3-[2-[4-(3-Chloro-2-methylphenylmethyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-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

Feng Han, Yasufumi Shirasaki, and Kohji Fukunaga

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (F.H., K.F.); New Product Research Laboratories II, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan (Y.S.); and Tohoku University 21st Century Center of Excellence Program, Comprehensive Research and Education Center for Planning of Drug Development and Clinical Evaluation (CRESCENDO), Sendai, Japan (F.H., K.F.)

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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)-1H-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 protein, and the neuroprotective activity of DY-9760e is rescued by pre-treatment with a calpain inhibitor (Shirakura et al., 2005). These observations suggest that ME-induced ischemic injury involves cross-talk between calpain and caspase-3 through calpastatin.

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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)-1H-indazole dihydrochloride 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, carbobenzyox-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 ± 0.7 μm in diameter) suspended in a 20% dextran solution were injected with a 26 G needle into the left 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 ± 0.7 μm in diameter) suspended in a 20% dextran solution were injected with a 26 G needle into the left

Rectal temperature was monitored and maintained at 37 ± 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 50 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 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 Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM 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 15,000g. 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 (SDSPAGE) (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 50% 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 over night 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 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).
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 MgCl2, 1 mM Na3VO4, and 30 mM sodium pyrophosphate plus protease inhibitors and centrifuged at 14,000g 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 Exogeous 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 15,000g 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 20 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 Ca2+/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-
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 Ca2+/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).

**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-
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 mm²; P < 0.05) compared with vehicle group (542 ± 199 per mm²) in the ipsilateral cortex 72 h after ME.

**Ca²⁺/CaM-Induced Proteolysis of Calpastatin by Caspase-3 and Its Inhibition by DY-9760e Treatment.** Finally, we confirmed that caspase-3 induced proteolysis of calpastatin in a Ca²⁺/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 Ca²⁺/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 Ca²⁺/CaM-dependent manner. Ca²⁺/CaM-dependent proteolysis of calpastatin was inhibited by DY-9760e in a dose-dependent manner. To verify whether DY-9760e...
Calpains are expressed as two isoforms (I or \( \mu \)-calpain and II or m-calpain), which have different requirements for \( \text{Ca}^{2+} \) in the micromolar and millimolar ranges, respectively. \( \mu \)-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, calpase-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). Calpase-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 \( \mu \)- 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, \( \mu \)-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 our studies, caspase-3-mediated calpastatin degradation was observed 12 h after ME induction. 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 (Markgraf 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
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.

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**


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...


Address correspondence to: Dr. Kohji Fukunaga, Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aramaki-Aoba Aoba-ku, Sendai 980-8578, Japan. E-mail: fukunago@mail.pharm.tohoku.ac.jp