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Inhibition of proteolysis protects hippocampal neurons from ischemia

(hippocampus/calpain/spectrin/hypoxia/neuroprotection)

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ABSTRACT **Intense proteolysis of cytoskeletal proteins occurs in brain within minutes of transient ischemia, possibly because of the activation of calcium-sensitive proteases (calpains). This proteolytic event precedes overt signs of neuronal degeneration, is most pronounced in regions of selective neuronal vulnerability, and could have significant consequences for the integrity of cellular function. The present studies demonstrate that (*i*) the early phase of enhanced proteolysis is a direct response to hypoxia rather than other actions of ischemia, (*ii*) it is possible to pharmacologically inhibit the *in vivo* proteolytic response to ischemia, (*iii*) inhibition of proteolysis is associated with a marked reduction in the extent of neuronal death, and (*iv*) protected neurons exhibit normal-appearing electrophysiological responses and retain their capacity for expressing long-term potentiation, a form of physiological plasticity thought to be involved in memory function. These observations indicate that calcium-activated proteolysis is an important component of the post-ischemic neurodegenerative response and that targeting this response may be a viable therapeutic strategy for preserving both the structure and function of vulnerable neurons.**

Elevated levels of intracellular calcium during and/or following transient ischemia are widely believed to trigger cellular events that lead to neuronal death (e.g., ref. 1). During ischemia, calcium enters vulnerable neurons through voltage-sensitive and receptor-operated channels and is released from intracellular stores. Consequently, treatments that reduce the entry of calcium into vulnerable neurons have achieved some success in protecting neurons (1, 2). However, conflicting results have been reported regarding the effectiveness of calcium antagonists (2), perhaps due to the many routes through which calcium can reach cytoplasmic pools. An alternative strategy for neuroprotection is to identify and target cellular events that are triggered by calcium and likely to be involved in neurodegeneration. An important biochemical mechanism satisfying both of these criteria is the activation of calcium-sensitive proteases (calpains). Several prominent cytoskeletal proteins are preferred substrates for calpain [e.g., spectrin, microtubule-associated protein MAP2, and neurofilament proteins (3–5)], and increased proteolysis of spectrin is associated with toxin-induced (6, 7) and lesion-induced neuropathologies (8). Moreover, a marked accumulation of spectrin breakdown products (BDPs) caused by calpain is one of the earliest biochemical changes occurring in vulnerable neurons after transient ischemia (9). Substantial proteolysis of any or all of the substrate proteins (9, 10) for calpain would presumably have severe consequences for the integrity of neuronal structure and function. Calpain is, therefore, in a position to provide a link between transient ischemia and cell death inasmuch as (*i*) it is associated with a variety of neurodegenerative responses, (*ii*) it is activated by an appropriate signal (elevated intracellular calcium), and (*iii*) it produces appropriate effects (breakdown of cytoskeleton). Here we report evidence indicating that calcium-activated proteolysis is an important event in the process of post-ischemic cell death and that targeting this mechanism is a viable therapeutic strategy for protecting vulnerable neurons.

MATERIAL AND METHODS

Histological and Electrophysiological Studies of Post-Ischemic Hippocampus. Adult gerbils [*Meriones unguiculatus*; 50–80 g (body weight)] received either saline ($n = 9$) or leupeptin, a protease inhibitor known to block calpain (20 mg/ml; $n = 9$), by infusion through a cannula implanted into the right lateral ventricle. Three days after the start of drug infusion with an Alzet microosmotic pump, transient ischemia was administered by clamping the carotid arteries bilaterally for 10 min (11). A treatment period of 3 days is sufficient to allow access of leupeptin to hippocampal neurons (12) and is the approximate life span of the microosmotic pumps. Animals survived for 14 days after carotid artery surgery and were sacrificed by Nembutal overdose and transcardial perfusion of a 10% (wt/vol) solution of paraformaldehyde in phosphate-buffered saline. Hippocampal neurons perish within 4 days after ischemia (13, 14); the 14-day survival period was, therefore, more than sufficient for the full expression of delayed neuronal death under normal conditions. Control animals (i.e., untreated unoperated, $n = 7$) were also sacrificed and analyzed in conjunction with the post-ischemic groups. Coronal sections of the brain stained with cresyl violet were examined (*i*) to quantify the number of pyramidal-like cells in the CA1b region of the hippocampus, a region selectively vulnerable to transient ischemia (13, 14) and (*ii*) to verify the placements of cannulae implanted in the lateral ventricles. Microscope slides containing sections of the septal aspect of the hippocampus were coded and analyzed in random order. The stratum pyramidale was examined on both sides of the brain for at least two sections per animal. Cells with well-preserved round-to-oval perikarya and nuclei were counted; only those cells with a minimum diameter of 10 μm were included in the counts.

For the examination of electrophysiological responses after ischemia, additional animals were subjected to transient ischemia with ($n = 8$) or without ($n = 7$) leupeptin pretreatment, as described above. Animals in these groups survived at least 14 and as many as 40 days after ischemia. Hippocampal slices were then prepared and synaptic responses were tested as described (15). Briefly, evoked synaptic responses [field excitatory postsynaptic potentials (fEPSPs)]

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Abbreviations: fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; BDP, breakdown product.

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were recorded in the CA1b region of the hippocampus in response to activation of the combined Schaffer collateral commissural afferents (see Fig. 3). The capacity for expressing long-term potentiation (LTP) was also examined by testing the effect of brief bursts of high-frequency stimulation on subsequent synaptic responses. fEPSPs were sampled at a rate of 1 per 30 sec prior to and after the delivery of high-frequency stimulation (three bursts at 100 sec^{-1} for a 0.5-sec duration delivered at 15-sec intervals).

Spectrin Proteolysis After Transient Ischemia *in Vivo*. In a related series of experiments, spectrin BDPs were measured 30 min after a 10-min period of transient ischemia in groups of untreated and leupeptin-treated animals. An additional group of untreated animals was subjected to 10 min of ischemia but sacrificed at the end of the ischemic period without unclamping the carotid arteries (i.e., no reflow). Leupeptin treatment and carotid artery surgeries were performed as described above. Animals were sacrificed by decapitation, the brains were rapidly removed and placed in cold homogenization buffer (see Fig. 1). The brain was then bisected in a coronal plane to separate the injection site from the hippocampus. The CA1 region of the hippocampus was dissected from the caudal aspect of the brain and homogenized. Sample preparation and immunoblot analysis for spectrin BPDs were performed as described in detail elsewhere (16). The remaining (i.e., rostral) aspect of the brain was frozen and sectioned with a cryostat to verify the placement of the injection cannula.

Spectrin Proteolysis After Hypoxia *in Vitro*. The effect of hypoxia on spectrin proteolysis was examined utilizing *in vitro* slices of the hippocampus. Hippocampal slices were prepared from control (i.e., untreated unoperated) animals as described (15). An equilibration period of 60 min was allowed after preparation of the slices. For hypoxic treatment of the slices, the atmosphere of the *in vitro* chamber, which normally contains 95% O_2/5% CO_2, was changed to 95% N_2/5% CO_2 for 10 min. Control slices were removed just prior to the hypoxic period, whereas "hypoxic slices" were removed at the end of the hypoxic period. Groups of slices were then homogenized and assayed for spectrin proteolysis as described above.

RESULTS

Spectrin Proteolysis After Transient Ischemia. Ischemia elicited a marked increase in the levels of spectrin BDPs, consistent with the findings of Seubert *et al.* (9). Fig. 1 shows immunoblots from control (lane 1) and ischemic (lane 2) gerbils and, as is evident, the ≈155-kDa BDP is elevated in the latter case. This BDP has been shown to be identical to that resulting when spectrin is incubated with calpain (16). Leupeptin infusion for 3 days prior to the ischemic episode greatly reduced the amount of BDP as indicated in lane 3. Fig. 1 also provides a quantitative estimate of the amount of spectrin BDPs (expressed as a percent of total immunoreactivity) in groups of control (i.e., untreated unoperated; $n = 5$), ischemic only ($n = 4$), and leupeptin-treated ischemic ($n = 5$) gerbils. The values for the first two groups are virtually identical to those reported by Seubert *et al.* (9) indicating the reliability of the ischemia-induced degradation of spectrin. Leupeptin reduced this effect to ≈25% of that seen in untreated ischemic animals. These observations indicate that the rapid post-ischemic phase of spectrin proteolysis can be significantly inhibited by *in vivo* treatment with the protease inhibitor.

Effects of Proteolytic Inhibition on Neuronal Structure and Function. Histological studies of the effect of protease inhibition on post-ischemic neuronal death revealed dramatic results. Fig. 2*A* shows a photomicrograph of the hippocampus from a typical saline-treated gerbil sacrificed 2 weeks after transient ischemia and illustrates the extensive damage typically found in the CA1 field. This is consistent with numerous previous studies demonstrating the selective vulnerability of neurons in this region (13, 14). The hippocampus

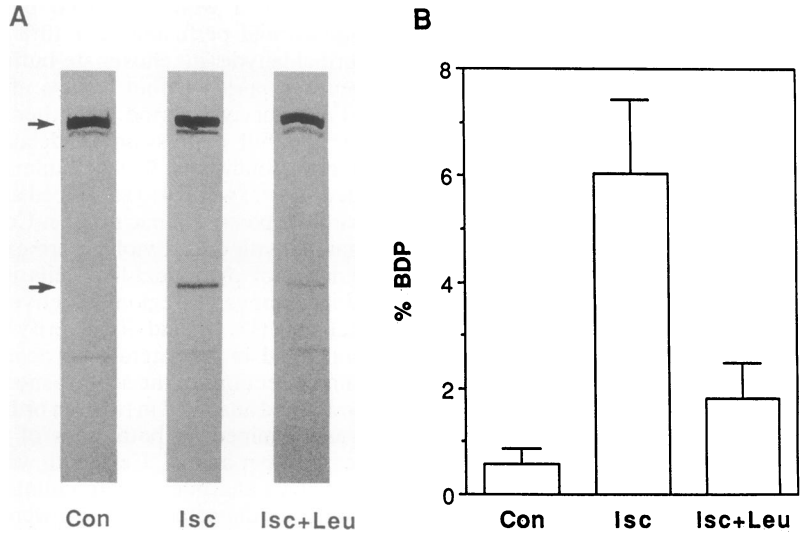

FIG. 1. Leupeptin inhibits post-ischemic proteolysis of spectrin in the hippocampus. (*A*) Immunoblots showing the native and partially digested spectrin molecule. Lanes: Con, control animal; Isc, post-ischemic animal; Leu + Isc, leupeptin-treated post-ischemic animal. The upper arrow indicates the position of the α- and β-spectrin subunits (240 kDa and 235 kDa, respectively). The lower arrow indicates the ischemia-induced 155-kDa BDP. (*B*) Histogram depicting the relative amounts of BDP (expressed as a percent of total immunoreactivity) from groups of control (Con; $n = 5$), ischemic (Isc; $n = 4$), and leupeptin-treated ischemic (Isc + Leu; $n = 5$) gerbils. The average of duplicate samples was calculated for each animal, these values were pooled in each category and the data are expressed as mean ± SD. Samples of the CA1 region were homogenized in ice-cold dissection buffer [0.32 M sucrose/10 mM Tris·HCl/2 mM EDTA/1 mM EGTA/100 μM leupeptin/L-1-tosylamido-2-phenylethyl chloromethyl ketone (20 μg/ml), pH 7.4], added to 1/3 vol of 3× SDS/PAGE buffer (150 mM Tris/6% SDS/30% glycerol/3.75 mM EDTA/3% 2-mercaptoethanol, pH 6.8 (adjusted with H_3PO_4), and boiled for 3–5 min. Equal amounts of protein were subjected to SDS/PAGE on a 3–10% linear gradient polyacrylamide with a 4.5% stacking gel. Electrophoretic transfer, immunodetection, and densitometric quantification of resolved spectrin species are described elsewhere (16), as is the characterization of affinity-purified anti-spectrin antibodies (17). All samples were electrophoresed in duplicate and samples from each experimental manipulation were loaded on each gel.

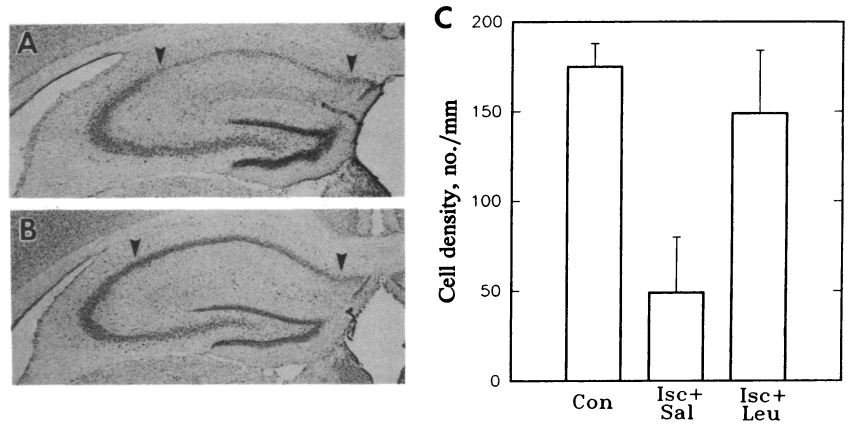

FIG. 2. Leupeptin attenuates neuronal death after transient ischemia. (*A* and *B*) Photomicrographs of the hippocampus from two animals subjected to transient forebrain ischemia. (*A*) Animal treated with saline shows extensive loss of CA1 pyramidal neurons (arrows). (*B*) In contrast, the leupeptin-treated animal has a normal-appearing layer of CA1 pyramidal neurons (arrows). This effect is quantified in *C* for the following groups of animals: (*i*) unoperated control (Con); (*ii*) saline-treated ischemic (Isc + Sal), and (*iii*) leupeptin-treated ischemic (Isc + Leu). Animals were sacrificed 14 days after transient ischemia, and cell density in the stratum pyramidale of region CA1b was measured. The density of intact cells is expressed per mm length of the stratum pyramidale. After ischemia, cell density was only slightly (and not significantly) reduced in the leupeptin-treated ischemic animals whereas the saline-treated ischemic animals showed a substantial loss of neurons. The protective effect of leupeptin (i.e., the difference between the saline-treated ischemic and leupeptin-treated ischemic groups) was highly significant ($P < 0.001$ on a two-tailed *t*-test). All values shown in *C* are the mean + SD.

from a leupeptin-treated animal (Fig. 2*B*) sacrificed at the same post-ischemic time point shows much less pathology. Cell density measurements showed 72% loss of neurons in the saline-treated group but only a 15% loss in the leupeptin-treated group (Fig. 2*C*). This effect was apparently not due to changes in thermoregulation; rectal temperature was monitored prior to, during, and for 30 min after ischemia and did not differ between groups of animals subjected to transient ischemia. In addition, previous studies have demonstrated that leupeptin treatment does not detectably alter excitatory synaptic responses *in vivo* (12) or *in vitro* (18). This indicates that an effect of leupeptin on excitatory transmission is not responsible for its neuroprotective actions.

Recent studies demonstrate that neurons can appear healthy by morphological criteria but be compromised functionally after ischemia (19). Another series of experiments was, therefore, undertaken to determine whether neurons that appear intact are also functionally "protected" by leupeptin. Transient ischemia (i.e., bilateral occlusion of the carotid arteries) was delivered to untreated ($n = 7$) and leupeptin-treated ($n = 8$) animals as described above. Hippocampal slices were then prepared from these animals after post-ischemic survival periods of 14–40 days. Synaptic responses (fEPSPs) recorded in area CA1 were normal appearing in slices from seven of the eight leupeptin-treated animals whereas these responses were virtually eliminated in slices from all of the untreated animals (Fig. 3). The absence of synaptic responses in the slices from untreated animals was not the result of a general loss of slice viability inasmuch as fEPSPs were readily obtained in the dentate gyrus of these slices. The CA1 region of slices from leupeptin-treated animals also exhibited an intact capacity for LTP (Fig. 3). Brief bursts of high-frequency stimulation resulted in a sustained enhancement of synaptic responses, a characteristic plastic response of synapses in this region.

Spectrin Proteolysis Elicited During Ischemia *in Vivo* and Hypoxia *in Vitro*. The protective effect obtained with the protease inhibitor emphasizes the importance of identifying the events occurring during and/or after ischemia that might activate calpain. Accordingly, the levels of spectrin BDPs were examined in three animals sacrificed at the end of the ischemic episode but prior to reoxygenation (i.e., without reflow). A typical increase in the concentration of spectrin BDPs was obtained in all three animals, indicating that the initial phase of proteolysis is a response to ischemia rather than to the secondary consequences that develop during reoxygenation. To determine whether hypoxia alone is sufficient to elicit proteolysis, we exposed *in vitro* slices of hippocampus prepared from control (i.e., unoperated untreated) animals to a 10-min period of hypoxia. This treatment produced a sizable increase in spectrin BDPs compared to adjacent control slices that were removed immediately prior to hypoxia. The relative amounts of spectrin BDPs (calculated as in Fig. 1) were 6.1 ± 1.3% (mean ± SEM) in control slices and 11.5 ± 2.1% in hypoxic slices ($P < 0.005$, paired *t* test, 16 pairs of slices). Preincubation of slices for 1 hr with calpain inhibitor I (200 μM; Boehringer Mannheim) a potent inhibitor of calpain, reduces the level of spectrin BDPs in slices and also prevents hypoxia-induced increases in spectrin proteolysis ($n = 4$; data not shown).

DISCUSSION

The present results provide evidence of the benefits (in terms of neuroprotection) that can be derived from targeting ischemia-induced proteolysis. *In vivo* treatment with leupeptin inhibits calcium-activated proteolysis and protects against the degeneration of vulnerable hippocampal neurons after ischemia. In contrast to therapies directed at calcium channels (2), this approach is not contingent on blocking a particular source of elevated intracellular calcium, be it through multiple membrane channels and/or from intracellular stores. The suitability of any neuroprotective strategy, however, will ultimately hinge on its capacity to preserve the functional repertoire of vulnerable neurons in addition to its success in preventing structural degeneration. In humans, severe anterograde memory deficits are associated with the specific loss of CA1 pyramidal cells (20), indicating a critical role for this region in memory formation. LTP in the hippocampus has also been implicated in the process of memory formation and storage (e.g., ref. 21). It is therefore of particular interest that the capacity to express LTP is preserved in the vulnerable CA1 region of "neuroprotected" animals. This indicates that proteolytic inhibition during ischemia is capable of preserving more delicate physiological features of neuronal function as well as attenuating the morphological manifestations of cellular pathology.

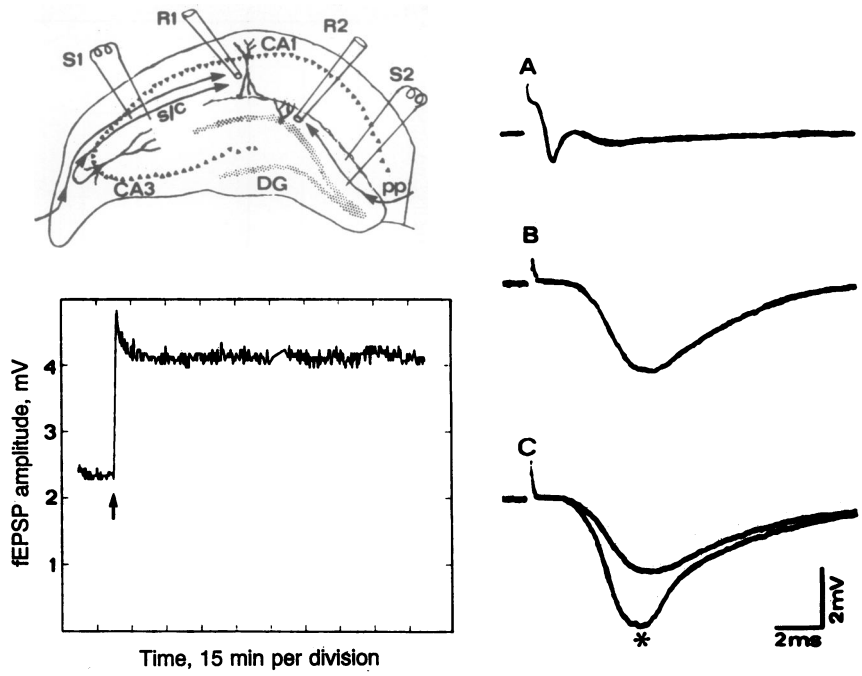

FIG. 3. Leupeptin prevents the post-ischemic loss of physiological responses. A schematic drawing of the *in vitro* hippocampal slice is shown in which the placements of stimulating (S) and recording (R) electrodes are depicted. The traces at the right of the figure are evoked potentials (fEPSPs) recorded in the stratum radiatum of CA1b (R1) in response to stimulation (S1) of the Schaffer collateral/commissural afferents (s/c). Trace A was recorded in a slice prepared from an untreated ischemic animal that was allowed to survive for 14 days after ischemia. The short latency negative potential (fiber volley) reflects the activity of axons passing through the CA1 region but little or no synaptic potential (fEPSP) was observed. A very high stimulation intensity was needed to evoke this fiber volley in the slices from untreated animals. Comparable results were obtained in each of seven experiments on slices from untreated ischemic animals. In contrast, trace B shows a fEPSP evoked in a slice prepared from a leupeptin-treated ischemic animal (also recorded by R1 in response to S1). The fEPSP in trace B represents a summation of synaptic potentials in the CA1b region and indicates that synaptic transmission is intact in these slices. The capacity for LTP was also intact in leupeptin-treated ischemic animals. Trace C was recorded in a slice from a leupeptin-treated ischemic animal that was allowed to survive 40 days after ischemia. Two superimposed fEPSPs are shown that were evoked prior to and 30 min after (asterisk) three brief bursts of high-frequency stimulation (three bursts at 100 sec^{-1} for a 0.5-sec duration delivered at 15-sec intervals). Responses were greatly enhanced after high-frequency stimulation. The graph summarizes a separate experiment in which responses to single-stimulation pulses were collected at 1 per 30 sec for 15 min before and 120 min after an episode of high-frequency stimulation (arrow); note that a sustained potentiation of the fEPSP was obtained. Preservation of synaptic responses and of the LTP induction–expression mechanisms was obtained in slices from seven of eight ischemic gerbils treated with leupeptin. Synaptic potentials evoked in the dentate gyrus [recorded in the stratum moleculare (R2) in response to activation (S2) of perforant path afferents (pp)] were intact in slices from both untreated ischemic and leupeptin-treated ischemic animals (data not shown). ms, msec.

The *in vivo* studies also demonstrate that calcium-activated proteolysis is initiated during ischemia and does not require vascular reflow. Complementary experiments utilizing *in vitro* slices indicate that hypoxia alone is a sufficient stimulus to elicit calcium-activated proteolysis in the hippocampus and that this event can be blocked by calpain inhibitor I, a highly specific inhibitor of calpain. This compound, along with leupeptin, partially protects slices from the acute pathophysiology that follows prolonged hypoxia and does so without affecting baseline synaptic potentials or the initial response of the slice to hypoxia (18). Thus, these *in vivo* and *in vitro* observations indicate that the rapid phase of proteolysis is initiated during ischemia and does not depend wholly on tissue reoxygenation. In addition, the absence of systemic factors (e.g., hypothermia and hypotension) in the *in vitro* slice experiments points to the conclusion that hypoxia is the agent through which ischemia causes spectrin proteolysis.

The therapeutic utility of modulating calcium-activated proteolysis will probably depend on the development of more permeable, potent, and specific protease inhibitors (22). In the present experiments, leupeptin was selected because it represents the only protease inhibitor that has previously been shown to block a trauma-evoked calpain response *in vivo* (8). Leupeptin inhibits a range of cysteine and serine proteases and additional effects on other proteases cannot be ruled out in the *in vivo* studies. However, companion *in vitro* studies examining the effects of hypoxia on hippocampal slices demonstrate that a more-specific calpain inhibitor (calpain inhibitor I) is also neuroprotective (18), strongly implicating calpain activation as a critical event in ischemic pathology. In addition to intervention with exogenous protease inhibitors, another possible approach to neuroprotection is suggested by the current observations. Calpain activity is regulated in part by calpastatin, an endogenous inhibitor of calpain's proteolytic activity. If calpastatin activity could be elevated or potentiated, then calpain-mediated proteolysis would be inhibited and cell loss could be presumably attenuated. Recent studies showing that nerve growth factor stimulates the synthesis of calpastatin and, thereby, reduces calpain activity in cultured cells (23) suggests that such an approach may be feasible.

Suppression of proteolysis thus represents a promising avenue for limiting functional and structural pathology after transient ischemia. The participation of calcium-activated proteolysis in a wide range of pathological conditions suggests that this approach may be useful in the treatment of a variety of neurodegenerative responses.

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