Improved Posthypoxic Recovery of Synaptic Transmission in Gerbil Neocortical Slices Treated With a Calpain Inhibitor

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Background and Purpose: Among the various calcium-induced biologic events occurring in hypoxic neurons, activation of the calcium-activated neutral proteinase (calpain) is a likely mediator of neuronal degeneration. In this study, we assessed the protective effects of a calpain inhibitor (Cbz-Val-Phe-H) against hypoxic damage to the neocortex.

Methods: An in vitro neocortical slice model from gerbils was used to study the delay to hypoxic depolarization during hypoxia and the recovery of synaptic responses after hypoxia. These responses were examined in control slices and slices treated with Cbz-Val-Phe-H.

Results: The delay to hypoxic depolarization did not differ between treated and control groups. In contrast, synaptic recovery after a fixed period of hypoxia (15 minutes) was significantly improved in the Cbz-Val-Phe-H-treated slices (P<.01). Concentrations of Cbz-Val-Phe-H of 50 µmol/L or greater were significantly more protective than a concentration of 20 µmol/L (P<.01).

Conclusions: The data indicate that calcium-activated proteolysis plays a critical role in hypoxic damage to the neocortex and that calpain inhibitors may be useful therapeutic agents. (Stroke. 1993;24:1725-1728.)

KEY WORDS • calcium • calpain • neuronal damage • gerbils

It is widely held that increased intracellular calcium concentrations play a pivotal role in neuronal injury associated with hypoxia and ischemia.1,2 However, the calcium-sensitive mechanisms responsible for this form of neuronal damage are not well understood and need to be elucidated. Understanding the roles of calcium-activated mechanisms will not only provide insights into the pathophysiology of hypoxic/ischemic damage but will also facilitate improvement of therapeutic strategies for stroke.

Among the various calcium-sensitive biologic events in neurons, calcium-activated proteolysis via the neutral protease calpain is a likely candidate to participate in hypoxic/ischemic neuronal degeneration. Calpain is activated by an appropriate signal (elevated calcium), and sustained activation can exert deleterious effects, such as the proteolysis of key cytoskeletal proteins. Interest in this concept has recently been intensified by two sets of observations. First, the levels of several calpain substrates, including neurofilament protein, microtubule-associated protein 2, spectrin, and calcium/calmodulin-dependent kinase II, are reduced substantially after hypoxia or ischemia.3,7 Second, calpain inhibitors attenuate both ischemic and hypoxic neuronal injury in the CA1 area of the hippocampus,8,10 a selectively vulnerable region of the central nervous system. Targeting calcium-activated proteolysis may therefore be a useful therapeutic approach for ischemic neuronal death.

One of the critical remaining issues concerning the therapeutic utility of calpain inhibitors is whether their

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neuroprotective effects are specific to hippocampal neurons or whether their effects generalize to other brain regions. To examine this issue, we employed an in vitro model of hypoxic cell death that uses neocortical brain slices.11 The findings presented here indicate that a membrane-permeable inhibitor of calpain, Cbz-Val-Phe-H (MDL-28170),12 significantly enhances the post-hypoxic recovery of synaptic transmission in the neocortex.

Materials and Methods

Adult Mongolian gerbils (Meriones unguiculatus) weighing 60 to 80 g were anesthetized with ether and killed by decapitation. The basic procedures for preparing cortical brain slices were similar to those described previously.13 Briefly, the brains were rapidly removed and placed in cold artificial cerebrospinal fluid (ACSF) consisting of the following (in mmol/L): 124 NaCl, 3.3 KCl, 1.25 KH2PO4, 2.4 MgSO4, 2.0 CaCl2, 25.7 NaHCO3, 10 glucose. Using a razor blade, the brain hemisphere was dissected into the superior-lateral quadrant and then cut in a coronal plane at a thickness of 400 µm. Although damage to the cut surfaces occurs in slice preparations, this damage is usually limited to approximately 50 µm from the surface; recording electrodes are not positioned in this compromised portion of the slices. Brain slices containing parietal cortex at the level of the striatum were transferred to an interface-type holding chamber maintained at 35.5°C with a humidified atmosphere of 95% O2/5% CO2. After a postanesthesia period of at least 1 hour, slices were transferred individually to an identical recording chamber as required. One to four slices were studied from a given preparation.

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A bipolar stimulating electrode was positioned in layer V of the slice, and stimuli were delivered once every 15 seconds. A glass microelectrode filled with 3 mol/L NaCl (1 to 5 M) was placed in layer III near the position of the stimulating electrode to record the evoked responses. The amplitude of evoked responses was measured on-line using a computer with an analog-to-digital acquisition board. Stimulation intensity was adjusted to elicit responses of approximately 60% to 80% maximum, and responses were measured for at least 15 minutes to verify the stability of the slice. A second recording electrode of the same construction was placed in layer III to record the DC potential. After the stability of the preparation was established under normoxic conditions, hypoxic conditions were achieved by substituting 95% N2/5% CO2 for the normal 95% O2/5% CO2 in the atmosphere of the recording chamber. In the present studies, and in almost all reported studies on brain slices, normoxic conditions (ie, 95% O2/5% CO2) are actually hyperoxic. This level of oxygen in the chamber is used because normal atmospheric air is incapable of sustaining synaptic responses in most brain slice preparations.

In the first set of experiments, hypoxic conditions were initiated and maintained until the occurrence of hypoxic depolarization (HD); at this time, oxygen flow was immediately reestablished. Control slices were perfused with ACSF for the entire experiment (n=10). Slices treated with the calpain inhibitor (n=10) were perfused with ACSF containing 100 μmol/L Cbz-Val-Phe-H for 90 minutes before the hypoxic insult. Cbz-Val-Phe-H (MDL-28170) was kindly provided by Marion Merrell Dow Research Institute (Cincinnati, Ohio). This particular calpain inhibitor was selected because of its membrane permeability, inhibitory characteristics, and proven ability to limit hypoxic damage in the hippocampus. The timing of the treatment was chosen based on previous studies by Arai et al14 in which calpain inhibitors were shown to block hypoxia-induced proteolysis. The delay to HD was measured in all slices (n=20 slices) for quantitative assessment. Statistical analyses of the two groups were performed using the unpaired Student’s t test. A two-tailed value of P<.05 was considered to be statistically significant.

In a second set of experiments (n=56 slices), hypoxia was sustained for a fixed period of 15 minutes. The maximum amplitude of evoked potentials was obtained before and 1 hour after hypoxia. In these experiments, electrodes were repositioned after hypoxia to ensure the recording of maximal responses. A recovery ratio for synaptic responses was calculated by dividing the maximal posthypoxic response by the maximal prehypoxic response and multiplying this value by 100. The magnitudes of the recovery ratios were then compared among the different groups, which included slices treated with: (1) ACSF only (control slices; n=26); (2) 20 μmol/L Cbz-Val-Phe-H (n=9); (3) 50 μmol/L Cbz-Val-Phe-H (n=9); and (4) 100 μmol/L Cbz-Val-Phe-H (n=12). Recovery values were assessed by analysis of variance, and statistical differences between experimental groups were determined by Tukey’s multiple comparison test (P<.05 was considered significant). All data in the text and figures are presented as mean±SEM.

Results

Evoked responses recorded in layer III of the parietal cortex exhibited a waveform similar to those described previously.11,13 Cortical evoked responses were characterized by a small, short-latency, nonsynaptic response followed by a larger and longer-latency synaptic potential (Fig I). There was no apparent change in the shape or amplitude of the evoked waveform when ACSF containing 100 μmol/L Cbz-Val-Phe-H was perfused, an observation that is consistent with the studies of Arlinghaus et al.9

Transient hypoxia produced a characteristic sequence of changes in electrophysiological responses (Fig 1). In most slices the synaptic responses declined first, followed later by an elimination of the nonsynaptic component. The complete loss of the nonsynaptic component coincided temporally with the occurrence of hypoxic depolarization. HD is a sudden, 10- to 17-mV shift of the DC potential. As shown in Fig 2, the average delays to HD in the control and the treated groups were virtually identical (1.71±0.09 and 1.88±0.09 minutes, respectively). In addition, the sequence of changes in the waveform during hypoxia was not affected by the drug treatment. After reoxygenation, the DC shift, the early nonsynaptic component, and the late synaptic component recovered in series. The DC potential recovered completely within 1 to 2 minutes in both groups. The time course, sequence, and magnitude of recovery of evoked potentials were similar in the Cbz-Val-Phe-H-treated and control groups.

In the second series of experiments, sustained hypoxia elicited the same set of intrahypoxic events as was observed in the first series of experiments. HD occurred within 1 to 3 minutes, and these depolarizations were sustained until the end of hypoxia in slices from each group. However, since hypoxic conditions were main-
Fig 2. Bar graph depicts delay to hypoxic depolarization in control slices and slices treated with Cbz-Val-Phe-H (100 μmol/L). There was no significant difference between these groups with respect to the delay to hypoxic depolarization. Cbz indicates calpain inhibitor–treated group.

obtained for a total of 15 minutes in this paradigm, the depolarized state persisted for a total of 12 to 14 minutes. The recovery of DC potentials after reoxygenation was complete in all groups. In contrast, the recovery of the evoked potentials was substantially reduced after sustained hypoxia. In the control group, 5 of 26 slices exhibited no detectable recovery of the synaptic potential 60 minutes after reoxygenation, and the average amount of recovery was 20.0±2.6% of the prehypoxic level.

Slices treated with Cbz-Val-Phe-H exhibited enhanced recovery of synaptic potentials after prolonged hypoxia. Treatment with 20, 50, or 100 μmol/L of Cbz-Val-Phe-H resulted in recovery levels of 41.4±5.9%, 67.4±4.2%, and 68.2±2.7%, respectively (Fig 3). All treated groups exhibited significantly enhanced recovery when compared with the control group; recovery of synaptic responses in the 50- and 100-μmol/L groups were significantly greater than that observed in the 20-μmol/L group (Fig 3).

Fig 3. Bar graph depicts effect of Cbz-Val-Phe-H on the posthypoxic recovery of synaptic responses. The percent recovery of the late negativity (synaptic component) of the evoked waveform is shown for slices subjected to a fixed period of hypoxia (15 minutes). Posthypoxic measurements were taken 60 minutes after reintroducing oxygen into the chamber. Treatment with the calpain inhibitor enhanced the posthypoxic recovery of responses. Values are mean±SEM. *P<.01 vs control; #P<.01 vs 20 μmol/L Cbz-Val-Phe-H (one-way analysis of variance followed by Tukey’s procedure). Cbz indicates calpain inhibitor–treated groups.

Discussion

Numerous studies have attempted to ameliorate ischemic and hypoxic neuronal damage by directly or indirectly limiting the elevation of intracellular calcium. The results of these studies have been mixed.13 The limitations of this approach probably stem from the multiple sources that contribute to the elevation of intracellular calcium after sustained neuronal depolarization. Calcium can enter neurons through a variety of different channels; in addition, substantial levels of calcium can be released from intracellular stores. It is therefore quite difficult, if not impossible, to antagonize all of these different sources of calcium. The present experiments indicate that interventions that target mechanisms activated subsequent to intracellular calcium elevation represent a viable therapeutic strategy, and that calcium-activated proteolysis is a critical component of the pathophysiological cascade leading to neuronal death.

The rationale for investigating calpain inhibitors as potential therapeutic agents for neocortical damage is based on several recent observations. First, key cytoskeletal proteins (ie, spectrin, microtubule-associated protein 2, and neurofilament proteins) are preferred substrates for calpain. Second, substantial changes in the levels of these cytoskeletal proteins have been observed after hypoxia.7,10,14 Presumably, the uncontrolled proteolysis of any or all of these structural proteins would jeopardize continued cellular viability. Finally, calpain inhibitors have been shown to exert a neuroprotective effect against hypoxic and ischemic damage in the hippocampal neurons.8-10 The concept that treatment with a calpain inhibitor would be neuroprotective therefore has a good theoretical and mechanistic basis.

The in vitro neocortical slice model used here simulates in vivo hypoxic neuronal insults, which can incur considerable morbidity in the cerebral cortex. The slice preparation is particularly useful because it facilitates both the identification of mechanisms contributing to cellular damage and the evaluation of treatments designed to protect against such damage. A common feature of hypoxic responses in both in vitro and in vivo systems is the precipitous and profound depolarization observed during the intrahypoxic period, ie, HD. The onset of HD is closely associated with a massive entry of calcium into neurons.16,17 This is a critical step in the hypoxic response because sustained calcium elevation triggers a cascade of events that can ultimately lead to cell death. Treatments prolonging the delay to HD have been shown to be useful for limiting hypoxic neuropathology18; these treatments are presumed to act by delaying the elevation of intracellular calcium. A complementary approach for achieving neuroprotection would be to reduce the deleterious impact of high concentrations of intracellular calcium. By attenuating harmful calcium-induced mechanisms, it might be possible to limit the extent of cellular damage at a later stage of the hypoxic cascade. A useful feature of the in vitro slice model is that it permits one to discriminate between therapeutic effects operating before or after calcium entry into the cell. The present studies took advantage of this feature to characterize the neuroprotective effect of a calpain inhibitor. The data presented here indicate that doses ranging from 20 to 100 μmol/L of Cbz-Val-Phe-H are protective. However, no greater neuroprotective effect was obtained in the 100-μmol/L group
than was seen with the 50-μmol/L group. The reason for this lack of a progressive effect is unclear. It is possible that the protection achieved at 50 μmol/L and 100 μmol/L Cbz-Val-Phe-H represents a maximal effect. Further studies will be required to clarify this issue.

In conclusion, our results demonstrate that treatment with Cbz-Val-Phe-H effectively improved posthypoxic synaptic recovery without altering the delay to HD. These observations indicate that the neuroprotective effect of the calpain inhibitor is not the result of delayed calcium entry. Rather, it appears to operate by mitigating against the impact of elevated intracellular calcium. These findings underscore the importance of calcium-activated proteolysis in the process of hypoxic/ischemic cell death and suggest that calpain inhibitors may be a useful therapeutic treatment for clinical situations involving ischemia of the central nervous system.

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References


**Editorial Comment**

Calcium influx following the activation of postsynaptic glutamate receptors has been identified as a major factor contributing to neuronal death after an ischemia insult. Various experimental strategies have aimed at the blockade of various glutamate receptors and the prevention of calcium influx. However, this approach may cause undesirable side effects, since it affects the normal neuronal function that is also associated with glutamate receptor activation. Other experimental approaches have aimed at modifying calcium cascades, including the alteration of the activities of phospholipases, proteases, protein kinase C, and Ca²⁺/calmodulin-dependent protein kinase II. Using well-defined, neocortical slices of adult Mongolian gerbils as a model system, Hiramatsu et al now report that posthypoxically induced synaptic transmission recovery was significantly improved by Cbz-Val-Phe-H, a specific inhibitor of the calcium-activated, neutral protease calpain. These data support the notion that Ca²⁺-associated protein degradation is an important event among the calcium cascades that would ultimately lead to synaptic dysfunction and neuronal death. Thus, further studies are warranted to extend this in vitro provocative observation of this compound to its possible use as neuronal protective agent in vivo so that therapeutic intervention can be developed to ameliorate ischemic brain injury.

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