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Matrix metalloproteinase-2 deletions protect against hemorrhagic transformation after 1 hour of cerebral ischemia and 23 hours of reperfusion

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Abstract

Although elevated MMP-2 levels were highly related to the degradation of tight junction (TJ) proteins and basal lamina and neuronal injury after ischemia, until very recently, little experimental evidence was available to test the role of the MMP-2 knockout (KO) in blood-brain-barrier injury and development of hemorrhage transformation (HT). Here, we assessed the role of the MMP-2 KO in blood-brain-barrier injury, HT and other brain injuries after 1 h of ischemia and 23 h of reperfusion. Middle cerebral artery occlusion (MCAO) was performed in MMP-2 KO mice. Reperfusion was started 1 hour after the onset of MCAO. All mice were sacrificed 24 hours after the MCAO. MMP-2 deficiency reduced the decrease in protein levels of collagen IV and cellular membrane occludin ($p < 0.01$ and 0.05 vs. WT, respectively) and attenuated increase in cytosol occludin level in ischemic brain ($p < 0.01$ vs. WT). The hemorrhage volume and brain infarction were significantly decreased in both the cortex and striatum in the MMP-2 KO mice ($p < 0.01$ vs. WT). The MMP-2 knockout also had reduced brain swelling in the cortex and improved neurological deficits ($p < 0.01$ vs. WT). These studies provide direct evidence that targeting MMP-2 will effectively protect against collagen and occludin loss and HT after ischemia and reperfusion.

Keywords

cerebral ischemia; hemorrhagic transformation; MMP-2 knockout; mice

Although elevated MMP-2 levels were highly related to the degradation of tight junction (TJ) proteins and the basal lamina, BBB disruption and neuronal injury after ischemia (Rosenberg et al., 1998, Heo et al., 1999, Yang et al., 2007), until very recently, little experimental evidence was available to test the role of MMP-2 knockout (KO) in blood-brain-barrier injury and HT development. Our recent publication observed the roles of MMP-2 and/or MMP-9 KO in the early stage of ischemia reperfusion. We found that the MMP-2 deficiency and MMP-2 and MMP-9 double deficiency were more protective against HT than the MMP-9 deficiency mice after 1 h of ischemia and 7 h of reperfusion but that the MMP-2/9 dKO did not show potentiation compared with the MMP-2 KO alone (Suofu et al., 2012). These results prompted us to further investigate the effects of the MMP-2 KO in

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protecting against reperfusion-induced HT and other brain injuries after 1 h of ischemia followed by 23 h of reperfusion. In the current study, a mouse suture middle cerebral artery occlusion model (MCAO) was used to produce the ischemia and mechanical reperfusion. Twenty four hours after the ischemia and reperfusion, we assessed the changes in the basal lamina and tight junction proteins, the incidence and amount of hemorrhage, brain infarct, edema and the neurological functions.

Experimental procedures

Animals

The University of Cincinnati Animal Care Committee approved all experimental procedures. All procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The MMP-2 KO (MMP-2^{-/-}) mice in the C57BL/6 J genetic background were a kind gift from Dr. L. Matrisian (Vanderbilt University, Nashville, TN). The homozygous MMP-2-deficient mice and control wild-type (WT, MMP-2^{+/+}) mice were the progeny of heterozygous breeding pairs of C57BL/6 J mice with a disruption in the MMP-2 gene. These breeding pairs were backcrossed for more than six generations (Itoh et al., 1997). The genotypes of all mice were verified using PCR. The animals were allowed access to food and water *ad libitum* and were housed with a 12-hour light–dark cycle.

In all experiments, we used male mice at 13 to 15 weeks of age with a body weight of 25 to 27 g.

The middle cerebral artery occlusion model (MCAO) and experimental groups

The standard intraluminal middle cerebral artery occlusion method was used to induce focal ischemia, as previously described (Lu et al., 2008, Suofu et al., 2012). Briefly, each mouse was anesthetized with 1.5% isoflurane in 28.5% oxygen and 70% nitrous oxide using a face mask. The rectal temperature of all animals was maintained at 37 ± 0.5 °C with a feedback-controlled heating blanket. The mice were placed in the supine position. Following a midline skin incision, the left common carotid artery, external carotid artery, and internal carotid artery were exposed. A 6.0 nylon monofilament coated with silicon was introduced into the left internal carotid artery through the external carotid artery to occlude the origin of the middle cerebral artery. The sutures were randomly assigned to the mice in the different groups. The wound was then closed, and the suture was kept in place. After 1 h of ischemia, the mice were reanesthetized, the neck skin was reopened, and the nylon suture was removed to achieve reperfusion. Intracranial ischemia and reperfusion were confirmed by laser Doppler flowmetry (5 mm lateral and 2 mm posterior to the bregma). To prevent hypothermia after surgery, the mice were transferred to a temperature-controlled incubator at 37 °C for 20 min until the animals woke up completely. The mice were then transferred to cages with Delta Phase Isothermal Pads (Braintree Scientific, Inc.). Three groups of animals were studied: (1) sham group, a sham operation in WT mice (MMP-2^{+/+}); (2) WT group, ischemia–reperfusion in WT mice (MMP-2^{+/+}); and (3) MMP-2 KO group, ischemia–reperfusion in MMP-2 KO mice (MMP-2^{-/-}). The number of animals for each group is listed in the figure legends. The surgical procedure of the sham operation group was the same as the other two groups, but there was no suture insertion and occlusion of the MCA. The mice were sacrificed 24 h after either the sham operation or the onset of ischemia. One animal died in the WT group and was excluded from the data collection.

Hemorrhagic rates and volumes

A previously reported visual method of estimating the cerebral hemorrhage was used (Lapchak et al., 2001, Suofu et al., 2012). The mice were sacrificed at 24 h. The transcatheter perfusion was performed with 0.9 saline at a rate of 2 ml/min for 15 min. Seven coronal slices per brain (1 mm thickness) were prepared using a brain matrix. The slices were scanned to quantify the hemorrhagic areas, which is small to more confluent red petechiae. H-E staining confirmed that there were red cells in these red areas in perfused brain sections in our preliminary studies. The hemorrhagic area on the surface of the slice was quantified with 10-fold magnification using an MCID digital image analysis system (Imaging Research, Inc., St Catharines, Ontario, Canada), and the hemorrhage volume of each slice was calculated as the hemorrhagic area on each surface times the slice thickness. The incidence of hemorrhage was also calculated.

Infarction volume, edema and neurological deficits

The brain slices from 24 h of ischemic mice were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 10 min in a 37 °C water bath (Suofu et al., 2012). Areas that were not stained red with TTC were identified as infarct areas. After staining, the brain slices were fixed with 4% paraformaldehyde for 30 min and scanned. The standard indirect method was used for measuring the areas of infarction on each slice surface, and the ischemic lesion volumes were quantified with the MCID automated imaging system. Brain edemas were calculated as follows: (volume of infarcted cortex or basal ganglia or whole hemisphere – volume of contralateral normal cortex or basal ganglia or whole hemisphere)/volume of contralateral side × 100% (Lu et al., 2009). TTC is a marker of tissue dehydrogenase and mitochondrial dysfunction, it may overestimate infarct size (Liu and McCullough, 2011).

A vibrissae-stimulated forepaw placing test was used to assess deficits in the sensorimotor cortex or striatum (Belayev et al., 2003). The animals were gently held by their neck skin with their forelimbs hanging freely. Stimulation of the right vibrissae by gently brushing against the edge of a table results in responses by the right forepaw. A successful placement response was determined by the ability of the mice to place a forepaw on the table. The test was conducted immediately before sacrifice. For each mouse, 10 trials were performed in triplicate.

Western blots

Western blots were performed as previously described (Lu et al., 2002, Stamatovic et al., 2006). Briefly, the brains were removed of meninges and sliced into four 2-mm coronal slices at 4 °C. The brain slices were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO) for 10 min at 37 °C. The infarction area was then dissected. To analyze the collagen IV changes, the tissue was homogenized in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 10 µL/mL Igepal CA-630, 5 mg/mL sodium deoxycholate, 1 mg/mL sodium dodecyl sulfate [SDS], pH 7.4) with 0.01% phenylmethylsulfonyl fluoride (PMSF), 3% aprotinin and 0.02% sodium orthovanadate. The samples were centrifuged at 10 000 g for 10 min, and the supernatant was removed and centrifuged again. To analyze the occludin, the tissue was homogenized in homogenization buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 300 mM sucrose, 5 mM EDTA, protease inhibitor cocktail). The samples were sonicated for 20 s and centrifuged at 600 g for 10 min at 4 °C. The supernatant was centrifuged at 16,000 g for 20 min at 4 °C to separate the crude membrane fraction (P1) and cytosolic fraction (S1). The P1 was washed and sonicated again in homogenates buffer with 0.5% triton, resulting in the washed crude membrane fraction (P2). The protein concentrations in the S1 and P2 were determined using BCA (Pierce, Rockford, IL, USA), and gel samples were prepared by adding sample buffer

containing final concentrations of 50 mM Tris (pH 6.7), 2% SDS, 2% β -mercaptoethanol, and bromophenol blue as a marker. The samples were boiled for 3 min, and then 50 μ g of protein was loaded onto 10% SDSpolyacrylamide gels. The proteins were transferred to nitrocellulose membranes using standard electroblotting procedures. The nitrocellulose membranes were blocked with 10% non-fat dry milk. The blocking solutions were prepared in a buffer containing 100 mM Tris-HCl, 0.9% NaCl, and 0.05% Tween-20 (TBST, pH 7.6). The blots were incubated overnight at 4°C with anti-occludin antibody (1:750, Invitrogen, Camarillo, CA, USA) or anti-rat collagen IV monoclonal antibody (M3F7; 1:200; Developmental Studies Hybridoma Bank at the University of Iowa, USA). The immunoblots were washed and incubated with anti-mouse Ig linked to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was detected with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's recommendations. The blots were quantified using densitometric analyses with the MCID digital image analysis system. The values for both collagen IV and occludin were normalized to β -actin.

Statistical analysis

Quantitative data were expressed as the mean $\pm$ SD. Statistical comparisons were conducted using t-test or ANOVA for group comparisons. Differences with $p < 0.05$ were considered statistically significant.

Results

Post-ischemic changes in regional cerebral blood flow are similar in MMP-2 KO and wild-type mice

Regional cerebral blood flow (rCBF) was measured in the cortex at baseline, 10 min after the onset of ischemia, and 10 min before and after reperfusion in all ischemic mice; the results are shown in Table 1. After ischemia, the rCBF decreased in all groups ($p < 0.01$ vs. baseline value). The degree of ischemia was not significantly different among the groups. After reperfusion, the values of the average rCBF recovered to the pre-ischemic levels (Table 1). The vascular boundaries of the MCA were not significantly different between the WT and MMP-2 KO mice, as we previously reported (Suofu et al., 2012)

MMP-2 KO protected against hemorrhagic transformations after 1 h of ischemia and 23 h of reperfusion

The ischemia for 1 h and reperfusion for 23 h caused hemorrhagic infarction (HI) in all mice in the WT and MMP-2 KO groups, revealed as multiple dots, small petechiae and more confluent petechial hemorrhage (Fig. 1d). Hemorrhagic volumes were significantly decreased in the MMP-2 KO mice ($p < 0.01$ vs. WT) in both the cortex (Fig. 1a,d) and basal ganglia (Fig 1b,d). Hemorrhage was mainly confined to basal ganglia in MMP-2 KO mice (Fig 1b,d).

Brain infarction volume, edema and neurological deficits were reduced in the MMP-2 KO mice after 1 h of ischemia and 23 h of reperfusion

Fig. 2 illustrates the volumes of infarction. At 24 h after the onset of ischemia, the infarction volumes were significantly reduced in the MMP-2 KO mice in both the cortex (Fig. 2a, $p < 0.01$ vs. WT) and basal ganglia (Fig. 2 b, $p < 0.05$ vs. WT). The MMP-2 KO mice also displayed decreased brain swelling in the cortex (Fig. 3a, $p < 0.01$ vs. WT). Compared with the wild-type mice, the neurological deficits were ameliorated in the MMP-2 KO mice (Fig. 3 b, $p < 0.01$ vs. WT).

MMP-2 KO protected against injuries to the proteins of the cerebral microvascular basal lamina and tight junction after 1 h of ischemia and 23 h of reperfusion

The collagen IV and occludin western blots were performed to assess injuries to the basal lamina and tight junction in the cerebral blood vessels in the mice. The collagen IV protein levels decreased in the WT ischemia-reperfusion mice (Fig. 4a, $p < 0.01$ vs. Sham), MMP-2 KO reduced the decrease in collagen IV levels (Fig. 4a, $p < 0.01$ vs. WT). The occludin protein levels decreased in the cellular membrane and increased in the cytosol in the WT ischemia-reperfusion mice (Fig. 5, $p < 0.01$ vs. Sham). These changes were also partially reversed in the MMP-2 KO mice (Fig. 5a, $p < 0.05$ vs. WT in the membrane fraction; Fig. 5b, $p < 0.01$ vs. WT in the cytosol fraction).

Discussion

Our previous studies indicated that the MMP-2 KO protects against injuries to the cerebral microvascular basal lamina in the early stage of ischemia and reperfusion (1 h of ischemia and 7 h of reperfusion) (Suofu et al., 2012). In the current study, we further demonstrated that the MMP-2 KO reduced the decrease in the basal lamina protein collagen IV and in the cellular membrane tight junction protein occludin in ischemic brain. The ischemia mediated the increase of occludin level in the cytosol was also attenuated. ProMMP-2 is derived from brain including astrocytes (del Zoppo et al., 2012) and endothelial cell (Reuter et al., 2013). It is induced within 1 hour post stroke in brain and remains elevated thereafter (Liu et al., 2012). It is possible that MMP-2 deficiency decreases the degradation of collagen IV and occluding. This is supported by facts that MMP-2 enzymatically processed collagen IV (Monaco et al., 2006) and cerebral ischemia initiated MMP-2-mediated occludin degradation and BBB disruption in an early stroke stage (Liu et al., 2012). It is also possible that the MMP-2 KO partially reduced the translocation of the tight junction protein occludin from the membrane to the cytosol after 1 h of ischemia and 23 h of reperfusion. Ischemia and reperfusion increased the secretion of CCL2 by both astrocytes and brain endothelial cells (Dimitrijevic et al., 2006). CCL2 induced MMP-2 secretion in the endothelial cells and the redistribution of tight-junction (TJ) proteins with an obvious enhancement of barrier permeability (Werle et al., 2002, Dimitrijevic et al., 2006). Cerebral ischemia elevated expression of PKC in the ischemic brain microvascular endothelial cells through NMDA receptor activation (Miettinen et al., 1996). Glutamate and PKC were related to increased MMP-2 secretion, occludin phosphorylation and redistribution and disruption of endothelial barrier function (Angelow et al., 2005, Andras et al., 2007, Harris and Friedland, 2008). Hydrogen peroxide exposure increased ERK1/ERK2 and MMP-2 activation and mediated serine phosphorylation, redistribution and degradation of occludin and elevated endothelial permeability (Kevil et al., 2000, Lischper et al., 2010). MMP-2 could be important effective molecules in CCL2, glutamate and reactive oxygen species-induced BBB injury after ischemia, but how MMP-2 is involved in occludin redistribution is not clear. The effect of MMP2 knock out on BBB permeability also need to be further studied. Previous publications report that elevated MMP-2 levels were related to the degradation of tight junction (TJ) proteins and of the basal lamina and to the disruption of the BBB (Rosenberg et al., 1998, Heo et al., 1999, Yang et al., 2007). Administering an MMP inhibitor reversed the degradation of the TJ proteins and blocked the opening of the BBB after ischemia and reperfusion (Yang et al., 2007). These results are in line with our findings, suggesting that targeting MMP-2 will effectively protect the blood brain barrier against ischemia and reperfusion injuries.

A previous publication from another group reported that an MMP-2 KO did not protect against brain infarct when the ischemia was severe (2 h post-ischemia and 22 h post-reperfusion with a total infarct volume of approximately 120 mm³ in the WT group) (Asahi et al., 2001). In the current study, we performed 1 h of ischemia followed by 23 h of

reperfusion in mice and induced less severe ischemic injuries (with a total infarct volume of $68.8 \pm 8.1 \text{ mm}^3$ in the WT group). The MMP-2 KO significantly protected against brain infarction in both the cortex and striatum in this model, suggesting that MMP-2 contributes to brain infarct formation. However, the different technical approaches in those experiments may also affect infarct volume, for example, Asshi, et al used halothane as anesthetic instead of isoflurane which we used. A previous publication reported that a significant delay in the recovery of adenosine triphosphate and intracellular pH in the halothane-treated rats compared with the isoflurane-treated animals after cerebral ischemia (Nakajima et al., 1997). Activated MMP-2 hydrolyzed the extracellular domain of fibroblast growth factor (FGF) receptor type 1 (FGFR1) (Levi et al., 1996). It also cleaved CXCL12/SDF-1 and produced a highly neurotoxic cleaved product (Zhang et al., 2003). The prevention of MMP-2 KO against brain infarct could be due to increasing FGF function and eliminating the cleaved product of CXCL12/SDF-1.

In the current study, we demonstrated that MMP-2 deficiency reduced the hemorrhagic volume in both the cortex and striatum after 1 h of ischemia and 23 h of reperfusion, which could be due to decreased injuries to the basal membrane and BBB and reduced brain infarction and edema in the MMP-2 KO mice. Importantly, the MMP-2 KO increased their forelimb placing scores after ischemia and reperfusion, demonstrating their improvements in neurological function.

Conclusion

In this study, we reported that the MMP-2 deficiency reduced the decrease in the collagen IV levels and membrane occludin levels after 1 h of ischemia and 23 h of reperfusion. The hemorrhagic volume and brain infarct were decreased in both the cortex and striatum in the MMP-2 KO mice. The MMP-2 deficiency also reduced brain edema and improved the neurological deficits. These findings provide direct evidence that targeting MMP-2 effectively protected against BBB injury and HT and improved neurological function after 1 h of ischemia and 23 h of reperfusion.

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Abbreviations

BBB	blood-brain barrier
MCAO	middle cerebral artery occlusion
MMP	matrix metalloproteinase
HT	hemorrhagic transformation
KO	knockout
WT	wild-type
MCAO	Middle cerebral artery occlusion
MCID	microcomputer image device
rCBF	Regional cerebral blood flow

TTC	2,3,5-triphenyltetrazolium chloride
WT	wide type

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Highlights

- MMP-2 deletions attenuated the decrease of collagen IV levels in ischemic brain.
- MMP-2 deficiency reduced membrane occludin loss after ischemia.
- MMP-2 knockout (KO) decreased the hemorrhagic volume in the cortex and striatum.
- MMP-2 KO protected against brain infarctions in the cortex and striatum.
- MMP-2 deficiency reduced brain edema and improved neurological function.

Figure 1. Effects of the MMP-2 knockout on brain hemorrhage transformation after 1 h of ischemia and 23 h of reperfusion ($n = 11$ in the WT group, $n = 13$ in the MMP-2 KO group)
a) Cortex hemorrhage, b) basal ganglia hemorrhage, c) total hemorrhage volume, d) representative images. ** $p < 0.01$ compared with the WT ischemia-reperfusion control. The MMP-2 KO protected against hemorrhage in both the cortex and basal ganglia.

Figure 2. Effects of the MMP-2 knockout on brain infarct in mice after 1 h of ischemia and 23 h of reperfusion ($n = 11$ in the WT group, $n = 13$ in the MMP-2 KO group)

a) Cortex infarction, b) basal ganglia infarction, c) total infarct volume, d) representative images. * $p < 0.05$, ** $p < 0.01$ compared with the WT ischemia-reperfusion control.

Figure 3. Effects of the MMP-2 knockout on brain edema and behavioral deficits after 1 h of ischemia and 23 h of reperfusion ($n = 11$ in the WT group, $n = 13$ in the MMP-2 KO group)
 The behavioral deficits were assessed using the forelimb placing test. Higher forelimb placing scores represent better improvements in function. a) Cortex swelling, b) forelimb placing score (%). ** $p < 0.01$ compared with the WT ischemia-reperfusion control.

Figure 4. Collagen IV western blot in the brains of adult mice after focal MCA ischemia ($n = 3$) Isch, ischemia-reperfusion; WT, WT group; MMP-2 KO, MMP-2 KO group. ## $p < 0.01$ compared with the sham group (%). ** $p < 0.01$ compared with the WT ischemia-reperfusion group. a) Collagen IV changes. b) representative images. MCA ischemia for 1 h followed by reperfusion for 23 h led to decreased levels of collagen IV. The MMP-2 KO prevented the decrease in collagen IV after focal MCA ischemia.

Figure 5. Occludin western blot in the brains of adult mice after focal MCA ischemia ($n = 3$)
 Isch, ischemia-reperfusion; WT, WT group; MMP-2 KO, MMP-2 KO group. ^{##} $p < 0.01$ compared with the sham group. (%). $*p < 0.05$, $**p < 0.01$ compared with the WT ischemia-reperfusion group. a) Occludin changes in the membrane, b) occludin changes in the cytosol,

c) representative images. The MMP-2 deficiency prevented occludin translocation from the membrane to the cytosol after 1 h of ischemia and 23 h of reperfusion.

Table 1

The change in regional cerebral blood flow after ischemia-reperfusion.

	before MCAO	10 min after MCAO	10 min before reperfusion	10 min after reperfusion
WT	100	13.5 ± 4.3 **	14.7 ± 4.6 **	98.7 ± 31.8
MMP-2 KO	100	11.2 ± 3.9 **	15.9 ± 6.9 **	113.5 ± 48.6

MCAO: middle cerebral artery occlusion.

**
 $p < 0.01$ compared with the levels before MCAO.