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The Post-ischemic Administration of 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 Calmodulin Antagonist, Prevents Delayed Neuronal Death in Gerbil Hippocampus

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Abstract. The novel calmodulin (CaM) antagonist DY-9760e (3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate) with an apparent neuroprotective effect in vivo preferentially inhibits neuronal nitric oxide synthase (nNOS), Ca^{2+}/CaM-dependent protein kinase IIα (CaMKIIα), and calcineurin in vitro. In the present study, we investigated the molecular mechanism underlying its neuroprotective effect with the gerbil transient forebrain ischemia model, by focusing on its inhibition of these Ca^{2+}/CaM-dependent enzymes. Post-ischemic DY-9760e treatment (5 mg/kg, i.p.) immediately after 5-min ischemia significantly reduced the delayed neuronal death in the hippocampal CA1 region. CaMKIIα was transiently autophosphorylated immediately after reperfusion with concomitant sustained decrease in its total amounts in the Triton X-100-soluble fractions. Calcineurin activity, accessed by the phosphorylation state of dopamine- and cAMP-regulated phosphoprotein of *Mr* 32,000 (DARPP-32) at Thr34, was elevated at 6 h after reperfusion. Post-treatment of DY-9760e had no effects on both CaMKIIα and DARPP-32 phosphorylation at 6 h after reperfusion. However, DY-9760e significantly inhibited nitrotyrosine formation, as a biomarker of NO, and in turn, peroxynitrite ($ONOO^-$) production. These results suggest that DY-9760e primarily inhibits Ca^{2+}/CaM-dependent neuronal NOS, without any effects on CaMKII and calcineurin, and the inhibition of NO production possibly accounts for its neuroprotective action in brain ischemic injury.

Keywords: cerebral ischemia, nitric oxide synthase (NOS), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), calcineurin, dopamine- and cAMP-regulated phosphoprotein of *Mr* 32,000 (DARPP-32)

Introduction

Ca^{2+} overload is thought to play a pivotal role in the pathological events following cerebral ischemia (1 – 3). During cerebral ischemia, elevated levels of extracellular glutamate cause excessive stimulation of its postsynaptic receptors and subsequently induce Ca^{2+} influx into neurons (1, 4, 5). The elevated Ca^{2+} concentration in neurons disrupts the ionic balance and activates various Ca^{2+}-dependent enzymes and Ca^{2+}-binding proteins. Calmodulin (CaM) is a major Ca^{2+}-binding protein found in the central nervous system (6, 7). CaM is implicated in a variety of cell functions through the activation of CaM-dependent enzymes, such as protein kinases, protein phosphatases, and nitric oxide synthases (NOSs) (7). Thus Ca^{2+} overload in neurons induced by

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ischemic insult may overactivate Ca^{2+}/CaM-dependent pathways, thereby leading to irreversible cell damage.

We recently characterized a novel CaM antagonist, DY-9760e (3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazole dihydrochloride 3.5 hydrate), which preferentially inhibits the indicated enzymes with the following order of potency: neuronal NOS (nNOS) > Ca^{2+}/CaM-dependent protein kinase II (CaMKII) > Ca^{2+}/CaM-dependent protein phosphatase 2B (calcineurin) (K_i = 0.9, 1.4, and 2.0 μM, respectively) in vitro (8, 9). DY-9760e shows powerful neuroprotective effects in focal cerebral ischemia with moderately wide therapeutic time windows (10 – 12). The protective effect was partly attributed to inhibition of nitric oxide (NO) production and subsequent protein tyrosine nitration by peroxynitrite ($ONOO^-$) (13). In addition, DY-9760e attenuated brain edema formation mediated by the inhibition of enhanced blood brain barrier (B.B.B) permeability after ischemic insults, in which CaM may be involved in the regulation of brain microvascular barrier function (14).

Since DY-9760e has similar inhibitory effects on neuronal NOS, CaMKII, and calcineurin in vitro (8, 9), we here investigated which enzymes were primarily inhibited by post-ischemic DY-9760e treatment in gerbil hippocampus after transient forebrain ischemia. We found that the post-treatment with DY-9760e predominantly inhibited Ca^{2+}/CaM-dependent NOS in vivo without any effects on CaMKII and calcineurin, implying that the DY-9760e inhibition against NOS accounts for its neuroprotective actions.

Materials and Methods

Animal experiments

All animal procedures were approved by the Committee of Animal Experiments at Kumamoto University School of Medicine. All efforts were made to minimize the number of animals used and their suffering. All experiments were performed on adult male Mongolian gerbils weighing 60 to 70 g. Anesthesia was achieved with 1% halothane in 70% N_2O / 30% O_2. Gerbils were subjected to 5 min of forebrain ischemia by bilateral occlusion of the common carotid artery with rectal temperature maintained at 37.0°C to 38.0°C as previously reported (13). Gerbils were intraperitoneally (i.p.) administered with 5 mg/kg DY-9760e (Daiichi Pharmaceutical Co., Tokyo) dissolved in saline with 10% dimethyl sulfoxide (vehicle) or vehicle alone immediately after reperfusion. For Western blotting, samples were prepared as previously described (n = 3 – 6 per group or time point) (13).

Immunoblot analyses

Samples for immunoblots were taken from the hippocampal CA1 regions, and protein extraction was performed with ice-cold RIPA lysis buffer as previously described (13). Insoluble materials were removed by a 10 min of centrifugation at 15,000 × *g*. Protein concentrations were determined by the Bradford method. The supernatant fractions were treated with Laemmli's buffer containing 2-mercaptoethanol and heated to 100°C for 3 min. Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred to a polyvinylidine difluoride membrane (Immobilon P; Millipore Corp., Bedford, MA, USA). Then, each immunoblot was incubated with primary monoclonal antibodies against CaMKIIα (1:1000, BD Biosciences, Franklin Lakes, NJ, USA) and β-tubulin (1:10000; Sigma, St. Louis, MO, USA) or polyclonal antibodies against phospho-Thr286/287 CaMKIIα (1:2000; Upstate, Biotechnology, Lake Placid, NY, USA) and dopamine- and cAMP-regulated phosphoprotein of *Mr* 32,000 (DARPP-32), phospho-Thr34 DARPP-32 (1:1000 each; Cell Signaling, Beverly, MA, USA). For immunobloting with anti-nitrotyrosine antibody (1:1000, Upstate Biotechnology), the supernatants were mixed with non-reducing sample buffer without 2-mercaptoethanol and heated to 60°C for 3 min as previously described (13), because nitrotyrosine is easily reduced to aminotyrosine by heating in 2-mercaptoethanol-containing buffer (15, 16). To quantitate immunoreactive bands, Western blots were scanned and optical densities of bands were analyzed using Scion Image software (Scion Corp., Frederick, MD, USA).

Measurement of body temperature and cerebral blood flow (CBF)

The rectal temperature was monitored before induction of anesthesia, at 30 min and 6 h after reperfusion, in gerbils with or without DY-9760e. Intra-ischemic CBF changes in the CA1 region were measured with a laser bloody flow meter (ALF21; Advance, Tokyo). Gerbils were anesthetized as above and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). A small burr hole was drilled through the skull for stereotaxic insertion of a 1.8-mm diameter laser-Doppler flow probe (Type-E, Advance) into the right hippocampus (2.5-mm posterior and 2-mm lateral to the bregma and 1-mm below the brain surface). After a stabilization period, 5-min ischemia was performed as above. Laser-Doppler flows were continuously recorded throughout the experiments. Proper probe placement was confirmed upon postmortem inspection.

Neuropathological evaluation

At 7 days after sham operation or 5 min of ischemia, histopathological evaluation was performed as previously described [13]. Cell viability in either condition with or without DY-9760e was expressed as percentages of the averaged number of viable cells from sham-operated animals.

Statistical analyses

Data were expressed as the mean ± S.D. Statistical evaluation was performed by ANOVA with Scheffé's test, or an unpaired Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Post-ischemic DY-9760e administration prevents delayed neuronal death in gerbil hippocampus after 5 min of forebrain ischemia

At 7 days after 5 min of forebrain ischemia, the viable neurons in the hippocampal CA1 regions of vehicle-treated ischemic gerbils were reduced to 4.5 ± 2.1% of sham-operated animals ($P<0.01$ for vehicle versus sham, n = 4 each; Fig. 1: A and B). Intraperitoneal injection (i.p.) of 5 mg/kg DY-9760e, immediately after reperfusion, significantly increased the number of viable neurons to 23 ± 8.9% that of the vehicle-injected animals ($P<0.01$ for DY-9760e versus vehicle, n = 4 each), whereas the number of viable neurons remained to be significantly reduced compared with sham-operated animals ($P<0.01$ for DY-9760e versus sham). There are no significant differences in the rectal temperature and intra-ischemic CBF in the hippocampal CA1 region between vehicle-treated and DY-9760e-treated animals (Fig. 1: C and D).

Phosphorylation states of CaMKIIα and DARPP-32 after transient forebrain ischemia in gerbil hippocampus

It has been reported that CaMKII translocates from the Triton X-100 soluble to the insoluble fractions with concomitant inactivation of the enzyme activity following brain ischemia (17 – 20). Consistent with these previous reports, the total amount of CaMKIIα in the Triton X-100 soluble fractions were significantly reduced throughout the reperfusion periods following brain ischemia ($P<0.05$ versus sham control, n = 3 each; Fig. 2: A and C). However, in the CA1 regions, the levels of CaMKIIα autophosphorylation significantly increased to 233 ± 76% of sham-operated animals immediately after reperfusion, followed by a gradual decrease to near basal levels ($P<0.01$ versus sham control, n = 3 each; Fig. 2: A and B).

Since calcineurin is known to be involved in ischemia- and glutamate-induced cell death (17, 21 – 25), we next investigated calcineurin activity in gerbil hippocampus after transient forebrain ischemia. To measure calcineurin activity, we examined phosphorylation levels of Thr-34 in DARPP-32, an excellent substrate for calcineurin, using a phospho-specific antibody (26). DARPP-32-Thr34 phosphorylation significantly increased to 161 ± 20% of sham-operated animals immediately after reperfusion and then significantly decreased to 22 ± 10% at 6 h after reperfusion ($P<0.01$ versus sham control, n = 3 each; Fig. 3: A and B). The total amounts of DARPP-32 were unchanged throughout the reperfusion periods. These results suggested that calcineurin is transiently activated at 6 h after reperfusion.

Post-ischemic DY-9760e treatment did not affect phosphorylation states of CaMKIIα and DARPP-32

To investigate the mechanisms of neuroprotection by post-ischemic DY-9760e treatment, we examined effects on phosphorylation states CaMKIIα and DARPP-32 at 6 h after reperfusion when these decreases were apparently significant after reperfusion (Figs. 2 and 3). However, i.p. injection of 5 mg/kg DY-9760e, immediately after reperfusion, had effects on neither the autophosphorylation state nor amount of CaMKIIα in the Triton X-100 soluble fractions (Fig. 4). Similarly, the drug treatment did not affect the phosphorylation state of DARPP-32 compared with vehicle-treated animals (n = 6 each, Fig. 5). These results indicated that DY-9760e did not affect CaMKII and calcineurin activities in vivo at least with the dose of 5 mg/kg.

Post-ischemic DY-9760e treatment prevents nitrotyrosine formation in the hippocampal CA1 region after 5 min of forebrain ischemia

We recently documented that transient forebrain ischemia significantly increases NO production, thereby eliciting protein tyrosine nitration in the gerbil hippocampal CA1 region from 2 to 24 h after reperfusion (13). We here examined whether post-ischemic DY-9760e treatment inhibited nitrotyrosine formation or not. Consistent with the previous results, 5 min of ischemia produced a marked increase in the immunoreactivity of several tyrosine nitrated proteins including 140 (Band-1) and 80 (Band-2) kDa proteins in the CA1 region 6 h after reperfusion (215 ± 52%, 259 ± 121% of corresponding bands of sham animals, respectively; $P<0.01$ versus corresponding bands of sham animals, n = 6 each; Fig. 6: A and B). The increased immunoreactivities of these two proteins were significantly blocked by DY-9760e treatment ($P<0.05$ for DY-9760e versus vehicle, n = 6 each; Fig. 6: A and B), but they remained still

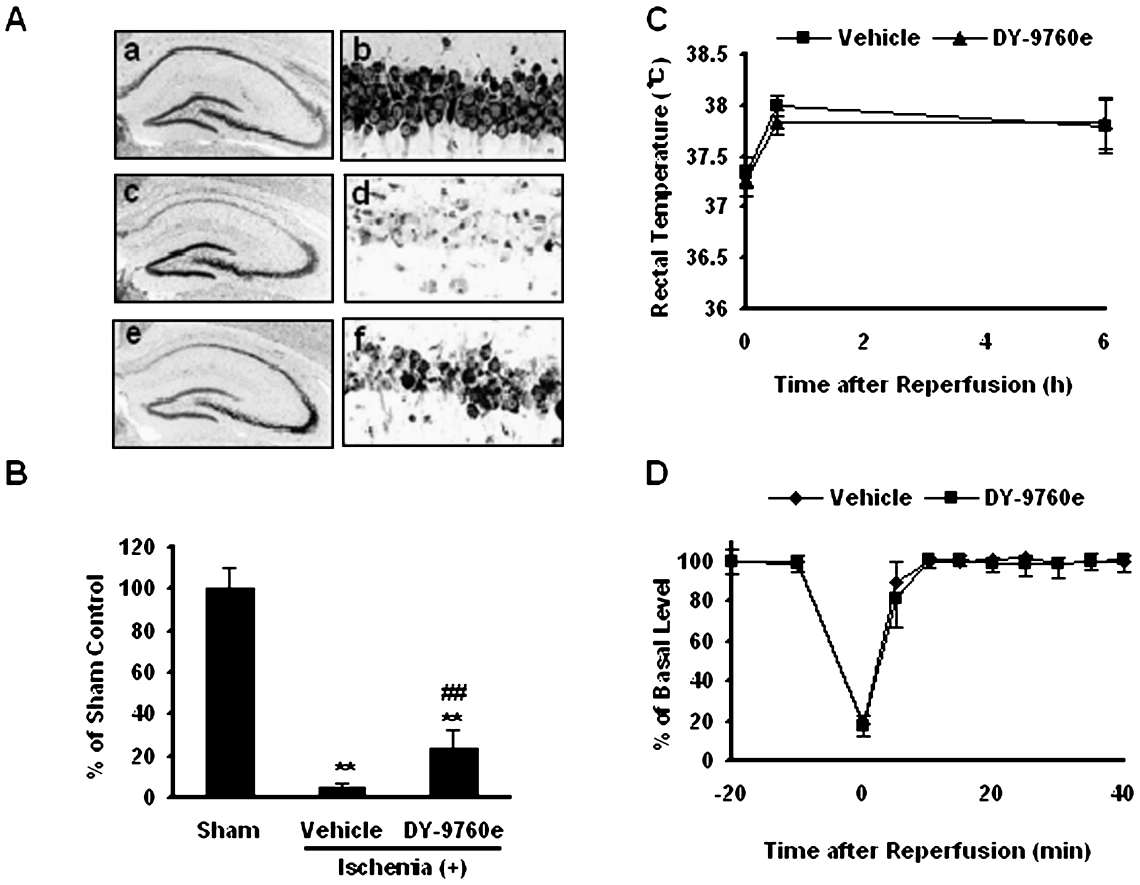

Fig. 1. Delayed neuronal death after 5 min of forebrain ischemia is prevented by DY-9760e. DY-9760e at 5 mg/kg or vehicle was intraperitoneally administered immediately after reperfusion, and neuropathological evaluation was performed at 7 days after reperfusion. A: Representative histological sections of hippocampus (a, c, and e) and the hippocampal CA1 region (b, d, and f) in sham-operated (a and b), vehicle-treated ischemic (c and d), or DY-9760e-treated ischemic gerbils (e and f) are shown. B: Post-ischemic DY-9760e treatment provided a neuroprotective effect. Cell viability was expressed as percentages of the averaged number of viable cells from sham-operated gerbils (n = 4 each). C: Changes in rectal temperature after transient forebrain ischemia. The rectal temperature was examined before induction of anesthesia, at 30 min and 6 h after reperfusion, in gerbils with or without DY-9760e (n = 3 or 4). D: Changes in CBF during ischemia. The CBF was monitored in 5-min ischemic animals with or without DY-9760e and expressed as percentages of the pre-ischemic basal level (n = 3 or 4). Data represent the mean ± S.D., $^{**}P<0.01$ versus sham-operated animals, $^{\#\#}P<0.01$ versus vehicle-treated ischemic animals.

significantly increased compared with sham-operated animals ($P<0.05$ for DY-9760e versus sham). These results suggest that DY-9760e preferentially inhibits Ca^{2+}/CaM-dependent NOS activity even in the post-ischemic treatment similar to that with pretreatment as described previously (13).

Discussion

Neuronal damage associated with cerebral infarcts and neurodegenerative diseases implies glutamate neurotoxicity acting via *N*-methyl-D-asparatate (NMDA) receptors (1, 3, 4). Ischemic insults induce massive Ca^{2+} influx through the NMDA receptors, which then excessively activate Ca^{2+}/CaM-dependent enzymes, thereby leading to irreversible neuronal cell damage. Therefore, Ca^{2+}/CaM complexes might be potential therapeutic targets to treat the acute phase of stroke. In fact, a novel CaM antagonist, DY-9760e, as well as other well-known CaM antagonists such as W-7, trifluoperazine, and calmidazolium, inhibit cell death or ischemic brain damage following brain ischemia (8, 10, 11 – 13).

CaMKII is a ubiquitously expressed protein kinase, whose α and β isoforms are particularly abundant in brain, and it regulates ion channel functions and transcriptions through Ca^{2+} signals. Elevated intracellular free Ca^{2+} triggers autophosphorylation of CaMKII at a threonine residue in the inhibitory domain (Thr286 in CaMKIIα), thereby converting the kinase to a persis-

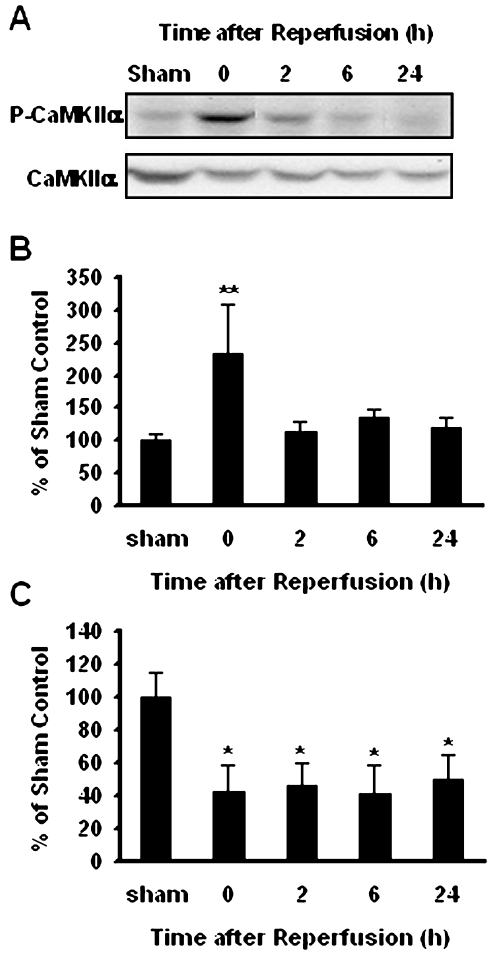

Fig. 2. Time course of CaMKIIα autophosphorylation and protein levels in the CA1 region after 5 min of forebrain ischemia. Protein extracts prepared from the CA1 region of gerbil hippocampus at indicated times after reperfusion were assessed by Western blot analysis with anti-phospho-Thr286/287 CaMKIIα or anti-CaMKIIα antibody. A: Representative image of immunoblot with anti-phospho-Thr286/287 CaMKIIα (upper panel) or anti-CaMKIIα antibody (lower panel). Semiquantitative analysis of CaMKIIα autophosphorylation (B) or its protein level in the soluble fractions (C) by densitometric scanning of immunoreactive bands (n = 3 or 4 per time point). Data represent the mean ± S.D., *P<0.05, **P<0.01 versus sham-operated animals.

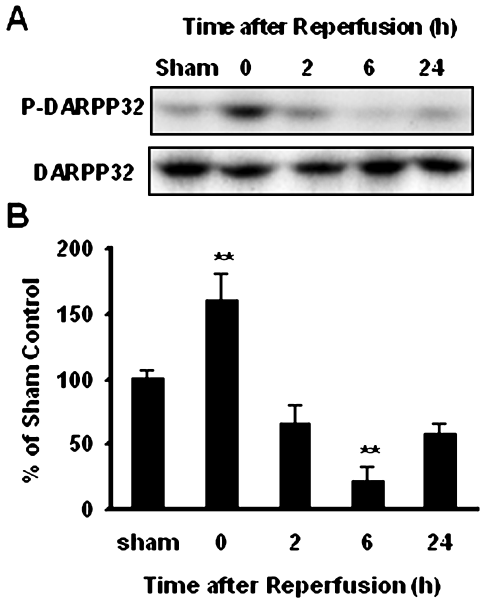

Fig. 3. Time course of DARPP-32 phosphorylation in the CA1 region after 5 min of forebrain ischemia. Protein extracts prepared from the CA1 region of gerbil hippocampus at indicated times after reperfusion were assessed by Western blot analysis with anti-phospho-Thr34 DARPP-32 or anti-DARPP-32 antibody. A: Representative image of immunoblot with anti-phospho-Thr34 DARPP-32 (upper panel) or anti-DARPP-32 antibody (lower panel). B: Semiquantitative analysis of DARPP-32 phosphorylation by densitometric scanning of immunoreactive bands (n = 3 or 4 per time point). Data represent the mean ± S.D., **P<0.01 versus sham-operated animals.

tently active state and CaMKIIα reversibly translocates to excitatory synapses under physiological conditions such as hippocampal long-term potentiation (27). However, massive and sustained increase in the intracellular free Ca^{2+} following brain ischemia leads to inactivation and irreversible translocation of CaMKII to the particulate fractions (Triton X-100 insoluble fractions) (17 – 20). The autophosphorylation of CaMKII also preceded the inactivation by over-loading of Ca^{2+} (28). Consistent with the previous results, CaMKIIα immediately translocated from the soluble fractions to the insoluble particulate fractions, thereby decreasing the amount of CaMKII in the soluble fractions as shown in Fig. 2C. Although the role of transient activation and in turn inactivation of CaMKIIα in ischemic neuronal damage is still controversial, CaMKIIα seems to play a protective role in ischemic neurons. Because cerebral infarction is larger in CaMKIIα-knockout animals compared with wild-type animals (29, 30). On the other hand, calcineurin is a Ca^{2+}/CaM-dependent serine/threonine phosphatase, which is abundant in brain regions, such as hippocampus and cortex, vulnerable to excitotoxic damage and ischemia (31). In neurons, calcineurin regulates neurotransmitter release and long-term depression (32). Since the immunosuppressant FK506, a calcineurin inhibitor, prevents ischemic cell damage and glutamate neurotoxicity (22 – 24, 33), calcineurin might also be involved in ischemic cell damage and/or apoptosis by glutamate neurotoxicity (17, 21 – 25). One major target protein for calcineurin is DARPP-32, which is dephosphorylated at Thr-34 through activated calcineurin by NMDA-receptor stimulation (26). Although DY-9760e preferentially inhibits CaMKIIα and calcineurin in vitro in addition to nNOS (8, 9), DY-9760e treatment altered

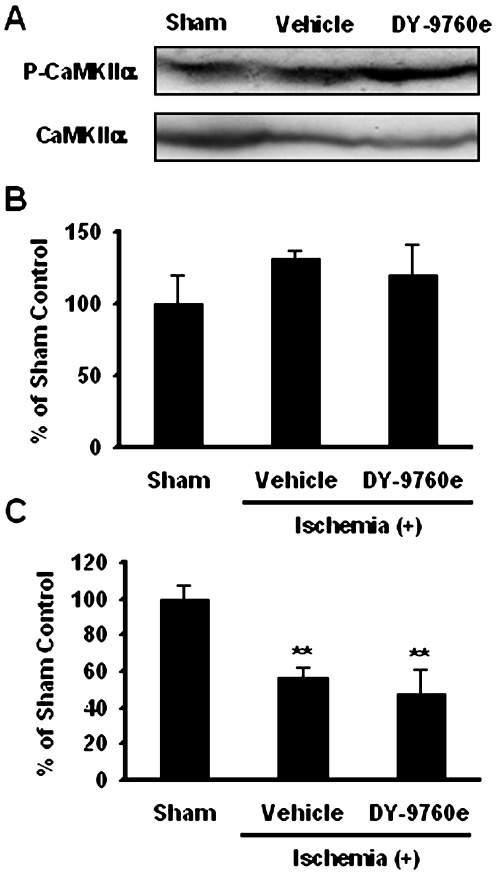

Fig. 4. DY-9760e affects neither autophosphorylation state nor protein level of CaMKIIα in the hippocampal CA1 region after 5 min of forebrain ischemia. Gerbils were treated (i.p.) with vehicle or 5 mg/kg DY-9760e immediately after reperfusion. Extracts from the CA1 regions of sham-operated and ischemic gerbils 6 h after reperfusion were subject to Western blot analysis with anti-phospho-Thr286/287 CaMKIIα or anti-CaMKIIα antibody. A: Representative image of immunoblot with anti-phospho-Thr286/287 CaMKIIα (upper panel) or anti-CaMKIIα antibody (lower panel). Semiquantitative analysis of CaMKIIα autophosphorylation (B) or its protein level in the soluble fractions (C) by densitometric scanning of immunoreactive bands (n = 6 each). Data represent the mean ± S.D., $^{**}P<0.01$ versus sham-operated animals.

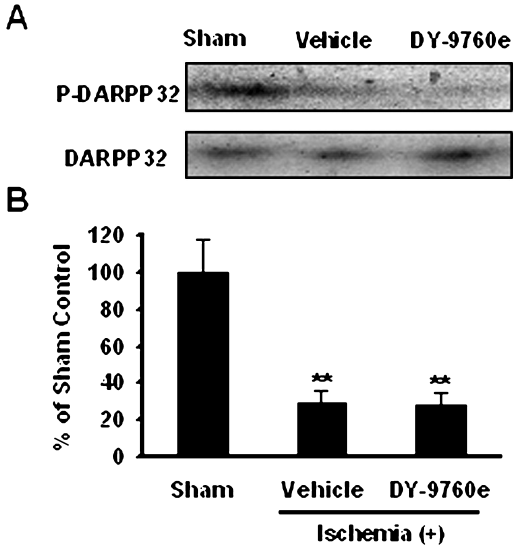

Fig. 5. DY-9760e has no effect on phosphorylation state of DARPP-32 in the hippocampal CA1 region after 5 min of forebrain ischemia. Gerbils were treated (i.p.) with vehicle or 5 mg/kg DY-9760e immediately after reperfusion. Extracts from the CA1 regions of sham-operated and ischemic gerbils 6 h after reperfusion were subject to Western blot analysis with anti-phospho-Thr34 DARPP-32 or anti-DARPP-32 antibody. A: Representative image of immunoblot with anti-phospho-Thr34 DARPP-32 (upper panel) or anti-DARPP-32 antibody (lower panel). B: Semiquantitative analysis of DARPP-32 phosphorylation by densitometric scanning of immunoreactive bands (n = 6 each). Data represent the mean ± S.D., $^{**}P<0.01$ versus sham-operated animals.

neither the phosphorylation state or translocation of CaMKIIα nor the phosphorylation state of DARPP-32. Taken together, the post-ischemic treatment of DY-9760e at least with the dose of 5 mg/kg inhibits neither CaMKIIα nor calcineurin in vivo. It is recently reported that calcineurin is directly cleaved by calpain and soon converted to a Ca^{2+}/CaM-independent active form in vitro and in vivo (25); however, we did not observe pronounced increase in the calcineurin activity throughout ischemia-reperfusion except for at 6 h after reperfusion. Further studies are required to define the pathophysiological relevance of biphasic phosphorylation and dephosphorylation of DARPP-32 in ischemic brain injury.

Studies of knockout mice suggest that nNOS activation is detrimental to the ischemic brain damage (34, 35). Indeed, we recently observed a marked and persistent increase in NO production following gerbil forebrain ischemia (13). Although the dose used in the post-ischemic DY-9760e treatment was lower than that in the previous work (5 versus 50 mg/kg, i.p.) (13), the post-treatment significantly rescued cells from delayed neuronal death. This is consistent with a wide therapeutic time window as previously reported (11, 12). Protein tyrosine nitration has pivotal roles in the cell injury and its inhibition possibly underlies the neuroprotective action of DY-9760e even in the post-treatment. Since neither the rectal temperature nor hippocampal CBF in the present study was affected by DY-9760e treatment, the drug preferentially inhibits nNOS rather than another Ca^{2+}/CaM-dependent isoform, endothelial NOS. Although the precise mechanisms of neuronal toxicity mediated by tyrosine nitration are unclear, protein nitration causes protein dysfunction and is implicated in the pathogenesis of stroke as well as other neurological disorders such as amyotrophic lateral sclerosis, Alzheimer disease, and Parkinson's disease

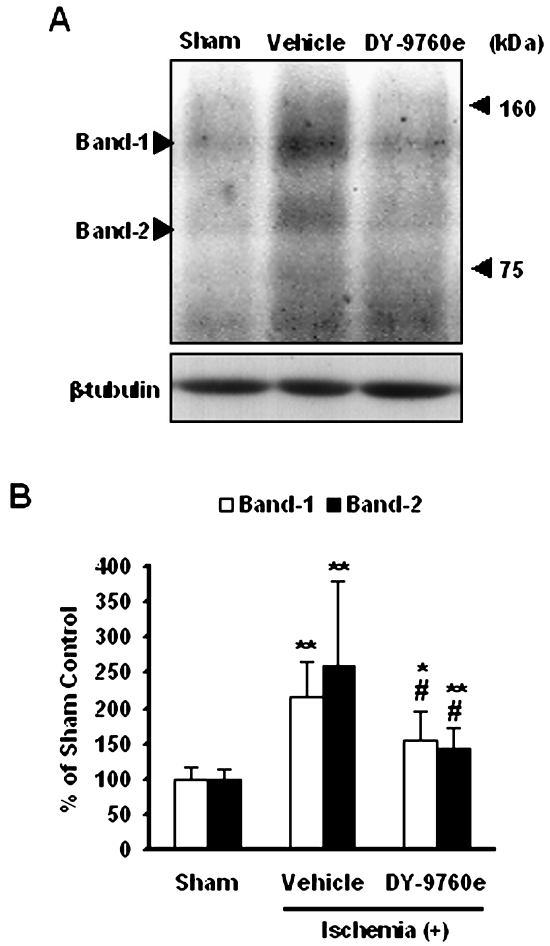

Fig. 6. DY-9760e inhibits increases in tyrosine nitration in the hippocampal CA1 region after 5 min of forebrain ischemia. Gerbils were treated (i.p.) with vehicle or 5 mg/kg DY-9760e immediately after reperfusion. Protein extracts from the CA1 regions of sham-operated and ischemic gerbils 6 h after reperfusion were subject to Western blot analysis with anti-nitrotyrosine antibody under the non-reducing condition as previously described (13). A: Representative image of immunoblot with anti-nitrotyrosine antibody shows several nitrotyrosine immunoreactive proteins (upper panel). β-Tubulin was used to normalize the amount of proteins applied in each lane (lower panel). B: Semiquantitative analysis of the levels of protein tyrosine nitration by densitometric scanning of nitrotyrosine immunoreactive Band-1 and Band-2 (arrowheads, n = 6 each). Data represent the mean ± S.D.; *P<0.05, **P<0.01 versus sham-operated animals; $^{\#}P$<0.05 versus vehicle-treated ischemic animals.

(36 – 38). Recent advances in proteomics indicate that nitrated proteins are involved in protein dysfunction under oxidative stress, apoptosis (39). We are now identifying the several tyrosine-nitrated proteins detected in the previous and present studies with proteomic methods (13) and will in future investigate the mechanisms of neuronal toxicity mediated by tyrosine nitration.

In conclusion, the aim of the present study was to investigate the molecular mechanism underlying the neuroprotective effects of a novel CaM inhibitor DY-9760e, especially by focusing on its inhibitory effects on CaMKIIα and calcineurin. Although inhibition of CaMKIIα and calcineurin activities by DY-9760e was evident in vitro (8), post-ischemic DY-9760e treatment had no apparent effects on these two enzymes at least at the dose of 5 mg/kg. The present study showed that inhibition of nitrotyrosine formation through nNOS inactivation by post-ischemic DY-9760e treatment, as well as in the pre-ischemic treatment (13), closely correlates with its neuroprotective effects.

Acknowledgments

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