Role of Protease-Activated Receptor-1 in Brain Injury After Experimental Global Cerebral Ischemia

Jinhua Wang, MD; Hang Jin, MD; Ya Hua, MD; Richard F. Keep, PhD; Guohua Xi, MD

Background and Purpose—Evidence suggests that the protease-activated receptor-1 (PAR-1), a thrombin receptor, mediates neuronal injury in experimental cerebral ischemia. The present study investigated whether PAR-1 plays a role in brain injury after global cerebral ischemia.

Methods—Adult male wild-type or PAR-1 knockout mice underwent a 20-minute bilateral common carotid artery occlusion or a sham operation. Behavior tests were performed before ischemia and 1, 2, and 3 days after bilateral common carotid artery occlusion. Mice were euthanized at different time points for thrombin activity, brain edema, Western blot analysis, and brain histology.

Results—Thrombin activity and PAR-1 expression were increased in the brain after bilateral common carotid artery occlusion. Compared with wild-type mice, PAR-1 knockout mice had less brain edema formation, neuronal death, and behavior impairment after bilateral common carotid artery occlusion. In addition, bilateral common carotid artery occlusion-induced activation of mitogen-activated protein kinases was absent in PAR-1 knockout mice.

Conclusion—PAR-1 contributes to the brain injury induced by global cerebral ischemia, which may be related to activation of mitogen-activated protein kinases. (Stroke. 2012;43:00-00.)

Key Words: brain edema ▪ global cerebral ischemia ▪ mitogen-activated protein kinases ▪ protease activated receptor-1 (PAR-1) ▪ thrombin

Global cerebral ischemia is caused by events such as cardiac arrest, cardiovascular surgery, and neurosurgical procedures. Severe brain damage occurs after global cerebral ischemia, and clinical outcomes remain poor. Therefore, it is critical to understand the mechanisms of neuronal death after global cerebral ischemia.

Evidence shows that thrombin activation plays a role in ischemic injury. This is mediated, at least in part, by protease-activated receptors (PARs). PARs are a superfamily that includes 4 G-protein coupled receptors (PAR-1 to PAR-4) and PAR-1 is considered as the main subtype. PAR-1 can be activated by thrombin and is known as a thrombin receptor. PAR-1 is expressed throughout the central nervous system and participates in brain injury after hemorrhagic and focal ischemic stroke. However, the role of PAR-1 in brain injury after global cerebral ischemia remains unclear.

The hippocampus shows vulnerability to global ischemia and it has been the focus of much research. However, the basal ganglia are also susceptible to damage after global ischemia, which has not been as well studied. Recent studies have described a transient global cerebral ischemia model with consistent basal ganglia injury in mice.

In this study, we investigated the role of PAR-1 in brain injury and activation of mitogen-activated protein kinases (MAPK) after global cerebral ischemia. PAR-1 knockout mice displayed markedly reduced brain injury and an absence of the MAPK activation found in wild-type mice.

Materials and Methods

Animal Preparation, Transient Global Cerebral Ischemia, and Intracerebral Injection of Thrombin

The University of Michigan Committee on the Use and Care of Animals approved the protocols for these animal studies. Male PAR1 knockout (PAR-1−/−) mice with C57BL/6J background and their wild-type littersmates (8–12 weeks; University of Michigan Breeding Core) were used. Mice were anesthetized with ketamine (100 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally). Blood glucose levels were measured in tail blood samples taken just before occlusion of bilateral common carotid arteries. Rectal temperature was maintained at 37.0 ± 0.5°C by a feedback-controlled heating pad. Regional cerebral blood flow was monitored by a laser Doppler flowmeter. The probe was fixed on the skull, 4 mm lateral to the bregma. Changes in regional cerebral blood flow after bilateral common carotid artery occlusion (BCCAO) were expressed as a percentage of the preischemic baseline value. Only animals whose regional cerebral blood flow decreased to <15% of preischemic value were included in this study. The BCCAO model was induced by using nontraumatic microaneurysm clips to occlude both common carotid arteries for 20 minutes. After clip removal, the skin incision was closed with suture, and animals were kept in a warm chamber (33–34°C) for 4 hours before being returned to their home cages at room temperature. Sham-operated animals underwent the same procedures described previously but without BCCAO. Normal saline
(0.5 mL) was administered subcutaneously in all animals 30 minutes and 24 hours after reperfusion.

In some mice, thrombin was injected intracerebrally. The mice received an injection of rat thrombin (1 U; Sigma) in 10 μL saline into the right basal ganglia (coordinates: 0.2 mm anterior, 3.5 mm ventral, and 2.5 mm lateral to the bregma).

**Experimental Groups**
This study was divided into 5 sets. In the first set, male wild-type mice underwent BCCAO or sham operation. They were euthanized at 4, 24, and 72 hours (n=3 each group) for thrombin activity determination. PAR-1 knockout mice (n=3) also had BCCAO and were euthanized at 24 hours for thrombin activity measurement. In the second set, male wild-type or PAR-1−/− mice underwent BCCAO or sham operation and they were euthanized at 4, 24, and 72 hours (n=4 each group) for Western blot analysis. In the third set, male wild-type or PAR-1−/− mice underwent BCCAO or sham operation and they were euthanized 24 hours later for brain water and ion content determination (n=5–6 each group). In the fourth set, male wild type or PAR-1−/− mice underwent BCCAO or sham operation and they were euthanized at 24 and 72 hours (n=5 each group) for histology. In the last set, male wild-type or PAR-1−/− mice had an intracaudate injection of thrombin (1 U) and were euthanized at 24 hours for Western blot analysis. All mice were subjected to behavior tests.

**Thrombin Activity Determination**
Thrombin activity was determined according to the method described by Chapman and coworkers with some modification. Briefly, animals were reanesthetized, perfused with saline, and the brain removed. The brain was cut into 6 to 8 coronal sections (at 1 mm thickness) using a mouse brain slicer matrix after weighing. Each brain section was then transferred to a well in a 96-well plate, which was prefilled with 50 μL buffer in each well. Another 100-μL buffer containing substrate Boc-Asp (OBzl)-Pro-Arg-AMC (final concentration 13 μmol/L, I-1560; Bachem) and protease inhibitor prolyl endopeptidase inhibitor II (final concentration 20 μmol/L, 537011; Calbiochem) was added to each well before measurement. The fluoresce was measured continuously for 40 minutes at 25°C using a fluorescence detection system (Glomax-Multi+ Detection System E8032; Promega, Madison, WI; excitation 365 nm, emission 410–460 nm). The hydrolysis of Boc-Asp (OBzl)-Pro-Arg-AMC·HCl was determined by the increase of fluorescence. Purified rat thrombin (TS7721 Sigma) was used as a calibration standard. Total thrombin activity was then calculated. Thrombin activity was expressed as the ratio of BCCAO/sham.

**Western Blot Analysis**
Western blot analysis was performed as described earlier. The primary antibodies were: rabbit polyclonal anti-PAR1 (1:1000 dilution; Abcam), rabbit polyclonal antiphospho-p38 and -p38 MAPKs, antiphospho-JNK and -JNK, and antiphospho-p44/42 and -p44/42 MAPKs (1:100; Cell Signaling Technology). The secondary antibody was goat antirabbit IgG (1:4000 dilution; Bio-Rad).

**Immunohistochemistry, Double Staining, and Brain Histology**
Immunohistochemistry and double staining were performed as previously described. The primary antibodies were: rabbit polyclonal anti-PAR1 (1:400 dilution; Abcam), sheep antihuman thrombin (1:500; Affinity Biologicales), and rabbit antiphospho-p38 MAPK, -phospho-p44/42 MAPKs (1:400; Cell Signaling Technology), chicken antimotigen-activated protein-2 (1:800 dilution; Abcam), goat antifibrillar acidic protein (1:200 dilution; Santa Cruz), and goat anti-Iba1 (1:200 dilution; Santa Cruz). Normal chicken, goat, rabbit, or sheep IgG was used as negative controls.

For immunofluorescent labeling, brain sections were reacted with rabbit anti-DARPP-32 monoclonal antibody (1:400; Cell Signaling Technology) and then incubated with Alexa 488-conjugated donkey antirabbit antibody (Invitrogen). DARPP-32 is a cytosolic protein highly enriched in medium-sized spiny neurons of the striatum and is used to reflect neuron viability in the striatum. Brain sections were also stained with Fluoro-Jade C, a marker of neurodegeneration.

**Brain Water and Ion Content Determination**
Animals were reanesthetized at 24 hours after BCCAO. The brains were removed and quickly divided into 3 parts: right hemisphere, left hemisphere, and cerebellum. Tissue samples were immediately weighed to obtain the wet weight. Samples were dried in a gravity oven at 95°C to 100°C for >24 hours to determine dry weight. Tissue water content (%) was calculated as [(wet weight−dry weight)/wet weight]×100. Dehydrated brain samples were digested in 1 mL of 1 N nitric acid for 2 weeks. The sodium ion content of this solution was measured by flame photometry and expressed in milli-equivalents per kilogram of dehydrated brain tissue (mEq/kg dry tissue).

**Behavior Tests and Body Weight**
We performed 3 behavioral tests in this study. All the animals were pretested 1 day before surgery to assess for baseline or abnormalities. Animals were restested at 24, 48, and 72 hours after BCCAO or sham surgery. All tests were done at a fixed time in the early evening in a quiet behavior tests special-use room with a dim red light. All behavioral testing equipment and surfaces were cleaned before and after tests. All animals were housed in a temperature- and humidity-controlled room with automatic 12-hour dark/light cycles.

**Open Field Test**
Mice were placed in the open field chamber (90×90 cm) for 5 minutes in each test. Locomotive behavior was monitored and analyzed by an autotracking system (Smart1.1), which quantified the total distance traveled. All mice were brought to the testing room 10 minutes before the beginning of the test and the activity was recorded immediately after the mice were placed in the open field apparatus.

**Hanging Wire**
The hanging wire tests both limb strength and balance after ischemia. A standard wire cage lid with its edges taped off was used for this experiment. The mouse was placed on the center of the wire lid and the lid was then slowly turned upside down and held at a height approximately 50 to 60 cm above a protective device. Latency to fall from the wire was recorded. The time-out period was 60 seconds. All animals with latency <60 seconds in the pretest were ruled out in this study.

**Neurological Disability Status Scale**
This part of the experiment was performed as described by Rodríguez et al. It is a method developed to access the neurological deficits after forebrain ischemia in mice. The neurological disability status scale has 10 progressive steps from 0 (normal) to 10 (death); the higher score means the greater neurological dysfunction. Animals with abnormalities found in pretest were all ruled out in this study.

The body weight of each mouse was measured before anesthesia and each day after behavior tests postsurgery.

**Cell Counting**
Cell counting was performed according to the method described by Yoshiioka et al. Five subregions of the caudate (central, dorsomedial, dorsolateral, ventromedial, and ventrolateral) were assigned for quantification of Fluoro-Jade C and DARPP-32 staining, each consisting of a rectangle of 250×174 μm.

**Statistical Analysis**
All the data in this study are presented as mean±SD. Data were analyzed by Student t test and one-way analysis of variance. A level of P<0.05 was considered statistically significant.
Thrombin (Prothrombin) and PAR-1 Immunoreactivity

Figure 1. Immunoreactivity of thrombin (prothrombin) and PAR-1 in the basal ganglia (A) and thrombin activity in the cerebrum (B) and protein levels of PAR-1 in the basal ganglia (C) at 4, 24, or 72 hours after BCCAO or sham operation. Scale bar=50 μm. Values are mean±SD, n=3 to 4. *P<0.05 versus the other groups. PAR-1 indicates protease-activated receptor-1; BCCAO, bilateral common carotid artery occlusion.

Results
Regional Cerebral Blood Flow, Blood Glucose, and Mortality After BCCAO
Regional cerebral blood flow reduction was no different in wild-type mice (percent of the baseline: 9.8%±3.3%) and PAR-1−/− mice (10.7%±2.2%) after BCCAO. Similarly, after reperfusion, blood flow returned to 86.5%±12.4 and 85.2%±20.9% of baseline in wild-type and PAR-1−/− mice, respectively. These values were not significantly different. Hyperglycemia was found in both wild-type (281±41 mg/dL) and PAR-1−/− mice (295±43 mg/dL) before BCCAO (P>0.05) because xylazine can cause an increase of blood glucose levels. The death rate was 4.1% (2 of 49) at 24 and 9.1% (2 of 22) at 72 hours in the wild-type mice, but no death was found in PAR-1−/− mice after BCCAO.

Thrombin Activity and PAR-1 Expression Were Upregulated After BCCAO
Thrombin-positive cells were found in the basal ganglia after BCCAO at 4 hours, reached the peak at 24 hours, and returned to a lower level at 72 hours. There were no thrombin-positive cells in sham-operated mice. Brain thrombin activity expressed as a ratio to that in sham was slightly increased at 4 hours (1.17±0.09, P>0.05), peaked at 24 hours (3.81±0.23, P<0.01), and declined at 72 hours (2.51±0.06, P<0.01) after BCCAO (Figure 1). Similarly, BCCAO induced PAR-1 expression in the basal ganglia. Basal ganglia PAR-1 protein levels were upregulated significantly at 4 and 24 hours (ratio to β-actin: 1.87±0.09 versus 0.39±0.17 in sham, P<0.01) but were back to normal at 72 hours after BCCAO (Figure 1).

We also measured thrombin activity after BCCAO in PAR-1 knockout mice. We found that brain thrombin activity is same 24 hours after BCCAO in wild-type and PAR-1 knockout mice (ratio: BCCAO/sham, 3.8±0.7 versus 3.3±1.4, P>0.05).

BCCAO Caused Less Brain Edema in PAR-1−/− Mice
BCCAO induced brain edema in both hemispheres. At 24 hours after BCCAO, brain water content in PAR-1−/− mice (78.4%±0.2%) was significantly lower than in wild-type mice (80.2%±1.4%, P<0.01; Figure 2). Also, there was less BCCAO-induced brain sodium accumulation in the PAR-1−/− mice (214±9 versus 313±109 mEq/kg dry wt in wild-type mice, P<0.01; Figure 2).

Neuronal Death Was Less in the Basal Ganglia of PAR-1−/− Mice After BCCAO
Fluoro-Jade staining and DARPP-32 immunofluorescence staining were used to examine neuronal death after BCCAO.
DARPP-32 was used as a marker of medium spiny neurons that represent 95% of striatal neurons. Five subregions showed in Figure 3 were used for the cells counting. There were less Fluoro-Jade C-positive cells in the caudate of PAR-1/H11002/H11002/H11002 mice at 24 (87 ± 41 versus 549 ± 232 cells/mm² in the wild-type mice, P<0.01) and 72 hours (41 ± 17 versus 365 ± 94 cells/mm² in wild-type mice, P<0.01) after BCCAO (Figure 3). More DARPP-32-positive cells were found in the caudate of PAR-1/H11002/H11002/H11002 mice at 24 (1018 ± 67 versus 690 ± 104 cells/mm² in the wild-type mice, P<0.01) and 72 hours (954 ± 26 versus 579 ± 215 cells/mm² in wild-type mice, P<0.01) after BCCAO (Figure 3).

**Neurological Deficits and Body Weight Loss Were Less in PAR-1−/− Mice**

For behavior tests, all animals were tested before global ischemia. We found no differences in behavior between wild-type and PAR-1−/− mice before ischemia (Figure 4).
Compared with the sham group, BCCAO resulted in shorter total traveled distance, reduced wire hang time, and a worse neurological disability score. Behavioral outcomes were better in PAR-1/H11002/H11002/H11002 mice compared with those in wild-type mice (Figure 4). Body weight loss (percent of initial) after BCCAO was also less in PAR-1/H11002/H11002/H11002 mice (Figure 4).

Activation of MAPKs After BCCAO Was Lower in PAR-1/H11002/H11002/H11002 Mice

Many phospho-p38 MAPK and phospho-p44/42 MAPK-positive cells were found in the caudate after BCCAO. Only a few of these positive cells were found in the caudate after sham operation (Figure 5). Western blots showed that protein levels of phosphorylated MAPKs were upregulated in the caudate 24 hours after BCCAO compared with those after sham operation (Figure 5). Levels of phospho-p38 and phospho-p44/42 MAPKs were less in PAR-1/H11002/H11002/H11002 mice compared to that in wild-type mice at 24 hours after BCCAO (Figure 5). It should be noted that PAR-1 gene knockout does not affect MAPK expression in nonischemic mice. For example, brain total (including nonphosphorylated) p44/42 MAPK levels were the same in normal wild-type mice and PAR-1/H11002/H11002/H11002 mice (16 440±1597 versus 18 761±1633 pixels, P<0.05). Both immunostaining and Western blotting did not detect phospho-p38 and phospho-p44/42 MAPKs in nonischemic mice. Double staining showed that most phospho-p38-MAPK-positive cells were neurons, whereas phospho-p44/42 MAPK-positive cells were either astrocytes or neurons (Figure 6). No microglia were activated p38- or p44/42 MAPK-positive (data not shown).

We hypothesized that increases of brain thrombin levels contribute to brain injury after global cerebral ischemia. We then injected thrombin intracerebrally and found that thrombin-induced increases of brain phospho-MAPK were much less in PAR-1 knockout mice (phospho-p44/42: 158±121 versus 6531±3710 pixels in the wild-type mice, P<0.01; phospho-p38: 47±54 versus 3772±3133 pixels, P<0.01).

Discussion

The major findings of the current study are: (1) thrombin activity was increased and PAR-1 expression was upregulated in the caudate after BCCAO; (2) BCCAO caused less brain edema, neuronal death, neurological deficits in PAR-1/H11002/H11002/H11002 mice; and (3) BCCAO- and thrombin-induced p38- and p44/42 MAPK activation was less in PAR-1/H11002/H11002/H11002 mice.
Thrombin plays crucial roles in brain injury after cerebral ischemia.\(^2\)\(^,\)\(^21\) Low concentrations of thrombin are neuroprotective, whereas high concentrations are detrimental to neurons or astrocytes.\(^21\)\(^,\)\(^22\) In our previous study, low concentrations of thrombin may also cause cell death after stroke. For example, a small dose of exogenous thrombin exacerbates ischemic brain injury.\(^3\)\(^,\)\(^23\)\(^,\)\(^24\) It has been shown that systemic thrombin inhibition attenuated neurodegeneration and brain edema formation after transient cerebral ischemia.\(^25\)\(^,\)\(^26\) In the current study, we found that thrombin activity after BCCAO increased 4-fold compared with sham-operated mice at 24 hours, which suggests that thrombin could be an important factor that causes brain injury after global ischemia. Future study should measure exact thrombin activity using a thrombin inhibition method.

PAR-1 is involved in thrombin-induced brain damage after cerebral ischemia. Brain PAR-1 expression was upregulated by BCCAO in the basal ganglia areas. Our previous study showed PAR-1 upregulation in a rat model of focal cerebral ischemia.\(^3\) In addition, increased brain PAR-1 levels were reported in hippocampal slice cultures after oxygen–glucose deprivation.\(^6\) In the present study, BCCAO induced less brain edema formation, neuronal death, and neurological deficits in PAR-1 knockout mice. In addition, BCCAO-caused animal death was only found in wild-type mice but not in PAR-1 knockout mice. Other studies also found that PAR-1 is associated with brain injury after focal cerebral ischemia. For example, brain infarct volume is reduced in PAR-1 knockout mice and intracerebroventricular injection of BMS-200261, a PAR-1 antagonist, reduces infarct volume in a mouse focal cerebral ischemia model.\(^12\) PAR-1 blockade using PAR-1 antagonists might be a promising therapy for global cerebral ischemia.

The results in the current study indicate that PAR-1 plays an important role in MAPKs activation after BCCAO. The MAPK family regulates a diverse array of functions such as neuronal death and survival, proliferation, and apoptosis in response to extracellular stimuli, including cerebral ischemia.\(^7\)\(^,\)\(^27\)\(^,\)\(^28\) MAPKs are associated with brain injury after cerebral ischemia.\(^29\)\(^–\)\(^31\) In our current study, the phosphorylation levels of p38 MAPK and p44/42 MAPK were enhanced markedly 24 hours after BCCAO or thrombin stimulation. The upregulation for phospho-p38 occurred in neurons, whereas phospho-p44/42 upregulation occurred in neurons and astrocytes. This upregulation of both was almost absent in the PAR-1\(^–/–\) mice.

Effects of PAR-1 deletion on MAPK upregulation after BCCAO and thrombin stimulation were significant. It is known that thrombin and PAR-1 agonist can markedly upregulate brain p44/42 MAPK.\(^3\)\(^,\)\(^32\) Intracerebral hemorrhage also causes an upregulation in phosphorylated p44/42 MAPK and there is some evidence that this is blocked by argatroban, a thrombin antagonist.\(^33\) This suggests that PAR-1 may also be involved in MAPK upregulation in hemorrhagic stroke. However, brain thrombin levels are higher in hemorrhagic stroke compared with cerebral ischemia and it is still uncertain whether thrombin is the sole cause of the MAPK upregulation after BCCAO. Although we did find 4-fold increases in the level of this serine protease, it should be noted that there are other naturally occurring PAR-1 agonists.\(^2\) This merits further investigation as does the potential role of PAR-1 in MAPK upregulation after focal cerebral ischemia.

In conclusion, PAR-1 activation has a major role in brain injury after transient global cerebral ischemia. That activation is probably due to thrombin production in the brain and it is responsible for the marked activation of MAPKs after global ischemia.

Sources of Funding
Supported by grants NS-039866 and NS-057539 from the National Institutes of Health and 0840016N from the American Heart Association.

Disclosures
None.

References
Role of Protease-Activated Receptor-1 in Brain Injury After Experimental Global Cerebral Ischemia
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Stroke. published online July 17, 2012;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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