Neuroprotection With a Calpain Inhibitor in a Model of Focal Cerebral Ischemia

Seung-Chyul Hong, MD; Yasunobu Goto, MD; Giuseppe Lanzino, MD; Scott Soleau, BA; Neal F. Kassell, MD; Kevin S. Lee, PhD

Background and Purpose  Excessive elevation of intracellular calcium and uncontrolled activation of calcium-sensitive events are believed to play a central role in ischemic neuronal damage. Calcium-activated proteolysis by calpain is a candidate mechanism in this form of pathology because it is activated under ischemic conditions and its activation results in the degradation of crucial cytoskeletal and regulatory proteins. The present studies examined the effects of a cell-penetrating inhibitor of calpain on the pathological outcome after transient focal ischemia in the brain.

Methods  Twenty-five male Sprague-Dawley rats were divided into four groups: a saline-treated group, a vehicle-treated group, and two calpain inhibitor-treated groups (Cbz-Val-Phe-H; 30-mg/kg and 60-mg/kg cumulative doses). Ischemia was induced by occluding the left middle cerebral artery and both common carotid arteries for 3 hours followed by reperfusion. Animals were killed 72 hours after surgery, and quantitative measurements of infarction volumes were performed using histological techniques. Eight additional rats were killed 30 minutes after ischemia and examined for the extent of proteolysis using immunoblot techniques. A final group of 12 animals was decapitated after injection of vehicle or calpain inhibitor, and the proteolytic response was measured after 60 minutes of total ischemia.

Results  Rats treated with Cbz-Val-Phe-H exhibited significantly smaller volumes of cerebral infarction than saline-treated or vehicle-treated control animals. Intravenous injections of cumulative doses of 30 mg/kg or 60 mg/kg of Cbz-Val-Phe-H were effective in reducing infarction, edema, and calcium-activated proteolysis. The proteolytic response to post-decapitation ischemia was also reduced by the calpain inhibitor.

Conclusions  These results demonstrate the neuroprotective effect of a cell-penetrating calpain inhibitor when administered systemically. The findings suggest that targeting intracellular, calcium-activated mechanisms, such as proteolysis, represents a viable therapeutic strategy for limiting neurological damage after ischemia. (Stroke. 1994;25:663-669.)

Key Words  • calpain inhibitor • cerebral ischemia, focal • neuroprotection • rats

Neurological damage resulting from a compromise of the cerebrovascular supply is a leading cause of death and disability. A central hypothesis concerning the etiology of ischemic neuronal death is that sustained elevation of intracellular calcium triggers a variety of intracellular events that can impair or harm cellular function. Under physiological conditions, the activation of calcium-sensitve events is carefully regulated by a variety of mechanisms, foremost among which is the precise maintenance of low intracellular calcium levels. The loss of calcium homeostasis during ischemia permits the unrestrained activation of several calcium-sensitive mechanisms, which then become detrimental to cellular function. A prime example of a mechanism of this type is calcium-activated proteolysis. Continuous stimulation of calcium-activated neutral proteases (calpains) during ischemia can result in the abnormal degradation of substrate proteins. Preferred substrates for calpain include cytoskeletal proteins such as microtubule-associated protein 2 (MAP2), spectrin, and neurofilament proteins. Other substrates include key regulatory enzymes such as protein kinase C and calcium/calmodulin-dependent protein kinase II.

Received May 20, 1993; final revision received September 20, 1993; accepted December 1, 1993.

From the Department of Neurological Surgery, Health Sciences Center, University of Virginia, Charlottesville, Va.

Correspondence to Dr Kevin S. Lee, Department of Neurological Surgery, Box 420, Health Sciences Center, University of Virginia, Charlottesville, VA 22908.
models of neuronal pathology. Brain slices from the hippocampus and neocortex exhibit significantly less damage after transient hypoxia when the hypoxic episode occurs in the presence of a calpain inhibitor.\textsuperscript{27-29} Hypoxic damage to primary cultures of telencephalic neurons is also reduced after treatment with a calpain inhibitor.\textsuperscript{30} The protease inhibitors that have been shown to be effective in these models include calpain inhibitor I, leupeptin, and zVF. The demonstrated ability of zVF to penetrate the brain and its effectiveness in limiting neuronal damage in vitro models of neuronal pathology raise the possibility that this calpain inhibitor might be of therapeutic value in an in vivo model of ischemic neuronal damage. The present studies examined this issue by administering zVF intravenously to rats subjected to transient focal ischemia of the brain. The findings presented here suggest that calpain inhibitors may provide a useful therapeutic treatment for ischemic neuronal damage.

**Materials and Methods**

**Surgical Procedures**

The surgical procedures used to induce temporary focal ischemia in the neocortex are identical to those described by Buchan et al.\textsuperscript{32} This ischemic challenge produces large and consistent infarctions, as described in detail elsewhere.\textsuperscript{31,32}

Male Sprague-Dawley rats (Hilltop Lab Animals Inc) weighing 250 to 350 g were given food and water ad libitum before and after surgery. Anesthesia was induced with a mixture of 3.0% halothane in oxygen. The right femoral artery and vein were cannulated to (1) monitor mean arterial blood pressure (model VT-15, WECO), (2) assay blood gases (P0\textsubscript{2}, P\textsubscript{CO\textsubscript{2}}, and pH; model 278 blood gas analyzer, CIBA Corning), and (3) administer drugs, saline, and vehicle. Both common carotid arteries (CCAs) were exposed by a vertical midline incision of the neck. A snare was placed around each CCA using 5.0 nylon suture and short segments of polyethylene tubing (PE-50); this permitted the occlusion and release of the CCAs at a later time. After orotracheal intubation, the rat was mechanically ventilated (Harvard rodent ventilator, model 683, Harvard Instrument Co). Artificial ventilation could be maintained without the use of muscle relaxant by adjusting the tidal volume and respiration rate to that of the self-respiration of the rat and by keeping arterial P\textsubscript{CO\textsubscript{2}} levels normal. The concentration of halothane in pure oxygen was maintained between 1.0% and 1.5% to keep the mean arterial blood pressure near 95 mm Hg. Rectal temperature was monitored by a digital thermometer (model 8402-20, Cole-Parmer Instrument Co) and maintained at 37.0°C by use of a heat pad, lamp, or fan. Atropine sulfate (0.1 mg) was injected intramuscularly every 3 hours during the operation.

The left eye was closed by tarsorrhaphy to avoid extrusion during retraction of the temporal muscles. A 1.5-cm incision was made between the left eye and the tragus. With the tympanic bone resected, the temporal muscle and the mandible were retracted to expose the temporal squamous bone. Using an operating microscope (OPMI-1FC, Carl Zeiss), a 3-mm-diameter burr hole was made with a dental drill, just rostral to the foramen ovale. Saline irrigation was performed during drilling to avoid thermal brain damage. The dura was retracted to expose the temporal branch of the MCA. A small piece of gelfoam was placed on the craniectomy site, and the wound was closed. After recovery from the anesthesia, the rats were kept in cages with free access to food and water.

**Drug Treatment**

Twenty-five rats were divided randomly into four groups. In the saline-treated group (n=6), intravenous injections of saline (4 mL/kg) were administered at the following times: 30 minutes before vascular occlusion, 90 minutes after beginning occlusion, and 30 minutes after reperfusion. In the vehicle-treated group (n=7), injections of vehicle (1% dimethyl sulfoxide and 10% emulphor in saline) were administered in the same manner. In the two groups treated with calpain inhibitor (zVF), injection protocols were identical to those described above. zVF was dissolved in vehicle at concentrations of 2.5 mg/mL and 5.0 mg/mL. Animals injected with the 2.5-mg/mL solution received a cumulative dose of 30 mg/kg; animals injected with the 5.0-mg/mL solution received a cumulative dose of 60 mg/kg. zVF was kindly provided by Marion Merrell Dow Research Institute. The surgeon was unaware of the composition of the injection solutions at the time of surgery.

**Histological Techniques**

Seventy-two hours after surgery, animals were administered an overdose of sodium pentobarbital and perfused intracardially with 100 mL of warm heparinized saline (10 U/mL [United States Pharmacopeia]). To verify patency of the MCA, warm saline containing 13% india ink and 5% gelatin was then perfused. Animals were decapitated, and their brains were removed. The patency of the MCA was confirmed by observing the black cast in the vessel under a microscope. Coronal sections of the brain (1 mm thick) were cut with a tissue chopper; this typically generated 11 to 12 sections. The sections were immersed in a 2% solution of 2,3,7-triphenyltetrazolium chloride (TTC) in saline at 37°C and kept in the dark for 30 minutes. The stained slices were then fixed by immersion in a solution of 10% formalin.

Normal tissue appears red with this staining procedure, while infarcted tissue appears white. TTC has been used previously at the 72-hour postischemia time point in studies of neuroprotection;\textsuperscript{31} the invasion of macrophages at this time does not obscure the demarcation of the infarcted zone. The infarcted area and the total hemispheric area of each section were traced using an image analysis system (IMAGE-1, Universal Imaging Co); the hemispheres ipsilateral and contralateral to the occluded MCA were measured separately. The identity of the sections was unknown to the investigator performing the measurements. The total volume of each hemisphere and the volume of infarction were calculated by multiplying each area measurement by section thickness and summing these values for all the sections from a given animal. The percentage of infarction in the hemisphere ipsilateral to the MCA occlusion was calculated by dividing the volume of infarction by the total volume of the ipsilateral hemisphere.\textsuperscript{31} An edema index was calculated in each animal as follows: total infarction volume—(left hemispheric volume—right hemispheric volume).

**Proteolysis Experiments**

Two additional experiments were performed to determine the effect of systemically administered zVF on ischemia-induced proteolysis. The proteolysis of spectrin, a preferred
By guest on April 13, 2017 http://stroke.ahajournals.org/ Downloaded from
were necessary, Tukey's test was applied. The volume of edema index were examined with a one-way ANOVA and infarction (both absolute and percentage values) and the above.

Statistical Methods

Physiological data (ie, blood pressure, gases, and pH) were analyzed with a two-way ANOVA. If multiple comparisons were necessary, Tukey's test was applied. The volume of infarction (both absolute and percentage values) and edema index were examined with a one-way ANOVA and the volume of hemorrhage were examined with a two-way ANOVA. If multiple comparisons were necessary, Tukey's test was applied. The incidence of hemorrhage (both absolute and percentage values) and the volume of edema index were examined with a two-way ANOVA. Statistical analyses for the proteolysis studies were performed using a one-way ANOVA and Student's t test.

Results

Proteolysis Data

The effect of transient focal ischemia on the proteolysis of spectrin in frontoparietal cortex was measured using Western blot techniques and scanning densitometry. Spectrin BDPs were increased after ischemia in both the vehicle-treated and calpain inhibitor-treated groups (Fig 1A). However, the levels of BDPs were significantly lower in the calpain inhibitor-treated group. The level of BDPs in the vehicle-treated group (n=4) was 48.5±3.7% (mean±SEM); the level in the calpain inhibitor-treated group (n=4) was 24.1±2.2%. This represents a 50.3% inhibition of the ischemia-induced proteolytic response in the inhibitor-treated group, an effect that was significant at P<.01 on Student's t test.

The proteolytic response to decapitation-induced ischemia was also examined in the frontoparietal cortex after middle cerebral artery occlusion. The ischemia-induced increase in BDPs was attenuated significantly in zVF-treated animals. *P<.01, Student's t test. B, Bar graph shows effect of zVF on proteolysis after decapitation-induced ischemia. The proteolytic response measured 60 minutes after decapitation was significantly inhibited in zVF-treated animals. *P<.01, Student's t test.

Student's t test. The results are presented in the text as mean±SEM. A value of P<.05 was accepted as significant. Statistical analyses for the proteolysis studies were performed with a one-way ANOVA and Student's t test.
Physiological and Morphometric Data

Measurements of physiological parameters are shown in Table 1. There were no significant differences observed in these data.

Cortical infarction was present in each rat examined in this study. The infarcted region was demarcated as a pale area that contrasted with the red appearance of intact brain tissue stained with TTC. Infarctions were confined to the neocortex in all but four rats that showed small subcortical infarctions in the dorsolateral striatum; these four cases exhibited striatal infarctions of 1%, 1%, 4%, and 6% of total hemispheric volume.

The volumes of infarction (in cubic millimeters) are shown for each animal in each group in Fig 2. The average values for the volume of infarction and percent infarction (see “Materials and Methods”) are shown for each group in Table 2. Although the infarction size was slightly larger in the vehicle-treated group than in the saline-treated group, this difference did not achieve statistical significance (P=.26, Student’s t test). Animals treated with zVF showed significant reductions in the volume of infarction (Table 2). The infarction volumes in the calpain inhibitor–treated groups receiving 30 mg/kg and 60 mg/kg were reduced relative to the control groups (Table 2). When compared with the vehicle-treated group, the infarctions in the calpain inhibitor–treated groups were reduced by 24.3% and 23.6%, respectively. These differences were statistically significant (P<.01). The cerebral edema index was also decreased significantly in both calpain inhibitor–treated groups (Table 2).

**Discussion**

Several lines of evidence support the hypothesis that calcium-activated proteolysis plays a central role in the degenerative responses to cerebral ischemia and hypoxia. First, substrate proteins for the calcium-activated
protease calpain are degraded after ischemic or hypoxic challenges.3-8,10-13,36-38 Preferred substrates for calpain include essential cytoskeletal proteins and regulatory proteins; the integrity of these proteins is necessary for continued cellular viability. Second, the initial proteolytic response to ischemia or hypoxia is rapid; degradation of MAP2 and spectrin has been shown to occur within minutes of hypoxic or ischemic challenge.5,36,39

The rapid onset of this response demonstrates that proteolysis is not restricted to the terminal phase of cell loss but occurs during a time frame that would permit direct participation in the degenerative response. Finally, calpain inhibitors are neuroprotective in models of hypoxic neuronal death in vitro. Hypoxia-induced cell damage in hippocampal slices is reduced substantially by the following protease inhibitors: calpain inhibitor I, leupeptin, and zVF.27,28 Similar neuroprotective results have been obtained in neocortical slices treated with zVF.29 In addition, a recent report demonstrates that hypoxic cytotoxicity in primary neuronal cultures of the telencephalon is reduced by calpain inhibitor 1 or leupeptin.30 Taken together, these observations support the hypothesis that calcium-activated proteolysis participates in the process of ischemic and hypoxic neuronal damage and that protease inhibitors may be useful for limiting this type of damage.

The potential utility of proteolytic inhibition as a therapeutic strategy for limiting ischemic damage in vivo also appears promising. Ischemia-induced proteolysis is inhibited by intracerebroventricular infusion of the protease inhibitor leupeptin after transient forebrain ischemia.5 Leupeptin reduces neuronal loss and preserves electrophysiological function of selectively vulnerable neurons in the hippocampus in this global ischemic model.5 Intraventricular administration of the calpain inhibitor E-64c reduces ischemia-induced proteolysis of the calpain substrate MAP2 in a model of permanent focal cerebral ischemia.9 Taken together, the above studies demonstrate that the proteolytic response to global and focal ischemia can be attenuated by treatment with protease inhibitors. Moreover, the functional and structural damage resulting from global ischemia can be inhibited by intracerebral application of a protease inhibitor. These observations provide direct support for the concept that targeting cellular proteolysis could represent a useful therapeutic strategy for limiting ischemic cell loss in vivo.

The findings presented here provide the first evidence that treatment with a calpain inhibitor can limit the extent of cerebral infarction after focal ischemia. In addition, the findings demonstrate that proteolytic inhibition and neuroprotection can be achieved when an inhibitor is administered systemically. Intravenous administration of the calpain inhibitor zVF inhibits post-ischemic proteolysis of spectrin by approximately 50%. This antiproteolytic effect of zVF can be ascribed to a direct influence on cerebral proteolysis for the following reasons. First, systemic factors that could influence ischemic outcome, such as temperature and blood pressure, were unchanged in the treated animals. Second, the antiproteolytic action of zVF was also observed under conditions in which changes in blood flow and brain temperature could not contribute to the proteolytic response (ie, after decapitation). These findings indicate that the inhibition of ischemia-induced proteolysis is due to a direct effect of zVF on the ischemic tissue and not the result of modifications in systemic factors.

Treatment with the calpain inhibitor also resulted in significant neuroprotection. zVF reduced the volume of infarction by approximately 25% compared with vehicle-treated animals. The largest reductions in infarct size in the inhibitor-treated animals were observed in the most rostral and most caudal sections of the brain. Although the precise volumes of the core and penumbra regions are difficult to identify unequivocally in this model, it is likely that the 25% reduction in overall infarct size reflects a substantial protection of the penumbra. It is also noteworthy that the magnitude of this neuroprotective effect is comparable to that which we have previously obtained using mild, intraischemic hypothermia.33 It remains unclear whether a more extensive inhibition of proteolysis (ie, >50% inhibition) would yield even greater neuroprotection. The resolution of this issue awaits further investigation.

The efficacy of targeting proteolysis as a therapeutic strategy will ultimately depend on the development of inhibitory compounds with optimal specificity and bioavailability.25,26 The protease inhibitor used in the present study, zVF, is a dipeptidyl aldehyde that is both membrane permeable and effective against calpain.24,25 However, the presence of valine in the P2 position of zVF suggests that this compound may be active against other cysteine proteases in addition to calpain.26 Since the impact of inhibiting other cysteine proteases is unknown, it will be important for future studies to evaluate therapeutic protease inhibitors for their specificity of action in addition to their permeability and efficacy for inhibiting calpain.

Protease inhibitors may have broader applications in the treatment of cerebral pathology that extend beyond the treatment of ischemic cell damage. Calpain is activated by excitatory amino acids in the brain.37,40,42 and excitotoxic damage has been implicated in a wide variety of disease states in the central nervous system, including Huntington's disease, Parkinson's disease,
Alzheimer's disease, and amyotrophic lateral sclerosis.\textsuperscript{43} Calpain inhibitors may therefore be useful in multiple pathogenic conditions in which excessive activation of excitatory amino acid receptors occurs. It is noteworthy in this context that a calpain inhibitor has recently been shown to be neuroprotective in a model of central nervous system excitotoxicity.\textsuperscript{44} Purkinje cell damage induced by the glutamate receptor agonist amino-3-hydroxy-5-methyl-4-isoxazole propionic acid is attenuated by 2VF in an in vitro model using cerebellar slices. This finding supports the general concept that proteolytic inhibition may be useful in treating degenerative states in which glutamate receptor-mediated toxicity is a common feature.

In conclusion, an inhibitor of the calcium-activated protease calpain was shown to be neuroprotective in a rat model of reversible cerebral ischemia. The continued development of peptide and nonpeptide inhibitors of calpain will provide the basis for testing the true extent of this therapeutic approach.

Acknowledgments

This study was supported by grant No. NS30671 (Dr Lee). We thank Dr Shujaat Mehdii and Marion Merrell Dow Research Institute for their advice and support. We thank Dr Frank Schottler for his contribution to the statistical analyses. The assistance of Mr Song Kang in preparing immunobots is appreciated. We also thank Mrs Sarah B. Hudson and Mrs Jennifer A. Marron for manuscript assistance.

References


Activation of postsynaptic glutamate receptors followed by increases in calcium influx and the biochemical amplification of calcium-dependent cascades have been considered to be major mechanisms underlying ischemic brain damage. Furthermore, calcium-activated proteolysis of cytoskeletal and regulatory proteins by calpain may be responsible for the pathogenesis of neuronal cell death after episodes of ischemia. Using a rat model of focal cerebral ischemia that mimics human stroke, Hong and colleagues have now demonstrated that Cbz-Val-Phe-H, a calpain inhibitor, reduces protein proteolysis, cerebral edema, and infarction in a dosage-dependent fashion.

This study supports the concept that calcium-dependent proteolysis plays an important role in the development of ischemic brain infarction and edema. In addition to this provocative finding, one important observation resulting from this study needs to be emphasized: the neuronal protective effect of this compound appears to be unrelated to cerebral blood flow, brain temperature, or blood-brain barrier permeability property, since the proteolytic response to postdecapitation ischemia is also reduced by this calpain inhibitor. This study offers a unique therapeutic approach to ameliorating focal stroke by targeting the proteolytic process in posts ischemic brain tissue.

Pak H. Chan, PhD, Guest Editor
Departments of Neurosurgery and Neurology
University of California
School of Medicine
San Francisco, Calif

References
Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia.
S C Hong, Y Goto, G Lanzino, S Soleau, N F Kassell and K S Lee

Stroke. 1994;25:663-669
doi: 10.1161/01.STR.25.3.663

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/25/3/663