of the 21.5kDa isoform of MBP following BB-94 treatment as compared to vehicle ($p < 0.05$) (Fig. 7C), suggesting myelin protection. Hence we proposed a reduction in OLG loss. However, following BB-94 treatment, there was no change in the immunostaining of CC-1 as compared to vehicle (Fig. 8A). In addition, caspase-3 Western blots of the white matter punches failed to show a significant change in caspase-3 expression following BB-94 treatment (Fig. 8B), suggesting that MMP inhibition had no effect on OLG death following 3 days of reperfusion.

Discussion

White matter damage is a component of various diseases and injury pathways. With the majority of research in transient global ischemia focused on neuronal loss, understanding the mechanisms of white matter injury to OLGs and myelin is important. We found increased expression of MMP-2, but not MMP-9, in reactive astrocytes after 3 days of reperfusion. Myelin basic protein loss was identified and quantified by Western blots. Cell counts of OLGs showed fewer cells in the ischemic brains. Using an MMP inhibitor we identified an MMP-mediated mechanism of myelin breakdown, which was protected by the inhibitor and was associated with expression of active MMP-2. On the contrary, we failed to show that the MMP inhibitor reduced the death of OLGs, which was linked to expression of caspase-3, suggesting an independent mechanism of caspase-mediated apoptosis.

Comparison of various models of white matter injury shows some distinct differences between species, strain, and insult (Hagberg et al., 2002; Farkas et al., 2007). Specifically in global ischemia, the permanent carotid occlusion model in the rat leads to white matter damage after 7 to 14 days (Tomimoto et al., 2003) lasting up to 13 weeks following injury (Farkas et al., 2004). Permanent occlusion of both carotid arteries causes hypoxic hypoperfusion, which simulates chronic vascular disease in an aging population. In contrast, the mouse model of transient global ischemia represents acute brain injury as would occur following hypotension with cardiac arrest (Harukuni and Bhardwaj, 2006).

MMPs have been implicated in various diseases in addition to stroke, including bacterial meningitis, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (Rosenberg, 2002; Kiaei et al., 2007). We identified a significant increase in MMP-2 in tissue samples from the ischemic white matter without a significant increase in MMP-9. Our findings are similar to those of others with MMP-2 emerging as the main candidate for white matter damage (Ihara

et al., 2001; Magnoni et al., 2004; Nakaji et al., 2006). A recent study suggested that MMP-9 was not involved in glial scarring following mild focal ischemia (Copin and Gasche, 2007).

We confirmed the increase in MMP-2 in the zymograms by Western blot and immunohistochemistry. Furthermore, we used a fluorogenic assay to demonstrate a significant increase in MMP-2 activity in the white matter. Inhibition of MBP breakdown with BB-94 supported the role for MMPs in white matter injury. Our studies are consistent with the earlier demonstration of reduced myelin damage in the MMP-2 knockout mouse (Nakaji et al., 2006). Although MMP-9 was not significantly increased in our model, we cannot rule out a role for MMP-9 in white matter injury since BB-94 is a broad-spectrum MMP inhibitor.

The ischemic/hypoxic injury led to expression of caspase-3 in the OLGs, as detected by immunohistochemistry, suggesting an apoptotic cell death. Detection of caspase-3 protein by Western blotting showed a significant increase in the pro-caspase-3 band but failed to detect the cleaved, active form of caspase-3 in the blots. Caspase-3 expression did not change following treatment with a MMP inhibitor. Our findings show divergent effects of BB-94, which blocked the action of the MMPs on myelin degradation, but had no effect on OLG death. Separating myelin injury by MMP-2 from OLG death processes was a novel finding in our study.

Several other pathways of OLG death could be occurring in our model including oxidative stress (Scott et al., 2003), inflammation (Barnett et al., 2006), or glutamate toxicity (Goldberg and Ransom, 2003). With MMP involvement in MBP breakdown but not OLG death, there appears to be alternative mechanisms that result in the increase in pro-caspase-3 protein expression observed in this study. In addition, hypoxia may lead to an increase in pro-survival factors that may decrease OLG death. A recent study has shown a potentially protective role of HIF-1 α in vitro (Yao et al., 2008). As various protective and detrimental factors have altered expression following hypoxic insult, separating these pathways becomes more important.

Independent mechanisms and timing of OLG death versus demyelination have been proposed in other models (Merrill and Scolding, 1999; Jamin et al., 2001), with our data supporting a distinction in the loss of OLGs and myelin. With the loss of OLGs and myelin both occurring at 3 days of reperfusion in our model, this time point appears to be critical in white matter injury following global ischemia. One potential cause for this timing of injury could be the increased reactivity of the astrocytes and microglia. These cells have been implicated in various inflammatory pathways (Mun-Bryce et al., 2002; Kim et al., 2005), with increased TNF α expression in the microglia (Walker and Rosenberg, 2009) as well as astrocyte regulation of MMPs and TIMPs (Liu et al., 2007). Further studies into the role of these glial cells in OLG and myelin injury will shed more light into the mechanisms of white matter injury.

The physiological differences between the OLG cell body and myelin processes may be one of the reasons for our findings of differential effects of MMP activity on OLG death and myelin breakdown. NMDA receptor subunits are primarily found on the processes of the

OLG and AMPA/kainate receptor subunits are found on the cell body (Salter and Fern, 2005). Blockage of AMPA/kainate receptors with antagonists in the in vitro optic nerve prevented white matter damage (Tekkok et al., 2007), but it was essential to block both the NMDA and AMPA receptors to improve conduction in the myelinated axon following in vitro ischemia (Bakiri et al., 2008). Distinguishing between the cell body and the process with OLG loss and myelin breakdown is crucial to understanding mechanisms of white matter injury.

In conclusion, our data implicate a hypoxia-induced delayed OLG death and provide evidence for an MMP-mediated MBP breakdown at 3 days of reperfusion in transient global ischemia in the mouse. There was increased reactivity of astrocytes and microglia, implicating an inflammatory pathway. MMP inhibition reduced astrocytosis, myelin breakdown and MMP-2 activity, suggesting involvement of MMPs in myelin disruption. On the contrary, caspase-mediated OLG apoptosis was unaffected by MMP inhibition, suggesting MMP-independent mechanisms. We propose that this novel mechanism of isolated myelin injury by MMPs may be relevant for any hypoxia-induced insults to the white matter, and that our results support further studies of the use of MMP inhibitors in disorders with white matter damage.

Acknowledgments

The authors would like to thank Jeffrey Thompson for technical assistance, and Eduardo Candelario-Jalil for advice on the MMP activity assay.

Contract grant sponsor: NIH; Contract grant number: 5R01NS04547; Contract grant sponsor: NIH; Contract grant number: R01NS052305 (to G.A.R.).

References

- Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH. Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. *J Cereb Blood Flow Metab.* 2000; 20(12):1681–1689. [PubMed: 11129784]
- Asahi M, Sumii T, Fini ME, Itoharu S, Lo EH. Matrix metalloproteinase 2 gene knockout has no effect on acute brain injury after focal ischemia. *Neuroreport.* 2001a; 12:3003–3007. [PubMed: 11588620]
- Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH. Effects of matrix metalloproteinase-9 gene knockout on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci.* 2001b; 21:7724–7732. [PubMed: 11567062]
- Bakiri Y, Hamilton NB, Karadottir R, Attwell D. Testing NMDA receptor block as a therapeutic strategy for reducing ischaemic damage to CNS white matter. *Glia.* 2008; 56:233–240. [PubMed: 18046734]
- Barnett MH, Prineas JW. Relapsing and remitting multiple sclerosis: Pathology of the newly forming lesion. *Ann Neurol.* 2004; 55:458–468. [PubMed: 15048884]
- Barnett MH, Henderson AP, Prineas JW. The macrophage in MS: Just a scavenger after all? Pathology and pathogenesis of the acute MS lesion. *Mult Scler.* 2006; 12:121–132. [PubMed: 16629415]
- Chandler S, Coates R, Gearing A, Lury J, Wells G, Bone E. Matrix metalloproteinases degrade myelin basic protein. *Neurosci Lett.* 1995; 201:223–226. [PubMed: 8786845]
- Copin JC, Gasche Y. Matrix metalloproteinase-9 deficiency has no effect on glial scar formation after transient focal cerebral ischemia in mouse. *Brain Res.* 2007; 1150:167–173. [PubMed: 17434457]
- D'Souza CA, Moscarello MA. Differences in susceptibility of MBP charge isomers to digestion by stromelysin-1 (MMP-3) and release of an immunodominant epitope. *Neurochem Res.* 2006; 31:1045–1054. [PubMed: 16871440]

- Farkas E, Donka G, de Vos RA, Mihaly A, Bari F, Luiten PG. Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. *Acta Neuropathol.* 2004; 108:57–64. [PubMed: 15138777]
- Farkas E, Luiten PG, Bari F. Permanent, bilateral common carotid artery occlusion in the rat: A model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain Res Rev.* 2007; 54:162–180. [PubMed: 17296232]
- Gijbels K, Proost P, Masure S, Carton H, Billiau A, Opdenakker G. Gelatinase B is present in the cerebrospinal fluid during experimental autoimmune encephalomyelitis and cleaves myelin basic protein. *J Neurosci Res.* 1993; 36:432–440. [PubMed: 7505841]
- Goldberg MP, Ransom BR. New light on white matter. *Stroke.* 2003; 34:330–332. [PubMed: 12574526]
- Hagberg H, Peebles D, Mallard C. Models of white matter injury: Comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment Retard Dev Disabil Res Rev.* 2002; 8:30–38. [PubMed: 11921384]
- Harukuni I, Bhardwaj A. Mechanisms of brain injury after global cerebral ischemia. *Neurol Clin.* 2006; 24:1–21. [PubMed: 16443127]
- Hisahara S, Okano H, Miura M. Caspase-mediated oligodendrocyte cell death in the pathogenesis of autoimmune demyelination. *Neurosci Res.* 2003; 46:387–397. [PubMed: 12871760]
- Ihara M, Tomimoto H, Kinoshita M, Oh J, Noda M, Wakita H, Akiguchi I, Shibasaki H. Chronic cerebral hypoperfusion induces MMP-2 but not MMP-9 expression in the microglia and vascular endothelium of white matter. *J Cereb Blood Flow Metab.* 2001; 21:828–834. [PubMed: 11435795]
- Jamin N, Junier MP, Grannec G, Cadusseau J. Two temporal stages of oligodendroglial response to excitotoxic lesion in the gray matter of the adult rat brain. *Exp Neurol.* 2001; 172:17–28. [PubMed: 11681837]
- Kiaei M, Kipiani K, Calingasan NY, Wille E, Chen J, Heissig B, Rafii S, Lorenl S, Beal MF. Matrix metalloproteinase-9 regulates TNF-alpha and FasL expression in neuronal, glial cells and its absence extends life in a transgenic mouse model of amyotrophic lateral sclerosis. *Exp Neurol.* 2007; 205:74–81. [PubMed: 17362932]
- Kim YS, Kim SS, Cho JJ, Choi DH, Hwang O, Shin DH, Chun HS, Beal MF, Joh TH. Matrix metalloproteinase-3: A novel signaling proteinase from apoptotic neuronal cells that activates microglia. *J Neurosci.* 2005; 25:3701–3711. [PubMed: 15814801]
- Kobayashi H, Chattopadhyay S, Kato K, Dolkas J, Kikuchi S, Myers RR, Shubayev VI. MMPs initiate Schwann cell-mediated MBP degradation and mechanical nociception after nerve damage. *Mol Cell Neurosci.* 2008; 39:619–627. [PubMed: 18817874]
- Lee SR, Lo EH. Induction of caspase-mediated cell death by matrix metalloproteinases in cerebral endothelial cells after hypoxia-reoxygenation. *J Cereb Blood Flow Metab.* 2004; 24:720–727. [PubMed: 15241180]
- Lee SR, Tsuji K, Lee SR, Lo EH. Role of matrix metalloproteinases in delayed neuronal damage after transient global cerebral ischemia. *J Neurosci.* 2004; 24:671–678. [PubMed: 14736853]
- Liu W, Furuichi T, Miyake M, Rosenberg GA, Liu KJ. Differential expression of tissue inhibitor of metalloproteinases-3 in cultured astrocytes and neurons regulates the activation of matrix metalloproteinase-2. *J Neurosci Res.* 2007; 85:829–836. [PubMed: 17279554]
- Magnoni S, Baker A, George SJ, Duncan WC, Kerr LE, McCulloch J, Horsburgh K. Differential alterations in the expression and activity of matrix metalloproteinases 2 and 9 after transient cerebral ischemia in mice. *Neurobiol Dis.* 2004; 17:188–197. [PubMed: 15474357]
- Merrill JE, Scolding NJ. Mechanisms of damage to myelin and oligodendrocytes and their relevance to disease. *Neuropathol Appl Neurobiol.* 1999; 25:435–458. [PubMed: 10632895]
- Miller AK, Alston RL, Corsellis JA. Variation with age in the volumes of grey and white matter in the cerebral hemispheres of man: Measurements with an image analyzer. *Neuropathol Appl Neurobiol.* 1980; 6:119–132. [PubMed: 7374914]
- Mun-Bryce S, Lukes A, Wallace J, Lukes-Marx M, Rosenberg GA. Stromelysin-1 and gelatinase A are upregulated before TNF-alpha in LPS-stimulated neuroinflammation. *Brain Res.* 2002; 933:42–49. [PubMed: 11929634]

- Nakaji K, Ihara M, Takahashi C, Itohara S, Noda M, Takahashi R, Tomimoto H. Matrix metalloproteinase-2 plays a critical role in the pathogenesis of white matter lesions after chronic cerebral hypoperfusion in rodents. *Stroke*. 2006; 37:2816–2823. [PubMed: 17008622]
- Pantoni L, Garcia JH, Gutierrez JA. Cerebral white matter is highly vulnerable to ischemia. *Stroke*. 1996; 27:1641–1646. [PubMed: 8784142]
- Park E, Velumian AA, Fehlings MG. The role of excitotoxicity in secondary mechanisms of spinal cord injury: A review with an emphasis on the implications for white matter degeneration. *J Neurotrauma*. 2004; 21:754–774. [PubMed: 15253803]
- Petito CK, Olarte JP, Roberts B, Nowak TS Jr, Pulsinelli WA. Selective glial vulnerability following transient global ischemia in rat brain. *J Neuropathol Exp Neurol*. 1998; 57:231–238. [PubMed: 9600215]
- Rosenberg GA. Matrix metalloproteinases and neuroinflammation in multiple sclerosis. *Neuroscientist*. 2002; 8:586–595. [PubMed: 12467380]
- Rosenberg GA, Cunningham LA, Wallace J, Alexander S, Estrada EY, Grossetete M, Razhagi A, Miller K, Gearing A. Immunohistochemistry of matrix metalloproteinases in reperfusion injury to rat brain: Activation of MMP-9 linked to stromelysin-1 and microglia in cell cultures. *Brain Res*. 2001; 893:104–112. [PubMed: 11222998]
- Salter MG, Fern R. NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature*. 2005; 438:1167–1171. [PubMed: 16372012]
- Schmitz T, Chew LJ. Cytokines and myelination in the central nervous system. *ScientificWorldJournal*. 2008; 8:1119–1147. [PubMed: 18979053]
- Scott GS, Virag L, Szabo C, Hooper DC. Peroxynitrite-induced oligodendrocyte toxicity is not dependent on poly(ADP-ribose) polymerase activation. *Glia*. 2003; 41:105–116. [PubMed: 12509801]
- Tekkok SB, Ye Z, Ransom BR. Excitotoxic mechanisms of ischemic injury in myelinated white matter. *J Cereb Blood Flow Metab*. 2007; 27:1540–1552. [PubMed: 17299453]
- Terayama R, Bando Y, Murakami K, Kato K, Kishibe M, Yoshida S. Neuropsin promotes oligodendrocyte death, demyelination and axonal degeneration after spinal cord injury. *Neuroscience*. 2007; 148:175–187. [PubMed: 17629414]
- Tomimoto H, Ihara M, Wakita H, Ohtani R, Lin JX, Akiguchi I, Kinoshita M, Shibasaki H. Chronic cerebral hypoperfusion induces white matter lesions and loss of oligodendroglia with DNA fragmentation in the rat. *Acta Neuropathol*. 2003; 106:527–534. [PubMed: 13680276]
- Walker EJ, Rosenberg GA. TIMP-3 and MMP-3 contribute to delayed inflammation and hippocampal neuronal death following global ischemia. *Exp Neurol*. 2009; 216:122–131. [PubMed: 19111539]
- Yao SY, Soutto M, Sriram S. Preconditioning with cobalt chloride or desferrioxamine protects oligodendrocyte cell line (MO3.13) from tumor necrosis factor- α -mediated cell death. *J Neurosci Res*. 2008; 86:2403–2413. [PubMed: 18438939]
- Yoshioka A, Yamaya Y, Saiki S, Kanemoto M, Hirose G, Beesley J, Pleasure D. Non-N-methyl-D-aspartate glutamate receptors mediate oxygen—glucose deprivation-induced oligodendroglial injury. *Brain Res*. 2000; 854:207–215. [PubMed: 10784123]

Fig. 1.

Inflammation of astrocytes and microglia in the white matter. **(A)** GFAP-positive astrocytes (FITC, green) increased at 3 days as compared to sham in white matter tract. DAPI (blue) counter stain for nuclei. Quantification of fluorescence intensity demonstrated increased GFAP reactivity at 1 day (* $p < 0.05$), 3 days (** $p < 0.01$) and 7 days (** $p < 0.01$) of reperfusion ($n = 10$ for 3d; $n = 8$ for Sham, 1d, and 7d). **(B)** Iba-1 positive microglia/macrophages increased at 3 days as compared to sham. Quantification of fluorescence intensity demonstrated increased Iba-1 reactivity at 3 days (** $p < 0.01$) and 7 days (* $p < 0.05$) of reperfusion ($n = 8$ /group). Values represent mean $\pm$ SEM. Scale Bars: 50 μ m.

Fig. 2.

MMP expression in astrocytes and gelatin zymography. **(A)** MMP-2 (Cy3 red) co-localized with GFAP-positive astrocytes (green) at sham and 3 days of reperfusion. **(B)** No MMP-9 (red) expression in sham and minimal MMP-9 expression at 3 days of reperfusion in GFAP-positive astrocytes (green). DAPI (blue) counter stain for nuclei. Scale Bars: 50 μ m. **(C)** Gelatin zymography showed increased expression of pro-MMP-9 (98 kDa), with a significant increase in pro-MMP-2 (72 kDa) expression following 3 days of reperfusion ($\star p < 0.05$). HT1080 is conditioned media from HT1080 human fibrosarcoma cells, a source of MMP-2 and -9. Sham and 3 day columns are from 3 representative mice ($n = 6$ /group). Relative optical density normalized to HT1080 band. Values represent mean $\pm$ SEM.

Fig. 3. MMP-2 expression and activity in white matter tissue punches. **(A)** Western blot quantification of 72 kDa MMP-2 protein demonstrated increased expression at 1 day (** $p < 0.01$), 3 days (***) ($p < 0.001$), and 7 days (** $p < 0.01$) of reperfusion. Protein loading normalized to actin, 2 representative animals per group are shown ($n = 5$ for Sham; $n = 4$ for 1d, 3d, 7d). **(B)** MMP-2 activity assessed with a fluorometric assay showed increased activity at 3 days reperfusion (* $p < 0.05$). Controls included blank with lysis buffer only and substrate control with FRET peptide only, not shown ($n = 10$ for Sham, 7d; $n = 7$ for 1d, 3d). Values represent mean $\pm$ SEM.

Fig. 4.

MBP staining and protein expression. (A) Immunohistochemistry for MBP decreased following 3 days reperfusion. Arrows indicate demyelination. Scale Bar: 50 μ m. (B) Western blotting quantification of 21.5 kDa and 18 kDa MBP isoforms show loss of myelin at 3 days ($\star\star p < 0.01$) and 7 days ($\star\star p < 0.01$) of reperfusion in both bands. Protein loading normalized to actin, 2 representative animals per group are shown ($n = 9$ for Sham, 3d; $n = 8$ for 1d; $n = 6$ for 7d). Values represent mean $\pm$ SEM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

OLG stereology and staining analysis. **(A)** White matter tracts stained with GFAP and Iba-1 (both DAB-positive, brown cells) and counterstained with nuclear stain CVA (purple) for histological analysis showed a decrease in purple OLG cell bodies at 3 days of reperfusion. Stereology of CVA-positive OLGs was reduced at 3 days (* $p < 0.05$) and 7 days (* $p < 0.05$) of reperfusion as compared to sham animals ($n = 14$ for Sham; $n = 10$ for 1d; $n = 12$ for 3d, 7d). **(B)** Confirmation of morphological analysis with immunohistochemistry and fluorescence intensity analysis showed decreased CC-1 positive cells (green) in the white matter and reduced fluorescence intensity at 3 days of reperfusion (** $p < 0.01$) as compared to sham ($n = 8$ / group). DAPI (blue) counter stain for nuclei. Values represent mean $\pm$ SEM. Scale Bars: 50 μm . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6. Caspase-3 staining and protein expression. **(A)** Co-localization of caspase-3 (red) with CC-1 (green) positive cells increased following 3 days of reperfusion. DAPI (blue) counter stain for nuclei. Scale Bar: 50 μ m. **(B)** Western blot quantification of 34 kDa pro-caspase-3 band in white matter punches showed increased expression at 3 days ($\star p < 0.05$) and 7 days ($\star p < 0.05$) of reperfusion as compared to sham. Protein loading normalized to actin, 2 representative animals per group are shown (n = 9 for Sham, 1d; n = 6 for 3d, n = 8 for 7d). Values represent mean $\pm$ SEM.

Fig. 7.

MMP inhibition reduced astrocytosis and protected myelin following 3 days of reperfusion. (A) GFAP-positive astrocytes (green) decreased following BB-94 treatment as compared to vehicle. DAPI (blue) counter stain for nuclei. Fluorescence intensity analysis confirmed significant reduction in GFAP intensity (*** $p < 0.001$) ($n = 8/\text{group}$). (B) Immunohistochemistry for MBP showed increased staining following BB-94 treatment. (C) Western blot quantification of MBP expression showed a significant increase in the 21.5 kDa band (* $p < 0.05$), with a non-significant increase in the 18 kDa band. Protein loading normalized to actin, 2 representative animals per group are shown ($n = 8/\text{group}$). Values represent mean $\pm$ SEM. Scale Bars: 50 μm .

Fig. 8.

MMP inhibition does not affect OLG death. **(A)** Immunohistochemistry of CC-1 positive cells (green) in the white matter showed no difference following BB-94 treatment as compared to vehicle. DAPI (blue) counter stain for nuclei. Scale Bar: 50 μ m. Fluorescence intensity analysis confirmed no significant difference between BB-94 and vehicle treated mice following 3 days reperfusion ($n = 8$ /group). **(B)** Western blot quantification of 34 kDa pro caspase-3 band in white matter punches also showed no significant difference between BB-94 and vehicle treated mice. Protein loading normalized to actin, 2 representative animals per group are shown ($n = 8$ /group). Values represent mean $\pm$ SEM. [Color figure can be viewed in the online issue, which is available at www.interscience.-wiley.com.]