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

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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 postdecapitation 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.

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

Several reports demonstrating the postischemic degradation of cytoskeletal proteins and reductions in regulatory enzymes have been presented during the last few years. It is clear that the uncontrolled proteolysis of any or all of these structural and regulatory proteins could have a severe impact on cellular viability. The above evidence suggests that inhibitors of calcium-activated proteolysis could serve a useful therapeutic function in ischemic cell damage. A key to testing this approach, however, is the development of compounds with adequate inhibitory strength and appropriate bioavailability. Since proteolysis by calpain is an intracellular event, a critical feature of any calpain inhibitor is its ability to penetrate cellular membranes. Recent efforts to identify effective calpain inhibitors have therefore focused on the permeability as well as the potency of target compounds. An important outcome of these efforts has been the design and synthesis of effective cell-penetrating inhibitors of calpain. One of the most efficacious of these compounds is the dipeptidyl aldehyde Cbz-Val-Phe-H (zVF). zVF exhibits a low Ki for calpain in both broken membrane preparations and intact cell systems; these findings indicate that the compound possesses both good membrane penetrability and potent inhibitory activity toward the protease.

Recent studies have begun to examine the potential neuroprotective effects of calpain inhibitors in vitro.
Immediately after clipping of the MCA, the CCA snares were positioned proximal to the temporal branch of the MCA to permit application of an arterial clip. The arterial clip (Sundt dissected from the exposed middle cerebral artery (MCA) to the foramen ovale. Saline irrigation was performed during drilling to avoid thermal brain damage. The dura was heat pad, lamp, or fan. Atropine sulfate (0.1 mg) was injected intramuscularly every 3 hours during the operation.

The concentration of halothane in pure oxygen was maintained between 1.0% and 1.5% to keep the mean arterial pattern of the rat and by keeping arterial PcO₂ levels normal. Artificial ventilation could be maintained consistent infarctions, as described in detail elsewhere. Hiramatsu et al. This ischemic model uses a 3-hour occlusion of three vessels and is similar to the model described by Buchan et al. Male Sprague-Dawley rats (Hilltop Lab Animals Inc) weighing 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, WECCO), (2) assay blood gases (Po₂, PcO₂, 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 pattern of the rat and by keeping arterial PCO₂ 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 tarsorraphy 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 then opened with a sharp needle, and the arachnoid membrane was dissected from the exposed middle cerebral artery (MCA) to permit application of an arterial clip. The arterial clip (Sundt AVM Microclip No. 1, Codman) was applied to the MCA at the lateral border of the olfactory tract and was always positioned proximal to the temporal branch of the MCA. Immediately after clipping of the MCA, the CCA snare was pulled to stop blood flow through the CCAs. Heparin sodium (50 U IV [United States Pharmacopeia]) was injected 30 minutes before and 90 minutes after beginning the occlusion. The three-vessel occlusion lasted for 3 hours, during which time the temporal muscle temperature was monitored with a digital thermometer (NIST 15-077-8, Fisher Scientific Co) and maintained between 36.0°C and 37.0°C with a heating lamp. The microclip and snare were then released, and recirculation through the MCA was confirmed by direct visual observation. 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; 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-I, 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. 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...
substrate for calpain, was used as an index of calpain activity. In the first experiment, rats were treated with vehicle (n=4) or zVF (30 mg/kg cumulative dose; n=4) and subjected to focal cerebral ischemia as described above. The animals were killed by decapitation immediately after the last injection of vehicle or zVF, ie, 30 minutes after ischemia. The brains were rapidly removed, and samples were taken from the frontoparietal cortex within the MCA supply field; this sample encompasses the core and periphery of infarction. The sample was homogenized in 600 μL of ice-cold buffer containing 0.32 mol/L sucrose, 10 mmol/L tris(hydroxymethyl)aminomethane (Tris) HCl, 2 mmol/L disodium ethylenediaminetetraacetic dihydrate (EDTA; Bio-Rad Lab), 1 mmol/L ethylene glycol-β-D-aminooxy ether) N,N,N′,N′-tetraacetic acid (EGTA; Sigma Chemical Co), 100 μmol/L leupeptin, and 1 μg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma Chemical Co) (pH 7.4). The protein concentration in the homogenate was determined by the method of Bradford.34 An aliquot of the homogenate was added to 1/3 volume of concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (150 mmol/L Tris HCl, 6% SDS, 30% glycerol, 3.75 mmol/L EDTA, and 3% β-mercaptoethanol, pH 6.8). This mixture was heated in a boiling water bath for 5 minutes and then stored at -80°C for later use. An aliquot containing 20 μg of protein was subjected to SDS-PAGE using a 6% gel. The proteins separated by SDS-PAGE were transferred in a Bio-Rad Transblot cell onto nitrocellulose membranes. Transfers were performed at 60 V over 12 to 15 hours in a buffer containing 25 mmol/L Tris, 190 mmol/L glycine, 0.01% SDS, and 20% methanol. Affinity-purified polyclonal antibody to spectrin was used for immunodetection of spectrin and its characteristic breakdown products (BDPs) as previously described.35 The blots were blocked for 2 hours in basic buffer solution (50 mmol/L Tris HCl, 2 mmol/L CaCl2, 80 mmol/L NaCl, pH 8.0) containing 5% nonfat milk and 0.2% nonionic detergent. Incubations were then performed for 3 hours at room temperature with a polyclonal antibody (1:1000 dilution) raised in rabbit (supplied by P. Vanderklish, University of California, Irvine). The blots were washed three times with the same buffer used during the blocking phase and were incubated with biotinylated anti-rabbit immunoglobulin G as a secondary antibody (1:1000) for 1 hour. After washing, the blots were stained with antiserum using an avidin-biotin complex peroxidase procedure. Development was performed using N,N-dimethylformamide in the presence of H2O2. Immunoreactivity of native spectrin (≈240 kD) and of spectrin BDPs (150 to 155 kD) was quantified using scanning laser densitometry (ImageQuant, Molecular Dynamics). The percentage of the amount of spectrin BDPs to the total amount of spectrin activity of native spectrin (≈240 kD) and of spectrin BDPs was measured 60 minutes after decapitation. The proteolytic response measured 60 minutes after decapitation was significantly inhibited 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.

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 IA). 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 of vehicle-treated and calpain inhibitor–treated animals (Fig IB). The levels of spectrin BDPs at 60 minutes after decapitation were 47.0±2.0% in the vehicletreated group (n=6). This proteolytic response was attenuated significantly (P<.01) in the calpain inhibitor–treated group (n=6), with BDP levels of 20.6±3.1% observed after 60 minutes.
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...
Table 2. Cerebral Infarction and Edema After Ischemia

<table>
<thead>
<tr>
<th>Drug</th>
<th>Saline-treated</th>
<th>Vehicle-treated</th>
<th>Drug (30 mg/kg)</th>
<th>Drug (60 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral infarction volume, mm³</td>
<td>203 ± 13</td>
<td>223 ± 11</td>
<td>169 ± 8t</td>
<td>170 ± 8t</td>
</tr>
<tr>
<td>Percent infarction</td>
<td>34.3 ± 1.9</td>
<td>36.3 ± 1.6</td>
<td>27.8 ± 1.5*</td>
<td>28.6 ± 1.4*</td>
</tr>
<tr>
<td>Edema index, mm³</td>
<td>150.5 ± 11.8</td>
<td>161.7 ± 14.1</td>
<td>109.7 ± 7.9t</td>
<td>126.9 ± 5.5*</td>
</tr>
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Values are mean ± SEM.

*P < .05, fP < .01, drug-treated vs vehicle-treated groups (Student's t test).

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. Leupeptin reduces neuronal loss and preserves electrophysiological function of selectively vulnerable neurons in the hippocampus in this global ischemic model. Intraventricular administration of the calpain inhibitor I also attenuates neuronal damage after hypotensive ischemia. Systemic administration of the calpain inhibitor E-64c reduces ischemia-induced proteolysis of the calpain substrate MAP2 in a model of permanent focal cerebral ischemia. 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 postischemic 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 infarction 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, intracerebral hypothermia. 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. The protease inhibitor used in the present study, zVF, is a dipeptidyl aldehyde that is both membrane permeable and effective against calpain. 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. 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 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, and Alzheimer's disease.
Alzheimer's disease, and amyotrophic lateral sclerosis.\(^\text{1}\) 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.\(^\text{2}\) Purkinje cell damage induced by the glutamate receptor agonist amino-3-hydroxy-5-methyl-4-isoxazole propionic acid is attenuated by 2 VF 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.

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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 postischemic brain tissue.

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