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# MATRIX METALLOPROTEINASE-2 DELETIONS PROTECT AGAINST HEMORRHAGIC TRANSFORMATION AFTER 1 H OF CEREBRAL ISCHEMIA AND 23 H OF REPERFUSION

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**Abstract—Although elevated matrix metalloproteinase (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 (BBB) injury and the development of hemorrhage transformation (HT). Here, we assessed the role of the MMP-2 KO in BBB 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 h after the onset of MCAO. All mice were sacrificed 24 h 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.* wild-type (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 KO 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. Published by Elsevier Ltd. on behalf of IBRO.**

**Key words: cerebral ischemia, hemorrhagic transformation, MMP-2 knockout, mice.**

## INTRODUCTION

Although elevated matrix metalloproteinase (MMP)-2 levels were highly related to the degradation of tight junction (TJ) proteins and the basal lamina, blood–brain barrier (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 BBB injury and hemorrhagic transformation (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 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 (MCAO) model was used to produce ischemia and mechanical reperfusion. Twenty-four hours after ischemia and reperfusion, we assessed the changes in the basal lamina and TJ 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/6J 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/6J 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-h light–dark cycle.

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

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*Abbreviations:* BBB, blood–brain barrier; EDTA, ethylenediaminetetraacetic acid; FGF, fibroblast growth factor; FGFR1, FGF receptor type 1; HT, hemorrhagic transformation; KO, knockout; MCAO, middle cerebral artery occlusion; MCID, microcomputer image device; MMP, matrix metalloproteinase; rCBF, regional cerebral blood flow; SDS, sodium dodecyl sulfate; TJ, tight junction; TTC, 2,3,5-triphenyltetrazolium chloride; WT, wild-type.

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### The MCAO model and experimental groups

The standard intraluminal MCAO 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 re-anesthetized, 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., Braintree, MA, USA). 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 transcardiac 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 microcomputer image device (MCID) digital image analysis system (Imaging Research, Inc., St Catherines, Ontario, Canada), and the hemorrhage volume of each slice was calculated as the hemorrhagic area on each surface time 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 TTC (Sigma, St. Louis, MO, USA) 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