0022-3565/06/3172-529–536$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 317:529–536, 2006

Vol. 317, No. 2
95018/3107514
Printed in U.S.A.

3-[2-[4-(3-Chloro-2-methylphenylmethyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole Dihydrochloride 3.5 Hydrate (DY-9760e) Is Neuroprotective in Rat Microsphere Embolism: Role of the Cross-Talk between Calpain and Caspase-3 through Calpastatin

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Received August 30, 2005; accepted February 6, 2006

ABSTRACT

Microsphere embolism (ME)-induced cerebral ischemia can elicit various pathological events leading to neuronal death. Western blotting and immunohistochemical studies revealed that expression of calpastatin, an endogenous calpain inhibitor, decreased after ME induction. Calpain activation after ME was apparently due to, in part, a decrease in calpastatin in a late phase of neuronal injury. The time course of that decrease also paralleled caspase-3 activation. In vitro studies demonstrated that calpastatin was degraded by caspase-3 in a Ca^{2+}/calmodulin (CaM)-dependent manner. Because CaM binds directly to calpastatin, we asked whether a novel CaM antagonist, 3-[2-[4-(3-chloro-2-methylphenylmethyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate (DY-9760e), inhibits caspase-3-induced calpastatin degradation during ME-induced neuronal damage. We also tested the effect of DY-9760e on degradation of fodrin, a calpain substrate. Consistent with our hypothesis, DY-9760e (25 or 50 mg/kg i.p.) treatment inhibited degradation of calpastatin and fodrin in a dose-dependent manner. Because DY-9760e showed powerful neuroprotective activity with concomitant inhibition of calpastatin degradation, cross-talk between calpain and caspase-3 through calpastatin possibly accounts for ME-induced neuronal injury. Taken together, both inhibition of caspase-3-induced calpastatin degradation and calpain-induced fodrin breakdown by DY-9760e in part mediate its neuroprotective action.

Microspheres infused into the internal carotid artery induce small widespread embolisms, thereby leading to multiple infarct areas in the brain. The pathogenesis of microsphere embolism (ME)-induced brain damage resembles clinical features of human territorial stroke or brain vascular dementia (Demura et al., 1993). ME-induced ischemic injury includes many pathological events, such as release of excitatory amino acids and deprivation of oxygen/glucose (Smith et al., 1990; Takeo et al., 1992). These events then trigger calcium influx into neurons, thereby leading to activation of Ca^{2+}-dependent signaling, such as Ca^{2+}/CaM-dependent enzymes and Ca^{2+}-dependent protease calpains (Hayashi et al., 1998; Takagi et al., 2001). Brain ischemia/reperfusion injury is associated with calpain-induced proteolysis of the cytoskeleton, including fodrin/spectrin (Fokuda et al., 1998; Sato et al., 1999; Yakota et al., 2003). We also documented nitric oxide production through activation of Ca^{2+}/CaM-dependent nitric-oxide synthase (NOS) in ME-induced ischemia, thereby producing protein tyrosine nitration (Shirakura et al., 2005). The novel CaM inhibitor DY-9760e rescues neurons from ME-induced neuronal damage with concomitant inhibition of both NO production and protein tyrosine nitration (Hashiguchi et al., 2003). Interestingly, the neuroprotective effect of DY-9760e is also highly correlated with inhibition of calpain-induced breakdown of fodrin, a cytoskeletal

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.095018.

ABBREVIATIONS: ME, microsphere embolism; NOS, nitric-oxide synthase; DY-9760e, 3-[2-[4-(3-chloro-2-methylphenylmethyl)-1-piperazinyl]-ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydro-chloride 3.5 hydrate; CaM, calmodulin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; FBDP, fodrin breakdown product; MDL28,170, carbobenzoxy-valinyl-phenylalaninal.

and CaM-binding protein (Sato et al., 1999). Therefore, we hypothesize that inhibition of fodrin breakdown by calpain is partly involved in the neuroprotective action of DY-9760e.

In addition to calpain activation, caspase-3 plays pivotal roles in apoptotic cell death by inducing Bid and poly(ADP-ribose) polymerase activation (Affar et al., 2001; Degli Esposti et al., 2003). Inappropriate imbalances between calpain and calpastatin activation may play critical roles in the delayed neuronal death including apoptosis (Rami et al., 2003). Inhibition of nitric oxide production and fodrin breakdown by DY-9760e may suppress apoptotic signals such as caspase-3 activation through maintaining membrane integrity and suppressing mitochondrial damage. Indeed, cross-talk between calpain and caspase-3 has been documented in brain ischemia. For example, calpain-mediated N-terminal truncation of caspase-3 to a p30 polypeptide enhanced caspase-3 activation in one study (Blomgren et al., 2001) and inhibited its activation in another (McGinnis et al., 1999). Calpain-mediated N-terminal truncation of caspase-9 results in the loss of ability to activate caspase-3 (Chua et al., 2000; Lankiewicz et al., 2000). Calpastatin, an endogenous calpain inhibitor, is cleaved by caspase-3, thereby leading to loss of its inhibitory effect on calpain (Rami et al., 2003). Calpastatin also inhibits translocation of calpain to the plasma membrane, thereby inhibiting its proteolytic activity toward membranous cytoskeletal proteins such as fodrin (Melloni et al., 1996; Kosower et al., 2000). In addition, calpastatin is a CaM-binding protein; therefore, it leads to the hypothesis that DY-9760e may affect proteolysis of calpastatin, which is mediated by caspase-3.

Little is known about the cross-talk between calpain and caspase-3 in brain ischemia-induced neuronal death in vivo. Therefore, we asked whether cross-talk of calpain/caspase-3 participates in ME-induced neuronal damage through calpastatin. We also determined that DY-9760e has an inhibitory effect on calpastatin degradation during ischemia and showed that inhibition of cross-talk between calpain and caspase-3 by DY-9760e probably underlies its neuroprotective action in brain ischemia. Therefore, we propose a novel neuroprotective mechanism of DY-9760e.

Materials and Methods

A Microsphere-Induced Permanent Focal Cerebral Ischemic Model. All experimental procedures using animals were approved by the Committee on Animal Experiments at Tohoku University. Male Wistar rats weighing 220 to 270 g were subjected to general anesthesia induced by 4% halothane in oxygen, which was maintained by spontaneous breathing of 1.5% halothane in oxygen via a face mask. After placing animals in a supine position, a lateral longitudinal midline neck incision was performed to identify and dissect the left common carotid artery and separate the internal carotid artery from the external one at the bifurcation as the base point. Both external carotid and pterygopalatine arteries were temporarily occluded by aneurysm clips. One thousand nonradioactive microspheres (48.4 $\pm$ 0.7 μm in diameter) suspended in a 20% dextran solution were injected with a 26 G needle into the left common carotid artery while visualizing flow into the internal carotid artery. The skin was closed, and halothane anesthesia was terminated. Rectal temperature was monitored and maintained at 37 $\pm$ 0.5°C with a heating blanket throughout the surgery. Sham-operated groups received the same treatment without microsphere injection. Behavioral changes such as lack of movement, truncal curvature, and forced circling during locomotion after microsphere injection were observed as described previously (Miyake et al., 1993). Neurologic scores were determined 0.5, 24, 48, and 72 h after microsphere injection. Scoring was as follows: grade 0, no deficit; grade 1, right side forelimb weakness and torso turning to the left side when held by tail; grade 2, circling to left side; grade 3, circling to left side and inability to bear weight on the right side; and grade 4, no spontaneous locomotor activity. Rats with grade 0 and grade 4 were not used for subsequent experiments. Rats were decapitated at 2, 6, 12, 24, 48, and 72 h after injection of microspheres, and the striatum and cortical regions of the ipsilateral hemisphere were dissected for immunoblotting analyses.

Drug Treatments. DY-9760e was dissolved with 100% DMSO and diluted in a 5% glucose solution to a final concentration of 1% DMSO. The microsphere-induced cerebral ischemic model was prepared as described above. After microsphere injection, rats were treated with DY-9760e at 12.5 or 25 mg/kg intraperitoneally. In each case, DY-9760e was injected twice 30 min and 6 h after microsphere injection. Vehicle-treated animals were administered the same volume of 1% DMSO in 5% glucose 30 min and 6 h after microsphere injection, and sham groups received the same experimental procedures without microsphere injection. The dose of DY-9760e used did not affect the mean of arterial blood pressure monitored 30 min after DY-9760e administration (data not shown).

Western Blotting Analysis. After decapitation, brains were removed and rinsed once with ice-cold 0.32 M sucrose. Coronal sections of 2 mm were prepared by a hand brain slicer, and ipsilateral and contralateral hemispheres including cortex and striatum were dissected and stored at −80°C. Frozen brain tissues were homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na_3VO_4, 30 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mM dithiothreitol (DTT). Insoluble material was removed by a 20-min centrifugation at 15,000*g*. After determining protein concentration in each fraction using Bradford's solution, samples containing equivalent amounts of protein were applied to 10 to 15% acrylamide-denaturing gel (SDS-PAGE) (Laemmli, 1970). Proteins were then transferred to an Immobilon polyvinylidene difluoride transfer membrane for 2 h at 70 V. Membranes were blocked in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 containing 5% fat-free milk powder for 1 h at room temperature and incubated with fodrin (rabbit polyclonal antibody, 1:2000) (Sato et al., 1999), cleaved caspase-3 (rabbit polyclonal antibody, 1:1000; Cell Signaling Technology, Beverly, MA), calpastatin (goat polyclonal antibody, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and β-tubulin (mouse monoclonal antibody, 1:10,000; Sigma, St. Louis, MO) antibodies overnight at 4°C. After washing, membranes were incubated 60 min at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 solution for 60 min at room temperature. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). Images were scanned and analyzed semiquantitatively using the Image Gauge Software (Fuji Film, Tokyo, Japan).

Immunohistochemical Studies. Rats were anesthetized and transcardially perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) as described previously (Kawano et al., 2002). Whole brains were immediately removed and postfixed overnight at 4°C in the same fixative. Then, coronal brain sections at the coordinates of 1 mm posterior to bregma (35-μm thick) were prepared using a vibratome. Sections were incubated at room temperature with 0.01% Triton X-100 in PBS for 30 min and for another hour in 3% bovine serum albumin in PBS. For immunolabeling, slices were probed with anticalpastatin (goat polyclonal antibody, 1:200; Santa Cruz Biotechnology), anticleaved caspase-3 (rabbit polyclonal antibody, 1:500; Cell Signaling Technology), and anti-NeuN (mouse monoclonal antibody, 1:500; Chemicon International, Temecula, CA) overnight at 4°C. After washing steps, sections were

incubated with biotinylated anti-goat or rabbit IgG (1:5000) in TNB buffer [0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.5% blocking reagent (supplied in TSA-Direct kit; NEN Life Science Products, Boston, MA)] for 1 h, followed by both streptavidin-horseradish peroxidase (1:5000) and Alexa 594 anti-mouse IgG (Molecular Probes, Eugene, OR) in TNB buffer (1:400) and labeled for 2 h. Sections were then stained with tetramethylrhodamine tyramide for 10 min using the TSA-Direct kit. Immunofluorescent images were taken with a confocal laser scanning microscope (TCS SP; Leica Microsystems). To normalize immunoreactivity with anticalpastatin antibody, we analyzed cortical slices without changing the confocal laser intensity and laser aperture. Furthermore, sections were double-stained with anti-NeuN antibody as a neuronal marker. We confirmed that the intensity of immunoreactivity with anti-NeuN antibody was the same between control and ischemic cortical slices. We further quantified the NeuN-positive neurons in six consecutive sections of the ipsilateral cortex under confocal laser microscope at 72 h after ME to evaluate neuroprotective action of DY-9760e. The photographed regions are borders of the infarction, in which neither obvious morphological nor immunohistochemical changes with anti-NeuN antibody were observed in neurons compared with sham-operated animals.

Capase-3 Activity Assay. Caspase-3 activity was measured by cleaving *N*-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin) (BioMol, Plymouth Meeting, PA), a selective substrate for caspase-3. The ipsilateral hemisphere containing both striatum and cortex was obtained 24 h after ischemia from both nonischemic and ischemic groups. Tissues were homogenized in a homogenizing buffer containing 15 mM Tris-HCl, pH 7.4, 320 mM sucrose, 1 mM EGTA, 2 mM EDTA, 50 mM NaF, 1 mM $MgCl_2$, 1 mM Na_3VO_4, and 30 mM sodium pyrophosphate plus protease inhibitors and centrifuged at 14,000*g* for 60 min at 4°C. Protein concentration of supernatants was measured by Bradford's method, and equal amounts of proteins (100 μg) were incubated in a total volume of 400 μl comprised of 20 mM HEPES, pH 7.4, 10% glycerol, and 2 mM DTT. The reaction was started by addition of caspase-3 fluorogenic substrate Ac-DEVD-AMC. After incubation for 60 min at 37°C, cleavage of the fluorogenic substrate was detected using a PerkinElmer LS50B luminescence spectrometer (PerkinElmer Life and Analytical Sciences, Boston, MA) with excitation and emission wavelengths of 380 and 460 nm, respectively. Activities of caspase-3 were expressed as changes in DEVDase activity.

Preparation of Brain Extracts and Incubation with Exogenous Caspase-3 Enzymes. Dissected brain tissue including cortex and striatum was homogenized in buffer containing 50 mM Tris-HCl, pH 7.4, 320 mM sucrose, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mM DTT. After centrifugation at 15,000*g* for 20 min, supernatants were incubated for 15 min in 60°C to denature endogenous protease activities. Brain extracts (20 μg of protein) were incubated for 30 min with 0.2 μg of purified caspase-3 at 30°C in a 50 μl-reaction medium containing 50 mM HEPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, and 10% glycerol. Under the same condition, heat-treated brain extracts were incubated with caspase-3 in the presence of 2.5, 5, or 20 μM DY-9760e with or without Ca^{2+}/CaM. After incubation with purified caspase-3, brain extracts were subjected to SDS-PAGE followed by immunoblotting with anticalpastatin and anticaspase-3 antibodies as described above.

Statistical Analysis. Data were represented as means ± S.D. and obtained from at least four independent animals. Significance differences between treated and control animals at each time point were assessed by Student's *t* test. Multiple comparisons between experimental groups were performed by one-way analysis of variance, followed by Dunnett's test. $P < 0.05$ was considered significant.

Results

Proteolysis of Fodrin and Calpastatin after Microsphere-Induced Cerebral Ischemia. To verify the temporal profile of calpain activation after ME, generation of a 150-kDa fodrin breakdown product (FBDP), which is a specific breakdown product for calpain, was measured in rat brain after ME. A significant increase in FBDP was seen 2 h after ME (Fig. 1A). Further significant increases in FBDP were also evident in 12 to 24 h compared with the amounts of FBDP at 6 h. Immunoblotting analysis with β-tubulin showed an equal amount of loaded protein in each lane (Fig. 1A). To determine whether changes in expression of calpastatin, an endogenous calpain inhibitor, underlie further activation of calpain, calpastatin levels were assessed by immunoblotting using anticalpastatin antibody. In sham-operated animals, a 110-kDa immunoreactive protein was detected by SDS-PAGE (Fig. 1B). Interestingly, levels of calpastatin were significantly reduced 6 to 24 h after ME induction. Such decreased levels in calpastatin were correlated with further increases in FDBP generation seen after 12 h, as shown in Fig. 1A.

Activation of Caspase-3 Accounts for Calpastatin Degradation. It is known that caspase-3 functions in the proteolysis of calpastatin. To confirm that reduced calpasta-

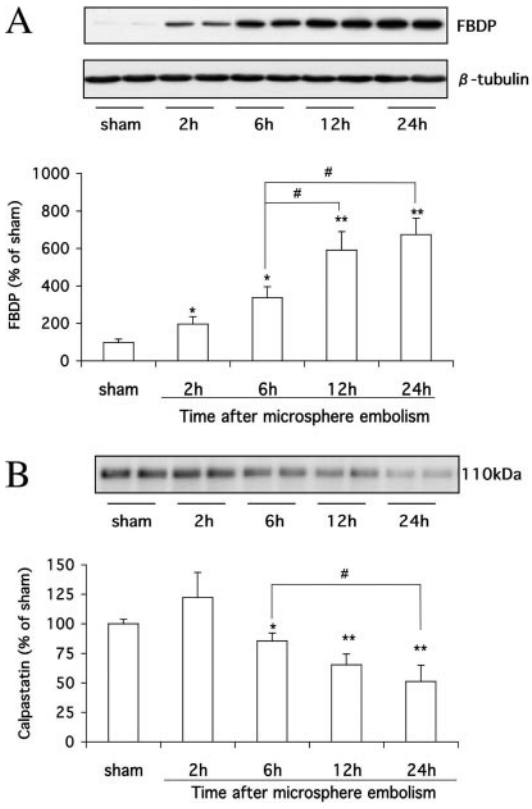

Fig. 1. Changes in expression of fodrin and calpastatin after ME induction in rat brain. A, calpain-mediated degradation of fodrin was determined by quantifying the 150-kDa FBDP after ME induction. The striatum and cortical regions of the ipsilateral hemisphere were dissected for immunoblotting analysis at the indicated times. Immunoblots with anti-β-tubulin antibody showed equal protein loading. Quantitative analysis of the 150-kDa FBDP levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 4$). *, $P < 0.05$; **, $P < 0.01$ versus sham-operated group. #, $P < 0.05$ versus the 6-h group. B, representative image of immunoblot using anticalpastatin antibody and the same samples described above. Quantitative analysis of the 110-kDa calpastatin levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 4$). *, $P < 0.05$; **, $P < 0.01$ versus sham-operated animals. #, $P < 0.05$ versus the 6-h group.

tin levels after ME were due to activation of caspase-3, we assessed caspase-3 activity by immunoblotting analysis using antibody recognizing active 17-kDa caspase-3 or by measurement of caspase-3 activity. In brain extracts from the ipsilateral hemisphere containing the cortex and striatum, a significant increase in cleaved 17-kDa active caspase-3 protein was observed 6, 12, and 24 h after ME induction (Fig. 2A). Likewise, a persistent and significant increase in caspase-3 activity assessed by Ac-DEVD-AMC as substrate was observed 12 to 24 h after ME induction (Fig. 2B).

Effects of DY-9760e on the Neurological Deficits. Neurological scores were determined to verify the outcome of neurological functions at 0.5, 24, 48, and 72 h after microsphere injection. There was no significant difference in scores between vehicle (2.90 ± 0.22) and DY-9760e-treated groups (3.10 ± 0.42) at 0.5 h after ME. However, the neurological scores in DY-9760e-treated rats significantly improved at 24, 48, and 72 h compared with vehicle-treated rats (Fig. 3).

Effects of DY-9760e on Fodrin Breakdown and Calpastatin Degradation after ME. Inhibition of fodrin breakdown by DY-9760e is due to inhibition of CaM binding to fodrin, which decreases its susceptibility to proteolysis by calpain. Therefore, we speculated that caspase-3-induced proteolysis of calpastatin possessing CaM-binding ability occurs in a Ca^{2+}/CaM-dependent manner and would be inhibited by DY-9760e. To confirm this possibility, rats were post-treated with DY-9760e and analyzed for effects on ME-induced calpastatin degradation as well as fodrin breakdown. Consistent with our hypothesis, proteolysis of calpastatin was significantly reduced by treatment with DY-9760e in a dose-dependent manner (Fig. 4A). Likewise, fodrin breakdown was inhibited by DY-9760e as reported in our previous study (Fig. 4B) (Sato et al., 1999).

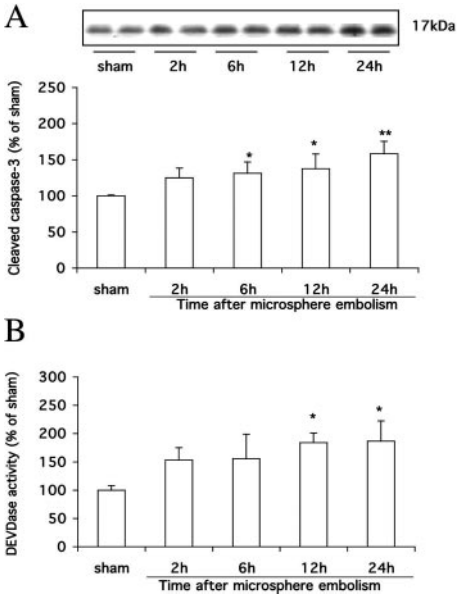

Fig. 2. Changes in levels of 17-kDa active caspase-3 after ME induction. A, representative immunoblots showing temporal changes in the 17-kDa active caspase-3 fragment after ME. Quantitative analysis of the 17-kDa active caspase-3 levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 4$). *, $P < 0.05$; **, $P < 0.01$ versus sham-operated animals. B, caspase-3 activity in brain extracts was assessed using *N*-acetyl-Asp-Glu-Val-Asp-AMC as a substrate. Caspase-3 activities were represented as percentage of values of sham-operated animals. Data are presented as mean ± S.D. ($n = 4$); *, $P < 0.05$ versus sham-operated group.

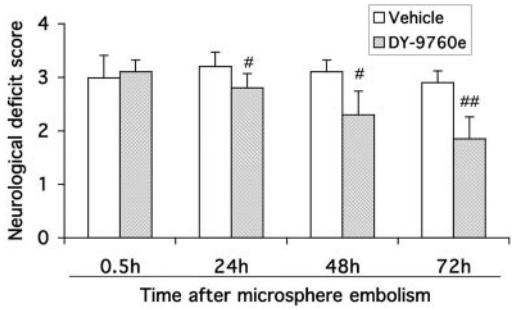

Fig. 3. Effects of DY-9760e on the neurological deficits. DY-9760e (12.5 mg/kg) was administered intraperitoneally twice 30 min and 6 h after ME induction. Neurological score was determined 0.5, 24, 48, and 72 h after ME with or without DY-9760e post-treatment. Data presented as mean ± S.D. ($n = 6$); #, $P < 0.05$; ##, $P < 0.01$ versus vehicle-treated rats.

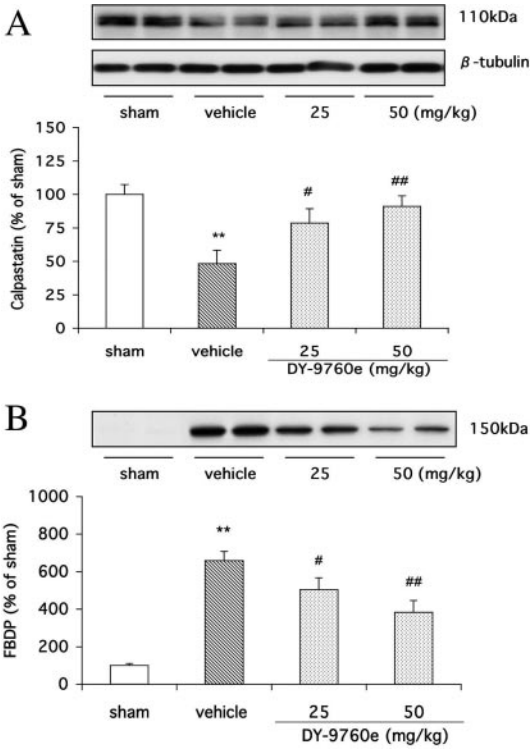

Fig. 4. Effects of postischemic treatment with DY-9760e on calpastatin degradation and fodrin breakdown. DY-9760e (12.5 or 25 mg/kg) was administered intraperitoneally twice 30 min and 6 h after ME induction. A, representative image of immunoblot using an anticalpastatin antibody. Quantitative analysis of levels of 110-kDa calpastatin protein was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 5$). **, $P < 0.01$ versus sham-operated animals; #, $P < 0.05$; ##, $P < 0.01$ versus vehicle-treated rats. B, representative image of immunoblot using the anti-150-kDa FBDP antibody. Quantitative analysis of 150-kDa FBDP levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 4$). **, $P < 0.01$ versus sham-operated rats. #, $P < 0.05$; ##, $P < 0.01$ versus vehicle-treated rats.

DY-9760e Treatment Blocked Activation of Caspase-3 after ME. We next asked whether DY-9760e suppresses caspase-3 activation after ME. As shown in Fig. 5, the level of 17-kDa caspase-3 active fragment significantly increased 24 h after ME and after treatment with DY-9760e (25 and 50 mg/kg) inhibited increased active caspase-3 pro-

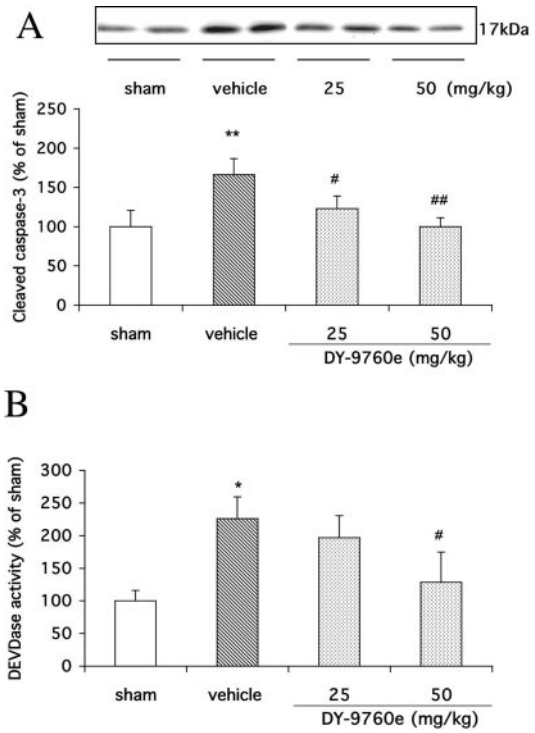

Fig. 5. Inhibitory effects of DY-9760e on ME-induced caspase-3 activation. A, cleaved active caspase-3 was measured 24 h after ME by immunoblotting with or without post-treatment with DY-9760e. Quantitative analysis of 17-kDa cleaved caspase-3 levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of sham-operated animals (mean ± S.D., $n = 5$). **, $P < 0.01$ versus sham-operated rats. #, $P < 0.05$; ##, $P < 0.01$ versus vehicle-treated rats. B, activity of caspase-3 assessed by *N*-acetyl-Asp-Glu-Val-Asp-AMC cleavage. DY-9760e significantly suppressed elevated caspase-3 activity 24 h after ME. Data are presented as mean ± S.D. ($n = 4$); *, $P < 0.05$ versus sham-operated rats. #, $P < 0.05$ versus vehicle-treated rats.

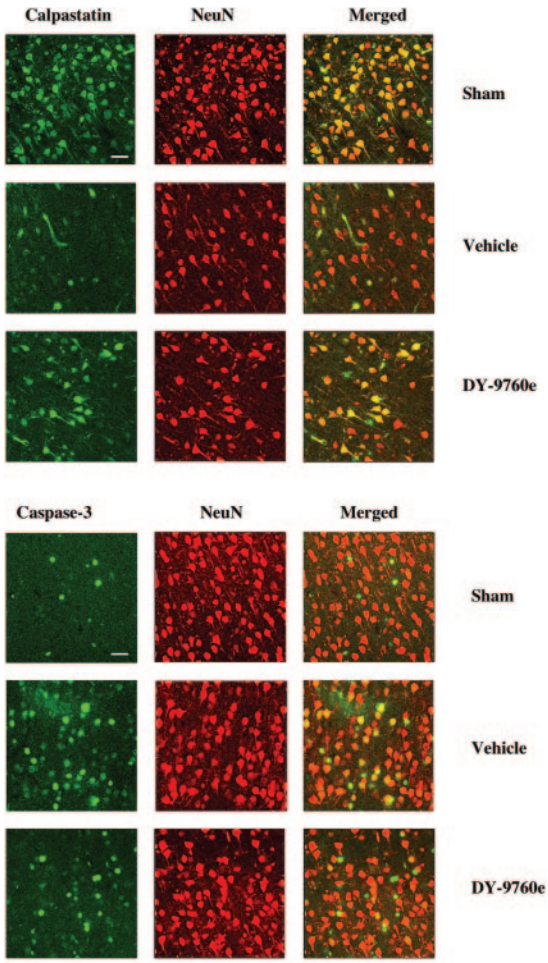

Fig. 6. Immunohistochemical localization of calpastatin and active caspase-3 in the cortex. A, confocal microscopic images of double staining with anticalpastatin (green fluorescence) and anti-NeuN (red fluorescence) antibodies, indicating predominant expression of calpastatin in neurons stained with anti-NeuN antibody. Markedly decreased calpastatin immunoreactivity was observed in cortex 24 h after ME. Calpastatin degradation in neurons was blocked by DY-9760e treatment. B, double staining with anti-active caspase-3 (green fluorescence) and anti-NeuN (red fluorescence) antibodies in the cortex showed that activation of caspase-3 occurred primarily in neurons of ME rats. The number of cells showing increases in active caspase-3 was significantly reduced by DY-9760e treatment. Scale bar, 30 μm.

duction in a dose-dependent manner (Fig. 5A). In accord with this, caspase-3 activity also increased at 24 h after ME, as production of 17-kDa active fragment and increased activity was reduced by DY-9760e treatment (Fig. 5B).

Immunohistochemical Calpastatin Expression Is Reduced in Neurons after ME-Induced Ischemia. To confirm that decreased calpastatin and caspase-3 activation occurs in neurons after ME, we performed immunohistochemical studies using anticalpastatin and active caspase-3 antibodies. When cortical sections were double-stained with anti-NeuN antibody, calpastatin immunoreactivity colocalized with NeuN immunoreactivity in both the cytosol and nucleus of cortical neurons (Fig. 6). Both immunoreactivities against calpastatin and NeuN markedly decreased 24 h after ME in cortical neurons compared with those seen in sham-operated animals. Immunoreactivity with calpastatin was markedly reduced, even in surviving NeuN-positive neurons. Finally, treatment with DY-9760e partially restored immunostaining patterns against both calpastatin and NeuN. Like cortical neurons, decreased calpastatin immunoreactivity was also evident in striatal neurons 24 h after ME (data not shown). In contrast with decreased calpastatin immunoreactivity, dramatic increases in active caspase-3 immunoreactivity were observed in cortical neurons. The appearance of active caspase-3 in cortical neurons was largely abolished by treatment with DY-9760e as expected. We further confirmed that DY-9760e treatment led to a significantly increase in the number of survival neurons (825 ± 225 per mm^2; $P < 0.05$) compared with vehicle group (542 ± 199 per mm^2) in the ipsilateral cortex 72 h after ME.

Ca^{2+}/CaM-Induced Proteolysis of Calpastatin by Caspase-3 and Its Inhibition by DY-970e Treatment. Finally, we confirmed that caspase-3 induced proteolysis of calpastatin in a Ca^{2+}/CaM-dependent manner in vitro using purified caspase-3 and calpastatin in brain extracts. After inactivation of endogenous caspases and proteases by heating, brain extracts (20 μg) were incubated with or without purified caspase-3 in the presence or absence of Ca^{2+}/CaM, and amounts of calpastatin were determined by immunoblotting. As shown in Fig. 7, the addition of caspase-3 to the incubation medium resulted in proteolysis of 110-kDa calpastatin in a Ca^{2+}/CaM-dependent manner. Ca^{2+}/CaM-dependent proteolysis of calpastatin was inhibited by DY-9760e in a dose-dependent manner. To verify whether DY-9760e

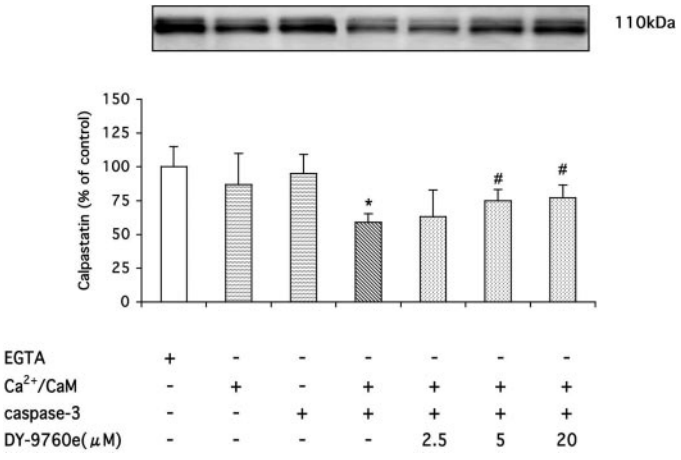

Fig. 7. Inhibition of caspase-3 mediated calpastatin degradation by DY-9760e in rat brain extracts. Brain extracts containing endogenous calpastatin were incubated with recombinant caspase-3 in the presence or absence of Ca^{2+}/CaM. The incubation was also performed in the presence or absence of DY-9760e. Calpastatin protein levels were measured by immunoblotting. Representative image of immunoblot using anti-110-kDa calpastatin antibody. Quantitative analysis of the 110-kDa calpastatin levels was performed by densitometric analysis of immunoblots. Data are expressed as percentage of values of EGTA (mean ± S.D., $n = 4$). *, $P < 0.05$ versus EGTA treatment. #, $P < 0.05$ versus Ca^{2+}/CaM plus caspase-3 treatment.

directly inhibits caspase-3 activity in vitro, we tested its effect on caspase-3 autolysis without substrate. The treatment with DY-9760e did not affect autolysis of caspase-3 up to 20 μM (data not shown).

Discussion

We recently introduced a novel neuroprotective drug, DY-9760e, that ameliorates ischemia/reperfusion-induced neuronal damage (Sato et al., 1999; Hashiguchi et al., 2003). In the previous study, we defined two possible neuroprotective mechanisms of DY-9760e using brain ischemia/reperfusion models. DY-9760e is a potent CaM inhibitor that primarily inhibits Ca^{2+}/CaM-dependent NOS (Hashiguchi et al., 2003) and fodrin breakdown (Sato et al., 1999) after ischemia/reperfusion in rat brain. Generation of NO after brain ischemia/reperfusion has detrimental effects on neurons by producing peroxynitrite (Hashiguchi et al., 2003). The fodrin breakdown probably mediates loss of membrane integrity, thereby leading to necrosis in neurons (Sato et al., 1999). Here, we found a novel mechanism of DY-9760e-induced neuroprotection, in which cross-talk between caspase-3 and calpain through calpastatin underlies its neuroprotective action. A key observation in the present study is that proteolysis of calpastatin by caspase-3 is dependent on Ca^{2+}/CaM in vivo. We also confirmed Ca^{2+}/CaM-dependent proteolysis of calpastatin in vitro by purified caspase-3 using brain extracts. We also found that DY-9760e, a novel neuroprotective agent, could rescue neurons in part by inhibiting cross-talk between caspase-3 and calpain after brain ischemia. Because cross-talk between caspase-3 and calpain plays crucial roles in apoptotic neuronal death after not only brain ischemia but also neurodegenerative disorders (Zhao et al., 2000; Blomgren et al., 2001; Pike et al., 2004), our results showing inhibition of this activity by a novel CaM antagonist is potentially important for development of novel neuroprotective drugs for these conditions.

Calpains are expressed as two isoforms (I or μ-calpain and II or m-calpain), which have different requirements for Ca^{2+} in the micromolar and millimolar ranges, respectively. μ-Calpain is known to function in both apoptotic and necrotic neuronal death after brain ischemia (Rami, 2000). In the case of apoptotic signaling, calpain-mediated cleavage of caspase-3, -7, -8, -9, and -12 is apparently involved in apoptotic signaling (Chua et al., 2000; Lankiewicz et al., 2000; Nakagawa et al., 2000; Blomgren et al., 2001). However, the functional relevance of cleavage of caspases by calpain is unclear. Calpain-mediated cleavage directly activated procaspase-7 and -12 (Ruiz-Vela et al., 1999; Nakagawa et al., 2000). In addition to the calpain-mediated caspases, caspase-mediated cleavage of calpastatin has been documented in cell culture models of apoptosis (Porn-Ares et al., 1998; Wang et al., 1998; Kato et al., 2000; Neumar et al., 2003). Caspase-mediated cleavage of calpastatin also results in activation of calpain. Therefore, the relevance of cross-talk between calpains and caspases should be tested in vivo ischemia models. We here show that caspase-mediated cleavage of calpastatin accounts for activation of calpain in an in vivo ischemia model. Furthermore, cleavage of calpastatin by caspase-3 occurred in a Ca^{2+}/CaM-dependent manner in the brain. Because calpastatin is ubiquitously expressed and is a potent inhibitor of μ- and m-calpain, the balance of calpain-calpastatin levels possibly controls pathological Ca^{2+}-induced events in mammalian tissues. Overexpression of calpastatin by gene transfer ameliorates contractile dysfunction in rat hearts subjected to ischemia/reperfusion (Maekawa et al., 2003). In rabbit hippocampus regions, μ-calpain is highly expressed in pyramidal neurons of both the CA1 and CA3 regions, and studies of loss of calpastatin in CA1 pyramidal neurons suggest vulnerability of CA1 neurons to ischemic injury (Fukuda et al., 1990). A previous report also showed that calpastatin is proteolyzed by calpain in the rat hippocampus after ischemia/reperfusion (Saido et al., 1997). In the present study, both calpastatin and caspase-3 activation after ME was almost completely abolished by treatment with DY-9760e (50 mg/kg), whereas fodrin breakdown by calpain was partly inhibited DY-9760e treatment. Taken together, DY-9760e could preferentially inhibited Ca^{2+}/CaM-dependent calpastatin breakdown compared with fodrin breakdown. Because the membrane-permeable calpain inhibitor MDL28,170 blocked brain injury in a rat model of focal stroke (Markraf et al., 1998), pathological activation of calpain probably leads to necrosis and apoptosis of neurons. On the other hand, physiological and transient activation of calpain in the limited compartment of the synapse is also essential for neuronal plasticity such as long-term potentiation (Jourdi et al., 2005). In our studies, caspase-3-mediated calpastatin degradation was closely associated with delayed increases in calpain activity, which were associated with irreversible neuronal death including apoptosis. Moreover, significant calpastatin degradation was observed 12 h after ME induction. The delayed calpastatin degradation is particularly important concerning the wide therapeutic time window for treatment of neuroprotective drugs.

Another interesting feature of the present study is finding that downstream targets of calpain and caspase-3, such as fodrin and calpastatin, respectively, are CaM binding proteins. Binding of Ca^{2+}/CaM to fodrin, which acts as a scaffold protein between ion channels and actin filaments, promotes

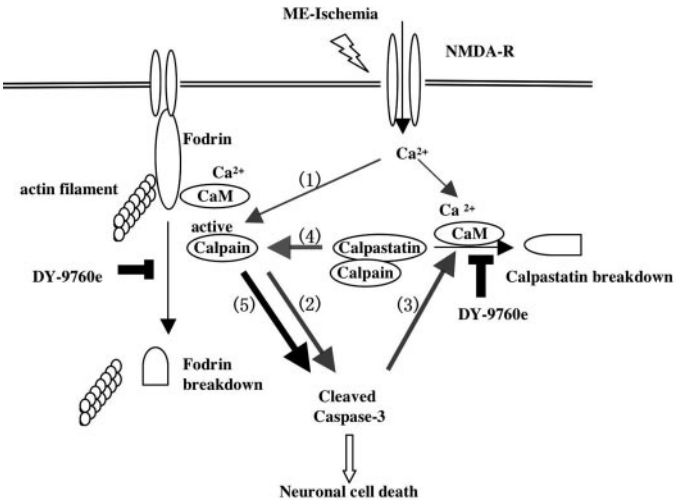

Fig. 8. Schematic model of Ca^{2+} signaling underlying neuronal death and possible site of action of DY-9760e. We have shown that Ca^{2+} elevation by *N*-methyl-D-aspartate (NMDA) activation promotes calpain activation (1). The earlier activation of calpain results in cleavage of caspase-3, leading to its activation (2). Cleaved caspase-3 mediates degradation of calpastatin in the presence of Ca^{2+}/CaM (3). The calpastatin degeneration further promotes calpain activation (4) and accelerates caspase-3 cleavage (5) during ME ischemia. In addition to inhibiting fodrin breakdown, DY-9760e inhibits Ca^{2+}/CaM-dependent calpastatin degradation, thereby eliminating delayed and marked activation of calpain, which is critical for activating apoptosis signals.

its degradation, thereby causing loss of membrane integrity and down-regulation of ion channel functions. The Ca^{2+}/CaM-mediated susceptibility of fodrin to calpain is reduced by DY-9760e, which has potent neuroprotective action (Sato et al., 1999; Takagi et al., 2001; Hashiguchi et al., 2003). We believe that ME-induced ischemia causes early and mild activation of calpain by Ca^{2+} influx through glutamate receptors such as the *N*-methyl-D-aspartate receptor (Fig. 8). The early calpain activation was evident 2 h after ME induction and probably leads to cleavage of caspase-3. Thereafter, Ca^{2+}/CaM accelerates calpastatin breakdown by the activated caspase-3. Thus, the inhibition of calpastatin proteolysis by DY-9760e treatment possibly accounts for suppression of further activation of calpain and caspase-3. Thus, we assumed that calpastatin is the key player in cross-talk between calpain and caspase-3, at least in rat ME-induced brain damage. In the rat transient forebrain ischemia model, calpain is predominantly activated 36 to 72 h after ischemia without significant elevation of caspase-3 activity (Zhang et al., 2002). By contrast, we show here that ME-induced permanent ischemia causes marked increases in both calpain and caspase-3 activities 12 h after ME induction. In particular, fodrin degradation by calpain was seen immediately after ME. Therefore, ME ischemic insults may induce aberrant and prolonged intracellular Ca^{2+} elevation compared with transient forebrain ischemia, resulting in prolonged enhancement of the Ca^{2+}/CaM-dependent fodrin breakdown. In ischemic brain damage, caspase-3 is activated not only by calpain but also by activation of apoptotic signal through extracellular regulated signals such as Fas ligand and tumor necrosis factor α. Delayed calpain activation through caspase-3 mediated calpastatin degradation possibly turns on a switch leading to irreversible apoptotic or necrotic neuronal death.

In summary, our results provide evidence that caspase-3-mediated calpastatin proteolysis accounts for delayed and pronounced activation of calpain, thereby leading to irreversible neuronal death. Like transient forebrain ischemia, DY-9760e has a potent neuroprotective effect against ME-induced ischemic brain damage. In addition to inhibition of NOS activity by DY-9760e as described previously (Fukunaga et al., 2000; Hashiguchi et al., 2003), DY-9760e-mediated inhibition of fodrin and calpastatin degradation possibly contributes its neuroprotective action.

References

Affar EB, Germain M, Winstall E, Vodenicharov M, Shah RG, Salvesen GS, and Poirier GG (2001) Caspase-3-mediated processing of poly(ADP-ribose) glycohydrolase during apoptosis. *J Biol Chem* **276:**2935–2942.

Blomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, Mallard C, and Hagberg H (2001) Synergistic activation of caspase-3 by *m*-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis"? *J Biol Chem* **276:** 10191–10198.

Chua BT, Guo K, and Li P (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J Biol Chem* **275:**5131–5135.

Degli Esposti M, Ferry G, Masdehors P, Boutin JA, Hickman JA, and Dive C (2003) Post-translational modification of Bid has differential effects on its susceptibility to cleavage by caspase 8 or caspase 3. *J Biol Chem* **278:**15749–15757.

Demura N, Mizukawa K, Ogawa N, Yamashita K, and Kanazawa I (1993) A cerebral ischemia model produced by injection of microspheres via the external carotid artery in freely moving rats. *Neurosci Res* **17:**23–30.

Fukuda S, Harada K, Kunimatsu M, Sakabe T, and Yoshida K (1998) Postischemic reperfusion induces α-fodrin proteolysis by *m*-calpain in the synaptosome and nucleus in rat brain. *J Neurochem* **70:**2526–2532.

Fukuda T, Adachi E, Kawashima S, Yoshiya I, and Hashimoto PH (1990) Immunohistochemical distribution of calcium-activated neutral proteases ans endogenous CANP inhibitor in the rabbit hippocampus. *J Comp Neurol* **302:**100–109.

Fukunaga K, Ohmitsu M, Miyamoto E, Sato T, Sugimura M, Uchida T, and Shirasaki Y (2000) Inhibition of neuronal nitric oxide synthase activity by 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate (DY-9760e), a novel neuroprotective agent, in vitro and in cultured neuroblastoma cells in situ. *Biochem Pharmacol* **60:**693–699.

Hashiguchi A, Kawano T, Yano S, Morioka M, Hamada J, Sato T, Shirasaki Y, Ushio Y, and Fukunaga K (2003) The neuroprotective effect of a novel calmodulin antagonist, 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate, in transient forebrain ischemia. *Neuroscience* **121:**379–386.

Hayashi H, Sato K, Kuruhara Y, and Takeo S (1998) Microsphere embolism-induced changes in noradrenaline release in the cerebral cortex in rats. *Brain Res* **783:** 241–248.

Jourdi H, Yanagihara T, Martinez U, Bi X, Lynch G, and Baudry M (2005) Effects of positive AMPA receptor modulators on calpain-mediated spectrin degradation in cultured hippocampal slices. *Neurochem Int* **46:**31–40.

Kato M, Nonaka T, Maki M, Kikuchi H, and Imajoh-Ohmi S (2000) Caspases cleave the amino-terminal calpain inhibitory unit of calpastatin during apoptosis in human Jurkat T cells. *J Biol Chem* **127:**297–305.

Kawano T, Morioka M, Yano S, Hamada J, Ushio Y, Miyamoto E, and Fukunaga K (2002) Decreased Akt activity is associated with activation of forkhead transcription factor after transient forebrain ischemia in gerbil hippocampus. *J Cereb Blood Flow Metab* **22:**926–934.

Kosower NS and Barnoy S (2000) Calpastatin (the endogenous calpain inhibitor) and membrane protein degradation in cell fusion. *Methods Mol Biol* **144:**181–194.

Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680–685.

Lankiewicz S, Marc Luetjens C, Truc Bui N, Krohn AJ, Poppe M, Cole GM, Saido TC, and Prehn JH (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. *J Biol Chem* **275:**17064–17071.

Maekawa A, Lee JK, Nagaya T, Kamiya K, Yasui K, Horiba M, Miwa K, Uzzaman M, Maki M, Ueda Y, et al. (2003) Overexpression of calpastatin by gene transfer prevents troponin I degradation and ameliorates contractile dysfunction in rat hearts subjected to ischemia/reperfusion. *J Mol Cell Cardiol* **35:**1277–1284.

Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, and Linnik MD (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke* **29:**152–153.

McGinnis KM, Gnegy ME, Park YH, Mukerjee N, and Wang KK (1999) Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochem Biophys Res Commun* **263:**94–99.

Melloni E, Michetti M, Salamino F, Minafra R, and Pontremoli S (1996) Modulation of the calpain autoproteolysis by calpastatin and phospholipids. *Biochem Biophys Res Commun* **229:**193–197.

Miyake K, Takeo S, and Kajihara H (1993) Sustained decrease in brain regional blood flow after microsphere embolism in rats. *Stroke* **24:**415–420.

Nakagawa T and Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* **150:**887–894.

Neumar RW, Xu YA, Gada H, Guttmann RP, and Siman R (2003) Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. *J Biol Chem* **278:**14162–14167.

Pike BR, Flint J, Dave JR, Lu XC, Wang KK, Tortella FC, and Hayes RL (2004) Accumulation of calpain and caspase-3 proteolytic fragments of brain-derived

αII-spectrin in cerebral spinal fluid after middle cerebral artery occlusion in rats. *J Cereb Blood Flow Metab* **24:**98–106.

Porn-Ares MI, Samali A, and Orrenius S (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Diff* **5:**1028–1033.

Rami A, Agarwal R, Botez G, and Winckler J (2000) μ-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. *Brain Res* **866:**299–312.

Rami A, Volkmann T, Agarwal R, Schoninger S, Nurnberger F, Saido TC, and Winckler J (2003) β2-Adrenergic receptor responsiveness of the calpain-calpastatin system and attenuation of neuronal death in rat hippocampus after transient global ischemia. *Neurosci Res* **47:**373–382.

Ruiz-Vela A, Gonzalez de Buitrago G, and Martinez-A C (1999) Implication of calpain in caspase activation during B cell clonal deletion. *EMBO (Eur Mol Biol Organ) J* **18:**4988–4998.

Saido TC, Kawashima S, Tani E, and Yokota M (1997) Up- and down-regulation of calpain inhibitor polypeptide, calpastatin, in postischemic hippocampus. *Neurosci Lett* **227:**75–78.

Sato T, Morishima Y, Sugimura M, Uchida T, and Shirasaki Y (1999) DY-9760e, a novel calmodulin antagonist, reduces brain damage induced by transient focal cerebral ischemia. *Eur J Pharmacol* **370:**117–123.

Shirakura T, Han F, Shiota N, Moriguchi S, Kasahara J, Sato T, Shirasaki Y, and Fukunaga K (2005) Inhibition of nitric oxide production and protein tyrosine nitration contribute to neuroprotection by a novel calmodulin antagonist, DY-9760e, in the rat microsphere embolism. *Biol Pharm Bull* **28:**1658–1661.

Smith CD, Thomas GS, Kryscio RJ, and Markesbery WR (1990) ^{31}P spectroscopy in experimental embolic stroke: correlation with infarct size. *NMR Biomed* **3:**259–264.

Takagi K, Sato T, Shirasaki Y, Narita K, Tamura A, and Sano K (2001) Post-ischemic administration of DY-9760e, a novel calmodulin antagonist, reduced infarct volume in the permanent focal ischemia model of spontaneously hypertensive rat. *Neurol Res* **23:**662–668.

Takeo S, Taguchi T, Tanonaka K, Miyake K, Horiguchi T, Takagi N, and Fujimori K (1992) Sustained damage to energy metabolism of brain regions after microsphere embolism in rats. *Stroke* **23:**62–68.

Wang KK, Posmantur R, Nadimpalli R, Nath R, Mohan P, Nixon RA, Talanian RV, Keegan M, Herzog L, and Allen H (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch Biochem Biophys* **356:**187–196.

Yokota M, Saido TC, Kamitani H, Tabuchi S, Satokata I, and Watanabe T (2003) Calpain induces proteolysis of neuronal cytoskeleton in ischemic gerbil forebrain. *Brain Res* **984:**122–132.

Zhang C, Siman R, Xu YA, Mills AM, Frederick JR, and Neumar RW (2002) Comparison of calpain and caspase activities in the adult rat brain after transient forebrain ischemia. *Neurobiol Dis* **10:**289–305.

Zhao X, Newcomb JK, Pike BR, Wang KK, d'Avella D, and Hayes RL (2000) Novel characteristics of glutamate-induced cell death in primary septohippocampal cultures: relationship to calpain and caspase-3 protease activation. *J Cereb Blood Flow Metab* **20:**550–562.

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