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Reperfusion Activates Metalloproteinase's That Contribute to Neurovascular Injury

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Abstract

In this study, we examine the effects of reperfusion on activation of matrix metalloproteinase (MMP) and assess the relationship between MMP activation during reperfusion and neurovascular injury. Ischemia was produced using suture-induced middle cerebral artery occlusion in rats. The MMP activation was examined with *in situ* and gel zymography. Injury to cerebral endothelial cells and basal lamina was assessed using endothelial barrier antigen (EBA) and collagen IV immunohistochemistry. Injury to neurons and glial cells was assessed using Cresyl violet staining. These were examined at 3 hours after reperfusion (8 h after initiation of ischemia) and compared with permanent ischemia at the same time points to assess the effects of reperfusion. A broad-spectrum MMP inhibitor, AHA (p-aminobenzoyl-gly-pro-d-leu-d-ala-hydoxamate, 50 mg/kg intravenously) was administered 30 minutes before reperfusion to assess the roles of MMPs in activating gelatinolytic enzymes and in reperfusion-induced injury. We found that reperfusion accelerated and potentiated MMP-9 and MMP-2 activation and injury to EBA and collagen IV immunopositive microvasculature and to neurons and glial cells in ischemic cortex and striatum relative to permanent ischemia. Administering AHA 30 minutes before reperfusion decreased MMP-9 activation and neurovascular injury in ischemic cerebral cortex.

Keywords

Cerebral ischemia; Brain damage; Neuroprotection; Rats

Introduction

Recently, the occluded intracranial large vessels were directly opened with endovascular embolectomy device (Merci Retriever) in acute stroke patients. These treatments promoted recanalization and produced favorable outcome in the patients within 8 hours of the onset of stroke symptoms. However, intracranial hemorrhage occurred in 35.8% to 46.7 % of patients. Symptomatic intracranial hemorrhage occurred in 6.7% -9.9% of patients (Smith et al., 2005; Smith, 2006). Although it has been known that prolonged ischemia is important in hemorrhage transformation (HT) occurrence, the role of reperfusion itself on HT and related mechanism

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have not been well studied (Molina et al., 2001). In this report, in order to investigate the HT mechanisms, we have studied the effect of reperfusion on activation of matrix metalloproteinases (MMPs) after prolonged ischemia and its link to neurovascular injury.

MMPs have an important role in ischemia reperfusion injury after transient ischemia. In non-human primates, increased proMMP-2 coincided with early extravasations of plasma constituents and neuronal injury and increased proMMP-9 was observed in subjects with HT (Heo et al., 1999; del Zoppo and Mabuchi, 2003). Nonspecific MMP inhibitors decreased Evans blue leakage and brain edema (Rosenberg et al. 1998; Gasche et al., 2001; Pfefferkorn and Rosenberg 2003). A targeted knockout of MMP-9 decreased infarct volume (Asahi et al., 2001a, b). However, it is controversial whether MMP-9 and -2 are activated after transient ischemia. In some studies, activated MMP-9 was observed in mice and rats after ischemia (Fujimura et al. 1999; Planas et al., 2000, 2001; Gasche et al., 2001), while in others, no activated form of MMP-9 was reliably detected in rat, mice and baboons (Rosenberg et al., 1998; Heo et al., 1999;Asahi et al. 2001a; Pfefferkorn and Rosenberg, 2003). A few studies observed increase of active MMP-2 after ischemia, especially in the SOD1 knockout mouse (Heo et al., 1999; Planas et al., 2000; Gasche et al. 2001), but other studies did not detect activated MMP-2 (Rosenberg et al., 1998; Fujimura et al., 1999; Asahi et al., 2001a; Pfefferkorn and Rosenberg, 2003). Moreover, MMP changes after transient ischemia are impacted by two factors: ischemia and reperfusion injury. It is unclear whether reperfusion potentiates MMP activation as compared to ischemia alone. Since administering protective drugs in conjunction with endovascular embolectomy (just before reperfusion) is the most practicable time point for preventing HT in clinical practice, it is important to distinguish between these two mechanisms.

In the present study, we therefore tested the hypothesis that reperfusion, after prolonged cerebral ischemia, potentiates MMP activation relative to permanent ischemia that causes neurovascular injury. A rat suture middle cerebral artery occlusion model (MCAO) was used to precisely control reperfusion time. Permanent ischemia was used as the control to assess the effects of reperfusion. A water soluble, broad-spectrum MMP inhibitor, AHA (MMP-1, -3, -8 and -9 blocker), was administered to a subset of rats 30 minutes before reperfusion to assess the effects of MMPs on neurovascular reperfusion injury and the feasibility of AHA protection.

Methods and Materials

Rat Focal Cerebral Ischemia Model

Animal protocols were approved by the University of Cincinnati animal Care Committee and conform to the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male Sprague-Dawley rats weighing approximately 290-320 grams had unrestricted access to food and water and were housed with a 12-hour light-dark cycle.

Focal ischemia was produced as described in our previous studies (Lu et al., 2002). Briefly, rats were anaesthetized with 3% isoflurane and maintained with 1.5% isoflurane in a mixture of 70% N_2 and 28.5% O_2. Rectal temperature was monitored and maintained at 37 ± 0.5° C with a feedback-controlled heating blanket. Blood pressure, blood gases (pO_2, pCO_2, and pH) and blood glucose concentration were monitored by tail artery catheter and maintained in the normal range. The left common carotid artery, external carotid artery, and internal carotid artery were isolated via a ventral midline incision. A 3/0 monofilament nylon suture was used to occlude the middle cerebral artery. The suture was inserted into the external carotid artery and advanced into the internal carotid artery until the tip occluded the junction of the middle cerebral artery and anterior cerebral artery. Cerebral blood flow was measured using Laser Doppler (5 mm lateral and 2 mm posterior to bregma) (Nito et al., 2004). The wound was closed and the suture was kept in place for 5 hours. Rats were re-anesthetized, and the filament was

pulled back to achieve reperfusion. After the operation, rats were transferred to a temperature-controlled incubator at 37 °C for 30 minutes until animals completely woke up, and then to cages with the Delta Phase Isothermal Pad (Braintree scientific, Inc) to prevent hypothermia. Four groups of animals were studied (n = 4). (1) The sham operation group was given 0.9 % saline intravenously at 4.5 h with an infusion pump over a period of 30 minutes after sham operation, and sacrificed at 8 h. The sham operation was the same as the MCAO procedure except that there was no suture insertion and occlusion of middle cerebral artery. (2) The permanent ischemia group was administered 0.9 % saline at 4.5 h after MCAO using an infusion pump over a period of 30 min, and sacrificed at 5 h or 8 h after the onset of ischemia. (3) The ischemia-reperfusion group was administered 0.9 % saline intravenously at 4.5 h after MCAO using an infusion pump over a period of 30 min, reperfused at 5 h after MCAO, and sacrificed at 3 h after reperfusion (8 h after the onset of ischemia). (4) The ischemia-reperfusion with MMP inhibitor group was administered AHA (Calbiochem, CA, USA. 50 mg/kg in 0.9%saline) intravenously at 4.5 h after MCAO using an infusion pump over a period of 30 min, with reperfusion at 5 h after MCAO, and sacrificed at 3 h after reperfusion (8 h after the onset of ischemia). The dose of the AHA is from previous publication (Gasche et al., 2001).

Immunohistochemistry and Cresyl violet staining

At designated time points after MCAO, rats were deeply anesthetized with isoflurane and sacrificed. The brains were removed and frozen in 2-methylbutane. Coronal sections (20 μm) were cut in a cryostat (-20 °C) and stored at -80 °C. Sections were thawed and fixed in cold acetone or 2% Paraformaldehyde for 10 min and quenched in 3% hydrogen peroxide in PBS for 5 min. The sections were blocked for 1.5 hours in blocking buffer (2% serum/0.2% Triton X-100/0.1% BSA in 0.1 mol PB) and then incubated with primary antibody. After PB washes, the sections were incubated with a biotinylated secondary antibody for 1.5 hours (Vector Labs). After three 5-min PB washes, ABC reagent (Vector Labs) was applied. Diaminobenzidine was used to visualize the HRP. The primary antibodies used for immunohistochemistry included Mouse anti-Rat Endothelial barrier antigen (EBA) antibody (SMI71, 1: 1000; Sternberger Monoclonals. Lutherville, MD, USA), and Mouse anti-rat collagen IV monoclonal antibody (M3F7; 1: 200; Developmental Studies Hybridoma Bank at the University of Iowa, USA). EBA serves as a marker of the injury to endothelial cells, and collagen IV as a marker of the injury to cerebral microvascular basal lamina. Some sections were stained in 1% filtered cresyl violet solution to assess neuronal and glial cell injury.

For tissue measurements, we focused on sections at 0.2 mm anterior to bregma. For counting, two images were taken through parietal cerebral cortex (arrow, Fig 1.) where AHA showed the best protection; and four images were taken from the striatum (area numbers 1-4 on Fig 1). The average was calculated for measurements. Based on previously published data (Lin and Ginsberg, 2000), the following measurements were made: (1) The total area of the microvasculature per square millimeter of tissue ($\mu m^2/mm^2$) was measured and calculated for the EBA and Collagen IV stained sections; and the total area of the cells per square millimeter of tissue ($\mu m^2/mm^2$) was measured on cresyl violet stained sections. (2) In addition, the total numbers of separate EBA and Collagen stained microvascular profiles were counted in each square millimeter of tissue (numbers/mm^2); and the total numbers of cresyl violet stained cells were counted in each square millimeter of tissue (numbers/mm^2). These measurements were performed for the regions of parietal cortex shown in Figure 1 and in the areas of striatum shown in Figure 1 using the MCID automated imaging system. Finally, we calculated the average size of each microvascular EBA and Collagen IV stained profile by dividing the total area of the profiles ($\mu m^2/mm^2$) by the total numbers of profiles (numbers/mm^2) to give the average area of each microvascular profile (μm^2/microvascular profile). Similarly, we calculated the average area of each Cresyl violet stained cell by dividing the total area of the

cells ($\mu m^2/mm^2$) by the total numbers of cells (numbers/mm^2) to give the average area of each cell (μm^2/cell).

In situ zymography

The frozen sections were thawed and incubated with reaction buffer containing 100μg/ml FITC-labeled DQ gelatin for 10 hours at room temperature, and 2 hours at 37 °C. The sections were rinsed in PBS and then mounted in ProLong® Gold antifade reagent (Invitrogen, Carlsbad, CA, USA). The images were observed and pictures were taken using fluorescence microscopy. FITC-labeled DQ gelatin is intramolecularly quenched. Proteolysis by gelatinases yields cleaved gelatin-FITC peptides that are fluorescent. Incubation could vary from 2 hours to 24 hours and the fluorescence intensity increases with prolonged incubation times (Gasche et al., 2001; Jourquin et al., 2003). A constant exposure condition was used and the fluorescent intensity was visually estimated (Thiyagarajan et al., 2004).

MMP gel zymogram

Rats were deeply anesthetized with isoflurane and sacrificed. The brains were quickly removed and sliced into six 2-mm coronal slices at 4 °C. The cortex and striatum in the 2^{nd}, 3^{rd}, and 4^{th} slices were dissected and frozen immediately in dry ice. Brain tissue extracts were prepared as previously described (Zhang and Gottschall, 1997). Briefly, brain samples were homogenized in “working buffer” (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, and 0.02% NaN_3) and 1% Triton X-100, and then purified with gelatin-Sepharose 4B. The pellets were eluted with “elution buffer” (“working buffer” plus 10% DMSO). The proteins in the eluant were separated by electrophoresis through 10% polyacrylamide zymogram gels containing gelatin (Invitrogen, Carlsbad, California, USA). After washing in 2.5% Triton X-100 to remove SDS and then in 50 mM Tris-HCl, pH 7.5, the gels were incubated overnight in developing buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1μM $ZnCl_2$, and 0.05% BRIJ-35) at 37°C. Gels were stained with 0.1% Commassie blue and destained before being photographed and analyzed by the MCID automated imaging system.

Statistical analysis

Quantitative data were expressed as mean + SEM. Statistical comparisons were conducted using ANOVA for group comparisons. Differences with $p < 0.05$ were considered statistically significant.

Results

The change of cerebral blood flow after ischemia and reperfusion

Regional cerebral blood flow (rCBF) is measured with Laser Doppler flowmetry (LDF) in ischemia-reperfusion cortex. After ischemia, perfusion dropped to approximately 20% of baseline value (Table 1). The degree of ischemia was not statistically different between the groups. Reperfusion produced more than 90% recovery of average rCBF. AHA has no effect on rCBF (Table 1).

MMP activation and reperfusion injury of endothelial cells in cerebral blood vessels

EBA immunohistochemistry was performed to assess the injury to endothelial cells in cerebral blood vessels in rats following focal ischemia-reperfusion, and the relationship between reperfusion-induced MMP activation and the endothelial injury was defined by administering MMP inhibitor AHA just before reperfusion. The anti-EBA antibody is specific for a rat endothelial protein found in areas with blood-brain or blood-nerve barriers and is a marker of its integrity. EBA is decreased after brain trauma, inflammation, ischemia and hemorrhage

(Lin and Ginsberg, 2000; Nishigaya et al., 2000; Fagan et al., 2003; Gursoy-Ozdemir et al., 2004). In the cortex, 8 hour PMCAO produced modest injury to cerebral blood vessel endothelium. Some EBA-immunoreactive microvessels appear short, with a tendency towards decreased microvascular area ($\mu m^2/mm^2$) and decreased average microvascular size relative to sham animals ($p < 0.07$, Fig 2B, E and F). In contrast, 5 hours of focal ischemia followed by 3 hour reperfusion (8 h total) accelerated and potentiated injury to blood vessel endothelium. Many of the EBA-immunoreactive microvessels disappeared or had degraded to short segments (Fig 2C). The total microvascular area and size of each microvascular profile were significantly decreased compared with permanent ischemia 8 hours ($p < 0.05$ and 0.01, respectively from Figs 2 E and F). When the broad-spectrum MMP inhibitor AHA (50 mg/kg) was injected intravenously 30 minutes before reperfusion (4.5 h after MCAO), reperfusion-induced injury of blood vessel endothelium was decreased in parietal cerebral cortex where the total microvascular area and size of each microvascular profile were significantly increased ($p < 0.01$) relative to PMCO 8 h (Figs 2 D, E and F). Injury in striatum evolved at a much faster rate. PMCAO 5h and 8h produced severe injury, and significantly decreased the total microvascular area ($p < 0.05$) and size of each microvascular profile ($p < 0.05$ or 0.01) compared with sham animals (Table 2). Reperfusion 3 h after 5 h MCAO worsened endothelial cell injury resulting in further decreases in the total microvascular area (Table 2: $p < 0.05$, relative to PMCAO 8 h group). The microvascular numbers were also decreased relative to PMCAO 8 h (data not shown, $p < 0.05$). This was not be prevented by administering AHA 30 minutes prior to reperfusion (Table 2, AHA 50 mg/kg, i.v. starting from 4.5 h after MCAO).

MMP activation and reperfusion injury of cerebral microvascular basal lamina

Injury to the cerebral microvascular basal lamina was assessed using collagen IV immunohistochemistry in rats, and the relationship between reperfusion-induced MMP activation and the basal lamila injury was defined by administering MMP inhibitor AHA just before reperfusion. Type IV collagen is one of the main components of the basal lamina (Petty and Wettstein, 2001), and this protein disappears following focal ischemia and reperfusion in rats (Hamann et al., 2004). The effects of PMCAO and ischemia-reperfusion on Collagen IV immunoreactive microvessels in cortex and striatum were similar to those already described. In the cortex, PMCAO 8 h only slightly degraded Collagen IV of the basal lamina in parietal cortex. The average microvascular profile size was decreased compared with sham group ($p < 0.05$, Figs 3 B and F). However, 5 h ischemia followed by 3 hours of reperfusion accelerated and worsened the injury. The total microvascular area ($\mu m^2/mm^2$) and size of each microvascular profile were decreased further compared to PMCAO 8 h ($p < 0.05$, Fig 3 C, E and F). When the MMP inhibitor AHA (50 mg/kg) was infused intravenously 30 minutes before reperfusion (4.5 h after MCAO), these changes were partially reversed ($p < 0.05$ for the microvascular area and $p < 0.01$for the average microvascular size relative to the Isch5 h/R 3 h group) (Figs 3 D, E and F). In striatum, PMCAO of 5 h and 8 h led to definite damage to microvascular basal lamina assessed using Collagen IV immunoreactivity (Table 2). The total microvascular area was decreased compared with sham group ($p < 0.05$; Table 2). However, 5 hours of focal ischemia followed by 3 hour reperfusion (Ischemia 5 h/R 3 h) accelerated and worsened the injury, and further decreased the total microvascular area ($p < 0.01$) and the size of each microvascular profile ($p < 0.05$) relative to PMCAO 8 hours (Table 2). AHA (50 mg/kg, i.v. starting at 4.5 h after MCAO) did not protect against the injury to the basal lamina in the striatum (Table 2).

MMP activation and reperfusion injury to neuronal cells

Injury to neuronal cells was assessed using Cresyl violet staining, and the relationship between reperfusion-induced MMP activation and neuronal cell injury was defined by administering MMP inhibitor AHA just before reperfusion. In cortex there was a modest decrease in the total area of Nissl stained cells following 8 h of permanent MCAO ($p < 0.08$ relative to sham animals,

Figs 4 B and E). In contrast, 5 h MCAO followed by 3 h reperfusion markedly accelerated and worsened damage to cortex. The total number of cells (numbers/mm^2) and the average size of each cell were significantly decreased relative to PMCAO 8 h group ($p < 0.01$ and 0.05, respectively, Figs 4 C, E and F). This was partially reversed by the MMP inhibitor AHA (50 mg/kg) when it was injected intravenously 30 minutes before reperfusion (4.5 h after MCAO; $p < 0.05$, Figs 4 D, E and F). These results in cortex again contrasted with those in striatum. There was more severe damage by 5h and 8 h PMCAO and decreased the total cell area and the average cell size relative to sham operation group ($p < 0.01$; Table 3). The 5 hours of focal ischemia followed by 3 h reperfusion worsened the damage and resulted in a further decrease relative to PMCAO 8 h ($p < 0.01$ for the cell area and $p < 0.05$ for the size). The total cell numbers were also decreased relative to PMCAO 8 h ($p < 0.01$; Table 3). AHA (50 mg/kg, i.v. starting at 4.5 h after MCAO) did not protect the Nissl substance loss in the neuronal cells in the striatum (Table 3).

Reperfusion and MMP activation after cerebral ischemia

The effects of ischemia, reperfusion and AHA on activation of gelatinolytic enzymes were estimated using *in situ* zymography in the parietal cortex and striatum (Fig 5 a). Permanent ischemia for 8 hours caused weak activation of gelatinolytic enzymes in cortex (Figs 5 B) and moderate activation in striatum (data not shown). In contrast, 5 hours of focal ischemia followed by 3 hours of reperfusion (Ischemia 5 h, R 3 h, 8 h total) markedly increased gelatinolytic enzyme activation in parietal cortex (Fig 5 C) and in striatum. The methods used for the *in situ* gelatin zymography are thought to mostly detect MMP-2 and -9 (Snoek-van Beurden and Von den Hoff, 2005). To confirm that activated gelatinolytic enzymes are MMPs that are blocked by AHA, AHA (50 mg/kg) was injected intravenously just before reperfusion (4.5 h after MCAO, reperfusion starting at 5 h after MCAO). AHA blocked the activation of gelatinolytic enzymes in parietal cortex (Fig 5 D), but AHA did not block their activation in striatum. The gelatinolytic activity was most likely located in neural cells (C, arrowhead) and the microvasculature (C, arrow).

To further evaluate and quantify MMP activity of the extracted samples, gelatin gel zymagrams were performed (Fig 5 b). Active and latent forms of MMP-9 and their dimers were found in both MMP-9 positive control and ischemic samples. In the cortex, active MMP-9 (dimer) was statistically increased after 5 h ischemia followed by 3 h reperfusion relative to PMCAO group ($p < 0.05$, Figs 5 E and G). Administration of AHA just before reperfusion inhibited MMP-9 activation compared with ischemia 5 h/reperfusion 3 h group ($p < 0.05$,; Fig 5 E and G). Pro-MMP-9 was also increased after 8 h ischemia compared with sham animals ($p < 0.01$) and further enhanced after 5 hischemia/3 h reperfusion ($p < 0.05$), and decreased with AHA administration ($p < 0.01$, Fig 5 E and F). No significant change of MMP-2 was observed. In the striatum, Pro- and active MMP-9 were increased in both PMCAO group and ischemia 5h/ reperfusion 3 h group compared with sham control ($p < 0.01$ and 0.05, Fig 5 H and I). The 3 h reperfusion after 5 h ischemia also increased active MMP-2 compared with sham control or PMCAO group ($p < 0.01$, Fig 5 H and J). AHA did not inhibit MMP-2 and MMP-9 activation in the striatum.

Discussion

Our results show that reperfusion worsens neurovascular injury as compared to ischemia alone. Previous studies have shown that specific degradation of collagen IV and laminin occurs after cerebral ischemia. The loss of extracellular matrix in microvessel is associated with the loss of endothelial cell reactivity during focal ischemia. Endothelial cell P-selectin and E-selectin, and β_1-integrin expression within the ischemic territory occur only on those microvessels with an intact (laminin and collagen-containing) basal lamina. The degradation of parenchymal

laminin may interrupt cell-matrix interactions and Akt survival-signaling pathways, which triggers endothelial cell and neuron anoikis or apoptosis (del Zoppo et al., 1998; Lo et al., 2002; del Zoppo and Mabuchi, 2003; Wagner et al., 2003; Hamann et al., 2004). These reports support our results. Especially, our results demonstrate that administration of AHA just before reperfusion decreased the injury to the microvessel endothelium, basal lamina and neural cells in the parietal cortex. This suggests that MMP activation is an important mediator of reperfusion-induced injury to the neurovascular unit, and blocking MMP activation caused by reperfusion is likely to be a feasible treatment to decrease injury to the neurovascular unit.

Various studies support the concept that reperfusion potentiates MMP activation. First, reactive oxygen radicals are a key mediator of tissue damage after reperfusion in many organs including heart, kidney and brain (Lo et al., 2003). Oxidative stress triggers activation of MMP-9 and MMP-2 within the ischemic mouse brain (Gasche et al., 2001). Second, reperfusion after focal cerebral ischemia caused neutrophil adhesion to blood vessel endothelium, infiltration into the ischemic area and provided a major source of pro-MMP9 (del Zoppo and Mabuchi, 2003; Justicia et al., 2003; Gidday et al., 2005). Neutrophil elastase breaks down TIMP and activates MMP9 and MMP2 (Itoh and Nagase, 1995; Rice and Banda, 1995; Delclaux et al., 1996; Okada et al., 1988; Kohyama et al. 2002). Third, reperfusion increased nNOS and iNOS expression and nNOS activation after focal ischemia (Sorrenti et al., 1999; Holtz et al., 2001). Nitric oxide can directly activate MMP9 through S-nitrosylation at the catalytic site (Gu et al., 2002). Fourth, focal ischemia increased plasminogen activation in the ischemic hemisphere, and plasmin has been shown to activate pro–MMP-2 and pro-MMP-9 (Pfefferkorn et al., 2000; del Zoppo and Mabuchi 2003). Our results provide the direct evidence of reperfusion accelerating and potentiating MMP-9 activation in the ischemic cortex and MMP-2 activation in the ischemic striatum relative to permanent ischemia. In our results (Fig 4), the MMP signal is most likely located in neural cells and the microvasculature. Previous studies showed that ischemia-reperfusion produced genatinolytic activity in capillary and neural cells. Many injured neural cells showed strong genatinolytic signals (Gasche et al., 2001; Rosell et al., 2006). These reports are consistent with our results reported here. It is conceivable that MMPs from neural cells may contribute to the injury of both neurons and microvessels. The current study also showed that stronger MMP activation in reperfusion group led to more severe loss of EBA, collagen IV and neural cells. The MMP blocker administered just before reperfusion decreased both MMP activation and neurovascular injury in the cortex. These results also support the concept that MMP activation during reperfusion exacerbates neurovascular injury.

Even with mechanical recanalization, symptomatic intracranial hemorrhage occurs in 6.7% -9.9% of patients (Smith et al., 2005; Smith, 2006). Our unpublished experiments showed that reperfusion after 5 hours of cerebral ischemia worsened intracerebral hemorrhage and neurological function relative to permanent ischemia. The peak time of the hemorrhage and animal death was between 8 to 12 hours after ischemia. Administration of AHA just before reperfusion decreased intracerebral hemorrhage and improved neurological function. However, neither infarction volumes and nor brain edema was significantly changed in ischemia (5 hours)/ reperfusion (19 hours) group as compared to permanent ischemia. This study showed that the neurovascular injury was accelerated by reperfusion, which may contributes to more severe hemorrhage with behavior exacerbation.

A few caveats are worth considering. First, AHA administered just before reperfusion did not protect the striatum from injury to the neurovascular unit. This may be because 5 hours of ischemia induced too severe an injury in striatum compared to the cortex in striatum, and/or the possibility that 3 hours of reperfusion after 5 hours of ischemia had induced a strong MMP-9 and MMP-2 activation that was not blocked by a dose of 50 mg/kg AHA in the striatum. Further studies are needed to answer these questions. Second, the assessments were done at 8 hour

after ischemia. It is unclear whether the protective effect of MMP inhibition could be sustained behind this time window. A repeated administration of AHA might be necessary to sustain longer protective effects. However, the half-life time of AHA is not available at this time, so we do not know the required interval(s) for AHA administration. Future studies are also needed to clarify these issues. Third, we focused on brain sections at 0.2 mm to bregma for evaluate neurovascular injury. Although there are a good morphology of both striatum and cortex and reliable neurovascular injury in this section, assessments of more brain sections with a stereological method may increase precision of the results.

In summary, this is the first evidence that reperfusion, after prolonged ischemia, accelerated and potentiated the activation of MMP-9 and MMP-2 relative to permanent ischemia. Administering AHA just before reperfusion decreased MMP-9 activation and neurovascular injury in ischemic cortex. This work is clinically relevant, since no matter how reperfusion is achieved –using drugs or mechanical devices, this study suggests that the reperfusion per se injures the neurovascular unit after prolonged ischemia and blocking MMPs can decrease this injury.

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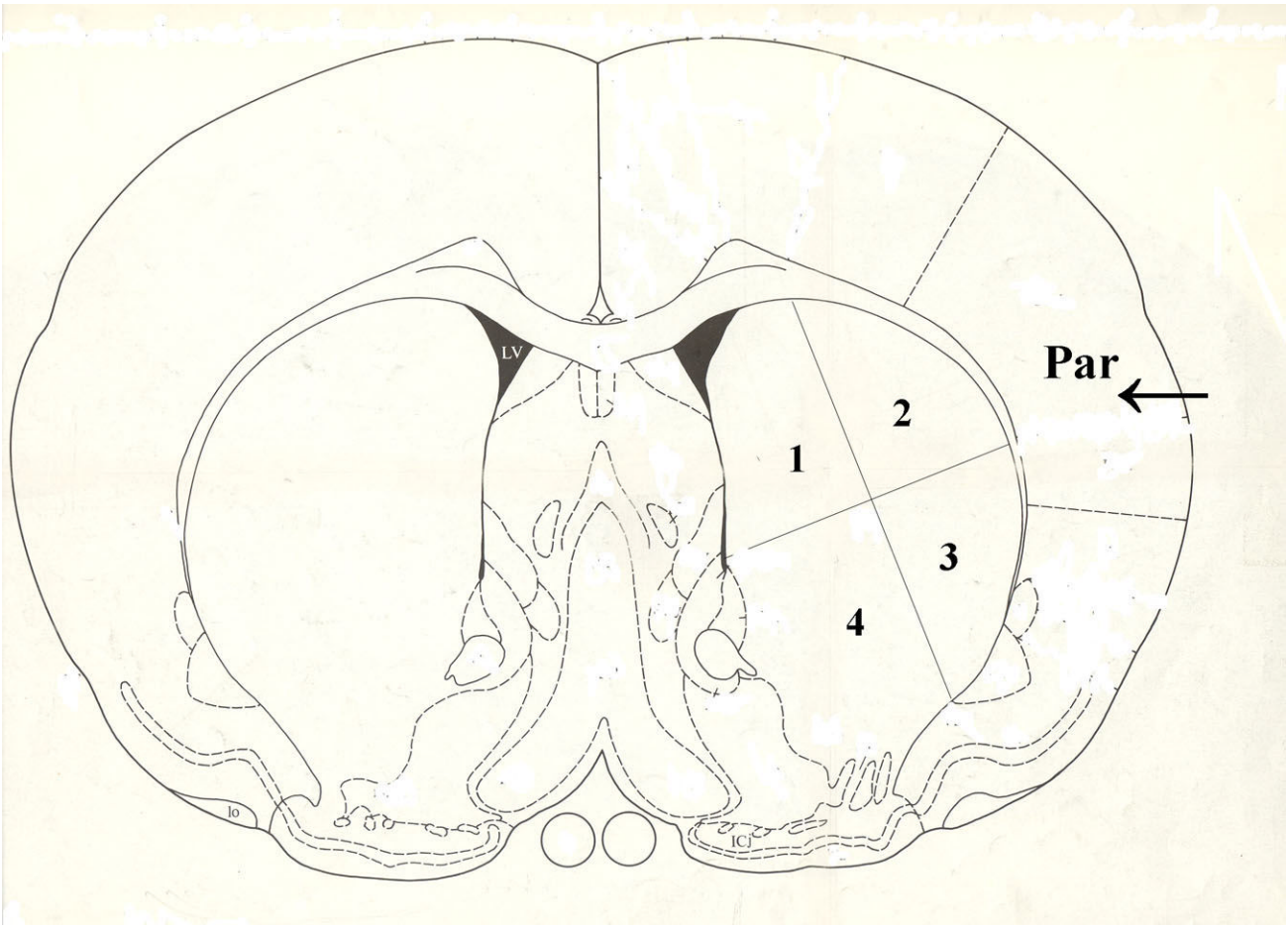


Fig 1.

Quantification was performed in the regions of parietal cerebral cortex (Par, arrow) and the striatum (area number 1-4) shown in this coronal section of the rat brain. In addition, all of the images in the following Figures were taken from these regions of parietal cerebral cortex (arrow in Par area) and striatum (area number 1-4).

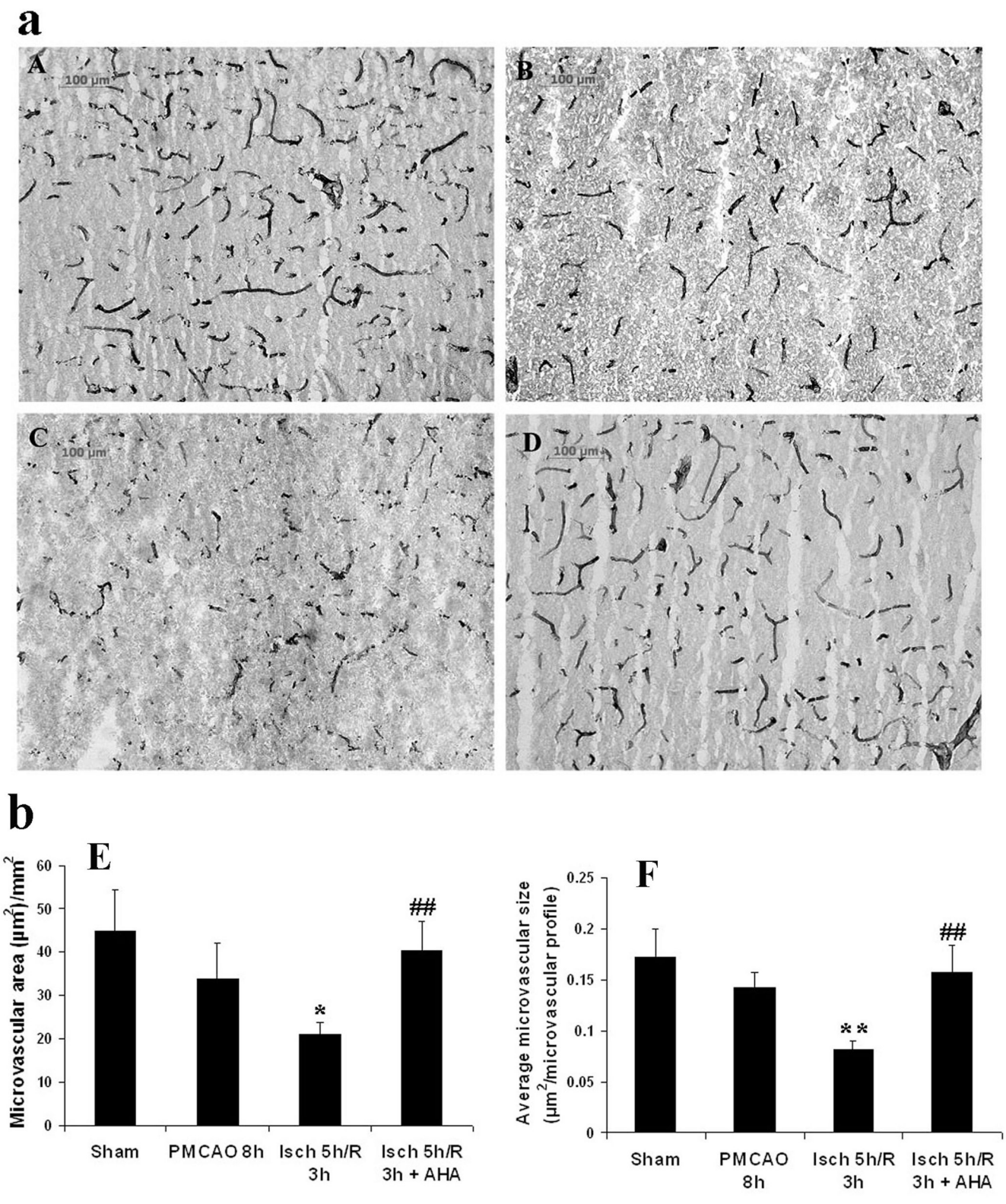


Fig 2.

EBA immunohistochemistry (Fig.2a; A-D) and quantification of the EBA staining (Fig.2b; E, F) in parietal cerebral cortex after focal MCA ischemia in adult rats. PMCAO: permanent middle cerebral artery occlusion. Isch: ischemia. R: reperfusion (n = 4). AHA: MMP blocker. (A) Sham. (B) PMCAO 8 h. (C) Isch 5 h/R 3 h. (D) Isch 5 h/R 3 h + AHA. *$p < 0.05$, **$p < 0.01$ compared with PMCO 8 h group. $^{\#\#}p < 0.01$ compared with Ischemia 5 h/R3 h group. Reperfusion 3 h after 5 h MCAO accelerated and potentiated injury to blood vessel endothelium relative to PMCAO 8 h in parietal cortex (C, E and F). Administering the MMP inhibitor AHA just before reperfusion (30 min before) decreased the injury (D, E and F).

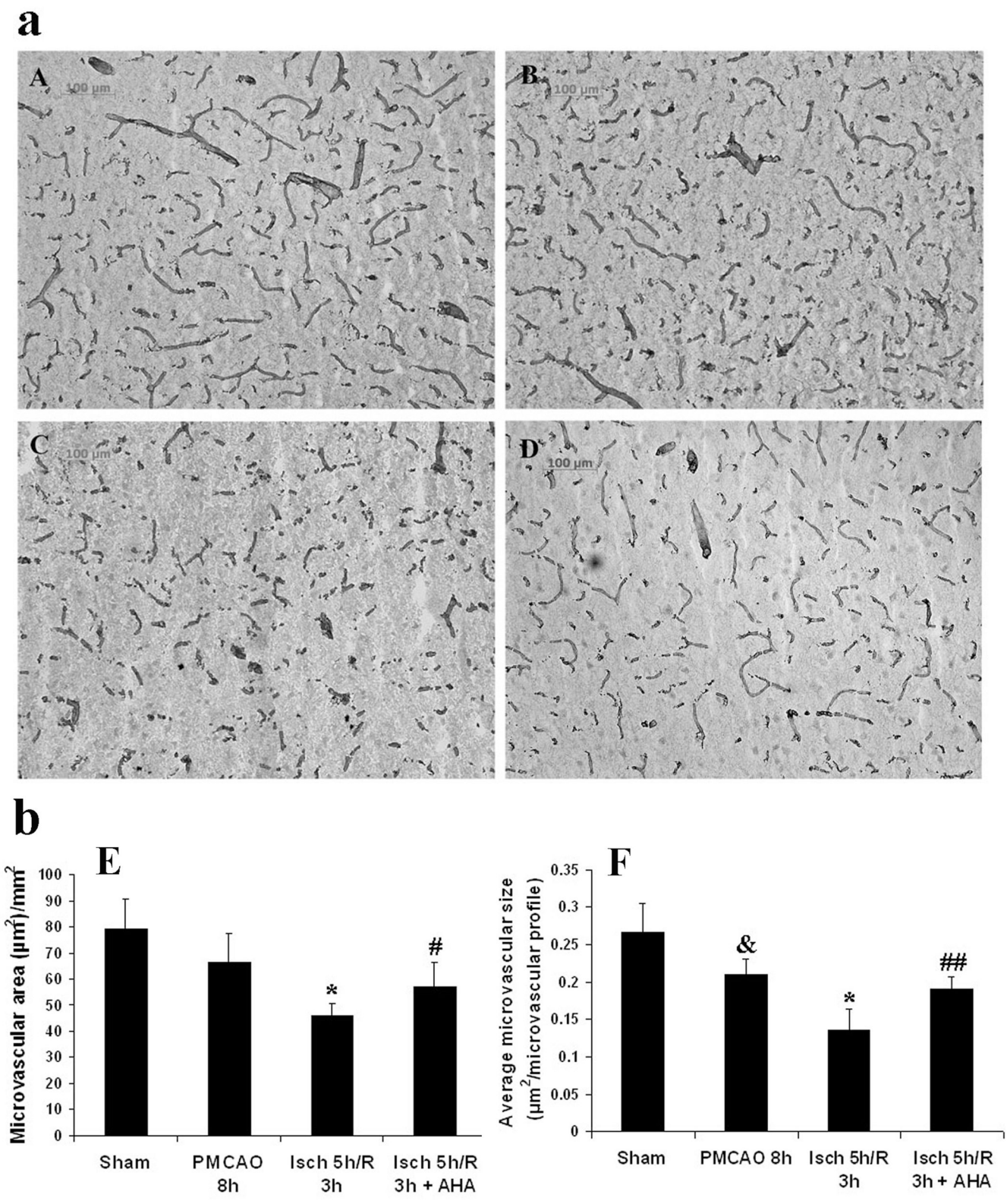


Fig 3.

Collagen IV immunostaining (Fig.3a; A-D) and quantification of the Collagen IV staining (Fig. 3b; E, F) in parietal cerebral cortex of adult rats following MCA focal ischemia. PMCAO: permanent middle cerebral artery occlusion (MCAO). Isch: ischemia. R: reperfusion (n = 4). (A) Sham. (B) PMCAO 8 h. (C) Isch 5 h/R 3 h. (D) Isch 5 h/R 3 h + AHA. $^{\&}p < 0.05$ compared with Sham group. $^{*}p < 0.05$ compared with PMCO 8h group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with Isch 5 h/R 3h group. Reperfusion 3 h after 5 h MCAO accelerated and worsened the injury to the basal lamina relative to PMCAO 8 h in parietal cortex (C, E and F). Administering the MMP inhibitor AHA just before reperfusion (30 min before) decreased the injury (D, E and F).

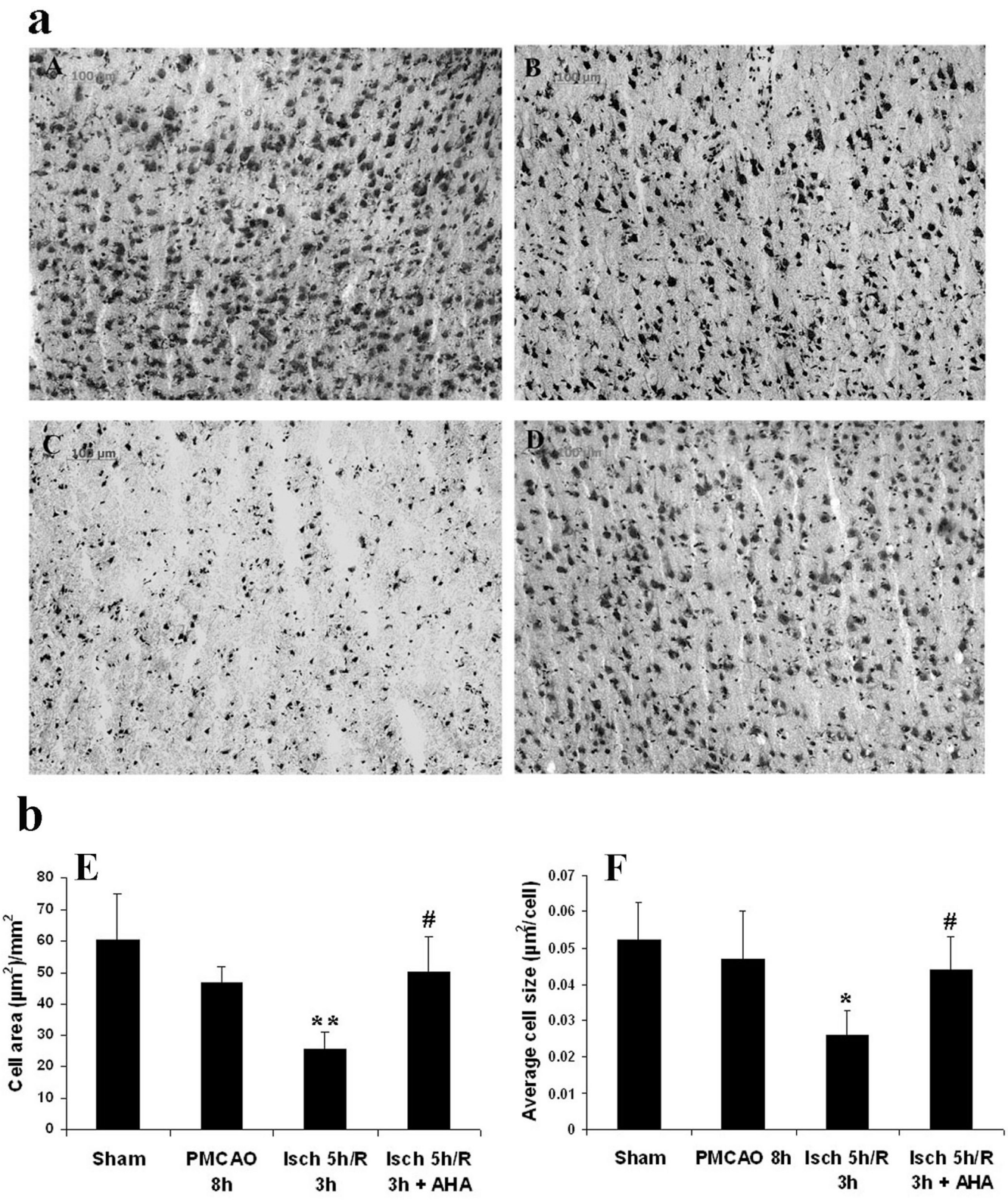
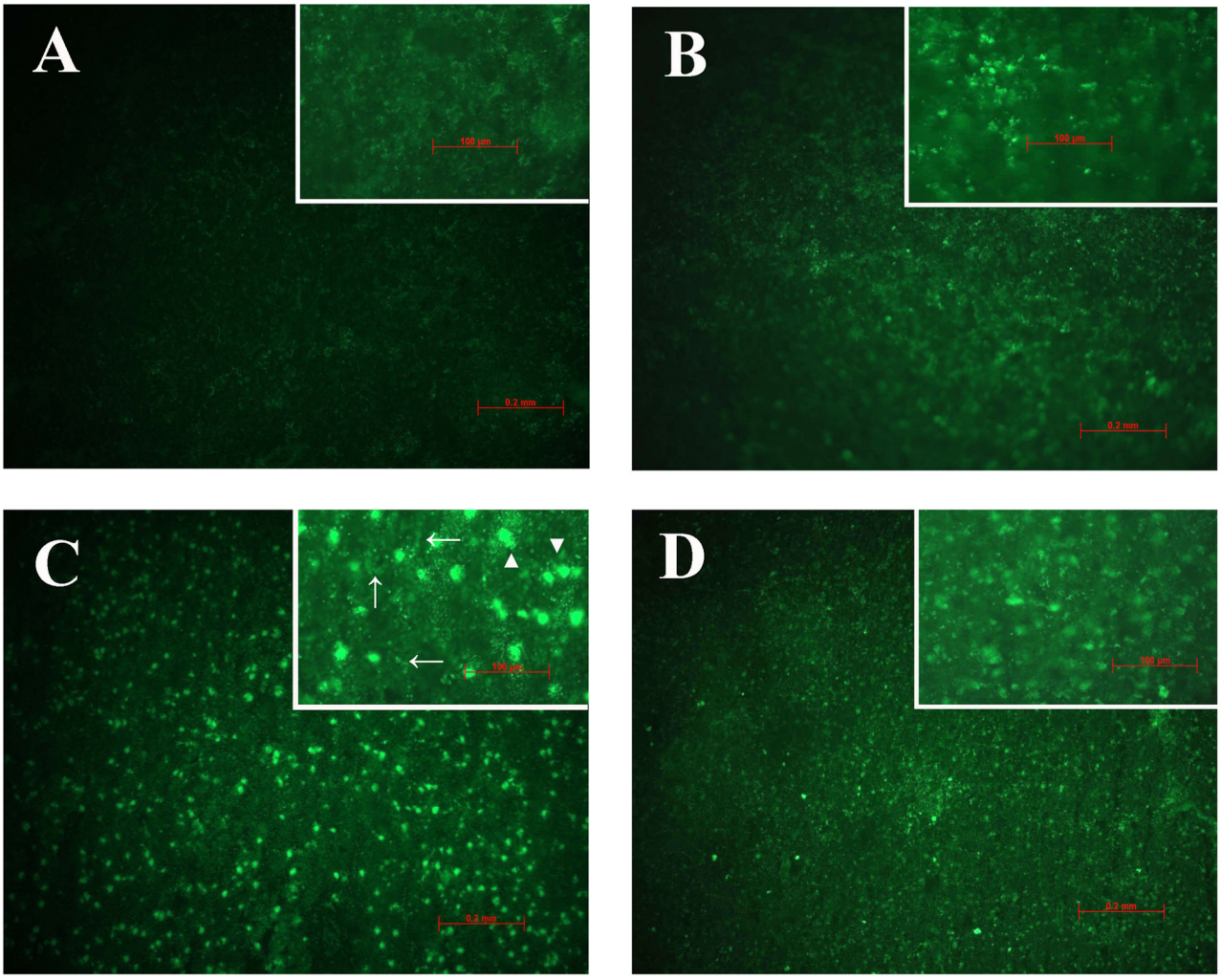


Fig 4.

Cresyl violet staining (Fig. 4a; A-D) and quantification of the Cresyl violet staining (Fig. 4b; E, F) in parietal cerebral cortex after focal ischemia in adult rats. PMCAO: permanent middle cerebral artery occlusion. Isch: ischemia. R: reperfusion (n = 4). (A) Sham. (B) PMCAO 8 h. (C) Isch 5 h/R 3 h. (D) Isch 5 h/R 3 h + AHA. *$p < 0.05$, **$p < 0.01$ compared with PMCO 8 h group. $^{\#}p < 0.05$ compared with Isch 5 h/R 3h group. Reperfusion 3 h after 5 h MCAO accelerated and worsened the injury to neuronal and glial cells relative to PMCAO 8 h in parietal cortex (C, E and F). Administering the MMP inhibitor AHA just before reperfusion (30 min before) decreased the injury (D, E and F).

a



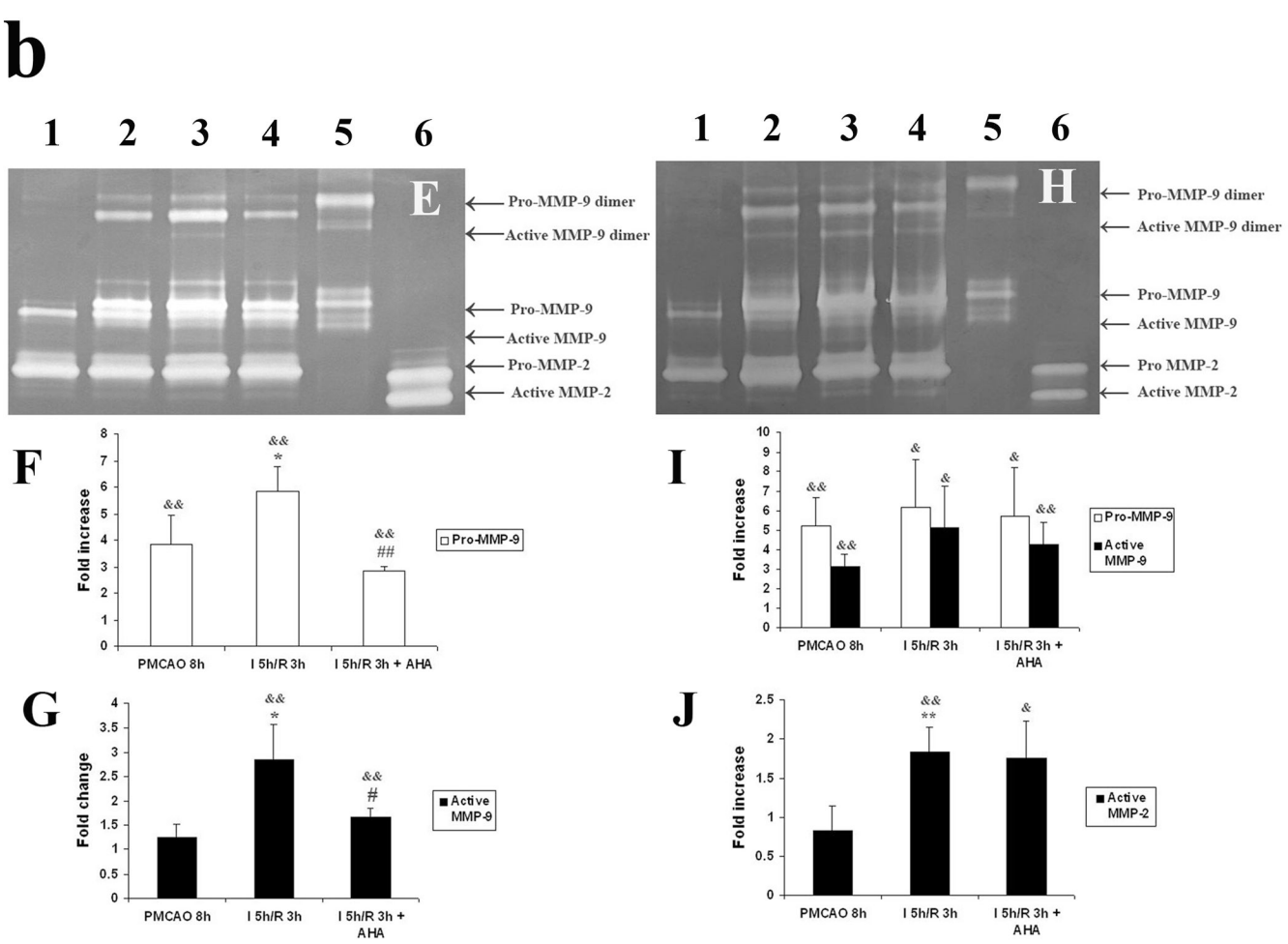


Fig 5.

a. *In situ* gelatinolysis assays in parietal cerebral cortex (Fig. 5a; A to D) of adult rats after focal MCA ischemia. PMCAO: permanent middle cerebral artery occlusion. Isch = ischemia. R = reperfusion (n = 3). (A) Sham. (B) PMCAO 8 h. (C) Isch 5 h/R 3 h. (D) Isch 5 h/R 3 h + AHA. The gelatinolytic activity (green fluorescence) was located in neural cells (C, arrowhead) and the microvasculature (C, arrow).

b. Gel zymogram and quantification of band density in cerebral cortex (Fig. 5b; E to G) and in striatum (Fig. 5b; H to G) of adult rats after focal MCA ischemia. PMCAO: permanent middle cerebral artery occlusion. I = ischemia. R = reperfusion (n = 4). (1) Sham. (2) PMCAO 8 h. (3) Isch 5 h/R 3 h. (4) Isch 5 h/R 3 h + AHA. (5) pro-MMP-9 positive control. (6) pro-MMP-2 positive control. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared with sham group. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with PMCAO 8 h group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with Isch 5 h/R 3 h group. 5 h of MCA ischemia followed by 3 h of reperfusion potentiated activation of MMP-9 in the cortex (E, G) and activation of MMP-2 in the striatum (H, J). Administering AHA just before reperfusion (30 min before) decreased MMP-9 activation in the cortex (E, G).

Table 1

The change of regional cerebral blood flow after ischemia-reperfusion

Group	Before MCAO	10 min after MCAO	5 h 10 min after MCAO (10 min after reperfusion)	8 h after MCAO (3 h after reperfusion)
PMCAO 8 h	100	20.6 ± 4.4**	17.9 ± 8.1**	17.2 ± 7.8**
Isch 5 h/R 3 h	100	17.4 ± 6.8**	93.6 ± 30.5	90.4 ± 23.8
Isch 5 h/R 3 h + AHA	100	21.9 ± 7.4**	95.7 ± 34.1	91.9 ± 23.0

PMCAO: permanent middle cerebral artery occlusion. Isch: ischemia. R: reperfusion (n=4). AHA: MMP blocker.

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

Table 2

The effects of reperfusion and the drug AHA on EBA- and collagen IV-immunoreactive microvessels in striatum.

Groups	EBA- immunoreactive		Collagen IV-immunoreactive	
	Total micro vascular area (μm^2)/mm^2 tissue	**Average microvascular size (μm^2/profile)**	**Total Micro vascular area (μm^2)/mm^2 tissue**	**Average microvascular size (μm^2/profile)**
Sham	39.4 ± 14.7	0.17 ± 0.03	55.5 ± 8.6	0.23 ± 0.06
PMCAO 5 h	21.2 ± 3.9$^{\&}$	0.11 ± 0.02$^{\&}$	43.2 ± 2.7$^{\&}$	0.19 ± 0.02
PMCAO 8 h	20.0 ± 3.6$^{\&}$	0.09 ± 0.02$^{\&\&}$	42.0 ± 3.8$^{\&}$	0.16 ± 0.01
Isch 5 h/R 3 h	12.9 ± 5.7*	0.08 ± 0.01	31.3 ± 3.6**	0.12 ± 0.02*
Isch 5 h/R 3 h + AHA	19.1 ± 6.9	0.08 ± 0.02	35.0 ± 1.8	0.14 ± 0.02

PMCAO: permanent middle cerebral artery occlusion. Isch: ischemia. R: reperfusion (n=4). AHA: MMP blocker.

$^{\&}p < 0.05$,

$^{\&\&}p < 0.01$ compared with Sham group.

$^{*}p < 0.05$,

$^{**}p < 0.01$ compared with PMCAO 8h group.

Table 3

The effects of reperfusion and AHA on the injury to neuronal and glial cells assessed with Cresyl violet staining in striatum.

Groups	Total cell area (μm^2)/mm^2 tissue	Cell numbers /mm^2 tissue	Average cell size (μm^2)/cell
Sham	67.9 ± 7.8	1237.6 ± 38.3	0.055 ± 0.005
PMCAO 5 h	34.7 ± 3.6&&	1205.4 ± 99.4	0.032 ± 0.004&&
PMCAO 8 h	34.1 ± 2.1&&	1239.6 ± 16.5	0.028 ± 0.002&&
Isch 5 h/R 3 h	19.2 ± 4.8**	991.1 ± 61.6**	0.019 ± 0.004*
Isch 5 h/R 3 h + AHA	22.5 ± 2.9	1114.6 ± 97.8	0.020 ± 0.003

PMCAO: permanent middle cerebral artery occlusion. Isch: ischemia. R: reperfusion (n=4). AHA: MMP blocker.

&& $p < 0.01$ compared with Sham group.

* $p < 0.05$,

** $p < 0.01$ compared with PMCAO 8h group.