Cathepsin and Calpain Inhibitor E64d Attenuates Matrix Metalloproteinase-9 Activity After Focal Cerebral Ischemia in Rats

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Background and Purpose—Matrix metalloproteinases (MMPs) and cysteine proteases (calpain and cathepsin B) play an important role in cell death and are upregulated after focal cerebral ischemia. Because there is a significant interaction between MMP-9 with calpain and cathepsin B, we investigated the role of E64d (a calpain and cathepsin B inhibitor) on MMP-9 activation in the rat focal ischemia model.

Methods—Male Sprague-Dawley rats were subjected to 2 hours of middle cerebral artery occlusion by using the suture insertion method followed by 22 hours of reperfusion. In the treatment group, a single dose of E64d (5 mg/kg IP) was administrated 30 minutes before the induction of focal ischemia, whereas the nontreatment group received dimethyl sulfoxide only. The neurological deficits, infarct volumes, Evans blue extravasation, brain edema, and MMP-9 activation in the brain were determined.

Results—Pretreatment with E64d produced a significant reduction in the cerebral infarction volume (353.1 ± 19.8 versus 210.3 ± 23.7 mm³) and the neurological deficits. Immunofluorescence studies showed MMP-9, calpain, and cathepsin B activation colocalized to both neurons and the neurovascular endothelial cells after ischemia, which was reduced by E64d.

Conclusion—These results suggest that E64d treatment provides a neuroprotective effect to rats after transient focal cerebral ischemia by inhibiting the upregulation of MMP-9.

Key Words: blood–brain barrier • calpain • cathepsin B • ischemia • matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-binding proteolytic enzymes involved in the remodeling of the extracellular matrix. MMPs, MMP-9 in particular, have been implicated previously in the pathogenesis of stroke. Activation of MMP-9 has been shown to increase blood–brain barrier (BBB) permeability, inflammatory infiltration, brain edema, and infarction volume after cerebral ischemia reperfusion injury.

Calpains and cathepsins are 2 families of cysteine proteases that play an important role in stroke-induced cell death. It has been reported that both calpain and cathepsin inhibitors prevent neuronal apoptosis after cerebral ischemia. Furthermore, evidence from recent studies suggested that calpain/cathepsin B and MMP-9 are interlinked. Inhibition of calpain or cathepsin B has been shown to prevent activation of MMP-9 in human glioblastoma and leukemic cell lines. A potential role for calpain/cathepsin B inhibitors in the prevention of MMP-9 activation after a neurological insult has not been studied. We therefore investigated the effect of E64d treatment on MMP-9 activation after focal cerebral ischemia and reperfusion injury.

Materials and Methods
The experimental protocol was approved by the local animal care and use committee of Loma Linda University. Sixty-five adult male Sprague-Dawley rats (Harlan, Inc; Ind) weighing between 270 and 350 g were divided randomly into 3 groups: sham, dimethyl sulfoxide (DMSO), and E64d.

Surgical Procedure
The animals were fasted overnight but were allowed free access to water. The body weight for each rat was calculated before the surgery. Anesthesia was induced with ketamine (80 mg/kg IP) followed by atropine at a dose of 0.1 mg/kg. A heating pad and a heating lamp were used to maintain the rectal temperature between 36.5°C and 37.5°C. Rats were intubated, and respiration was maintained with a small animal respirator (Harvard Apparatus). Rats were subjected to middle cerebral artery (MCA) occlusion as described by Yin et al but with some added modifications. Briefly, the left common carotid artery, internal carotid artery, and external carotid artery was occluded. The external carotid artery was then isolated and coagulated. A 4-0 nylon suture with silicon (Doccol Co.) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. The mean arterial blood pressure, heart rate, arterial blood gases, and blood glucose levels before, during, and after ischemia.
were analyzed. After 2 hours of MCA occlusion (MCAO), the suture was carefully removed to restore blood flow, the neck incision was closed, and the rats were allowed to recover. The body temperature was carefully monitored during the postoperative period and until the complete recovery of the animal from the anesthetic. After the experiment, the animals were housed individually until euthanized. All animals had free access to food and water.

**Treatment**

The treatment group was injected with E64d ([l-3-trans-carboxybene2]; 5 mg/kg; Biomol Inc) intraperitoneally 30 minutes before focal ischemia was induced. E64d was diluted with 1% DMSO to a concentration of 15 mg/mL. In the DMSO group, rats were treated with the same volume of DMSO that was delivered intraperitoneally 30 minutes before the focal ischemia.

**Neurological Scores**

The neurological scores were evaluated 24 hours after the MCAO using a scoring system reported by Garcia et al with modifications. The neurological examination was performed in a blinded fashion.

**2,3,5-Triphenyltetrazolium Chloride Staining and Evaluation of Infarction Volume**

Twenty-four hours after the MCAO, the rats (n=5 for each group) were deeply anesthetized with ketamine and then decapitated, after which the brains were rapidly removed. The brains were then carefully evaluated macroscopically for hemorrhagic transformation. The tissue was sliced into 2-mm-thick coronal sections, and the slices were stained in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) for 30 minutes at 37°C in the dark. The infarction areas were traced and were analyzed by Image J software (NIH), version 1.32.

**Brain Water Content**

Twenty-four hours after the MCAO, the brains (n=5 for each group) were removed and immediately separated into right and left hemispheres. The hemispheres were weighed after removal (wet weight) and again after drying in an oven at 105°C for 24 hours as described by Xi et al. The percentage of water content was calculated as [(wet weight – dry weight)/wet weight]×100%.

**Evans Blue Dye Extravasation**

Disruption of the BBB was analyzed 24 hours after the MCAO (n=3 for sham; n=5 for the DMSO and the E64d groups) using Evan’s blue (EB) dye as reported previously by Park et al with some modifications. Briefly, EB dye (4%; 2.5 mL/kg) was injected over 2 minutes into the left femoral vein and allowed to circulate for 60 minutes. Rats were deeply anesthetized and transcardially perfused with PBS until a colorless perfusion fluid was obtained from the right atrium. The amount of extravasated EB in the brain was determined by spectrofluorometry. Measurements were conducted at an excitation wavelength of 610 nm, an emission wavelength of 680 nm, and a bandwidth of 10 nm.

**Morphological Assessment**

Twenty-four hours after MCAO, rats were anesthetized and transcardially perfused with PBS and 10% paraformaldehyde as described previously. Brains were quickly removed and postfixed in 10% paraformaldehyde and 30% sucrose for 3 days. Coronal tissue sections 10 µm thick were cut with the aid of a cryostat (Leica LM3050S). For hematoxylin and eosin (H&E) staining, slices were stained with hematoxylin for 3 minutes and eosin for 30 seconds. Brain immunolocalization of IgG was conducted according to the protocols described previously. Briefly, sections were incubated with biotinylated anti-rat IgG antibody 1:100 (Santa Cruz Biotechnology) and then treated with an avidin-biotin peroxidase conjugate staining kit (Santa Cruz Biotechnology). Double and triple fluorescent labeling was performed as described previously. Briefly, brain sections were incubated with primary antibodies, namely MMP-9 (Santa Cruz Biotechnology, 1:200; Chemicon International Inc., 1:200), von Willebrand factor, anti-neuronal nuclei (neuronal marker; BD Biosciences; 1:200), cathepsin B, and calpain (Santa Cruz Biotechnology; 1:200) at 4°C for 24 hours. After rinsing with PBS, the sections were incubated for 1 hour in fluorescein isothiocyanate-, Texas red-, or aminomethyl-coumarin–conjugated secondary antibodies (Jackson ImmunoResearch; 1:100). The sections were then visualized using a fluorescent microscope (Olympus), and photographs were taken. Analysis of the pictures was performed using MagnaFire SP 2.1B software.

**Zymography**

Animals were euthanized 24 hours after MCAO for zymography (n=4 for each group). Rats were deeply anesthetized and transcardially perfused from the left ventricle. Brains were then divided into sections, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. The ipsilateral brain cortex was used to analyze MMP-9. Briefly, samples were homogenized in lysis buffer including protease inhibitors. After centrifugation, the supernatant was collected, and the total protein concentration was determined using the Bradford assay (Bio-Rad). Samples were loaded and separated by 10% Tris-tricine gel with 0.1% gelatin as a substrate. After separation by electrophoresis, the gel was renatured and then incubated with development buffer at 37°C for 24 hours. After development, the gel was stained with 0.5% compassion blue R-250 for 30 minutes and then destained appropriately. MMP activity was quantified with Image J, version 1.32. Optical densities were normalized to positive controls and calculated as percent above sham.

**Statistics**

Data are presented as means±SEM. Statistical differences between the various groups were assessed by a 1-way ANOVA followed by the Tukey and Mann–Whitney rank sum test. Comparisons between the 2 groups for the infarction volume and the neurological scores were assessed by the unpaired t test. A value of P<0.05 was considered statistically significant.

**Results**

**Physiological Data**

No statistical differences were observed between the DMSO and the E64d groups with regard to mean arterial blood pressure, heart rate, arterial blood gases, or glucose levels before, during, or after ischemia (data not shown).

**Effect of E64d on Brain Infarction Volume and Neurological Score**

Representative samples of TTC-stained brain sections in the DMSO and E64d groups are shown in Figure 1A. The mean infarction volume in the DMSO group was 353.1±19.8 versus 210.3±23.7 mm³ in the E64d group at 24 hours after the MCAO. E64d treatment significantly reduced the cerebral infarction volume compared with the DMSO group (P<0.05; Figure 1B).

The neurological scores in the DMSO and E64d groups were 9.2±0.4 and 11.6±0.4, respectively (Figure 1C). E64d treatment was found to increase the neurological scores significantly (P<0.05).

**BBB Permeability, Brain Edema, and Hemorrhagic Transformation**

IgG staining demonstrated BBB leakage in the MCA area, represented by IgG passing through a disrupted BBB and penetrating into the brain parenchyma. In the E64d group,
IgG staining was observed to a lesser extent compared with DMSO rats (Figure 2A).

The EB contents of the brain tissue for the sham-operated, DMSO, and E64d groups were 3.2±0.1, 9.2±0.9, and 4.9±0.4 ng/mg of tissue, respectively (Figure 2B). There was a significant increase in the permeability of the BBB in the DMSO group compared with the E64d group (P<0.01).

A significant increase in brain water content was revealed in rats 24 hours after the MCAO when compared with the sham group in the left hemisphere. The mean water content of the left hemisphere was 80.5±0.6% in the E64d group and 83.9±0.3% in the DMSO group (P<0.05; Figure 2C). No statistical difference in the groups for the right hemisphere water content was observed (sham versus DMSO versus E64d; 79.1±0.4 versus 79.8±0.2 versus 79.3±0.3).

Hemorrhagic transformation was not observed in the sham group. Hemorrhagic transformation was seen macroscopically in the ipsilateral MCA territory as shown in the coronal sections in the ischemic core (Figure 2D). In the DMSO group, the hemorrhagic transformation ratio was 17.4% (4 of 23 rats) at 24 hours after the MCAO. In contrast, the hemorrhagic transformation ratio was significantly decreased in the E64d group, with a ratio of 4.3% (1 of 23 rats; P<0.05). The H&E staining revealed extravasation of blood from the microvessels into the surrounding brain parenchyma (Figure 2D).

**MMP-9 Expression and Activity**

To demonstrate the interaction between MMP-9, cathepsin B, and calpain activation, we performed triple fluorescent staining in the tissue from the periphery of the ischemic cortical lesion at 2, 6, and 24 hours after the MCAO. An initial rise in calpain and cathepsin B activity was detected as early as 2 hours after the reperfusion by immunohistochemistry. However, at this time point, MMP-9 activation was not detected. By 6 hours after the reperfusion, there was a progressive expansion of the protease activity as well as the MMP-9 activation. Twenty-four hours after MCAO, extensive increases in calpain, cathepsin B, and MMP-9 were observed in both neurons and the neurovascular structures in the DMSO group, and merge imaging showed activated calpain, cathepsin B, and MMP-9 on concomitant cells by a white color (Figures 3 and 4). A significant reduction in both neural and vascular MMP-9 expression was observed in the E64d treatment group (Figure 4).

To determine the effect of E64d treatment on MMP-9 activity, zymography using gelatin as a substrate was performed at 24 hours after MCAO. The DMSO group showed increased expression of both pro–MMP-9 and activated MMP-9 (Figure 5B). Compared with DMSO, E64d significantly reduced the intensity of the band 24 hours after MCAO. Total MMP-9 levels were quantified together. Densitometric analysis of these bands showed that the reduction by E64d was significant (P<0.05).

**Discussion**

Evidence from recent studies strongly implicated a role for MMP-9 in the development of cerebral vascular degradation after focal cerebral ischemia. Activated MMP-9 damages neurovascular substrates such as type IV collagen, laminin, and fibronectin, which are the major components of the basal lamina around the vessel. Hence, MMP-9 activation leads to a breakdown of the BBB, ultimately hemorrhage, and edema, which increases cerebral infarction volume. However, it
is important to note that MMP-9 activation occurs in a variety of cells in the central nervous system, including neurons\textsuperscript{15,16,18} and endothelial cells.\textsuperscript{4} Consistent with previous reports, our results clearly showed the activation of MMP-9 in both the vascular endothelium and in cortical neurons after MCAO (Figures 3, 4, and 5A).

Recent studies showed that there is a link between calpain, cathepsin B, and MMP-9 upregulation. However, the precise mechanism by which these proteases activate MMPs is not known. Expression of cathepsin B is elevated in malignant tumors, particularly around the invasive edge of the tumor.\textsuperscript{19,20} Cathepsin B antisense transfection reduces inhibition of MMP-9 activity in vivo.\textsuperscript{10} Furthermore, calpain inhibitor CP1B has been shown to reduce mRNA expression of MMP-9 and MMP-2 in leukemic cells.\textsuperscript{9} In the present study, we showed that the activation of MMP-9, calpain, and cathepsin B were extensively colocalized to both the neuronal cells and the neurovascular structures at 24 hours after the ischemic insult. However, analysis of the protease and MMP-9 activation at different time points showed distinct patterns of activation, indicating the involvement of multiple steps in the process. Activation of the proteases occurred at early time points compared with MMP-9 activation. However, the inhibition of calpain and cathepsin B activation by E64d treatment reduced subsequent MMP-9 activation in both cell types, suggesting that calpain and cathepsin B could act directly on MMP-9 activation.

Because this is a proof of principle study, we administered E64d as a pretreatment. E64d is highly selective for cysteine proteases; however, the pharmacological properties of E64d, including its passage through the intact BBB, have not yet been fully investigated. Because the endothelium is located at the interface between the blood and the vessel wall, and E64d is a cell membrane-permeable drug, we speculated that E64d could penetrate the endothelial cell membrane and could inhibit proteases and subsequent

\begin{figure}
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\caption{A, Negative IgG staining was observed in the sham group, with extensive strong staining seen in DMSO group, and confined staining was observed in E64d group. B, The E64d group demonstrated a reduced brain EB content compared with the DMSO-treated group ($P<0.05$). C, The E64d group had a significantly decreased water content compared with the DMSO group ($P<0.05$ vs sham; #P<0.05 vs DMSO). D, Hemorrhagic transformation is observed in the DMSO group with an incidence of 17.4% at 24 hours after MCAO. Hemorrhage is noted in the lateral caudoputamen and frontoparietal cortex. H&E staining showed that erythrocytes leak from the microvessels into the surrounding brain parenchyma. Bars=40 $\mu$m.}
\end{figure}
MMP-9 activation, which consequently resulted in decreased BBB leakage and brain edema. However, the inhibition of neural proteases and MMP-9 activation may be related to the preservation of the BBB integrity or the direct effect of E64d on neurons. Further investigations are needed to clarify these issues.

The inhibiting effect of E64d on MMP-9 activation may be related to the Fas/FasL system. For example, E64d blocks aberrant apoptosis of cultured peripheral blood lymphocytes from HIV-infected individuals by inhibiting the upregulation of FasL. Recently, Ogier et al. demonstrated that the activation of Fas stimulates MMP-9 release by astrocytes. They suggested that the activation of tumor necrosis factor superfamily members triggers inflammatory signals in astrocytes and that MMP-9 could act as an inflammatory factor downstream of Fas activation. It has also been demonstrated that the expression of Fas and FasL plays an important role in both neural and endothelial cell apoptosis.

**Figure 3.** Activation of calpain, cathepsin B, and MMP-9, 2 and 6 hours after the insult in the peri-infarct penumbra cortex. Triple immunofluorescence staining was performed for calpain (red), cathepsin B (blue), and MMP-9 (green). An initial rise in calpain and cathepsin B activity is detected as early as 2 hours after the reperfusion (A and B), but MMP-9 activation was not detected (C). By 6 hours after the reperfusion, there was a progressive expansion of the protease activity as well as MMP-9 activation, which began to occur (E through H). E64d treatment reduced the number of protease positive cells (I through L). Bar=40 μm. Arrows show vascular structures.

**Figure 4.** Calpain, cathepsin B, and MMP-9 were observed in concomitant cells and vascular structures in the peri-infarct penumbra cortex. Triple immunofluorescence staining was performed for calpain (red), cathepsin B (blue), and MMP-9 (green; A through C and E through G). Merge imaging is shown in white (D and H). Bars=40 μm. E64d treatment reduced calpain, cathepsin B and MMP-9 activation in both neurons and vascular structures (I through P).
Lee et al\textsuperscript{4} demonstrated that the upregulation of MMP-2 and MMP-9 triggers apoptosis in human cerebral endothelial cells after hypoxia reoxygenation by interrupting cell matrix interactions and disrupting integrin signaling. Besides the disruption of the basal lamina of the vascular structures by MMP-9, vascular endothelial apoptosis is also involved in decreased vascular integrity after ischemic insults. It can be hypothesized that E64d can protect BBB and decreases brain edema by preventing MMP-9 activation in endothelium, which can trigger endothelial cell apoptosis. Moreover, activation of MMP-9 not only induces endothelial cell death but also neural apoptosis. Recently, Gu et al\textsuperscript{16} determined that MMP-9 degrades the extracellular matrix protein laminin, which induces neuronal apoptosis after focal cerebral ischemia.

In summary, we have shown that a single dose of 5 mg/kg E64d 30 minutes before the induction of focal ischemia not only prevents activation of calpain and cathepsin B but also reduces MMP-9 activation in both neuronal cells and neurovascular endothelial cells after transient MCAO in rats. However, a poststroke application of calpain and cathepsin B inhibitors on MMP activation after focal cerebral ischemia should be tested in further investigations.

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**Disclosures**

None.

**References**


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